CELLULAR SENESCENCE: CAUSES, CONSEQUENCES AND THERAPEUTIC OPPORTUNITIES

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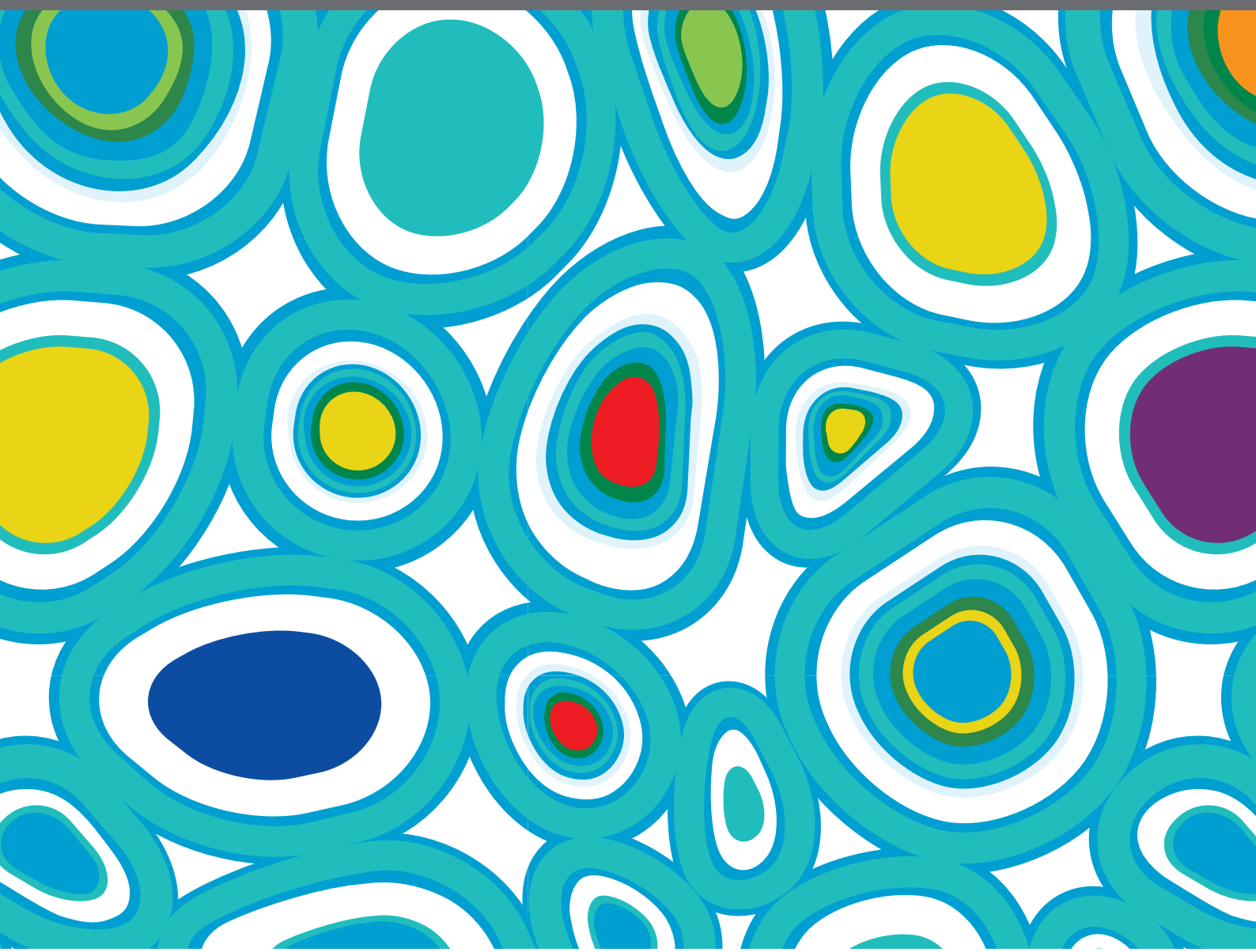

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CELLULAR SENESCENCE: CAUSES, CONSEQUENCES AND THERAPEUTIC OPPORTUNITIES

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EDITORIAL
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Editorial: Cellular Senescence: Causes, Consequences and Therapeutic Opportunities

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Editorial on the Research Topic

Cellular Senescence: Causes, Consequences and Therapeutic Opportunities

Once regarded as a tissue culture phenomenon, cellular senescence has long come of age. It is one of the hallmarks of aging and widely accepted as an important driver of the progression of age-related degenerative pathologies such as cardiovascular disease, neurodegenerative disease and type 2 diabetes (Muñoz-Espin and Serrano, 2014; Franceschi et al., 2018). The concept of reducing the burden of senescent cells or suppressing senescence-associate secretory phenotype (SASP) to delay age-related changes, improve tissue functions and subsequently increase health and life span were introduced and proven by many studies (Xu et al., 2015; Zhu et al., 2015, 2017; Chang et al., 2016; Fuhrmann-Stroissnigg et al., 2017). These ground-breaking findings underpin the rapidly expanding field of senotherapeutics to identify drugs that can eliminate senescent cells (senolytics) or suppress SASP (senomorphics) as a novel pharmacological approach to concurrently treat age-related multi-morbidities. In this Research Topic, we showcase both fundamental and translational advances in our understanding of cellular senescence, which will form the basis of future therapies for age-related degenerative changes. Schroth et al. discussed how senescent cell accumulation as a result of defective innate and adaptive immune responses leads to the progression of chronic kidney diseases (CKD), rationalizing the use of various immunomodulation approaches to enhance immunosurveillance and clearance of senescent cells for CKD treatment. Currently, strategies enhancing the cytotoxic activity of NK cells against senescent cells, activating the antibody-dependent cell-mediated cytotoxicity (ADCC) and dampening SASP-derived inflammatory milieu by targeting NF-κB and c/EBPβ transcriptional activities are being studied intensively (Paez-Ribes et al., 2019). Wilkinson and Hardman offered their new insight on the physiological and pathophysiological roles of cellular senescence in diabetic would healing. While short-lived tissue senescence is essential for effective repair, sustained tissue senescence involving the senescence-linked chemokine receptor, CXCR2, impairs diabetic wound healing. The blockage of CXCR2 promoted wound repair, thus, bolstering the notion of suppressing SASP for chronic wound healing treatment. Indeed, metformin, an effective anti-diabetic drug, via its SASP suppression activity has been shown to accelerate wound healing (Qing et al., 2019). Ramasamy et al. in their review on chondrocyte senescence and cartilage degeneration, highlighted the use of senolytics such as FOXO4-p53-interfering peptides or dasatinib/quercetin to selectively eliminate senescent cells during the *in vitro* expansion of cells used for autologous chondrocyte implantation (ACI). ACI is a targeted approach used to treat articular cartilage injuries and prevent the onset of post-traumatic osteoarthritis. Although effective in eliminating senescent cells within the expanded cell population and thus, increasing cell differentiation efficiency, whether these drugs could produce greater repair potential and promote cartilage repair clinically warrants further investigations (Huang et al., 2021). Similarly,

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Rayson et al. noted that not all geroprotectors produced expected clinical outcomes for osteoarthritis and osteoporosis. Although these compounds can alleviate pathologies following disease induction in animal models, they are in fact less effective in aged animals, suggesting that geroprotectors may be ineffective once the disease is well established. Metformin, for example, has been reported to attenuate cartilage degradation when administered in mice with early OA and in rhesus macaques exhibiting very early stage of cartilage damage (Rayson et al.).

To date, successful clinical applications of senotherapeutics remain limited by adverse effects and insufficient efficacy arising from the lack of selectivity and sensitivity of the current senotherapeutics. Ironing out these issues and optimization of dosing regimen would require extensive research and a thorough understanding on the mechanisms of cellular senescence. One of the challenges of senotherapeutics identification is to convincingly demonstrate senolytic or senomorphic effects *in vivo*. This is in view of cell-type specificity of senescence markers, SASP factors and senescent cell anti-apoptosis (SCAPs) mechanism. Alternative detection methods of senescent cells are needed to obtain reproducible and high quality *in vitro* and *in vivo* data. In his review on the different markers available for the detection of senescent cells, Faragher cautioned false positive arising from the timing of detection and deviations from the typical senescent cell physiology. In addition to SA-β-gal, visualisation of lipofuscin, ferritin and advanced glycation end products (AGEs) can be applied to reduce discrepancies in detection. Novel systems such as pigs and horses with higher clinical relevance to human disease and human progeria can be used to develop better detection techniques or drug screening models (Faragher). There are also challenges in differentiating quiescent and replicative senescent cells. Mehta et al. elegantly demonstrated the application of chromosome repositioning to differentiate senescence from quiescence and young from senescent cells. They observed that chromosome 10 relocation to the nuclear periphery was retarded in senescent cells. Chromosomal repositioning requires nuclear myosin 1β (NM1β). Its organization and distribution in the nucleoplasm were also altered in senescent cells, suggesting that NM1β could be a potential marker for senescence (Mehta et al.).

Targeting SASP with senomorphics could be a credible strategy to suppress the bystander effects of the senescent cell. Nonetheless, SASP is highly heterogenous and nonspecific, with its exact composition remains unknown since many different pathways are involved in SASP regulation. Kumari and Jat reviewed the different pathways in SASP regulation which converge to activate the transcription factors NF-κB and c/EBPβ in senescent cells. Identification of novel SASP mechanisms such as HMGA-NMPT-NAD^+, cGAS-STING and NOTCH1 pathways may present new druggable candidates for novel senomorphic development. Whether these pathways are interconnected in driving SASP and the inflammatory milieu in age-related pathologies requires further investigations (Kumari and Jat). It is also critical to identify SASP factors that are distinct from inflammatory mediators commonly associated to many diseases. Till then, the use of SASP as an unequivocal cellular senescence marker remains restricted and may limit the therapeutic potentials of senomorphics.

In the last decade, the complexity of the mechanisms of cellular senescence is beginning to unravel. Beyond the classical pathways, p53/p21WAF1/CIP1 and p16INK4A/pRB tumor suppressor in mediating cell cycle arrest, recent research shows the involvement of the dimerization partner, retinoblastoma (RB)-like, E2 factor (E2F) and multi-vulval class B (DREAM) complex working in concert with p53 in repressing genes to halt cell cycle progression. Updates on the role of DREAM complex in cellular senescence is summarized by Kumari and Jat. Cell cycle arrest can be triggered by ribosomal DNA (rDNA) repeats instability by activating DNA damage response (DDR). The association of rDNA stability with cellular senescence and ageing has been reported in human, as evidenced by rDNA copy loss in senescent cells and blood from aged individuals (Ren et al., 2017). Lee and Ong provided an update on rDNA instability caused by the accumulation of extrachromosomal rDNA circles (ERCs) in yeasts. ERCs are derived from rDNA through intra-molecular homologous recombination of the chromosome. In yeast, ELL-associated factor 3 (Eaf3) was reported to promote rDNA instability, whereby its absence resulted in less ERCs accumulation (Morlot et al., 2019). Its human homolog, MORF4-related gene on chromosome 15 (MRG15) has also been reported to be involved in DNA repair, cell cycle progression and cellular senescence through the association with nuclear protein complexes, including RB (Chen et al., 2010). The updates provided by the two reviews have exemplified the added layers of complexity in cellular senescence mechanisms and that there are considerably more unknown layers remain to be uncovered. FoxOs are key regulators of longevity downstream of insulin and insulin-like growth factor signaling (IIS). Of the four mammalian FoxO isoforms (FoxO1, 3, 4 and 6), only FoxO3 has been consistently correlated with longevity in population studies and preclinical models (Martin et al., 2015). FoxOs are involved in autophagy and the regulation of the ubiquitin–proteasome system, yet the exact mechanism involved is still unclear. Kapetanou et al. demonstrated the role of FoxO1 in proteostasis maintenance by binding onto the promoter region of β5 proteasome subunit to regulate its expression in mice. FoxO1 knockout but not FoxO3 severely impairs proteasome activity in mice tissues. Knockdown of insulin receptor substrate 1 (IRS1) is known to enhance FoxO1 activity and they showed that depletion of IRS1 also enhances both the transcriptional activity of FoxO1 and proteasome function (Kapetanou et al.). Findings from their study has provided new evidence of FoxO1's role in regulating proteosome function downstream of IIS.

Clearly from the above, there is still much more to learn about the mechanisms of cellular senescence and its pathophysiological implications. As research progresses, new modulators or

druggable targets or pathways may be unveiled to offer novel intervention approached to delay age-related pathologies. Complete understanding the intricacy of cellular senescence and innovative drug delivery methods are warranted to address current issues related to drug specificity and sensitivity for considerations of the design of future therapeutics targeting senescence. Overall, the field of senescence and ageing is tremendously exciting with the possibility of finding drugs that can delay ageing-related pathologies but warrants caution in data interpretations and clinical applications.

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P-FW drafted and finalised the final manuscript.

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REVIEW
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Senescence in Wound Repair: Emerging Strategies to Target Chronic Healing Wounds

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Cellular senescence is a fundamental stress response that restrains tumour formation. Yet, senescence cells are also present in non-cancerous states, accumulating exponentially with chronological age and contributing to age- and diabetes-related cellular dysfunction. The identification of hypersecretory and phagocytic behaviours in cells that were once believed to be non-functional has led to a recent explosion of senescence research. Here we discuss the profound, and often opposing, roles identified for short-lived vs. chronic tissue senescence. Transiently induced senescence is required for development, regeneration and acute wound repair, while chronic senescence is widely implicated in tissue pathology. We recently demonstrated that sustained senescence contributes to impaired diabetic healing via the CXCR2 receptor, which when blocked promotes repair. Further studies have highlighted the beneficial effects of targeting a range of senescence-linked processes to fight disease. Collectively, these findings hold promise for developing clinically viable strategies to tackle senescence in chronic wounds and other cutaneous pathologies.

Keywords: senescence, ageing, diabetes, wound healing, senolytics

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INTRODUCTION

Senescence, a seminal discovery of Hayflick and Moorhead (1961), is a defined process that globally regulates cell fate. Cellular senescence is traditionally described as a terminal stress response, whereby cells are triggered to undergo stable and essentially irreversible cell cycle arrest following initiation by a diverse range of stress-inducing stimuli (Hernandez-Segura et al., 2018). Indeed, this process acts as an autonomous anti-tumour mechanism, halting incipient neoplastic transformation (Faget et al., 2019). Yet, senescent cells can be found in non-cancerous tissues, accumulating exponentially with increasing chronological age (Hudgins et al., 2018; McHugh and Gil, 2018). These non-proliferative cells retain metabolic capabilities, exhibiting a hypersecretory phenotype (Coppé et al., 2010). It has recently been shown that some senescent cells may even engulf their neighbouring cells, for a survival advantage (Tonnessen-Murray et al., 2019). These profound functional behaviours, identified in cells long thought to be non-functional, pose new questions around their tissue roles and consequences. This review will explore emerging roles for cellular senescence in normal and pathological wound repair, highlighting areas of potential therapeutic opportunity.

SENESCENCE AS AN ANTI-PROLIFERATION MECHANISM

It was originally thought that only mitotic cells, which may be highly proliferative, or spend large periods of time in quiescence, undergo senescence (Campisi and di Fagagna, 2007). This view has since been challenged, as features of senescence are observed in some differentiated cells (Jurk et al., 2012; von Zglinicki et al., 2020). The major age- and stress-related processes that induce cellular senescence include replicative exhaustion (Hayflick and Moorhead, 1961), mitogenic signals (Tchkonia et al., 2013), oxidative stress (Passos et al., 2010), DNA breaks (Di Micco et al., 2006), and epigenomic damage (Pazolli et al., 2012). These stressors subsequently initiate anti-tumourigenic networks, controlled by transcriptional regulators such as p53 (Vousden and Prives, 2009). p53 directly transactivates the cyclin dependent kinase (CDK) inhibitor, p21, to inhibit CDK2, CDK4, and CDK6-mediated retinoblastoma protein (pRb) phosphorylation (He et al., 2007). p16 similarly prevents pRb inactivation, but in a p53-independent manner (Chen et al., 2006). pRb naturally binds E2F/DP transcription factor complexes to block transcription of E2F target genes, thus failure to phosphorylate pRb halts cell cycle progression from the G1 to S phase (Narita et al., 2003).

It is important to note that, while simplified here, the role for p53 in cell survival is complex and somewhat contradictory, as p53 activation can actually suppress senescence, instead causing cell quiescence (Demidenko et al., 2010) or apoptosis (reviewed in Salminen et al., 2011). In this regard, a cell's fate might be decided by the amount of damage sustained, and the expression of other senescence-linked factors. Molecular understanding of senescence is complicated further by the fact that the relative contribution of p21, p16, and other cell cycle regulators is thought to be context dependent (van Deursen, 2014).

SENESCENT CELL CHARACTERISTICS

Morphologically, senescent cells exhibit flattened, elongated features, and may have multiple nuclei and enlarged vacuoles (Rhinn et al., 2019). Senescence-associated beta galactosidase is often used as an archetypal senescence biomarker (Dimri et al., 1995; Debacq-Chainiaux et al., 2009), yet its specificity has come under criticism (Krishna et al., 1999; Lee et al., 2006). For that reason, it is most often used in conjunction with other key biomarkers, such as p16 and p21, to confirm senescence (Baker et al., 2016; Matjusaitis et al., 2016; Biran et al., 2017).

Senescent cells may also possess regions of highly condensed chromatin (senescence-associated heterochromatic foci; Zhang et al., 2007) and DNA damage-induced chromatin alterations, including γH2AX and H3K9Me3 (Rodier and Campisi, 2011). Loss of histones, centrosome aberrations and the breakdown of the nuclear envelope (e.g., degradation of lamin B1) similarly occur in many senescent states to enable rearrangement of heterochromatin (Tigges et al., 2014; Wang et al., 2017). These chromatin modifications sequester E2F target genes to potentiate senescence (Shah et al., 2013). Moreover, senescence is reinforced by microRNA-mediated silencing of E2F target genes (Benhamed et al., 2012).

Experimental manipulation of epigenetic marks has demonstrably shown their importance in controlling the molecular induction of cellular senescence. H3K27me3, for example, represses p16 and p14 expression by silencing the INK4a-ARF locus (Kotake et al., 2007). Removal of H3K27me3, by JMJD3-induced demethylation (Agger et al., 2009; Sui et al., 2019) or pharmacological inhibition of the histone lysine methyltransferase, EZH2 (Ito et al., 2018), promotes p16 expression and senescence. Inhibition of EZH2 also leads to SASP production via enrichment of H3K27ac, and loss of H3K27me3, at SASP-related loci (Ito et al., 2018). Overexpression of another histone demethylase, UTX, can also silence H3K27me3 to promote cellular senescence (Perrigue et al., 2020).

Stressed cells are repressed at the transcriptional level to prevent the expansion of potentially harmful mutations. It is therefore understandable that regulators, such as p53, are not only responsible for initiating senescence, but also decide whether cells should instead enter temporary quiescence or undergo apoptosis (Salminen et al., 2011). Intriguingly, senescent cells may actually retain heightened resistance to apoptosis, first demonstrated in fibroblasts (Wang, 1995), possibly due to altered p53 signalling (Childs et al., 2014) and upregulation of pro-survival pathways (e.g., BCL-2 and ephrins, Zhu et al., 2015). Indeed, senescent keratinocytes are resistant to ultraviolet radiation-induced apoptosis (Chaturvedi et al., 2004) and senescent fibroblasts to thapsigargin-induced apoptosis (Ryu et al., 2007). Senescent endothelial cells, on the other hand, are more likely to undergo apoptosis than their non-senescent counterparts (Hampel et al., 2004). Clearly, this senescence trait is situational, and not ubiquitous to all cell types.

The hypersecretory phenotype of senescent cells is most often referred to as the senescence-associated secretory phenotype (SASP), an attribute closely linked to the positive or negative outcomes of tissue senescence that appears to be cell-type and context-dependent. Even though studies have characterised the SASP in multiple cell types, its detailed composition remains elusive. Broadly, the SASP comprises a collection of pro-inflammatory cytokines and chemokines, growth factors, proteases, lipids and extracellular matrix components (Freund et al., 2010; Elzi et al., 2012; Acosta et al., 2013; Lopes-Paciencia et al., 2019). It is thought to mainly be a feature of senescent cells that have undergone a DNA damage response, as a SASP is not apparent in cells that naturally senesce due to overexpression of p16 and p21 (Coppé et al., 2011). However, a DNA damage-independent SASP can occur in fibroblasts via p38MAPK phosphorylation, challenging previous preconceptions (Freund et al., 2011). Collectively, the secretome is the characteristic of senescent cells that confers most of their biological effects, significantly contributing to age-related functional decline (Rodier et al., 2009) and chronic disease (Zhu et al., 2014) in autocrine and paracrine manners.

The SASP is dynamically regulated by a number of factors that mostly converge on the NF-κB complex (Sun et al., 2018). Inflammatory cytokines, such as IL-1α, can form positive feedback loops with NF-κB and partner cascades to reinforce

SASP release and senescence (Kuilman et al., 2008; Orjalo et al., 2009). In fact, multiple authors have demonstrated activation of NF-κB gene sets following senescence (Kuilman et al., 2010; Lujambio et al., 2013), while p53 and NF-κB are linked in coregulatory (in macrophages, Lowe et al., 2014) and antagonistic (HeLa cells, Huang et al., 2007) manners. The importance of NF-κB in senescence is highlighted by studies where NF-κB suppression allows oncogene-induced IMR-90 fibroblasts to bypass senescence (Chien et al., 2011) and reduces senescence in osteoarthritic cartilage (Wu et al., 2015).

Indeed, the SASP (e.g., TGFβ) can potentiate senescence in neighbouring cells (Acosta et al., 2013), but also promote senescent cell clearance by attracting immune cells (Kang et al., 2011; Tasdemir et al., 2016). The induction of senescence in, and clearance of, premalignant cells consequently reinforces tumour suppression. Paradoxically, the SASP can also drive pre-cancerous development in proximal tissues as many SASP proteins are potent mitogenic factors (e.g., VEGF, Coppé et al., 2006, 2010; Collado et al., 2007). The plasticity of the SASP across different microenvironments, cell types and stimuli (Campisi, 2013; Lupa et al., 2015; Maciel-Baron et al., 2016; Sun et al., 2018) further complicates our understanding of its role within tissues. However, it is clear that senescence, and the SASP, remain important regulators of normal physiology and pathology. Tissue consequences of senescent cells and their SASP are summarised in **Figure 1**.

ROLES FOR SENESCENCE DURING DEVELOPMENT AND REGENERATION

Many crucial biological processes require cells to undergo cell cycle arrest and differentiation to terminal states. For example, developmental lineage-specification requires cells to differentiate in a temporospatial manner in order to properly form tissues (Da Silva-Álvarez et al., 2019). In the skin, basal keratinocytes first proliferate, and then differentiate, transiting through the epidermis to replenish the non-viable stratum corneum (Fuchs and Byrne, 1994). In fact, to allow effective keratinocyte differentiation, p21 is activated initially (Missero et al., 1996), but then suppressed (Di Cunto et al., 1998). It is therefore unsurprising that tumour suppressor genes also aid development and regeneration through control of quiescence, terminal differentiation, apoptosis and senescence (e.g., Di Giovanni et al., 2006; Watkins et al., 2013).

Three main roles have been put forward for the presence of senescent cells during embryogenesis: (1) to promote the regression of transient structures; (2) to balance cell populations and/or; (3) to act as a signalling hub to regulate tissue morphogenesis (Da Silva-Álvarez et al., 2019). In embryonic development, temporal induction of senescence is required for tissue patterning in the developing limb bud. Here, p21 induction leads to SASP factor expression (e.g., FGF), stimulating cell proliferation and tissue formation. Resulting senescent (and apoptotic) cells are then effectively cleared by macrophages, prior to tissue remodelling. Indeed, genetic knockdown of p21 to attenuate senescence leads to mild patterning defects in murine limbs (Storer et al., 2013). In a corroborating study, p21 was shown to contribute to senescence-linked development in a p53-independent manner in human and murine embryos (Muñoz-Espín et al., 2013). In this case, however, loss of p21 was compensated for by increased apoptosis. Thus, p21-regulated senescence and apoptosis can perform synergistic roles during organismal development.

Akin to development, lower organisms and anamniotes are able to regenerate their tissues to full form and function, either as juveniles or throughout their lives (Brockes and Kumar, 2005). In fact, it has recently been shown that senescence may play an important role in these regenerative processes. In salamanders, senescence induction occurs at the intermediate stages of limb regeneration and then diminishes due to effective clearance by macrophages (Yun et al., 2015). Senescence is similarly invoked during pectoral fin regeneration in zebrafish, and impaired when senescence is blocked with the senolytic compound, ABT-263 (Da Silva-Álvarez et al., 2020). Given the importance of senescence in regulating tissue formation throughout development and regeneration, it is logical to ask whether senescence could play a role in the reparative responses of higher vertebrates.

SENESCENCE IN NORMAL TISSUE REPAIR

Tissue repair is necessary for all life. While it seldom leads to full regeneration, the process prevents exsanguination and infection, and aids structural and functional restoration required for survival. Tissue repair is rapid and highly dynamic, comprising multiple cell types and overlapping processes that broadly include haemastasis, inflammation, cell proliferation and dermal remodelling (Wilkinson and Hardman, 2017). During haemastasis, an insoluble blood clot is formed and endothelial cells from damaged vasculature enter the wound, depositing a temporary fibrin scaffold and releasing factors to attract both circulating immune cells and resident skin cells (Velnar et al., 2009). Inflammatory cells are rapidly recruited to the site of damage, first dominated by neutrophils and pro-inflammatory macrophages to remove bacteria and necrotic tissue (Young and McNaught, 2011). Later stage healing is characterised by a switch to anti-inflammatory macrophages, which phagocytose any remaining pro-inflammatory cells, supporting fibroplasia and wound resolution (Korns et al., 2011). To allow effective repair, keratinocytes undergo partial epithelial-to-mesenchymal transition and begin migrating to close the wound gap, a process known as re-epithelialisation (Shaw and Martin, 2016). Formation of new vasculature (angiogenesis) is essential to provide sustenance during the highly proliferative stage of healing (Baum and Arpey, 2005). Finally, the immature matrix laid down during early healing is replaced by stronger scaffold proteins, such as mature collagen fibres produced and remodelled by fibroblasts (Li et al., 2007). Each stage of wound repair involves extensive cellular communication, orchestrated by cytokines, chemokines, growth factors and components of the extracellular milieu. The plasticity of the response, and the cellular behaviours that occur, are homologous

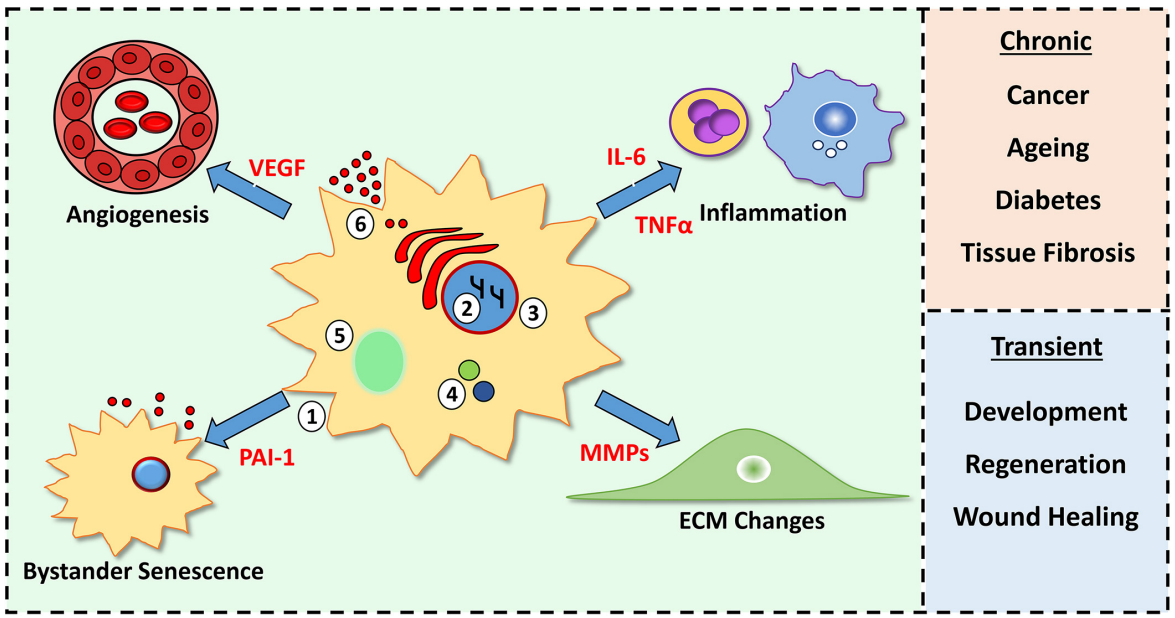

FIGURE 1 | Characteristics of senescent cells and their tissue consequences. Senescent cells feature morphological changes **(1)**, chromatin modifications **(2)**, loss of nuclear envelope lamin B1 **(3)**, increased p16 and p21 **(4)**, senescence-associated beta galactosidase activity **(5)** and production of a senescence-associated secretory phenotype (SASP, **6**). SASP components (red) alter cellular processes (e.g., inflammation and angiogenesis) within the microenvironment. A chronic SASP causes negative outcomes, while a short-lived, transient SASP is beneficial.

to those observed in cancer (e.g., immune cell infiltration, invasion and epithelial-to-mesenchymal transition, Schäfer and Werner, 2008). It is therefore not unreasonable to suggest that senescence, and associated mechanisms, could significantly contribute to wound healing.

Indeed, pertinent roles for senescence in tissue injury have been emerging, largely focusing on the beneficial, transient initiation of senescence during repair. Here, induction of senescence following liver damage (Krizhanovsky et al., 2008) and cutaneous injury (Jun and Lau, 2010) was shown to prevent excessive fibrosis that would otherwise cause tissue dysfunction. Krizhanovsky et al. (2008) confirmed that reduced fibrosis was the result of senescence-linked fibrolytic enzyme production, and immune-regulated clearance of injury-expanded cell populations that would otherwise contribute to excessive matrix deposition. Likewise, senescence decreased fibrosis in a model of cardiac injury, where genetic ablation of p53 and p16 accelerated fibrosis (Meyer et al., 2016). Ectopic expression of Ccn1, which increased cardiac senescence, also limited fibrosis in this model. Interestingly, Jun and Lau (2010), the first authors to observe transient senescence during skin repair, revealed that Ccn1 causes fibroblast senescence via an oxidative-stress dependent mechanism. Upregulation of Ccn1 was vitally important to prevent excessive fibrosis. More recently, the same authors demonstrated that topical application of another Ccn family member, Ccn2, similarly actuates senescence and reduces fibrosis in cutaneous murine wounds (Jun and Lau, 2017).

By contrast, when Demaria et al. (2014) ablated p16- and p21-expressing cells in mice they observed impaired extracellular matrix deposition and a decreased rate of wound closure. Intriguingly, by day 15 post-injury, these senescent-deficient wounds were excessively fibrotic. Similar to previous research (Jun and Lau, 2010), transient senescence appeared limited to fibroblast-like cells, which produced a PDGFA-enriched SASP to stimulate appropriate skin repair (Demaria et al., 2014). Studies continue to explore the importance of transient senescence during acute wound healing, with Hiebert et al. (2018) recently reporting that overexpression of nrf2 promotes fibroblast senescence, which is accompanied by accelerated wound re-epithelialisation and extracellular matrix deposition. Although at present limited to murine models, these key investigations provide clear evidence that temporal induction of senescence is necessary for effective skin repair. Yet, many questions remain unanswered. For instance, does transient wound-induced senescence arise through intrinsic cell factors or environmental influences? And how are these senescent cells so effectively cleared once they are no longer required?

SENESCENCE IN AGED AND DIABETIC WOUND HEALING

The above studies provide substantial insight into the importance of senescence for the healing of experimental wounds. What they do not address is the potential differential influences of acute vs. chronic senescence to tissue repair, nor how senescence could be involved in pathological healing. These are important considerations for the clinical setting, where effective healing can mean the difference between life or death (Han and Ceilley, 2017). Chronic, non-healing wounds are a huge socioeconomic

burden, reducing quality of life and costing billions each year to treat (Guest et al., 2015). Considered a "silent epidemic" (Lindholm and Searle, 2016), chronic wounds display diverse aetiology, with incomplete molecular and cellular understanding (Frykberg and Banks, 2015). Inadequate current treatments mean it is fundamentally important to further understand why chronic wounds fail to heal, and ultimately develop more effective therapies.

It has long been appreciated that chronic wound pathology is almost entirely restricted to those who are elderly and/or diabetic. This is fascinating, as the biological processes of ageing and diabetes are themselves notably linked to senescence (Wilkinson and Hardman, 2020). Senescence is both a characteristic feature of Baker et al. (2013), Xu et al. (2015), and Helman et al. (2016) and contributor to Baker et al. (2008, 2011) widespread tissue ageing. Epigenetic modifications are one feature of ageing that is linked to senescence. Genomic instability and DNA methylation changes correlate with chronological ageing in mice (Stubbs et al., 2017) and humans (Horvath, 2013). Interestingly, the repressive mark, H3K27me3, showed altered DNA coverage on aged vs. young stem cells (Liu et al., 2013; Sun et al., 2014), which may contribute to their reduced renewal capacity.

Another attribute of normal metabolic ageing that is experimentally linked to senescence is cumulative oxidative damage. For example, human diploid fibroblasts (Duan et al., 2005) and endothelial cells (Ruan et al., 2014) undergo senescence in the presence of heightened reactive oxygen species (ROS), while replicative lifespan can be extended in cell culture by lowering oxygen tension (Parrinello et al., 2003). More notably, exposure to ultraviolet radiation simulates photoageing by increasing ROS production in skin (Herrling et al., 2006), while ROS upregulates p16 in skin cells (Jenkins et al., 2011). Skin ageing is also characterised by cell accumulation of p16 (Waaijer et al., 2012) and senescence-associated beta galactosidase (Dimri et al., 1995; Ressler et al., 2006). This association is causally corroborated by Xu et al. (2018), who demonstrated that transplantation of senescent cells to young mice accelerated ageing, while Baker et al. (2011) revealed that eradication of p16-positive cells alleviated features of premature ageing in a murine progeroid model.

The link between diabetes and senescence is less well-established, but is an area of intense current research. As previously mentioned, senescent cells cause widespread disruption to normal tissue architecture by virtue of their SASP (Coppé et al., 2010). Major SASP constituents influence senescence by targeting immunological pathways, such as NF-κB (Salminen et al., 2011). This leads to matrix proteolysis and increased inflammation, primary features of aged and diabetic wounds (Makrantonaki et al., 2017; Wilkinson et al., 2019c). Indeed, growing evidence suggests that a heightened intrinsic immune response, or "sterile" inflammation, contributes to age- and diabetes-related pathology (reviewed in Prattichizzo et al., 2016). Characteristic features of diabetes that drive immune cell accumulation, and therefore potentiate senescence, include obesity and hyperglycaemia (Yokoi et al., 2006; Minamino et al., 2009; Maeda et al., 2015; Schafer et al., 2016). These processes most likely promote senescence via increasing advanced glycation end-products and causing widespread oxidative damage (Coughlan et al., 2011; Fang et al., 2016).

Turning specifically to the skin, it is clear that in diabetic and aged tissue, accumulation of senescent cells extends to both uninjured skin and wounds (Ressler et al., 2006; Waaijer et al., 2012; Wilkinson et al., 2019a). Previous authors have demonstrated that chronic venous leg ulcers harbour senescent fibroblasts (Mendez et al., 1998; Vande Berg et al., 1998; Agren et al., 1999; Wall et al., 2008). The presence of senescent fibroblasts in chronic wounds may even exacerbate pathology, where it was shown that ulcers containing over 15% senescent cells were hard to heal (Stanley and Osler, 2001). We recently reported a novel mechanistic link between senescence and healing in diabetic wounds (Wilkinson et al., 2019a). Here, intrinsically senescent macrophages were observed to promote impaired wound healing in a non-aged, murine model of diabetic pathological repair.

Indeed, many SASP factors attract monocytes and macrophages (e.g., MCP-1; Kamei et al., 2006; Coppé et al., 2008; Prattichizzo et al., 2018), often with a pro-inflammatory phenotype (Mosser and Edwards, 2008; Lujambio et al., 2013). Excessive immune cell recruitment and inappropriate retention is a hallmark of chronic wound pathology. This may be even be exacerbated by other local factors, such as iron, which induces a pro-inflammatory phenotype in macrophages and leads to fibroblast senescence in chronic venous leg ulcers (Sindrilaru et al., 2011). Thus, macrophages are likely a nexus for uncontrolled local inflammation in both diabetic pathogenesis and senescence, ultimately delivering poor wound healing. Moreover, the impaired function of macrophages (and other immune cell types) in aged (Swift et al., 2001) and diabetic (Wilkinson et al., 2019b) wounds likely contributes to prolonged, rather than transient, senescence due to ineffective clearance mechanisms.

Senescence in the wound environment is probably not limited to fibroblasts and macrophages, as other wound cells, including keratinocytes (Smirnov et al., 2016) and endothelial cells (Ruan et al., 2014), are capable of undergoing senescence in response to environment cues. Senescent keratinocytes are certainly observed in aged skin (Velarde et al., 2012) and are suggested to influence the reduced regenerative capacity of aged epidermis (Zouboulis et al., 2008). Chronic wounds also harbour pathogenic microorganisms (Kalan et al., 2019) that may contribute to senescence by stimulating ROS production in keratinocytes and exacerbating inflammation (Grange et al., 2009). Indeed, this may occur via specific bacterial virulence factors, as pyocyanin from *Pseudomonas aeruginosa* can induce senescence in fibroblasts (Muller et al., 2009).

It is clear that the chronic ulcer milieu, which is rich in pro-inflammatory factors, indirectly causes senescence via exacerbating inflammation. However, as wound fluid from venous leg ulcers directly induces senescence in neonatal fibroblasts (Mendez et al., 1999), it is likely that the local microenvironment also stimulates cellular senescence. Intrinsically senescent wounds cells, such as fibroblasts, are similarly capable of potentiating senescence across neighbouring cell types in a paracrine manner, via their SASP

(Acosta et al., 2013; Wilkinson et al., 2019a). Moreover, in local environments where the SASP is insufficient to directly induce cellular senescence, it may still promote pathological cellular phenotypes, such as epidermal hyperproliferation (Albanesi et al., 2018) and excessive dermal proteolysis (via MMPs; Caley et al., 2015).

To add a further level of complexity, evidence for the disparities between transient and chronic senescence is beginning to emerge, with clear implications for wound healing. For instance, stemness and reprogramming in keratinocytes is promoted by a transient SASP, yet inhibited when the SASP becomes chronic (Ritschka et al., 2017). Transient senescence also encourages matrix deposition following tissue injury (Demaria et al., 2014), but prevents excessive fibrosis (Jun and Lau, 2010), while chronic senescence is linked fibrotic disease (Yanai et al., 2015). Taken together, published and emerging studies are certainly challenging the dogma that senescence is primarily limited to age-related dysfunction and cancer. Indeed, evolving understanding of the concept of transient vs. chronic senescence is likely to deliver important new insight into the processes that occur during acute and pathological repair. Current understanding of senescence contribution to normal and pathological wound healing is summarised in **Figure 2**.

CELLULAR SENESCENCE AS A THERAPEUTIC TARGET IN PATHOLOGICAL WOUNDS

The widespread causative biological effects of cellular senescence in tissue ageing pathology make the therapeutic modulation of senescence an attractive target for a plethora of age-related diseases. Genetic studies positively support this idea, with inducible knockdown of p16 alleviating hallmark features of ageing in progeroid murine models (Baker et al., 2011; Sato et al., 2015). In fact, the well-documented effects of caloric restriction, which both extends mammalian lifespan (Sohal and Weindruch, 1996) and delays the onset of age-related disease (Weindruch and Walford, 1982; Colman et al., 2014), may be a physical manifestation of tissue senescence modulation. Caloric restriction has been shown to reduce cardiac senescence (Shinmura et al., 2011), and senescence in hepatocytes and intestinal crypt cells *in vivo* (Wang et al., 2010). At the epigenetic level, caloric restriction protects against age-related changes in DNA methylation (Hahn et al., 2017). Caloric restriction also decreases senescence partly by upregulating the epigenetically linked sirtuin pathway, promoting anti-apoptosis and anti-inflammatory mechanisms (Bonda et al., 2011). Subsequent effects include slowing metabolic processes that contribute to cellular ageing (e.g., oxidative stress, Yang et al., 2016), increasing antioxidant production (Meydani et al., 2011), and increasing autophagy to remove damaged and unimportant intracellular components (reviewed in Cuervo, 2008). Moreover, sirtuins may play important roles in preventing age-related decline in skin repair, as SIRT1 deficiency exacerbates healing pathology in diabetic wounds (Thandavarayan et al., 2015).

Although caloric restriction (without malnutrition) provides a multitude of health benefits, it retains poor feasibility as a clinical intervention, requiring high compliance and patient discipline. Many lifestyle choices, such as obesity, are actually strongly associated with social status (Drewnowski and Specter, 2004). Similarly, those suffering from uncontrolled type II diabetes and severe chronic wounds are often from socially deprived backgrounds (Anderson et al., 2018), a difficult population in which to manage compliance. For all of these reasons, a considerably more attractive proposition is the use of senescence-targeted drugs, otherwise known as senolytics. These drugs affect unique features of senescent cells, such as resistance to apoptosis (Salminen et al., 2011). Senescent cells upregulate prosurvival pathways, particularly BCL-2 (Ovadya and Krizhanovsky, 2018). This opens up drug repurposing opportunities around the numerous BCL-2 inhibitors that were developed for the treatment of cancer (Roberts et al., 2016; Montero and Letai, 2018). Results have been promising. Targeting BCL-2 *in vivo* induces apoptosis and thus eliminates senescent cells in the lung following irradiation (Yosef et al., 2016) and throughout the body following irradiation or natural ageing (Chang et al., 2016). Chang et al. (2016) further established that senescent human and murine fibroblasts, and human renal epithelial cells, are more susceptible to BCL-2 inhibitor (ABT-263) than non-senescent cells, proposing potent and specific effects. Unfortunately, traditional BCL-2 inhibitors possess activity against other BCL class proteins, such as BCL-XL, raising questions around off-target effects in the clinic, including thrombocytopenia and neutropenia. As a result, more specific BCL-2 inhibitors with lower toxicity are being tested (King et al., 2017). It has even been suggested that low-dose, combinatorial use of senolytics may be an effective and less harmful alternative (Ovadya and Krizhanovsky, 2018).

Other senolytics that have demonstrated experimental efficacy include the tyrosine kinase inhibitor, Dasatinib, used to treat leukaemia (Keskin et al., 2016), and the flavonoid p53 activator, Quercetin (Khan et al., 2016). Combinatorial treatment with Dasatinib and Quercetin extends lifespan, alleviates frailty (Xu et al., 2018), and improves vasomotor function (Roos et al., 2016) in aged mice. Dasatinib and Quercetin have also shown promise in a phase I trial in diabetic kidney disease patients, where reduced senescent cells and circulating SASP factors were observed following administration (Hickson et al., 2019). Alternative flavonoids are now being tested for their potential senolytic effects, such as Fisetin, which is able to eliminate senescent cells and, crucially, restore tissue function in aged mice (Yousefzadeh et al., 2018).

The importance of transient senescence for effective healing should not be underestimated. As noted previously, temporary induction of senescence aids rapid tissue reformation (Demaria et al., 2014; Hiebert et al., 2018). During a normal damage response, these senescent cells are effectively cleared by natural killer cells (Krizhanovsky et al., 2008) and macrophages (Yun et al., 2015). Nevertheless, in chronic situations, senescent cells persist, likely due to elevated immunosenescence and resulting impaired immunological functions (Hall et al., 2016). It follows that treatments to boost immune system function, for instance

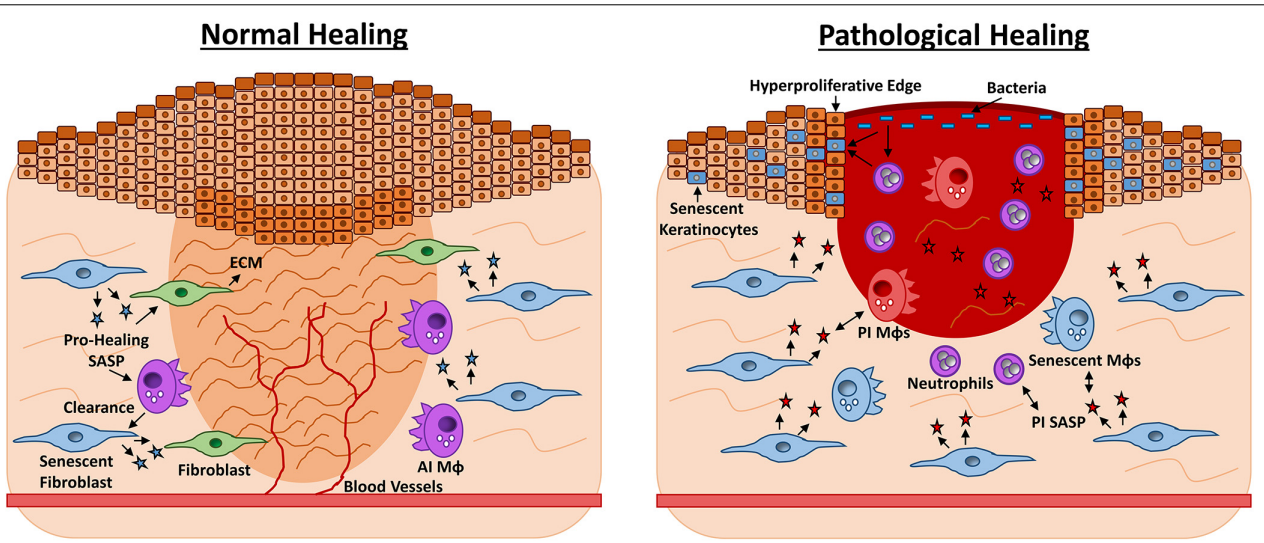

FIGURE 2 | Roles for senescence in acute vs. chronic wound repair. Late-stage wound healing is characterised by extracellular matrix (ECM) deposition, full wound closure and blood vessel perfusion **(left)**. Senescent fibroblasts appear during late-stage healing and contribute to ECM deposition by producing a pro-healing senescence-associated secretory phenotype (SASP, blue stars) containing PDGFA. Senescent fibroblasts prevent excessive fibrosis via CCN1 and CCN2. The senescent cells are then effectively cleared by anti-inflammatory macrophages (AI Mϕs) and the skin is restored. Chronic healing wounds feature wound edge hyperproliferation and excessive inflammation **(right)**. Here senescent fibroblasts and Mϕs (blue) exacerbate inflammation via a pro-inflammatory (PI) SASP of cytokines and proteases. The SASP may also exert a paracrine effect, causing senescence in other wound cell types. High bacterial load stimulates further inflammation and oxidative stress, which can cause hyperproliferation or senescence in keratinocytes. Senescent cells are not effectively cleared by the dysfunctional chronic wound immune cells, thus tissue damage persists and the wound fails to heal.

by aiding senescent cell recognition, could be beneficial in the context of transient senescence and tissue repair. Generally, senescent cells express stimulatory ligands that bind to NK2GD receptors on natural killer cells, thus initiating a killing response (Sagiv et al., 2016). However, senescent fibroblasts in aged skin have recently been shown to express HLA-E, which bypasses recognition and clearance by natural killer and T cells (Pereira et al., 2019). Here, approaches developed in the cancer field may also be useful, for example engineering T cells to express receptors that target specific cellular (tumour) proteins (reviewed in June et al., 2018). Studies to identify and validate new senescent cell receptors will be essential to the development and clinical application of such immune-regulated approaches.

Indeed, the emergence of global profiling methodologies, such as single-cell RNA sequencing, could provide the basis to understanding senescence-linked changes in ageing and pathology by identifying unique cell-based transcriptomic signatures within tissues. Kimmel et al. (2019) used this approach to compare cell frequency, heterogeneity and age-related transcriptomic changes between aged and young murine tissues. Similarly, Angelidis et al. (2019) combined transcriptomics and proteomics to not only identify the epigenetic and transcriptional consequences of ageing in the lung, but also determine their functional implications. Future harnessing of these technologies could therefore facilitate the identification and targeting of key senescence-linked receptors and biomarkers in a tissue and pathology-specific manner.

Alternative strategies to diminish or limit senescence and alleviate pathology instead target the SASP or specific senescence-linked receptors directly (summarised in **Figure 3**). Certainly, the SASP is transcriptionally regulated by NF-κB and others (Salminen et al., 2011), and contributes heavily to tissue deterioration, both driving widespread destruction and reinforcing senescence (Rodier et al., 2009; Acosta et al., 2013). SASP inhibitors affect key transcriptional mediators, blocking signalling and preventing SASP production (Moiseeva et al., 2013). Interestingly, Metformin, a widely used anti-diabetic drug, is an effective SASP inhibitor (reviewed in Rena et al., 2017) able to directly accelerate healing in diabetic mice (Han et al., 2017). Rapamycin, another SASP inhibitor, was the first drug revealed to extend lifespan in mice (Harrison et al., 2009), and enhance the replicative lifespan of human keratinocytes (Horvath et al., 2019) and skin fibroblasts *in vitro* (Sodagam et al., 2017). Although these studies suggest potential skin-related benefits of SASP inhibitors, removal of the SASP could be deleterious, impairing the healing response and preventing senescent cell clearance (von Kobbe, 2019). Consequently, it may be more advantageous to target particular SASP components known to impact tissue function, either with antibodies (e.g., IL-1α, Orjalo et al., 2009), or specific inhibitors (e.g., against CXCR2, Wilkinson et al., 2019a).

We remain a long way from implementing senescence-targeted treatments for pathological wound healing, yet it is reassuring to see that current senolytic drugs display efficacy across a wide range of tissues and pathologies. In a number of studies, systemic senolytic treatments have been shown to have clear effects in peripheral target tissues across a range of treatment regimens. For example, a single dose of BCL inhibitor

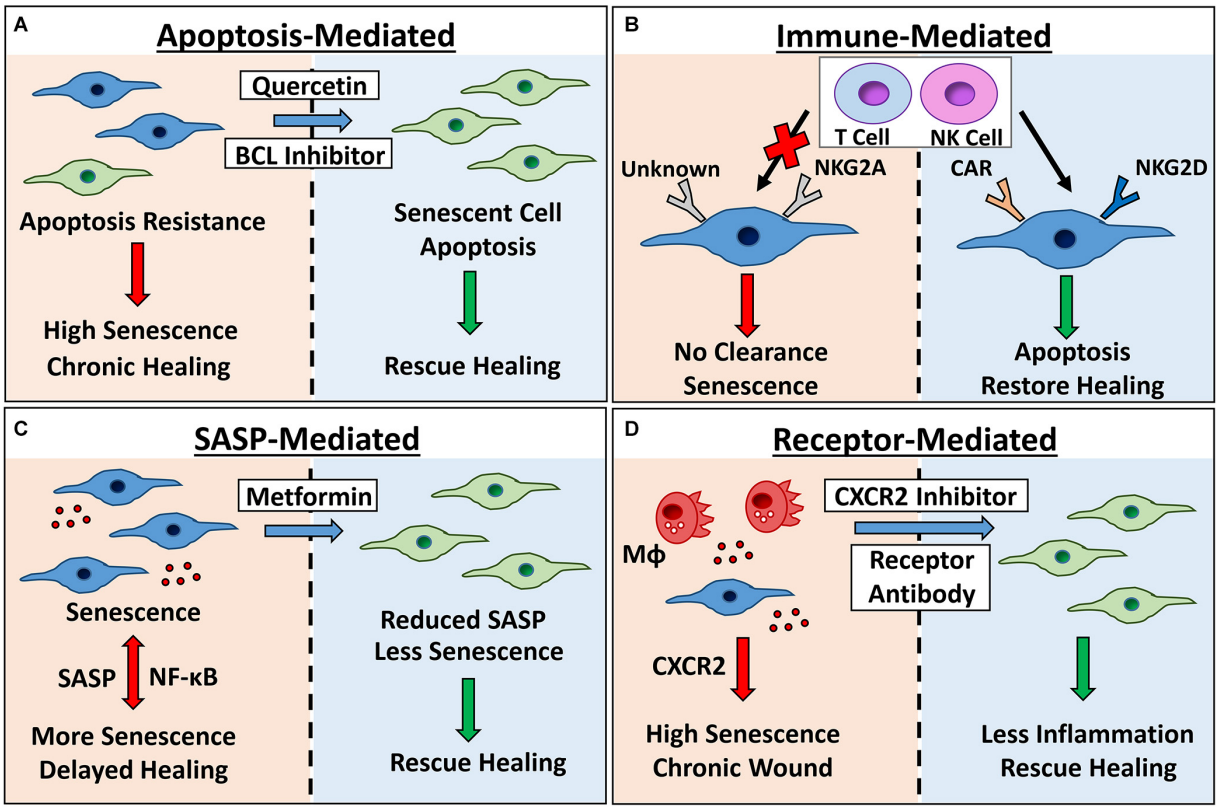

FIGURE 3 | Therapeutic targeting of senescence for chronic healing wounds. Senescent cells accumulate in chronic healing wounds, contributing to inflammation and poor healing. Senescence can be targeted by: **(A)** inhibiting pro-survival pathways with BCL inhibitors and broad spectrum drugs (e.g., quercetin) to cause apoptosis; **(B)** engineering chimeric antigen receptor (CAR) T cells to target senescent cell receptors, or modulating expression of natural killer (NK) cell receptors NKG2A and NKG2D to increase clearance; **(C)** using Metformin or other SASP inhibitors to reduce NF-κB-mediated inflammation and bystander senescence and; **(D)** inhibiting receptors known to potentiate wound senescence (e.g., CXCR2). Red arrows/left panels = negative outcomes. Green arrows/right panels = positive outcomes. Mϕ = macrophage. Senescent cells = blue.

(Yosef et al., 2016), and dosing over consecutive days (Chang et al., 2016), was able to reverse irradiation-induced senescence in different tissues. In the work by Xu et al. (2018), aged mice showed improved physical performance following biweekly oral treatments of Dasatinib and Quercetin for 4 months, yet reduced SASP was observed in human *ex vivo* cultured adipose tissue within 48 h of treatment. Moreover, a single 3 day oral treatment of Dasatinib and Quercetin was able to reduce senescence in the adipose tissue of diabetic patients in a phase I trial (Hickson et al., 2019). These studies therefore suggest that senolytic treatments not only have rapid effects in target peripheral tissues, but can overcome established tissue senescence.

Experimental studies do show beneficial effects of modulating senescence in the skin. For example, elimination of senescent cells from the epidermis restored proliferative capacity in hair follicle stem cells (Yosef et al., 2016), known to participate in wound healing (Joost et al., 2018). Further, blockade of the potential senescence receptor, CXCR2 (Acosta et al., 2008), directly accelerated healing in human *ex vivo* skin wounds and diabetic murine wounds *in vivo* (Wilkinson et al., 2019a). Here, a CXCR2 antagonist was administered to wounds topically (*ex vivo*) and subcutaneously (*in vivo*), suggesting direct delivery to the wound site as a viable administration route. Indeed, elevated CXCR2 has previously been observed in diabetic wounds (Wetzler et al., 2000), and more recently in T cells from human diabetic patients (Lau et al., 2019). We note with interest that pharmacological inhibition of CXCR1/2 additionally prevents inflammation-mediated damage to pancreatic islets, thus prohibiting streptozocin-induced diabetes in mice (Citro et al., 2015). Therefore, CXCR2 appears a common factor in both the ontology and local pathology of diabetes. Senolytics should certainly be considered for the treatment of human chronic wounds characterised by high levels of senescence (Stanley and Osler, 2001). However, given that knockdown of CXCR2 (Devalaraja et al., 2000) and ablation of senescent cells (Demaria et al., 2014) actually delays acute wound healing, future senescence-targeted therapies should be reserved for the treatment of chronic conditions.

CONCLUSION

Despite seemingly contradictory roles in many cancers, the detrimental contribution of cumulative senescence to ageing

and age-related disease is now well-established. By contrast, the short-lived, transient senescence observed to benefit tissue development, regeneration and repair, remains significantly less well-characterised. In wound repair, a paradigm is emerging where local transient senescence predominately constrains fibrosis, while chronic senescence drives diabetic wound pathology. Indeed, experimentally blocking the senescence-linked receptor, CXCR2, *in vivo* reverses pathology and accelerates diabetic healing. These observations now pave the way to explore the beneficial effects of senescence-targeted therapies for the treatment of chronic wounds.

AUTHOR CONTRIBUTIONS

HW wrote the manuscript and prepared the figures. MH provided critical appraisal. Both authors contributed to the article and approved the submitted version.

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REVIEW
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Senescence and the Aging Immune System as Major Drivers of Chronic Kidney Disease

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Chronic kidney disease (CKD) presents an ever-growing disease burden for the world's aging population. It is characterized by numerous changes to the kidney, including a decrease in renal mass, renal fibrosis, and a diminished glomerular filtration rate. The premature aging phenotype observed in CKD is associated with cellular senescence, particularly of renal tubular epithelial cells (TECs), which contributes to chronic inflammation through the production of a proinflammatory senescence associated secretory phenotype (SASP). When coupled with changes in immune system composition and progressive immune dysfunction, the accumulation of senescent kidney cells acts as a driver for the progression of CKD. The targeting of senescent cells may well present an attractive therapeutic avenue for the treatment of CKD. We propose that the targeting of senescent cells either by direct inhibition of pro-survival pathways (senolytics) or through the inhibition of their proinflammatory secretory profile (senomorphics) together with immunomodulation to enhance immune system surveillance of senescent cells could be of benefit to patients with CKD.

Keywords: aging, kidney, T cell, immune system, senescence

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INTRODUCTION

Age-related pathologies are a major global disease burden, with potentially half of all morbidities being attributable to aging (Chang et al., 2019). Inflammation (or "inflammaging") is one of the main causative factors contributing to disease progression, and has been described in various age-related pathologies, including type 2 diabetes (T2D) and cardiovascular disease (Franceschi et al., 2018). While being beneficial in the acute stages of an insult, inflammation increasingly fails to resolve with age, leading to changes in both cellular phenotypes and immune system composition. Senescence pathways are induced by, as well as potentiate, chronic inflammation, with increased cellular senescence being observed in various age-related diseases. Cellular senescence is characterized by a stable growth arrest and a proinflammatory secretome, which potentiates low grade chronic inflammation, thereby building a positive feedback loop, gradually exacerbating its effects on the body. With a prevalence of approximately 44% in the elderly population (>65 years), chronic kidney disease (CKD) presents a major disease burden in an aging population (Stevens et al., 2010). Therapies for late stage CKD including dialysis and renal transplantation carry a significant burden for patients, and the outcome is often poor (Roberti et al., 2018); therefore, there is a significant need for early diagnosis and novel therapies targeting mechanisms driving the disease. CKD is associated with chronic inflammation, elevated levels of cellular senescence, as well

as immune system dysfunction. Their characterization as phenotypes or primary drivers of disease progression is crucial for the development of novel CKD therapies. The role of inflammation, senescence, and the immune system, with their potential modulation through therapeutics are discussed below.

CHRONIC KIDNEY DISEASE—INCIDENCE AND RISK FACTORS

The human kidney undergoes various structural and functional changes with age. These include a decrease in renal mass, patterns of microstructural sclerosis (renal fibrosis), as well as changes in nephron number and a diminished glomerular filtration rate (Hommos et al., 2017). The global prevalence of CKD (at all stages) was estimated to be 9.1% in 2017 (Bikbov et al., 2020). Various risk factors, both inherited and environmental, have been identified to contribute to the development of CKD. Genetic risk factors include gene variants affecting creatine clearance (Bachmann et al., 2005; Köttgen et al., 2009), and glomerular filtration rates (Xu et al., 2018). Non-genetic risk factors such as smoking and nephrotoxins, as well as gender, ethnicity, and socioeconomic status increase the lifetime risk of developing CKD. Excessive comorbidity driven inflammation, for example, through type 2 diabetes (T2D) or obesity, is known to aggravate these processes, accelerating kidney dysfunction, leading to CKD and ultimately end stage renal disease (ESRD) (Liu et al., 2017). Diabetic kidney disease (DKD) is a major cause of ESRD and accounted for almost a third of disease adjusted life years of CKD in 2017 (Bikbov et al., 2020). Initiated by metabolic dysregulation, key contributors to DKD progression include the loss of tubular epithelial cells (TECs) and podocytes due to metabolic injury and ROS induced apoptosis (Susztak et al., 2006; Verzola et al., 2008). Frequent episodes of acute kidney injury (AKI) are also associated with progression to stage 5 CKD (ESRD) (Coca et al., 2012). AKI is defined by a sudden decrease in kidney function, often due to reduced renal blood flow and pre-existing health conditions, and is an independent risk factor of CKD incidence and progression (Coca et al., 2012). The mechanisms underlying the progression from AKI to CKD are not fully understood; however, production of fibrotic extracellular proteins mediated by TGF-β signaling in renal cells (Böttinger and Bitzer, 2002), as well as the p53 cell death signaling pathway has been described in tubular cells (Jiang et al., 2004). Crucially, proinflammatory/senescence and pro-apoptotic pathways have also been implicated in the development of CKD.

SENESCENCE AND INFLAMMATION IN PREMATURE AGING

Premature aging is a phenotype observed in many age-related pathologies such as T2D, rheumatoid arthritis, as well as in CKD (Franceschi and Campisi, 2014; Franceschi et al., 2018). Notably, individuals affected by these age-related diseases display similar phenotypes, including muscle wasting, vascular disease, osteoporosis, frailty, and immune dysfunction (Weiskopf et al., 2009; Crowson et al., 2010; Kooman et al., 2013; Schram et al., 2014). Given the similarity of these phenotypes, many of the underlying mechanisms are shared between them, in particular, genomic instability, changes in epigenetic modification, metabolic dysregulation, and cellular senescence (Kubben and Misteli, 2017). These changes are also observed in CKD, with patients exhibiting cellular alterations characteristic of increased inflammation and senescence. Additional causes of the premature aging observed in CKD include dialysis, interstitial sodium accumulation, uremia, as well as increases in angiotensin II and phosphate pools, which are expertly reviewed in Kooman et al. (2014, 2017).

Cellular Senescence

Cellular senescence is a conserved mechanism by which cells exit the cell cycle in response to both intrinsic and extrinsic stresses. Two forms of senescence exist, replicative senescence and stress-induced premature senescence, discussed further below. Activation of senescence causes alterations in cell morphology, secretory phenotype, cell metabolism, and composition (Van Deursen, 2014). Senescent cells can be characterized by several defining markers, which have also been observed in CKD patients. Among these, the aging murine and human kidney express increased levels of senescence-associated-beta-galactosidase (SA-β-Gal), p16, and Ki-67 (Melk et al., 2005; Clements et al., 2013). Senescent cells also secrete a proinflammatory milieu termed the senescence associated secretory phenotype (SASP), implicating them in the progression of kidney dysfunction toward senescence. This has been shown in Wistar rats, where CKD progression induced multi-organ genomic damage and an increased expression of inflammatory markers (IL-1, IL-6, and TNFα) (Hirotsu et al., 2011). Senescence is further exacerbated by a reduction in anti-aging renoprotective factors and processes, including Klotho, mitophagy, vitamin D, and bone morphogenetic protein (Koh et al., 2001; Gould et al., 2002; Levin et al., 2007; Zhan et al., 2015). Klotho contributes to a cells ability to resist oxidative stress and reduces senescence when overexpressed in mice (Haruna et al., 2007). CKD patients produce significantly less Klotho (Koh et al., 2001), thereby accelerating the process of renal senescence. In addition, mitophagy is downregulated, particularly in renal tubular cells of DKD patients, resulting in dysfunctional mitochondria secondary to significant increase in mitochondrial ROS, which, in turn, drive stress-induced premature senescence (Zhan et al., 2015). Overexpression of the mitophagy inducing protein optineurin reduces cellular senescence in high glucose stimulated renal TECs (Chen et al., 2018). Accumulating senescent cells are normally cleared by the immune system. However, due to the age-associated decline in immune function, senescent cells remain in local tissues and contribute to tissue dysfunction and chronic inflammation. The underlying cellular mechanisms which lead to senescence in CKD are described further below.

Sources of Senescence in CKD

Replicative senescence is caused by the progressive shortening of telomeres, which form the non-protein coding ends of

human chromosomes. Telomeres shorten with every cell division, protecting protein-coding DNA from shortening, and can be elongated by the telomerase enzyme. At critically short lengths, telomeres reach their "Hayflick limit" (Hayflick and Moorhead, 1961), and recruit DNA damage repair machinery, which activates the p21 cyclin-dependent kinase inhibitor and causes cell cycle exit and senescence. In CKD, senescence is observed in a variety of cells, including TECs, podocytes, interstitial cells, and mesangial cells. The senescent cell type may vary between different types of CKDs. As such, the development of diabetic nephropathy is associated with the acceleration of replicative senescence in TECs, as well as an increased expression of p16 in podocytes and mesangial cells (Verzola et al., 2008).The involvement of the p21 pathway in CKD has been illustrated in a telomerase-deficient mouse model, which had a lower 30-day recovery rate after ischemia reperfusion injury (IRI) than controls (Westhoff et al., 2010). IRI, tissue damage caused by the reoxygenation of a tissue, is one of the most common causes of AKI and its frequency as well as duration determines the progression from AKI to CKD. Patients suffering from CKD routinely undergo hemodialysis treatment to replace renal function (renal replacement therapy). In mononuclear cells of hemodialysis patients, telomeres are shorter than in those from age-matched controls (Ramírez et al., 2005). These findings were replicated in renal transplant patients, showing that renal transplantation leads to greater telomere attrition than that observed in CKD patients undergoing dialysis (Luttropp et al., 2016). These findings show that the treatment of CKD with either hemodialysis or renal transplantation further contributes to peripheral cell senescence and, possibly to the dysfunction of the immune system. Alternatively, senescence can be induced in a premature manner, due to both intrinsic and extrinsic stressors (**Table 1**), including increased genomic damage, mitochondrial dysfunction, and oxidative stress, all of which are observed during CKD (Corredor et al., 2010; Gamboa et al., 2016).

Senescence in the context of kidney function, however, is not all detrimental. Distinctions have to be made between acute and chronic senescence, as acute senescence has been observed to have beneficial effects. Anti-fibrotic mechanisms have been observed in murine models subjected to unilateral ureteral obstruction (UUO), which causes renal fibrosis due to tubular injury. INK4 knockout mice subjected to UUO reveal that $p16^{INK4A}$ plays a pivotal role in limiting both inflammation and cell proliferation (Wolstein et al., 2010). Additionally, patients suffering from polycystic kidney disease have reduced expression of the senescence marker p21. In contrast, restoration of p21 expression in mice via cyclin-dependent kinase inhibition reduces disease progression (Bukanov et al., 2006). This beneficial effect of pro-senescence pathways has been recapitulated in p21 knockout mice subjected to renal IRI, exhibiting greater impairment in renal function as well as an increase in mortality when compared to their wild-type litter mates (Megyesi et al., 2001). These senescence pathways crucially halt the cell cycle and prevent the replication of damaged DNA. The sustained presence of senescent cells due to a failure to clear the senescent cell burden in the kidney leads to the continuous expression of profibrotic factors and a gradual deterioration of renal function. However, senescent cells may also accumulate owing to increased evasion strategies, which are discussed in subsequent sections.

Inflammaging and the Senescence Associated Secretory Phenotype

The homeostatic regulation of inflammatory responses becomes aberrant in old age, favoring a chronic pro-inflammatory environment (Franceschi et al., 2006). The age-associated increase of a sterile, low-grade chronic inflammation is termed "inflammaging" and has been implicated in several age-related pathologies. Although normal aging is associated with an increase in chronic inflammation, various genetic and environmental factors may exacerbate inflammation and could explain the disparity between chronological and biological age in CKD patients (Kooman et al., 2014). Additionally, oxidative stress, acidosis, chronic infections, altered metabolism, and microbiome dysbiosis may also contribute to the inflammatory insult in patients with CKD. Together, these sources of inflammation compose the network theory of aging; where one component may lead to the exacerbation of another and thereby accelerates the overall aging process of the organism. Senescent cells secrete pro-inflammatory cytokines, the SASP, which is temporally dynamic and heterogeneous among cell types (Hernandez-Segura et al., 2017). Although the SASP can be beneficial by facilitating the recruitment of immune cells and promoting tissue damage repair, its inflammatory mediators also contribute to chronic inflammation, angiogenesis, and induce senescence of adjacent cells in a paracrine manner.

Chronic inflammation has long been implicated in CKD. Raj et al. analyzed plasma levels of pro-inflammatory mediators

TABLE 1 | Inducers of cellular senescence.

Inducer	Mechanism	References
Telomere attrition	Critically short telomeres induce a DNA damage response which leads to the activation of senescence pathways	Hastings et al., 2004
Genotoxic agents/ Irradiation	Irreparable DNA damage in response to genotoxic agents or irradiation	Yentrapalli et al., 2013
Oncogenes and tumor suppressor genes	Oncogene activation as well as suppression of tumor suppressor genes	Di Micco et al., 2006
Oxidative stress	Metabolic derived oxidative products induce DNA damage	Ziegler et al., 2015
Mitochondrial dysfunction	Decreased NAD+/NADH ratios drive senescence in an AMPK-dependant manner	Wiley et al., 2016
Epigenetic perturbations	Inhibition of DNA methylases and histone deacetylases induce senescence	Petrova et al., 2016
Paracrine mediators	SASP, particularly IL-1, induced senescence	Acosta et al., 2013

in 899 participants, finding that elevated levels of fibrinogen, TNFα, and IL-6 were associated with a more rapid progression of CKD (Amdur et al., 2016). Other studies have identified further inflammatory cytokines such as vascular endothelial growth factor (VEGF) and IL-10, as well as reporting a decreased renal clearance of these cytokines, likely due to uremia-induced lymphocyte dysfunction. Urea also induces endothelial progenitor cell senescence, contributing to the SASP in a ROS-dependent manner (D'Apolito et al., 2017). Upon kidney injury, factors such as VEGF and fibroblast growth factor-2 (FGF2) facilitate tissue remodeling, while pro-inflammatory cytokines facilitate the recruitment of immune cells. When the immune cell clearance of apoptotic or damaged cells fails, these cytokines are expressed chronically and contribute to the involvement of the SASP in the age-related pathological damage observed in CKD patients (Wang et al., 2017). Together, these findings describe the multifactorial nature of inflammation in CKD. Dysregulation of signaling-, metabolic-, and inflammatory-pathways, as well as decreased proliferation are features of senescent cells in CKD.

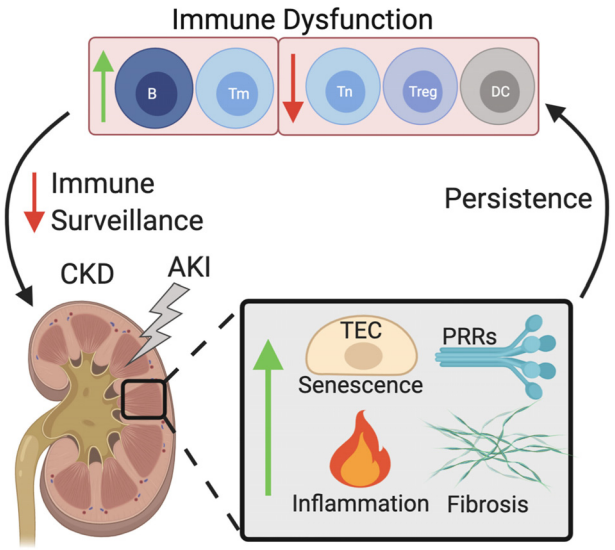

FIGURE 1 | Dysfunctional immune senescence caused by persistent senescence, inflammation, and fibrosis of the kidney. AKI increases senescence of TECs, kidney fibrosis, inflammation, as well as increased PRR expression. Persistence of these effects leads to immune dysfunction which in turn decreases immune surveillance of the kidneys, exacerbating the acute effects of AKI which then manifest as CKD. Abbreviations: AKI, acute kidney injury; TEC, tubular epithelial cell; PRRs, pattern recognition receptors; DC, dendritic cell; B, B cell; Treg, regulatory T cell; Tm, memory T cell; Tn, Naïve T cell; CKD, chronic kidney disease.

IMMUNE SYSTEM ALTERATIONS

Both the innate and adaptive immune systems are implicated in CKD progression (**Figure 1**). Senescence of tubular cells is particularly driven by the innate immune system, where AKI causes the infiltration of innate immune cells via Toll-like receptors (TLRs), and IL-1R signaling promotes senescence, mainly of TECs (Jin et al., 2019). In patients suffering from chronic renal failure, sera levels of pattern recognition receptors are also dysregulated, with increased mannose binding lectin levels (Atsushi et al., 2002), and increases in the macrophage scavenger receptors (Ando et al., 1996; Chmielewski et al., 2005). Monocytes and monocyte-derived dendritic cells (moDC) cultured in high concentrations of urea show decreased levels of endocytosis and poor maturation (Lim et al., 2007) and the terminal differentiation of moDCs is impaired in CKD patients, resulting in impaired antigen presentation and a decreased production of antigen-specific T cells (Verkade et al., 2007). FGF is elevated in CKD and contributes to impaired leukocyte recruitment to inflamed tissues via interference of leukocyte-integrin activation (Rossaint et al., 2016). These signaling pathways also induce a local SASP and are thereby implicated in aiding the progression of AKI toward CKD. As such, the senescence driven by CKD is mainly affected by local tissue changes, while senescent cell effector molecules (the SASP) contribute to both local and systemic changes in immune dysfunction. Additionally, CKD associated conditions such as uremia further potentiate this response by dysregulating immune cell function.

The adaptive immune response is also affected in CKD, with an increased presence of CD4+CD28− highly differentiated T cells, a reduced number of Tregs, and an overall decrease in proliferation rates all being observed (Lisowska et al., 2012). Patients in ESRD also have a reduced CD4:CD8 T cell ratio, suggestive of poor outcomes, as well as a selective depletion of naïve and central memory T cells (Yoon et al., 2006). A progressive decrease in renal function is associated with a selective loss of naïve and memory CD4 T cells, as well as an increase in CD8 memory T cells, which lack CD45RO and CCR7. A shift toward the pro-inflammatory Th1 differentiation is also observed (Litjens et al., 2006). In addition to the cellular immune response, humoral immunity is also impaired in CKD patients. Hemodialysis patients exhibit peripheral B cell lymphopenia (Fernández-Fresnedo et al., 2000), while a decrease in immature B cells has been reported in pre-dialysis ESRD patients (Kim et al., 2012). Notably, this immature B cell population inhibits the differentiation of proinflammatory CD4 T cells (Blair et al., 2010), illustrating the interlinked nature of cellular and humoral immune response dysregulation in CKD patients. Thereby the defective immune system leads to the accumulation of senescent cells in inflamed tissues that cannot efficiently be cleared.

The accumulation of senescent cells with age is not only due to reduced immune surveillance but also immune evasion. Perforin knockout mice which exhibit an impairment in immune cytotoxicity accumulate larger amounts of senescent cells (in all tissues) than their wild-type litter mates, which is accompanied by chronic inflammation and tissue fibrosis (Ovadya et al., 2018). Senescent cells, much like cancer cells, express receptors which activate inhibitory immune cell receptors, thereby evading immune cell clearance. Senescent fibroblasts accumulate the non-classical MHC receptor HLA-E in response to SASP signaling, which interacts with the inhibitory NK receptor, NKG2A expressed by NK cells, and highly differentiated CD8

T cells (Pereira et al., 2019). Both immune dysfunction and immune evasion contribute to the accumulation of senescent cells; however, discerning between the driver and product of CKD is crucial for identifying therapies.

THE CHICKEN OR THE EGG

Senescent cells may both be a phenotype of age-related inflammatory disease, as well as the cause for disease progression. Thereby two models of disease progression exist: One in which senescent cells arise from local tissue injury, promoting senescence in neighboring cells in a paracrine manner. Alternatively, immune clearance may be impaired, thereby allowing the accumulation of senescent cells. Distinguishing between these two models becomes pivotal when exploring potential new treatments of CKD.

Various renal diseases including AKI, diabetic nephropathy, and glomerulonephritis may lead to CKD. AKI is the main driver of CKD, where a rapid deterioration in kidney function often results in incomplete tissue repair. As described above, senescence is protective in response to AKI, with its SASP facilitating immune cell clearance and tissue repair. However, failure to clear these cells may lead to chronic SASP signaling and CKD. The importance and often bifunctional role of the immune system in AKI to CKD progression have been reported extensively. This includes proinflammatory infiltrating T and B cells which in response to tissue injury, contribute to a pro-fibrotic milieu, activating pericytes and inducing renal fibrosis and thereby CKD (Lee et al., 2017). Alternatively, CD4 and CD8 T cell depletion studies have illustrated their reno-protective effects in an acute aristolochic acid nephropathy model (Baudoux et al., 2018). Anti-inflammatory immune cells generally prevent the progression to CKD. M2 macrophages promote epithelial tissue repair process following IRI (Lee et al., 2011), as well as regulatory T cells promoting kidney recovery following AKI (Kinsey et al., 2013). The transcription of proinflammatory cytokines is largely regulated by the NF-kB transcription factor. Johnson et al. (2017) have reported that inhibition of the activation of NF-kB reduces renal fibrosis after AKI.

Much of the immune dysregulation is governed by the presence of proinflammatory cytokines, and uremia, or by pre-existing comorbidities such as high blood pressure or diabetes. In addition to the levels of inflammatory markers, an elevated white blood cell count is predictive of CKD development (Shankar et al., 2011). This suggests various modes of pathogenesis, with the immune system, senescence, and inflammation at its core. Cellular senescence and immunosurveillance occur in conjunction, increased cellular senescence due to chronic inflammation increases the demand for immune clearance; however, as described above, uremia and inflammation lead to dysfunction of the immune system, thereby establishing a positive feedback loop in which more senescent cells drive inflammation and immune dysregulation which, in turn, cause more senescence.

THERAPEUTIC TARGETING OF SENESCENT CELLS IN CKD

There has been a growing appreciation of the therapeutic potential of eliminating senescent cells to treat or prevent the onset of age-related disease. The first proof of this concept came with the engineering of a transgenic mouse with a drug inducible transgene of $p16^{INK4a}$, showing that elimination of $p16^{INK4a}$ positive senescent cells delayed the onset of age-related pathologies in tissues particularly prone to senescence (Baker et al., 2011). Inhibition of the SASP may also have reno-protective effects, dampening local tissue inflammation and preventing the paracrine signaling mediated induction of senescence in neighboring cells. And finally, the treatment of age-related immune dysfunction may be the most effective strategy, as it would activate the body's natural targeting of senescent cells, promoting tissue repair and dampening inflammation (**Figure 2**).

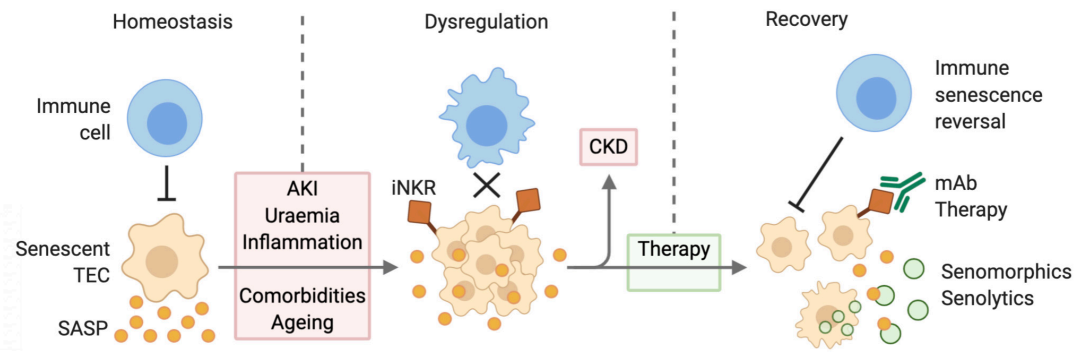

FIGURE 2 | Senescent cell accumulation due to defective immune surveillance leading to CKD. During homeostatic conditions, immune cells target senescent TECs and are thereby able to mitigate SASP expression. AKI, uremia, inflammation, comorbidities, and aging lead to the dysregulation of this mechanism causing senescent cell accumulation and increased SASP expression. Senescent TEC expression of inhibitory receptors further decreases their clearance. Over time this may progress to CKD. Therapy via senolytic and senomorphic therapies, as well as immunomodulation improve senescent cell clearance and thereby contribute to reduced inflammation and recovery. Abbreviations: TEC, tubular epithelial cell; SASP, senescence associated secretory phenotype; AKI, acute kidney injury; iNKR, inhibitory natural killer cell receptor; CKD, chronic kidney disease; mAb, monoclonal antibody.

Senomorphics

Drugs which inhibit SASP inducing pathways (senomorphics) alleviate the aging phenotype of various age-related diseases (**Table 2**). Clinical trials have begun to show efficacy of such interventions in CKD. Among these, sodium-glucose cotransporter 2 inhibitors affect renal hemodynamics and have reno-protective effects (Yaribeygi et al., 2018). In addition, p38 MAPK inhibitors reduce SASP cytokines in a variety of cell lines (Alimbetov et al., 2016) including kidney glomerular and mesangial cells (Stambe et al., 2003; Wang et al., 2013), while oral administration of the p38 MAPK inhibitor SB203580 improves renal function and decreases proteinuria in a systemic lupus erythematosus (MRL/lpr) mouse model (Jin et al., 2011). Resveratrol, an anti-inflammatory and antioxidant polyphenol, inhibits renal fibrosis via the activation of *SIRT1* and deacetylation of *SMAD3* in UUO mice (Li et al., 2010), and decreases the renal fibrosis caused by diabetic hyperglycemia activated renal fibroblasts via the inhibition of AMPK/NOX4/ROS signaling (He et al., 2016). However, clinical trials in CKD patients have thus far failed to replicate these beneficial preclinical effects (Saldanha et al., 2016). The SASP activating transcription factor NFκB is a primary target for inhibition in CKD. In a rat model receiving adenine overload, administration of the IκB kinase inhibiting compound pyrrolidine dithiocarbonate markedly reduces macrophage infiltration and attenuates renal interstitial fibrosis (Okabe et al., 2013). The naturally occurring NFκB inhibitor parthenolide ameliorates renal injury and inflammation in cisplatin-induced renal damage models (Francescato et al., 2007), as well as reducing renal monocyte and macrophage infiltration in UUO models (Esteban et al., 2004). Its antifibrotic properties have shown efficacy in patients suffering from diabetic nephropathy and glomerulosclerosis (Cho et al., 2007; Sharma et al., 2011). In addition, inhibition of the activation of NF-kB with an inhibitor of IKK after AKI (peak in creatinine) prevents the subsequent development of renal fibrosis, a key driver of the development of CKD (Johnson et al., 2017).

While senomorphics have shown to be potent inhibitors of the SASP, several challenges remain. Primarily, the clearance of SASP inhibited senescent cells proves difficult, as immune cells may fail to recognize these for clearance. *In vitro* senomorphic treatment of senescent fibroblasts has resulted in the downregulation of SASP mediators involved in immune cell recruitment, such as CXCL1 and GM-CSF (Lim et al., 2015). Additionally, treatment would require chronic administration of senomorphic agents, which would likely result in undesirable side effects due to their non-specific targeting of senescent cells. A brief and efficacious treatment course would thereby have to be achieved.

TABLE 3 | Senolytics.

Inhibitor(s)	Function	References
Dasatinib and quercetin	Bcl-2, PI3K, TK inhibitors	Zhu et al., 2015; Hickson et al., 2019
Fisetin	Bcl-2 family inhibitor	Zhu et al., 2017
Navitoclax (ABT-263)	Bcl-2 family inhibitor	Chang et al., 2016
ABT-737	Bcl-2 family inhibitor	Yosef et al., 2016
FOXO4-DRI	FOXO4-p53 interaction inhibitor	Baar et al., 2017

Senolytics

Pharmacological agents targeting characteristic cellular mechanisms and molecular features of senescent cells have been termed "senolytics." Identification of senescent-cell anti-apoptotic pathways (SCAPs) has allowed the development of specifically targeted senolytics for each of these. Additionally, senescent cells are highly metabolically active, and metabolically constrained, thereby providing further targeting phenotypes (Zhu et al., 2015). Several murine studies have shown that the clearance of senescent glial cells prevents cognitive decline (Bussian et al., 2018), while the elimination of $p19^{ARF}$ expressing cells enhances pulmonary lung function in 12-month-old mice (Hashimoto et al., 2016). Pre-clinical studies have identified a variety of SCAP interfering molecules, such as various BCL family inhibitors, ABT-263 (navitoclax) (Chang et al., 2016), ABT-737 (Yosef et al., 2016), and fisetin (Pal et al., 2013; Zhu et al., 2017), as well as dasatinib and quercetin which have a wide variety of pro-apoptotic and anti-survival effects (Zhu et al., 2015) (**Table 3**). In patients suffering from DKD, coadministration of dasatinib and quercetin reduces senescent cell burden in adipose and skin epidermal tissues, as well as circulating SASP factors (Hickson et al., 2019). Additionally, inhibition of the FOXO4-p53 interaction, which mediates senescent cell specific p53 nuclear exclusion and hence, apoptosis, restores fitness and renal function in naturally aged mice (Baar et al., 2017).

TABLE 2 | Senomorphics.

Inhibitor(s)	Function	References
SGLT2 inhibitors: Empagliflozin Dapagliflozin Canagliflozin	Affect renal hemodynamics and inhibition of proinflammatory cytokine production	Yaribeygi et al., 2018
BIRB796 UR-13756 SB203580	p38 MAPK inhibition	Alimbetov et al., 2016
Resveratrol	Pleiotropic effects, including *SIRT1/SMAD3* inhibition	Li et al., 2010
Pyrrolidine dithiocarbonate Parthenolide	NFκB inhibitors	Francescato et al., 2007; Okabe et al., 2013

Similar to senomorphic treatments, the senolytic targeting of senescent cells has resulted in undesirable off target effects. As shown in mouse models, this may be overcome by using nanocapsules, which target senescence specific proteins, thereby only releasing the senolytic upon senescent cell contact (Muñoz-Espín et al., 2018). Additionally, it is unclear whether apoptotic senescent cells can be efficiently cleared by a dysfunctional immune system. While senolytics may improve immune function by targeting senescent immune cells, immune mediated CKD may be exacerbated further via increased immune activation and inflammation. Thereby it is likely that senolytic treatment needs to be occur in conjunction with immunomodulatory treatments to achieve the specific targeting and clearance of senescent cells in the kidney.

Immunomodulation

Given the assumption that defective immune mediated senescent cell clearance is at the root of senescent cell accumulation and, thereby, its proinflammatory environment, modulation of the immune system would be the most effective treatment in age-related diseases. This modulation may come in various categories, including reversal of the proinflammatory senescent state of immune cells, induction of tolerance in response to acute injury, as well as improving senescent cell clearance by modulation of homing and targeting capabilities. Inhibition of p38 MAPK in senescent CD8 T cells, for example, increases their proliferation, telomerase activity, and mitochondrial biogenesis (Henson et al., 2014). Peripheral tolerance is largely controlled by DCs via the induction of Treg cells as well as T cell anergy. Induction of tolerogenic DC populations, via *ex vivo* adenosine 2A receptor agonist treatment, has reno-protective effects in experimental IRI by suppressing NKT cell activation (Li et al., 2012). Various immunotherapies may be applicable in the clearance of senescent cells, such as vaccines, reinfusion of *ex vivo* derived DCs, and chimeric antigen receptor (CAR) T cells. In a proof of concept study, Truong et al. engineered a chimeric IL-6 receptor which under IL-6 stimulation generated a Ca^{2+} signal. This was co-expressed with a Ca^{2+} activated RhoA, enabling migration of cells toward IL-6 and subsequent fusion of cells, leading to targeted cell death (Qudrat et al., 2017). Additionally, improvement of immune clearance of senescent cells can be achieved by blocking the inhibitory HLA-E:NKG2A signaling axis between senescent fibroblasts and NK cells and late differentiated CD8 T cells (Pereira et al., 2019).

Immunomodulation is likely a key therapeutic method to target senescent cells in CKD. Mitigation of age-related immune dysfunction in conjunction with improved targeting of senescent cells ensures a non-exaggerated immune response as well as the elimination of senescent cells. However, this strategy relies on the presence of conserved senescence specific markers. Tissue specific evaluation of senescent cell markers is therefore required.

CONCLUSION

Chronic kidney disease presents an ever-increasing global health burden. Its association with age and chronic inflammation identifies senescent cells as the main culprit of the deterioration of kidney structure and function. Both changes in the immune system as well as the accumulation of senescent cells within the kidney are associated with the progression of kidney disease. Targeting of both aspects will be necessary to preserve long-term beneficial effects of therapy. However, with advancing insights come major challenges, which will need to be tackled before these therapies will show efficacy in patients. The pleiotropic effects of senomorphics will need to be studied extensively to avoid unwanted effects. Optimization of dosing and the limitation of adverse effects still present significant challenges. Finally, the involvement of the immune system in the progression of CKD due to immune-senescence and immune-evasion need to be studied extensively to achieve the ultimate trifecta of therapy, by eliminating senescent cells, blocking their SASP signal, and promoting immune cell targeting of senescent cells.

AUTHOR CONTRIBUTIONS

JS wrote the manuscript. CT and SH proofed and advised on the content. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Genomic Instability and Cellular Senescence: Lessons From the Budding Yeast

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Aging is a complex biological process that occurs in all living organisms. Aging is initiated by the gradual accumulation of biomolecular damage in cells leading to the loss of cellular function and ultimately death. Cellular senescence is one such pathway that leads to aging. The accumulation of nucleic acid damage and genetic alterations that activate permanent cell-cycle arrest triggers the process of senescence. Cellular senescence can result from telomere erosion and ribosomal DNA instability. In this review, we summarize the molecular mechanisms of telomere length homeostasis and ribosomal DNA stability, and describe how these mechanisms are linked to cellular senescence and longevity through lessons learned from budding yeast.

Keywords: aging, longevity, rDNA stability, *Saccharomyces cerevisiae*, senescence, telomere length homeostasis

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INTRODUCTION

Mammalian cells possess a finite replicative capacity known as "Hayflick limit" (Hayflick and Moorhead, 1961) which when reached initiates replicative senescence upon irreversible cell cycle arrest (Demidenko and Blagosklonny, 2008). Replicative senescence is a natural process that occurs due to telomere shortening with every cell division (Stewart et al., 2003). However, premature senescence can be induced by cellular exposure to stresses such as oxidative stress or DNA damage (Debacq-Chainiaux et al., 2016). Cellular senescence is thought to be a beneficial protective system against cancer because it limits the proliferation of damaged cells and progression of malignant cells (Muñoz-Espín and Serrano, 2014). Senescent cells remain metabolically active and are viable for a long period of time (Blagosklonny, 2003), and exhibit phenotypes such as enlarged intracellular organelles and increased cell size (Cristofalo et al., 2004; Matsui and Matsuura, 2010)

While aging is caused by structural deteriorations at the organismal level, at the cellular level aging is caused by replicative or premature senescence *via* genomic instability amongst other factors (Lidzbarsky et al., 2018; Lagunas-Rangel and Bermúdez-Cruz, 2019). In this mini review, we summarize our current understanding of telomere length homeostasis and maintenance of ribosomal DNA (rDNA) stability, the two major contributors to genomic instability.

MAINTENANCE OF TELOMRE LENGTH HOMEOSTASIS PREVENTS CELLULAR SENESCENCE AND AGING

Telomeres at eukaryotic chromosome ends protect the chromosome ends from end-to-end fusion, degradation, and prevent misrecognition of the ends as double-stranded DNA breaks (DSBs)

(Tham and Zakian, 2002; Dieckmann et al., 2016). Telomeres have terminal single-stranded (ss) DNA overhangs with 3′ repetitive guanine-rich sequences (termed G tail or G-overhang) (Giraud-Panis et al., 2010; Eugène et al., 2017). Telomeres are marked by tandem repeats such as G_3T_2A in vertebrates and TG_{1-3} in budding yeast *Saccharomyces cerevisiae* (Tran et al., 2011; Wellinger and Zakian, 2012). In budding yeast, telomeres consist of subtelomeric repeats known as X′ element found in all telomeres and Y′ element found in two-thirds of telomeres (Louis and Haber, 1992; Teng and Zakian, 1999; **Figure 1A**).

Telomere Shortening

Telomere shortening (or erosion) causes genomic instability through the breakage-fusion-bridge cycle. The progressive loss of telomere end after cell division (Soudet et al., 2014) initiates DNA break repair (DBR) machinery that repairs shortened telomeres through DNA replication creating a fusion of two sister chromatids. During cell division, the segregation of fused chromosomes will cause a random break, leading to inheritance of deleted or amplified chromosomes by daughter cells. The continuous breakage-fusion-bridge cycle with every cell division leads to genomic instability (McClintock, 1938; Tanaka and Yao, 2009).

Telomere Length Homeostasis

Lagging and leading telomeres are synthesized in the progression of replication fork during telomere replication. Due to the end-replication problem, telomeres are replicated incompletely by DNA polymerases and end with a 3′ overhang. Excessive critically short telomeres will elicit a DNA damage signal causing permanent cell cycle arrest, subsequently cellular senescence and death (Shay and Wright, 2005; Aubert and Lansdorp, 2008).

Additionally, dysfunctional telomeres due to telomere uncapping can cause cellular senescence in an indirect manner. The unprotected telomeres undergo degradation (Vodenicharov and Wellinger, 2006; Ghadaouia et al., 2018) and induce a weak DNA damage response (DDR). Unprotected telomeres are prone to chromosomal end fusion resulting in secondary DNA breaks and genomic instability, eliciting a strong DDR. Consequently, the additional DNA damage causes permanent growth arrest and cellular senescence (Ghadaouia et al., 2018). Therefore, telomere length homeostasis must be maintained by telomere elongation to compensate for the end-replication problem and protect telomere ends. The two pathways involved in telomere elongation are telomerase-dependent pathway and homologous recombination (HR) pathway.

Telomerase-Dependent Pathway

Telomerase is a reverse transcriptase that depends on its internal RNA subunit Tlc1 (Gilson and Géli, 2007) as a template to extend telomeric repeats. Telomerase preferentially extends short telomeres in the late S phase (Wellinger and Zakian, 2012) by applying dNTP synthesized by ribonucleotide reductase (RNR) to add nucleotides at the telomeric 3′ overhang (Maicher et al., 2017) while the complementary strand is synthesized by DNA polymerases (Hug and Lingner, 2006; **Figure 1B**). A telomerase consists of four subunits Est1, Est2, Est3, and Tlc1 which positively regulate telomerase activity for telomere extension (Lendvay et al., 1996). Est1 enables the access of telomerase to telomere by interacting with Tlc1 and telomeric ssDNA-binding protein Cdc13 (Virta-Pearlman et al., 1996; Evans and Lundblad, 1999; Zhou et al., 2000; Li et al., 2013). Additionally, Est1 stimulates the generation of G-quadruplex at telomeric overhang for telomere extension and protection (Zhang et al., 2010; Tong et al., 2011; Li et al., 2013). Est2 (Counter et al., 1997) and Tlc1 (Singer and Gottschling, 1994; Cohn and Blackburn, 1995) catalyze telomere extension. Est3 which associates with Est1 and Est2, induces Est2's catalytic activity for telomere extension (Zhang et al., 2010; Mariasina et al., 2018).

Telomerase-Independent Homologous Recombination Pathway

Telomerase deficiency leads to progressive telomere shortening, and consequently cell death (Le et al., 1999). Nevertheless, a subset of cells with telomerase deficiency can still survive and have extended telomeres. These survivors are classified as type I or type II; with their telomeres extended *via* Rad51-dependent or Rad51-independent homologous recombination (HR) pathways respectively. Both recombination pathways involve Rad52, Exo1, and Pol32 which is a non-essential subunit of DNA polymerase δ (Chen et al., 2001; Maringele and Lydall, 2004; Lydeard et al., 2007; **Figure 1C**).

The telomeres of type I survivors contain tandemly amplified Y′ elements and short telomeric repeats TG_{1-3} at their ends while telomeres of type II survivor have amplified telomeric repeats TG_{1-3} at their ends with heterogenous length (**Figure 1C**). Although the survivors can depend on HR pathway to maintain telomere length, they possess shorter replicative life spans (RLS). The reactivation of telomerase activity can restore the reduced RLS, revealing the role of telomerase in sustaining cellular RLS possibly by suppressing telomere recombination and maintaining telomere length (Chen et al., 2009). Additionally, other proteins involved in the regulation of telomere length homeostasis in *S. cerevisiae* are summarized in **Table 1**.

Telomere Shortening and Its Effects on Aging

Telomere shortening decreases life span in mice and humans (Muñoz-Lorente et al., 2019; Whittemore et al., 2019). In mice, reactivation of telomerase activity can rescue premature aging phenotype through restoration of short telomere length and its function (Samper et al., 2001). Telomerase overexpression which promotes telomere extension can prolong life span in mice (Bernardes de Jesus et al., 2012). Furthermore, mice with overlengthened telomeres exhibited less DNA damage, less metabolic aging and an increased life span (Muñoz-Lorente et al., 2019). These findings show that the telomerase-mediated telomere extension can promote life span extension in animal models. Interestingly, telomerase overexpression in mice also reduced cancer incidences (Bernardes de Jesus et al., 2012; Muñoz-Lorente et al., 2019). This highlights the complex link between senescence and cancer suppression because senescence is thought to be a safeguard against cancer.

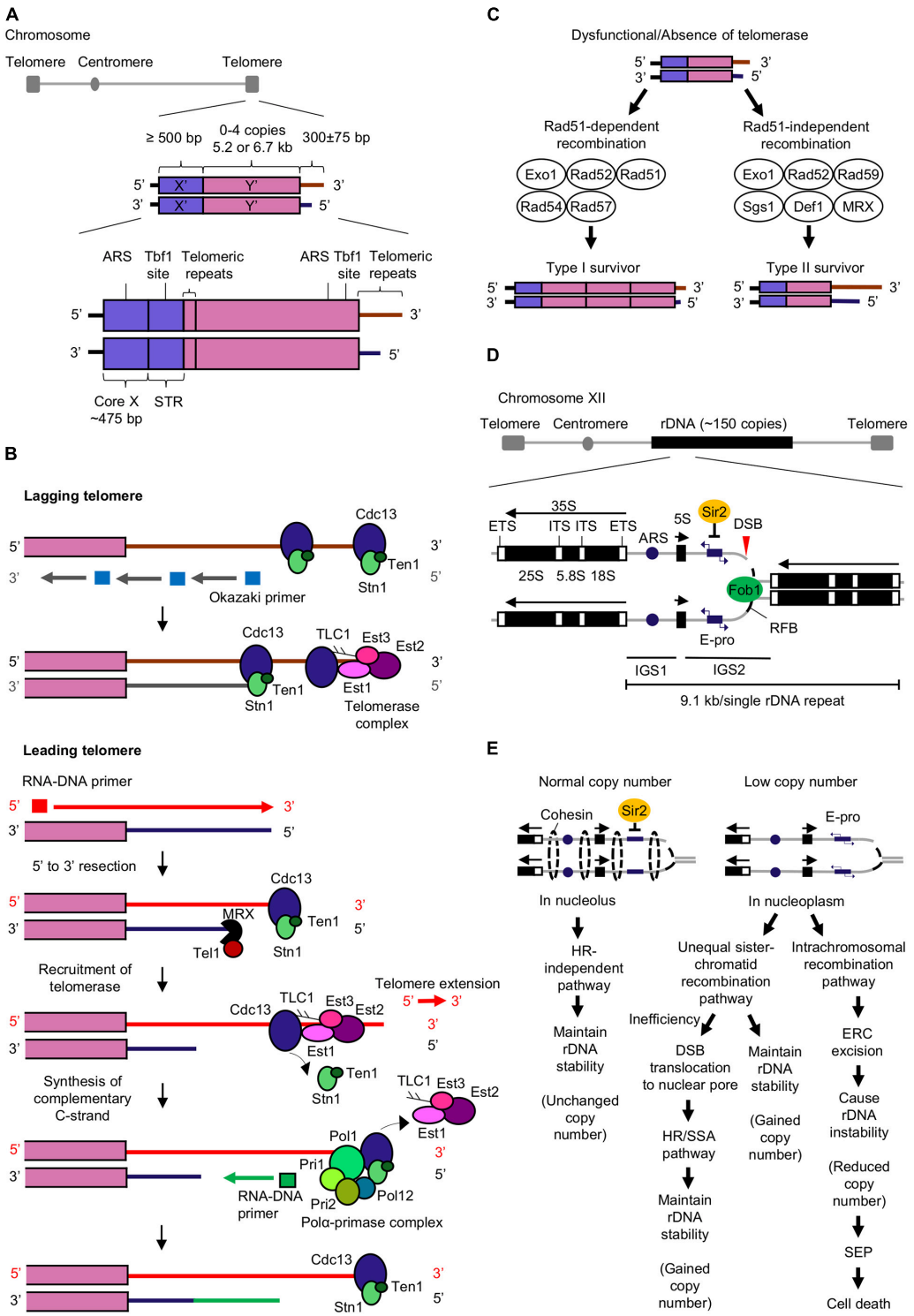

FIGURE 1 | Mechanisms of telomere extension and rDNA copy number maintenance that prevent cellular senescence and aging. **(A)** The budding yeast telomere structure consists of X′ and Y′ elements, and telomeric repeats. Core X that contains an autonomously replicating sequence (ARS) which is an origin of replication, and subtelomeric repeated elements (STR) that contains a Tbf1 binding site, are found in the X′ element. The ARS and Tbf1 binding site are also found in the Y′ element (Louis et al., 1994; Tham and Zakian, 2002; Wellinger and Zakian, 2012). **(B)** Telomerase-dependent pathway for telomere extension. Cdc13 and telomerase complex (Est1, Est2, Est3, and Tlc1) bind to both leading and lagging telomeres (Faure et al., 2010). DNA polymerase α (Polα)-primase complex generates RNA-DNA primers that initiate the synthesis of Okazaki fragments by DNA polymerase δ (Pol δ) (McElhinny et al., 2008; Perera et al., 2013) at lagging strand. After removal of the primers, the Okazaki fragments are ligated by DNA ligase I to form complementary lagging strand (Faure et al., 2010; Perera et al., 2013; *(Continued)*

FIGURE 1 | Continued
Liu et al., 2017). Telomere extension mediated by telomerase occurs primarily at the leading telomere (Faure et al., 2010). The RNA-DNA primer is required for initiating synthesis of complementary leading strand by DNA polymerase ε (Pol ε) (Pursell et al., 2007; McElhinny et al., 2008; Perera et al., 2013). CST complex (Cdc13-Stn1-Ten1) bound at telomere end restricts the access of telomerase to telomere end. MRX complex (Mre11-Rad50-Xrs2) induces the binding of Tel1 (Nakada et al., 2003; McGee et al., 2010) to short telomere and executes 5′ to 3′ exonuclease activity to synthesize 3′ overhang (Diede and Gottschling, 2001). Both MRX and Tel1 promote Cdc13-mediated telomerase tethering to telomere (Tseng et al., 2006; Yang et al., 2017). Extension of telomere by telomerase is completed upon the synthesis of the CST complex (Pfeiffer and Lingner, 2013) and telomerase departure from the telomere. Polα-primase complex (Pol1, Pol12, Pri1, and Pri2) (Lue et al., 2014) interacts with CST complex at the telomere (Churikov et al., 2013) and generates an RNA-DNA primer for synthesis of complementary C-strand (Churikov et al., 2013; Pfeiffer and Lingner, 2013). **(C)** Homologous recombination pathways for telomere extension in the absence of telomerase or when telomerase is dysfunctional. The Rad51-dependent recombination pathway for the generation of type I survivors requires Exo1, Rad52 (Chen et al., 2001; Maringele and Lydall, 2004), Rad51, Rad54, and Rad57 (Chen et al., 2001; Claussin and Chang, 2015) while the Rad51-independent recombination pathway for the generation of type II survivors includes Exo1, Rad52 (Chen et al., 2001; Maringele and Lydall, 2004), Rad59, Sgs1, Def1, and MRX complex (Huang et al., 2001; Signon et al., 2001; Chen et al., 2005). **(D)** The structure of budding yeast rDNA (Poveda et al., 2010; Chand Dakal et al., 2016; Kobayashi and Sasaki, 2017). Abbreviations: ETS, external transcribed spacer; ITS, internal transcribed spacer; IGS, intergenic spacer; ARS, autonomous replication sequence; E-pro, rDNA non-coding promoter; RFB, replication fork barrier site; DSB, double-stranded DNA break. **(E)** Regulation of rDNA stability by homologous recombination (HR)-independent and -dependent pathways (unequal sister-chromatid or intrachromosomal recombination pathway) (Kobayashi and Sasaki, 2017; Sasaki and Kobayashi, 2017; Horigome et al., 2019; Morlot et al., 2019). Abbreviations: DSB, double-stranded break; ERC, extrachromosomal rDNA circles; SEP, senescence entry point; SSA, single strand annealing.

Overlengthened telomeres has no impact on yeast chronological life span (CLS) (Harari et al., 2017). Conversely, telomere shortening causes RLS extension in budding yeast, possibly due to the relocalization of Sir2/3/4 complex to non-telomeric sites for heterochromatin structure maintenance and increased genomic stability (Austriaco and Guarente, 1997; Liu et al., 2019). Sir2 inhibits extreme CLS extension in yeast (Fabrizio et al., 2005), unlike its importance for RLS maintenance (Kaeberlein et al., 1999). Hence, the opposite roles of Sir2 in CLS and RLS may explain the different effects of telomere length on yeast aging. Furthermore, yeast telomeres do not shorten with age like the mammalian phenotype (D'Mello and Jazwinski, 1991). Therefore, while yeast is useful for telomere maintenance mechanism studies, it may not be the best model to study the telomere-aging link.

MAINTENANCE OF rDNA STABILITY PREVENTS CELLULAR SENESCENCE AND REPLICATIVE AGING

The ribosomal RNA gene (rDNA) is the most abundant RNA gene that encodes for ribosomal RNA. Ribosomal RNA is essential to form ribosomes with ribosomal proteins for protein synthesis (Kobayashi, 2008). *S. cerevisiae* has ∼150 tandem repeats of a 9.1 kb rDNA unit (Ganley and Kobayashi, 2014), found on chromosome XII locus (Petes, 1979; **Figure 1D**). These rDNA repeats are the most prone to DNA damage by external (ultraviolet light) and internal factors (replication errors and ROS), causing regional genomic instability. According to rDNA theory of aging, rDNA instability induces an aging signal that triggers DDR and initiate cellular senescence thus limiting cellular life span (Kobayashi, 2008, 2011a, 2014).

Maintenance of rDNA Copy Number for rDNA Stability

In yeast, Fob1 and Sir2 are major regulators of the rDNA maintenance system. Fob1 binds at the replication fork barrier (RFB) site in rDNA repeat to block the progression of the replication fork to the opposite direction of 35S rRNA gene transcription and form a DSB at the blocking site (Kobayashi, 2003). The blockage of the replication fork initiates the DBR either through a HR-independent or -dependent pathway depending on cellular rDNA copy number (Kobayashi and Sasaki, 2017; Sasaki and Kobayashi, 2017; **Figure 1E**).

The histone deacetylase Sir2 inhibits E-pro transcription when rDNA copy number is maintained at wild-type level. The inhibited E-pro transcription strengthens cohesion association to the broken DNA end and enables DBR which is independent of HR to occur in nucleolus (Saka et al., 2016; Sasaki and Kobayashi, 2017; Horigome et al., 2019). As a result, this repair leads to an unchanged rDNA copy number, maintaining rDNA stability and generating little to no aging signal (Saka et al., 2016).

In contrast, Sir2 does not inhibit E-pro transcription when rDNA copy number is less than wild-type level. The active transcription of E-pro dissociates the cohesins from the broken DNA end, stimulating the unequal sister chromatid recombination to occur between the misaligned rDNA repeats for DBR. The unequal sister chromatid recombination is Rad52-dependent (Torres-Rosell et al., 2007) and occurs in nucleoplasm (Horigome et al., 2019). As a result, unequal sister chromatid recombination duplicates and restore rDNA copy number (Kobayashi and Sasaki, 2017).

However, when the unequal sister chromatid recombination mediated DBR is inefficient, the rDNA break ends move to the nuclear pore and interact with nuclear pore complex. These DSBs at the nuclear pore may be repaired either by the HR or single strand annealing pathway. DNA damage checkpoint-associated Mec1/Tel1 kinases, replisome component Tof1 and proteins Tof2, Csm1, and Lrs4 that tether condensins to rDNA assist in the translocation of DSBs to nuclear pore for DBR and maintain rDNA stability (Horigome et al., 2019).

Intrachromosomal recombination can also occur upon activation of E-pro transcription, whereby the broken DNA end recombines with the rDNA copy within the same chromosome. Intrachromosomal recombination is mediated by Rad52 (Park et al., 1999) and results in the formation of extrachromosomal rDNA circles (ERCs) which are segregated from rDNA strand,

TABLE 1 | Functions of proteins involved in the regulation of telomere length homeostasis in *Saccharomyces cerevisiae*.

Proteins		Functions
Telomere capping complexes:		
Cdc13-Stn1-Ten1	CST complex	Protects telomere end from degradation (Grandin et al., 2001) Restricts telomerase access to telomere at the end of S phase (Churikov et al., 2013).
Ku70-Ku80	Yku complex	Protects telomere end from telomere-end resection (Vodenicharov et al., 2010; Shi et al., 2013).
Rap1-Rif1-Rif2		Restricts telomerase access to telomere to inhibit telomere extension of overlengthened telomere (Wotton and Shore, 1997; Goudsouzian et al., 2006; Hirano et al., 2009). Inhibits exonucleolytic degradation of telomere by preventing the association of Mre11-Rad50-Xrs2 (MRX) complex with telomere (Bonetti et al., 2010).
Telomere capping proteins:		
Npl3, Cdc2	hnRNP-related proteins	Prevents telomere end from being recognized as DNA break (Lee-Soety et al., 2012)
Rad6–Bre1–H2Bub1		Promotes telomere extension by inducing telomere-end resection (Wu et al., 2017)
Mre11-Rad50-Xrs2	MRX complex	Executes 5′ to 3′ exonuclease activity to synthesize 3′ overhang for Cdc13 binding at telomere (Diede and Gottschling, 2001) Mediates telomerase tethering to telomere (Tsukamoto et al., 2001). Protects uncapped telomere from exonucleolytic degradation during telomere extension (Vodenicharov and Wellinger, 2007; Wu et al., 2018).
Cdc13	ssDNA-binding protein	Protects telomere end (Nugent et al., 1996; Pennock et al., 2001). Promotes telomerase tethering to telomere (Nugent et al., 1996; Chandra et al., 2001)
Mec1	Phosphoinositide 3-kinase-related kinases (PIKKs)	Phosphorylates Cdc13 to mediate telomerase tethering to telomere (Tseng et al., 2006; Yang et al., 2017). Phosphorylates Rap1 to strengthen the interaction of Rap1 with Rif1 for promoting telomere end protection (Yang et al., 2017).
Tel1	Phosphoinositide 3-kinase-related kinases (PIKKs)	Recruited by MRX complex to short telomere (Nakada et al., 2003, 1; McGee et al., 2010) to promote telomerase tethering to telomere (Goudsouzian et al., 2006; Sabourin et al., 2007). Phosphorylates Cdc13 to mediate telomerase tethering to telomere (Tseng et al., 2006; Yang et al., 2017). Phosphorylates Rap1 to strengthen the interaction of Rap1 with Rif1 to promote telomere end protection (Yang et al., 2017).
Mre11	Double-strand break repair protein	Promotes telomerase tethering to telomere (Goudsouzian et al., 2006).
RPA	ssDNA-binding protein (replication protein A)	Promotes telomerase activity during telomere extension (Schramke et al., 2004; Luciano et al., 2012).
Cdk1	Cyclin-dependent kinase	Regulates telomere extension (Frank et al., 2006).
Pif1	Helicase	Unwinds G-quadruplex at telomere end to enable telomerase-mediated telomere extension and avoid DNA break (Paeschke et al., 2011)
Sgs1	Helicase	Unwinds G-quadruplex at telomere end to enable telomerase-mediated telomere extension (Huber et al., 2002). Generates type II telomerase-deficient survivors (Huang et al., 2001).
Elo3	Fatty acid elongase	Synthesizes very long-chain fatty acids (VLCFAs) (Kvam et al., 2005) to maintain telomere length through mediation of Yku (Ponnusamy et al., 2008).
Kcs1	Inositol hexakisphosphate and inositol heptakisphosphate kinase	Synthesizes inositol phosphates, which negatively affect telomere-maintaining role of Elo3 through mediation of Yku (Ponnusamy et al., 2008).
Ipk2	Inositol polyphosphate multikinase	
Sit4	Protein serine/threonine phosphatase	Synthesizes protein phosphatase 2A (PP2A) which dephosphorylates Sir3 to maintain the heterochromatin structure for telomere stabilization (Chan and Blackburn, 2002; Hayashi et al., 2005).
Def1	RNA polymerase II degradation factor	Positive regulator in telomere maintenance and required for the generation of type II telomerase-deficient survivors (Chen et al., 2005).
Tsa1	Thioredoxin peroxidase	Major reactive oxygen species (ROS) scavenger (Iraqui et al., 2009) that prevents telomere overextension due to ROS (Lu et al., 2013).
Rnr1	Major subunit of ribonucleotide reductase (RNR)	Provides precursors for synthesis of deoxynucleoside triphosphates (dNTPs) required for telomerase-mediated telomere extension (Maicher et al., 2017).
ESCRT-0, –I,–II, and –III	Endosomal sorting complex required for transport (ESCRT)	Maintains telomere length by participating in telomerase-dependent telomere extension (Dieckmann et al., 2016).
Pol ϵ and Pol δ	DNA polymerase	Maintain telomere length (Ohya et al., 2002) by synthesizing chromosomal DNA strands (McElhinny et al., 2008) Exhibits 3′-5′ exonuclease activity for telomeric ssDNA repair during cell cycle arrest (Ohya et al., 2002; Henninger and Pursell, 2014).
Pol α-primase complex		Synthesizes RNA-DNA primer required for synthesis of chromosomal DNA strands (Churikov et al., 2013; Pfeiffer and Lingner, 2013).
Yra1	RNA-binding protein required for mRNA export from nucleus	Overexpression causes telomere shortening (Gavaldá et al., 2016).

leading to rDNA copy loss (Kobayashi and Sasaki, 2017). The loss of rDNA copy causes rDNA instability and stimulates cellular senescence (Hein et al., 2012; Saka et al., 2016; **Figure 1E**).

rDNA Stability and Its Effects on Aging

Extrachromosomal rDNA circles were previously shown to cause aging in yeast and speculated to be the molecular cause of aging in higher species, including mammals (Sinclair and Guarente, 1997). This ERC theory of aging was later disputed by the rDNA theory of aging (Kobayashi, 2008) which affirms that rDNA instability is a major cause of aging independent of ERC accumulation level. Although the accumulation of ERCs and other episomes (plasmids) can shorten yeast RLS, they stimulate rDNA instability, reaffirming rDNA stability as a major life span-determinant (Falcón and Aris, 2003; Ganley et al., 2009; Saka et al., 2013). Nevertheless, ERCs can be markers for rDNA instability.

Asymmetrical segregations of instable rDNAs and ERCs occur more frequently to yeast mother cells, resulting in cellular senescence and aging while stable rDNAs are segregated to daughter cells, allowing daughter cells to undergo rejuvenation (Kobayashi, 2011b; Morlot et al., 2019). However, rejuvenation of daughter cells produced by old mother cells (after their first 40% of RLS) would be affected, thus exhibiting decreased RLS. This is likely due to impaired asymmetrical segregation of aging factors which are constrained as well in daughter cells (Kennedy et al., 1994).

More recently, Morlot et al. (2019) proposed a model that links the accumulation of ERCs to senescence and longevity. ERC-linked senescence is categorized into three stages: ERC excision, ERC self-replication, and post-SEP interval. Extensive ERC excision from rDNA during intrachromosomal recombination and self-replication of ERC leads to the accumulation of ERC. Even though accumulating ERC can upregulate rDNA transcription, ribosome synthesis is not enhanced. Loss of coordination between rDNA transcription and ribosome biogenesis could negatively affect cell growth. When the amount of ERCs reaches a threshold, the cells reach a senescence entry point (SEP). In post-SEP interval (an interval between SEP and cell death), the cells experience a loss of nuclear homeostasis [an increase in nucleus-to-cell area ratio (N/C ratio), increase in histone content and genomic defect] which causes cell death (Morlot et al., 2019; **Figure 1E**). The relationship between rDNA stability to RLS has been shown with yeast mutants of rDNA stability regulators. Yeast cells lacking *SIR2* with more ERCs causing rDNA instability exhibited a ∼50% decrease in RLS (Kaeberlein et al., 1999). Unlike *SIR2* mutants, *FOB1* mutants has less ERCs, thus enhancing rDNA stability and showed a 70% extension of RLS (Defossez et al., 1999). More recently, a new regulator of rDNA copy number, Eaf3 was discovered (Wakatsuki et al., 2019). Yeast cells lacking Eaf3 exhibited less ERCs which enhances rDNA stability leading to a 30% extension of RLS. Eaf3 likely activates transcription of E-pro to induce unequal sister chromatid recombination and intrachromosomal recombination which results in the formation of ERCs.

The link of rDNA stability to senescence and aging has also been established in mammalian cell studies. For example, genomic imaging revealed rDNA copy loss in senescent human cells and blood from aged individuals, validating the link between human aging and rDNA instability (Ren et al., 2017). The mammalian Sir2 homolog SIRT7 was also found to function like Sir2 to prevent rDNA instability and consequently cellular senescence *via* chromatin silencing (Paredes et al., 2018).

In summary, DNA replication-based telomere elongation and DBR-based rDNA copy number maintenance are fundamental mechanisms that minimize DNA loss and damage for maintenance of genomic stability, thus inhibiting the onset of cellular senescence and aging. Studies in yeast have identified proteins regulating telomere length homeostasis and the understanding of rDNA copy number maintenance. Current knowledge shows that rDNA instability possibly plays a bigger role than ERCs in aging in yeast and especially higher organisms. Still, yeast will continue to serve as a versatile model for studying rDNA instability and telomere length maintenance mechanisms.

AUTHOR CONTRIBUTIONS

JL drafted the manuscript. EO revised and edited further. Both authors approved the final version of the manuscript for submission.

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FoxO1 Is a Novel Regulator of 20S Proteasome Subunits Expression and Activity

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Proteostasis collapses during aging resulting, among other things, in the accumulation of damaged and aggregated proteins. The proteasome is the main cellular proteolytic system and plays a fundamental role in the maintenance of protein homeostasis. Our previous work has demonstrated that senescence and aging are related to a decline in proteasome content and activities, while its activation extends lifespan *in vitro* and *in vivo* in various species. However, the mechanisms underlying this age-related decline of proteasome function and the down-regulation in expression of its subunits remain largely unclear. Here, we demonstrate that the Forkhead box-O1 (FoxO1) transcription factor directly regulates the expression of a 20S proteasome catalytic subunit and, hence, proteasome activity. Specifically, we demonstrate that knockout of FoxO1, but not of FoxO3, in mice severely impairs proteasome activity in several tissues, while depletion of IRS1 enhances proteasome function. Importantly, we show that FoxO1 directly binds on the promoter region of the rate-limiting catalytic β5 proteasome subunit to regulate its expression. In summary, this study reveals the direct role of FoxO factors in the regulation of proteasome function and provides new insight into how FoxOs affect proteostasis and, in turn, longevity.

Keywords: FOXO factors, insulin signaling, proteostasis, proteasome, aging, longevity

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INTRODUCTION

Proteostasis is a pivotal process indispensable for the majority of cellular functions, including DNA replication, the regulation of the cell cycle, metabolism, maintenance of cellular architecture, signaling pathways, development and immune responses (Powers et al., 2009; Labbadia and Morimoto, 2015). Proteostasis collapse has been documented as a key factor contributing to the progression of aging (López-Otín et al., 2013), caused by a gradual failure of the respective defense systems (Taylor and Dillin, 2011). Furthermore, several studies have demonstrated that chronic exposure to aggregated or denatured proteins contributes to the development of age-related diseases, such as Alzheimer's and Parkinson's disease (Chondrogianni et al., 2015b; Labbadia and Morimoto, 2015). The proteasome plays a pivotal role in maintaining proteostasis and as such, is

Abbreviations: ChIP, chromatin immune precipitation; DBE, Daf-16 family binding element; FoxO, forkhead box-O; IIS, *insulin*/IGF-1 *signaling*; IRE, insulin response element; IRS1, insulin receptor substrate 1; KO, knockout; MEFs, mouse embryonic fibroblasts; RNAi, RNA inhibition; RT-PCR, real time polymerase chain reaction; TSS, transcription starting site.

involved in a multitude of biological processes (Vilchez et al., 2014). The 30S/26S proteasome is the main proteasome complex consisting of the 19S regulatory "cap" and the 20S catalytic "core" (single capped: 26S, double capped: 30S). The 20S core proteasome has barrel-like configuration and is comprised by seven different α subunits and seven distinct β subunits. Three β subunits, namely β1, β2, and β5, possess proteolytic activities with different substrate specificities (Chondrogianni et al., 2014). Our previous work has established a direct association between proteasome-mediated proteolysis and aging. Specifically, we have demonstrated that the accumulation of damaged proteins during aging is connected to an age-related downregulation of proteasome expression and activity. In addition, pharmacological or genetic induction of the proteasome improves both cellular and organismal lifespan and alleviates the pathological phenotype of protein aggregation-related diseases, such as Alzheimer's disease (Chondrogianni et al., 2015b; Mladenovic Djordjevic et al., 2021). Moreover, we have shown that human mesenchymal stem cells (hMSCs) exhibit a senescence-related decline of proteasome content and aberrations in physiological assembly of proteasome complexes during prolonged *in vitro* expansion, while proteasome activation via overexpression of the catalytic β5 subunit can enhance their stemness and lifespan (Kapetanou et al., 2017). Therefore, we hypothesize that the mechanisms and molecular factors that control proteasome subunit expression are crucial regulators of longevity. However, the respective underlying mechanisms remain largely obscure.

Numerous studies have identified an extensive array of genes that can alter the lifespan of several organisms. Despite this enormous volume of research, we still do not completely comprehend how these genes influence the aging process of an organism (Flatt and Partridge, 2018). A proteomic study in *D. melanogaster* (Tain et al., 2017) and a genetic approach in *C. elegans* (Vilchez et al., 2012) have suggested a potential interplay between the regulation of the proteasome and the Forkhead box-O (FoxO) transcription factors. FoxO factors control several cellular processes like autophagy and apoptosis in response to signals emanating from the environment and are important longevity determinants, downstream of insulin and insulin-like growth factor signaling (IIS). FoxO factors, under conditions of low IIS, translocate into the nucleus and bind to promoters of pro-longevity genes to regulate transcription. Nevertheless, their complex role in life-expectancy determination has not been fully elucidated yet. Here, we have dissected further the mechanism of IIS action on mammalian proteasome regulation and demonstrate for the first time that FoxO1 directly regulates the expression and activity of the 20S proteasome.

MATERIALS AND METHODS

Mouse Models and Husbandry

All mice were maintained at 22°C under a 12-h light/dark cycle (lights on from 7:00 am to 7:00 pm). Mice were housed in groups of three to five same-sex littermates under specific pathogen-free conditions within individually ventilated cages (Tecniplast UK Ltd., Kettering, Northamptonshire, United Kingdom). Mice had *ad libitum* access to normal chow [ssniff® R/M-H phytoestrogen-poor (9% fat, 34% protein, and 57% carbohydrate) ssniff Spezialdiäten GmbH, Soest, Germany] and water. *Irs1* global knockout mice were generated as described previously (Selman et al., 2008). The conditional FoxO1 (from Ron DePinho, MD Anderson Cancer Center) total knockouts were induced by tamoxifen treatment for 6 weeks using a ROSA26-CreERT2-mediated recombination (**Supplementary Figure 1**, mouse line from Thomas Langer, MPI for Biology of Aging) (Paik et al., 2007). *Foxo1* (n = 8, 6 females and 2 males) and *Irs1* (n = 7, females) KO and their littermate controls (+/+, n = 7, 5 females and 2 males and n = 7, females, respectively) were sacrificed at 16 weeks. *Foxo3* total knockouts were generated by using germline Cre-mediated recombination (actin-Cre) with the conditional *Foxo3* allele (from Ron DePinho, MD Anderson Cancer Center) producing the null allele. Mice were dissected and tissues were snap-frozen in liquid nitrogen.

Mouse Embryonic Fibroblast Isolation and Culture

Primary mouse embryonic fibroblasts (MEFs) were isolated from *Irs1*+/+ or *Irs1*−/− animals and cultured according to standard procedures (Qiu et al., 2016). Briefly, MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (v/v; Invitrogen), 2 mM glutamine and 1% non-essential amino-acids at 37°C, 5% CO_2 and 95% humidity.

Reagents and Antibodies

LLVY-AMC, MG132 and the primary antibody against the proteasomal subunits β5 (X, MB1, ϵ; PW8895; 22.9 kDa) and α6 (C2; PW8100; 33 kDa) were purchased from Enzo Life Sciences, Inc. The ChIP Grade antibody against FoxO1 (ab39670) and HRP-conjugated anti-rabbit and anti-mouse antibodies were purchased from Abcam.

Proteasome Peptidase Assays

Liver and brain tissues were lysed in 25 mM Tris/HCl lysis buffer, pH 7.6 containing 5 mM ATP, 10% glycerol, 20 mM KCL, 1 mM EDTA, 1 mM DTT, 0.2% Nonidet P-40, 10 mM phenylmethylsulfonyl-fluoride and 10 μg/ml aprotinin (Rivett et al., 1994). The CT-L activity of the proteasome was assayed after the incubation of 10 μg of total protein for 30 min at 37°C with the fluorogenic peptide LLVY-AMC, as previously described (Georgila et al., 2014). Proteasome activity was determined as the difference between total fluoresce and fluoresce in the presence of 20 μM of the proteasomal inhibitor MG132. AMC fluorescence was measured at 360 nM excitation and 460 nM emission using a spectrofluorimeter (Tecan). A Bradford assay was used to determine protein concentration, using bovine serum albumin as standard.

Immunoblot Analysis

Twenty μg protein of isolated protein were separated by 10% SDS-PAGE under non-reducing conditions according to standard procedures (Palmer, 2000). Following electrophoresis,

protein loading was analyzed using the Stain-free™ (Bio-Rad) imaging technology that allows the visualization of the proteins directly in the gel after a short photoactivation. Proteins were then transferred to nitrocellulose membrane (Amersham Biosciences) to be treated with the blocking buffer and were subsequently incubated with the appropriate antibodies. The primary antibodies were detected with horseradish peroxidase conjugated secondary antibodies. The detection with enhanced chemiluminescence was performed using ECL or ECL prime chemiluminescence kits (GE Healthcare) and a ChemiDoc station (Bio-Rad).

Real Time PCR Analysis

For the characterization of proteasome genes, total RNA was isolated using TRIzol (Invitrogen) and transcripted into cDNA with the cDNA iScript synthesis kit (Bio-Rad). The Real time PCR were run on the CFX Connect Real-Time PCR System (Bio-Rad). The RT-PCR primers are summarized in **Supplementary Table 1**. For the evaluation of FoxO1 knockout efficiency, RNA was isolated using TRIzol (Thermo-Fisher), treated with DNAse (Qiagen) and purified by isopropanol precipitation. cDNA was prepared using the SuperScript III reverse transcriptase kit (Invitrogen) as per manufacturer's instructions. TaqMan probes against Foxo1 and beta2-microglobulin were obtained from Applied Biosystems and run on a 7900HT real-time PCR system.

ChIP Analysis

Chromatin immune precipitation experiments were performed using the ChIP-IT® Express Enzymatic kit (Activemotif, cat. no 53009), as per manufacturer's instructions. Chromatin was sheared enzymatically for 5 min and precipitated with a ChIP-Grade Anti-FOXO1 antibody (ab39670, Abcam). Prior to amplification, the samples were subjected to DNA clean-up step using the Nucleospin R Gel and PCR Clean-up kit (740609.10, Macherey-Nagel). The ChIP products were then analyzed by Real-time PCR and the products were confirmed by agarose gel electrophoresis. An anti-IgG antibody was used as a negative control.

RNA Interference

The small interfering (si)RNAs targeting murine FoxO1 and IRS1 were obtained from Thermo Fisher Scientific (s80620) and Sigma (EMU061331 MISSION® esiRNA), respectively. The siRNA targeting murine FoxO3 has been purchased from Thermo Fisher Scientific (Silencer®, 100380). Briefly, the siRNA duplexes were transfected into 70–80% confluent MEFs cultured in a in a 6-well or 96-well plate format at a final concentration of 50 nM, in presence of the TransFectinTM Lipid Reagent (1703351, Bio-Rad) at a ratio of 1:2. Transfection complexes were prepared in Opti-MEM® Reduced Serum Medium, GlutaMAX™ Supplement (51985-034, Thermo Fisher Scientific). After 24 h, the cells were harvested or transfected with the LightSwitch™ plasmids without media change.

FoxO1 Activity Assay

The isolation of nuclear proteins and the subsequent examination of FOXO1 transcriptional activity were performed with the Nuclear Extraction Kit (ab113474, Abcam) and the FOXO1 Transcription Factor Assay Kit (Colorimetric; ab207204, Abcam), respectively, according to manufacturer's instructions.

Luciferase Assay

The promoter region of β5 and a mutagenized version (**Supplementary Table 2**) were cloned into the LightSwitch™ Promoter Reporter Vector (Active Motif). MEFs were transfected using the Transfectin reagent (1703351, Bio-Rad) and the luciferase assay was performed with the LightSwitch™ Luciferase Assay Kit (32031, Active Motif) in quadruplicates, according to manufacturer's instructions. Each well was read for 10 s and the signal of the empty vector (32021, Active Motif) was deducted from the values of sample wells.

Ethics Statement

This study was performed in strict accordance with the recommendations and guidelines of the Federation of European Laboratory Animal Science Associations (FELASA). The protocol was approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen.

In silico Identification of FoxO Binding Motifs

Genomic DNA sequences were downloaded in FASTA format from www.ensembl.org/ and individual matches of known FoxO binding motifs were scanned using FIMO[1] in 1 kb regions upstream of the TSS of murine 20S proteasome genes.

Statistical Analysis and Quantifications

Statistical analysis and the graphical representation of data was performed using the GraphPad Prism 5 (GraphPad Software, San Diego, CA, United States). All values were reported as mean ± SE, unless otherwise indicated. Densitometry analysis for the quantification of immunoblots was performed with Bio-Rad's Image Lab software 6.0.1. The average signal of control mice was arbitrarily set to 100% or 1.

RESULTS

Foxo1 Knockout Mice Exhibit Reduced Proteasome Activity and Expression in Liver and Brain

Firstly, we evaluated the proteasome status in various tissues of *Foxo1* KO mice sacrificed at 16 weeks of age, in comparison to their respective control animals. We found that *Foxo1* mutant mice displayed a substantial decrease in chymotrypsin like (CT-L) activity. Specifically, the data for liver and brain that are shown in **Figure 1A** and **Supplementary Figures 2A,3A** revealed a statistically significant reduction of proteasome activity by 25.8% and 22.6% in liver and brain, respectively. In support, the mRNA (**Figure 1B**) and protein levels (**Figure 1C** and **Supplementary Figures 2B,3B**) of representative β and α 20S subunits (catalytic

[1]http://meme-suite.org/tools/fimo

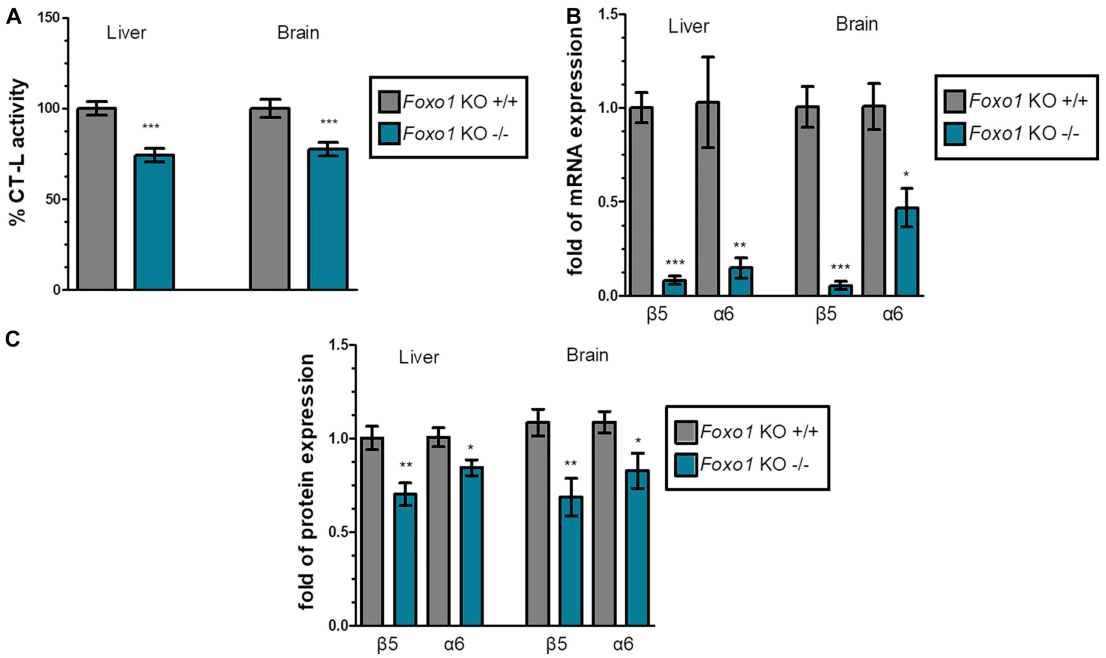

FIGURE 1 | FoxO1 depletion downregulates the proteasome: **(A)** Mean% CT-L activities, **(B)** mRNA expression, and **(C)** Mean values immunoblot analysis of the indicated proteasome subunits in the liver and brain of the indicated control and FoxO1-depleted mice. The total protein load was used as a control for equal protein loading and GAPDH was used for RT-PCR normalization. 100% or 1 has been arbitrarily set to the average values of the control samples. Data information: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. All error bars show SEM. Number of animals: $Foxo1^{-/-}$ $n = 8$, 6 females and 2 males and $Foxo1^{+/+}$ $n = 7$, 5 females and 2 males.

β5 and α6) declined considerably in absence of FoxO1 in these tissues. However, FoxO3 depletion affected neither proteasome expression nor its activity (**Supplementary Figure 6**).

Irs1 Global Knockout Enhances Proteasome Expression and Function

FoxO factors are activated when IIS activity is low to induce the expression of their target genes. To examine the effect of FoxO activation on the proteasome-mediated proteolysis, we characterized the proteasome status of the long-lived *Irs1* KO mice (sacrificed at 16 weeks of age) and observed that the CT-L activity was increased (see, **Figure 2A** and **Supplementary Figures 4A,5A**) by 55.3% in the liver and by 88.2% in the brain. Additionally, there was a consistent pattern of induced mRNA (**Figure 2B**) and protein expression (**Figure 2C** and **Supplementary Figures 4B,5B**) of the β5 subunit in *Irs1* KO mice in comparison to their relative control littermates. However, the expression of α6 was not consistently upregulated by *irs1* knockdown, suggesting that IIS has a more prominent role in the regulation of β5.

FoxO1 Directly Binds to the Murine Promoter Region of β5

Our data indicated that the expression of 20S proteasome subunits is positively regulated by FoxO1. To shed light on these observations, we performed IRS1, FoxO1, FoxO3 and double IRS1 + FoxO1 and IRS1 + FoxO3 silencing assays, using small interfering RNAs and tested the CT-L proteasome activity and the levels of the relevant β5 subunit. In support to the described *in vivo* data, IRS1 silencing led to doubled CT-L activity (**Figure 3A**) as well as to significantly increased β5 levels (**Figure 3B**), while FoxO1 silencing led to a downregulation of both proteasome activity and β5 content. In addition, the beneficial effects of lowered IRS1 levels on the proteasome were mediated by FoxO1 as double IRS1 + FoxO1 silencing abolished the increase in CT-L activity and β5 expression. In contrast, we observed similar effects of IRS1 and double IRS1 + FoxO3 silencing on proteasome status. Notably, we confirm that IRS1 silencing significantly enhances FoxO1 transcriptional activity (**Figure 3C**).

To determine if proteasome subunit promoters are occupied by FoxO1 *in vivo*, we analyzed several putative binding sites of FoxO1 containing its consensus sequences 5′-TT[G/A]TTTTG-3′ (Insulin Response Element, IRE) or 5′-TT(G/A)TTTAC-3′ (Daf-16 family binding element, DBE) (Furuyama et al., 2000) that were *in silico* identified in the promoter regions of β1, β2, β5, and β7 subunits, using chromatin IP. Specifically, we examined putative binding motifs that were localized at −374, −577, −237, and −770 upstream of the Transcription Starting Site (TSS) of each gene, respectively. The precipitated fragments were detected using quantitative real-time PCR primers that specifically amplify DNA encompassing the putative FoxO1 binding sites (**Supplementary Table 1**). As demonstrated in **Figure 3D** a putative IRE-containing site at −237 upstream of the β5 TSS promoter was detected in FoxO1 immunoprecipitants,

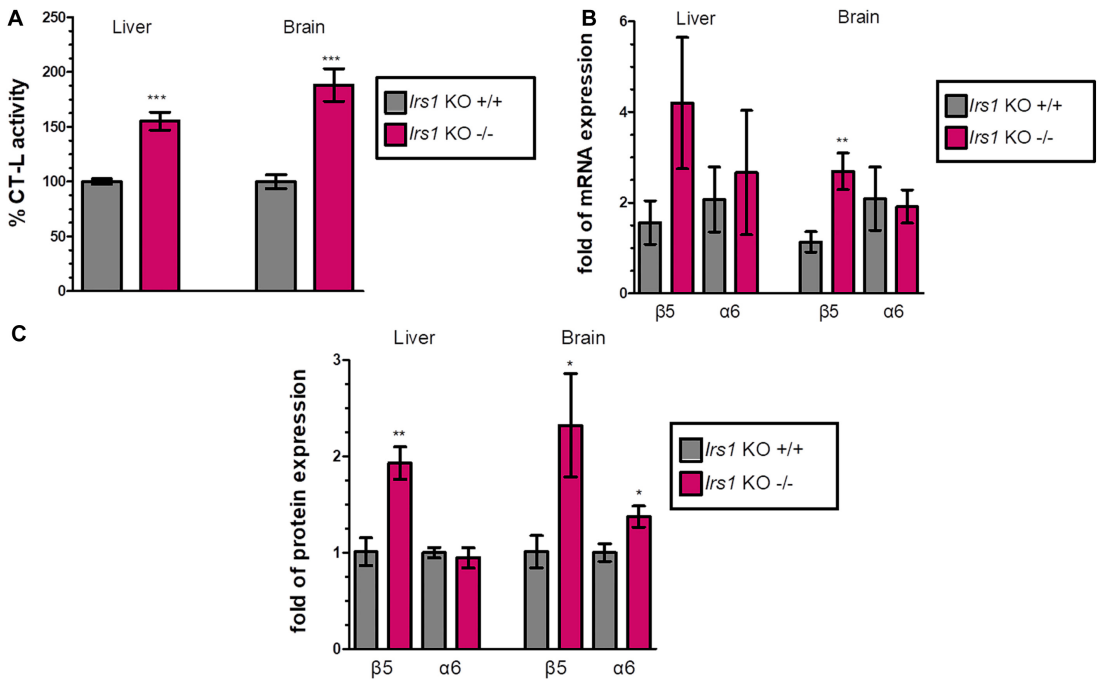

FIGURE 2 | Global *Irs1* knockdown induces the proteasome: **(A)** Mean% CT-L activities, **(B)** mRNA expression and **(C)** Mean values immunoblot analysis of β5 and a6 proteasome subunits, in the liver and brain of the indicated control and IRS1-depleted mice. The total protein load was used as a control for equal protein loading and GAPDH was used for RT-PCR normalization. 100% or 1 has been arbitrarily set to the average values of the control samples. Data information: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. All error bars show SEM. Number of animals: $Irs1^{-/-}$ $n = 7$, females and $Irs1^{+/+}$, $n = 7$, 5 females and 2 males.

using a primer pair that amplified a 180 bp region starting at −281 (Other indicated proteasome subunits promoters were not amplified; data not shown). The non-specific IgG antibody failed to precipitate *in vivo* the proteins bound to this sequence, suggesting that mouse FoxO1 has the potential to bind to the promoter of β5. Specifically, the ChIP-to-Input ratio was 2.4% in control MEFs, suggesting that FoxO1 may be an important factor participating in the regulation of gene expression (**Figure 3E**). Importantly, there was a 3-fold increase in PCR detection of the FoxO1 binding site in *Irs1* KO MEFs, demonstrating that low IIS activity enhances FoxO1 binding to the promoter of β5 to regulate gene expression.

To assess the functional consequences of FoxO1 binding on the putative IRE (5′-TTATTTTG-3′), the wild type β5 promoter region (−1,000 to 0 upstream the β5 TSS) or a mutagenized version with 8 bp substitutions on the detected FoxO binding motif at −237 were cloned into the pLightSwitch_Prom reporter vector, which utilizes the RenSP luciferase gene (see **Supplementary Table 2**). As shown in **Figure 3F**, the mutation on the putative IRE (mut-pβ5-lightswitch) significantly repressed luciferase activity by 26.3% compared to the wild type (pβ5-lightswitch), indicating that the respective sequence promotes transcription. Furthermore, co-transfection of siRNAs targeting FoxO1 markedly attenuated the reported activity of the pβ5-lightswitch construct by 25.2%, while IRS1 silencing yielded a 27.5% induction of the luciferase activity. Mutation of the detected IRE completely abolished the effects of FoxO1 or IRS1 silencing on promoter activity. These results indicate that the identified site is essential for the FoxO1-mediated regulation of β5 subunit expression.

DISCUSSION

FoxO transcription factors are conserved regulators of longevity downstream of insulin and insulin-like growth factor signaling. They integrate signals emanating from nutrient deprivation and stress stimuli to coordinate programs of genes involved in cellular metabolism and quality control. The evolutionary conserved function of reduced IIS in organismal lifespan extension has fueled research to understand the mechanisms underlying this pro-longevity function of FoxOs (Webb and Brunet, 2014; Fontana and Partridge, 2015; Martins et al., 2016). However, we still do not fully understand how these factors affect lifespan. In this study, we dissect the contribution of FoxO transcription factors in longevity and demonstrate for the first time using mouse models the ability of reduced IIS to promote 20S proteasome function in mammalian tissues.

The gradual age-related decline of proteostasis maintenance is considered to be an important hallmark of aging (López-Otín et al., 2013). Emerging evidence from various biological systems indicate that FoxOs orchestrate the expression of genes involved in the proteostasis network (Webb and Brunet, 2014). These findings suggest that the maintenance of proteostasis may in part underlie the ability of FoxOs to extend lifespan and to delay signs

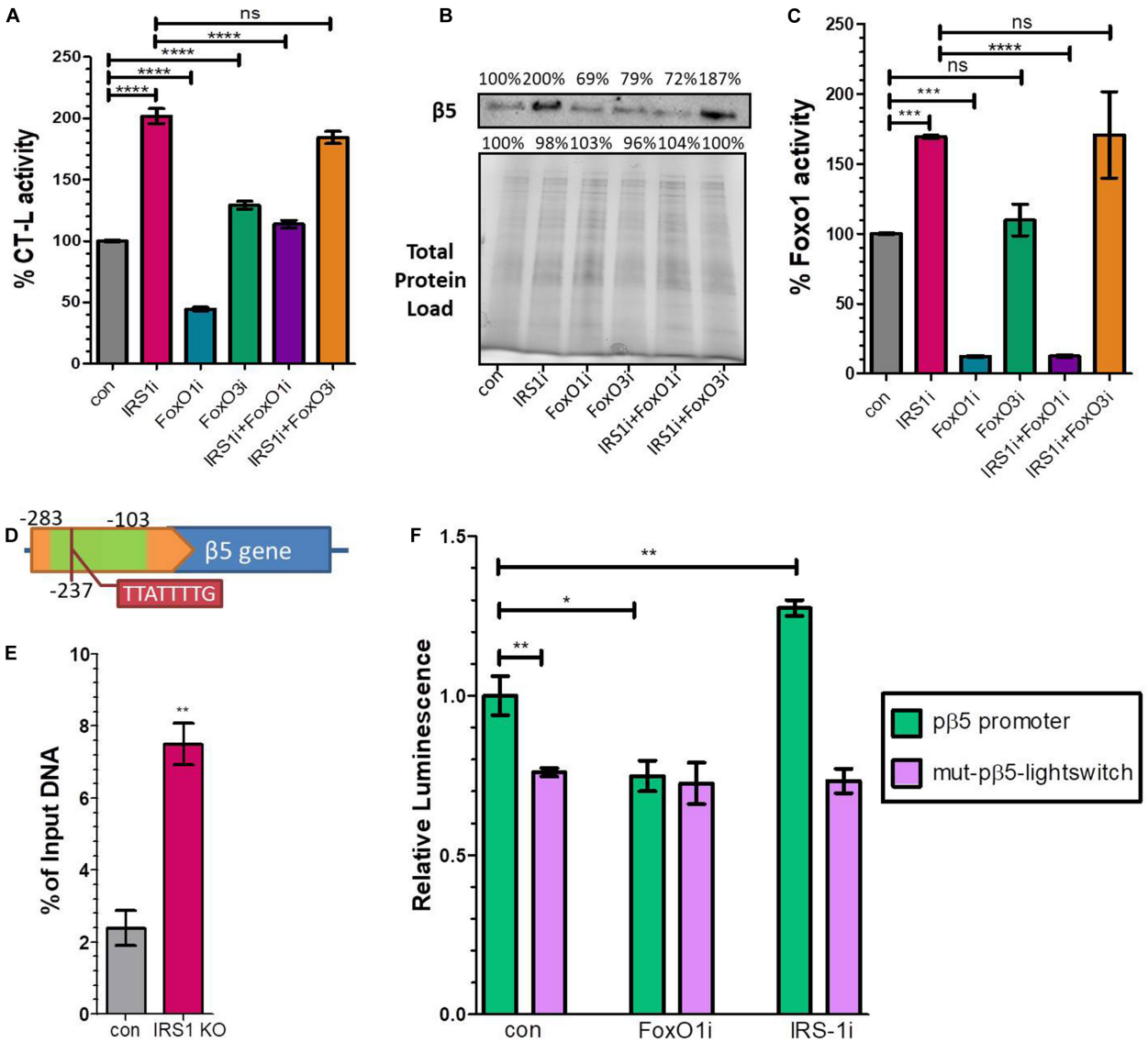

FIGURE 3 | FoxO1 binds to the murine β5 promoter to regulate gene expression. **(A)** % CT-L activities, **(B)** Immunoblot analysis of the β5 subunit and **(C)** FoxO1 activity in the presence of siRNAs targeting IRS1, FoxO1, FoxO3 or control as indicated. 100% has been arbitrarily set to the average values of the control samples and the total protein load was used as a control for equal protein loading. **(D)** Schematic diagram of the promoter region of β5 subunit. The green area indicates the amplified chromatin immunoprecipitation products, while the tab indicates the speculatory FoxO binding site. **(E)** Chromatin immunoprecipitation analysis of the β5 promoter region from control or *Irs1* depleted MEFs, using an anti-FoxO1 antibody. The amount of immunoprecipitated DNA was evaluated by RT-PCR. Histogram was constructed by the ratios of the immunoprecipitated DNA to the input (arbitrarily set to 100%). **(F)** Luciferase signal driven by the proximal wild type β5 promoter region (pβ5-lightswitch) or by a mutagenized version (mut-pβ5-lightswitch) with 8 bp substitutions on the detected FoxO binding motif in the presence of siRNAs targeting FoxO1, IRS1 or control as indicated. The signal of the empty vector has been deducted from raw values. The luminescence of wild type promoter under control conditions has been arbitrarily set to 1. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

of age-related diseases. Specifically it has been found that FoxO factors promote the expression of genes involved in autophagy and the ubiquitin–proteasome system. Whereas autophagy is thought to be relatively specific to long-lived proteins and degradation under chronic starvation conditions (Mizushima and Klionsky, 2007), the proteasome system is responsible for the degradation of most short-lived and regulatory proteins (Löw, 2011). Proteasome activity declines during aging in several tissues, including brain, heart, liver, muscle and skin, accounting for the observed accumulation of damaged proteins and the inclusion bodies (Chondrogianni et al., 2015b). Conversely, an intact proteasome is correlated with extreme longevity in humans (Chondrogianni et al., 2000), while proteasome activation can increase cellular and organismal lifespan, alleviate aggregation-related pathologies and enhance stemness (Chondrogianni et al., 2000; Kapeta et al., 2010; Chondrogianni et al., 2015a; Kapetanou et al., 2017). Interestingly, studies in brain have demonstrated the link between proteasome activity and aging (Triplett et al., 2015;

Kelmer Sacramento et al., 2020), while Tropea and co-workers have proposed the involvement of IIS in these processes (Wrigley et al., 2017). Other studies on skeletal muscle atrophy have addressed the role of mammalian FoxOs in the positive regulation of ubiquitin ligases. FoxO3 is a strong and direct regulator of the muscle-specific E3 ubiquitin ligases atrogin-1 and Murf-1 transcription (Sandri et al., 2004, 2006; Stitt et al., 2004). In addition to acting upstream of ubiquitination, emerging evidence suggests that FoxO factors are linked to the regulation of proteasome assembly by modulating the expression of a 19S subunit. In human embryonic stem cells (hESCs) and induced pluripotent stem cells, FoxO4 is both necessary and sufficient for expression of the 19S regulatory cap subunit PSMD11 (Rpn6) (Vilchez et al., 2012). The high proteasome activity that ESCs exhibit is considered to be critical for the prevention of senescence. Upon differentiation, PSMD11 expression declines as it is no longer under FoxO4 regulation. This is accompanied by a reduction of proteasome activity and an increase in the levels of polyubiquitinated proteins. Interestingly, FoxOs appear to have a conserved role in regulating proteasome activity. In *C. elegans*, FoxO/DAF-16 promotes various types of stress resistance via the activation of the PSMD11 ortholog, rpn-6 (Vilchez et al., 2012). Similarly, worms overexpressing pbs-5, the ortholog of the catalytic β5 subunit, display a daf-16 dependent increase in lifespan and resistance to proteotoxicity (Chondrogianni et al., 2015a).

However, the mechanisms underlying the exact role of FoxOs in the regulation of 20S proteasome expression and activity remained elusive. In different cell types, FoxO1 and FoxO3 factors modulate various cellular activities, while also having functional redundancies. Herein, we demonstrate that the depletion of FoxO1, but not of FoxO3, leads to a significant decline of proteasome activity in two murine tissues. Notably, the proteasome is an integral part of the cellular function and its inhibition above the observed levels is toxic to cells and tissues, while knockdown of distinct proteasome subunits is embryonic lethal (Tanaka, 2009). The detected reduction of CT-L activity is linked to the reduced mRNA and protein expression of 20S subunits, including the catalytic β5 subunit. Contrariwise, the knockdown of IRS1, which enhances FoxO1 activity and ameliorates lifespan (Taguchi et al., 2007; Kappeler et al., 2008), induces proteasome function and 20S subunits expression. In support, IIS reduction in the fly resulted in an enhanced proteasome assembly and activity in the gut accompanied by a reduction in the aberrant age-related accumulation of proteasome substrates and an increase in gut integrity with age (Tain et al., 2017). Proteasome activity was necessary for IIS-mediated longevity as treatment with low concentrations of a proteasome inhibitor abolished the beneficial effects of IIS reduction in lifespan and gut integrity. Interestingly, proteasome activation was sufficient to increase gut integrity and lifespan in *D. melanogaster*. Likewise, proteasomal inhibition abolished the beneficial effect of reduced IIS on the circuit function in old flies (Augustin et al., 2018).

Furthermore, we demonstrate that silencing of IRS1 leads to enhancement of FoxO1 transcriptional activity, which is in accordance with other studies in mice showing that IRS1 and IRS2 knockout prevents the repressive FoxO1 phosphorylation (Taniguchi et al., 2005; Dong et al., 2006; Cheng et al., 2009; Qi et al., 2013). Importantly, we demonstrate that FoxO1 mediates the beneficial effects of IRS1 downregulation on proteasome CT-L activity and on the protein expression of β5. Supporting the notion that FoxOs may serve discrete or tissue-specific functions (Paik et al., 2007), FoxO3 repression did not downregulate proteasome activity and was not required for the enhanced proteasome activity under conditions of diminished IRS1 expression. These results indicate that FoxO1 is a potent regulator of proteasome function downstream of IIS. FoxO transcription factors target either a conserved DNA binding sequence, 5′-TT(G/A)TTTAC-3′ (daf-16 family binding element, DBE) or the insulin response element (IRE), 5′-TT[G/A]TTTTG-3′ in the promoter regions of their target genes and subsequently regulate gene expression (Furuyama et al., 2000). Importantly, after analyzing putative FoxO1 binding sites in several 20S proteasome subunits, we show that FoxO1 directly binds to the promoter region of β5, in a region containing a candidate IRE element (5′-TTATTTTG -3′). As expected, FoxO1 binding to the β5 promoter was found increased in MEFs lacking IRS1. The functionality of the binding site detected through the ChIP-based experiment was further analyzed by cloning the wild type or a mutagenized β5 promoter into a luciferase reporter system. Supporting the notion that FoxO1 upregulates β5 expression, silencing of FoxO1 led to a significant decrease of β5 promoter activity, while IRS1 silencing led to an increase. Furthermore, the site specific mutagenesis of 8 bp of the detected IRE at position −237 downregulated gene expression and completely blunted the response to FoxO1 or to IRS1 silencing. Notably, mutation of the identified FoxO binding sequence yielded a reduction of luciferase signal similar to FoxO1 silencing. The detected variations of luciferase activity due to mutagenesis or silencing are biologically significant and fall within the typically observed range (Xiong et al., 2012; Singh et al., 2017). Moreover, as β5 is also regulated by other transcriptional factors such Nrf1 (Li et al., 2018), we did not expect that the mutation of the FoxO1 binding site would totally abolish β5 expression. Importantly, β5 in addition to being responsible for the rate-limiting chymotrypsin-like proteolytic activity of the proteasome (Kisselev et al., 1999), was shown to be sufficient to induce the expression of additional proteasome subunits and consequently to enhance 26/30S proteasome assembly and activity when overexpressed in human fibroblasts (Chondrogianni et al., 2005), in human mesenchymal stem cells (Kapetanou et al., 2017) and in *C. elegans* (Chondrogianni et al., 2015a). Hence, the stimulation of the β5 subunit by Foxo1 has implications on the regulation of the whole proteasome machinery. Collectively, these data demonstrate that FoxO1 binds to a functional IRE at β5 promoter to activate gene expression and enhance proteasome activity.

Taken together these data demonstrate that IIS in mice regulates proteostasis and, in turn, lifespan. Our findings provide new insights about the mechanisms regulating the activity of the proteasome and expand our knowledge of how the nutrient signaling pathways affect proteostasis and ultimately longevity. Understanding the mechanisms that mediate the beneficial effects of reduced IIS activity is of major importance

for the development of effective treatments to improve health span in humans.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen.

AUTHOR CONTRIBUTIONS

MK: conceptualization, methodology, data collection and analysis, and writing the manuscript. TN: methodology, data collection and analysis, and editing the manuscript. LT: methodology and editing the manuscript. AP: data collection and analysis. LP: conceptualization, supervision, editing the manuscript, and funding acquisition. EG: conceptualization, supervision, writing and editing the manuscript, and funding acquisition. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Mechanisms of Cellular Senescence: Cell Cycle Arrest and Senescence Associated Secretory Phenotype

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Cellular senescence is a stable cell cycle arrest that can be triggered in normal cells in response to various intrinsic and extrinsic stimuli, as well as developmental signals. Senescence is considered to be a highly dynamic, multi-step process, during which the properties of senescent cells continuously evolve and diversify in a context dependent manner. It is associated with multiple cellular and molecular changes and distinct phenotypic alterations, including a stable proliferation arrest unresponsive to mitogenic stimuli. Senescent cells remain viable, have alterations in metabolic activity and undergo dramatic changes in gene expression and develop a complex senescence-associated secretory phenotype. Cellular senescence can compromise tissue repair and regeneration, thereby contributing toward aging. Removal of senescent cells can attenuate age-related tissue dysfunction and extend health span. Senescence can also act as a potent anti-tumor mechanism, by preventing proliferation of potentially cancerous cells. It is a cellular program which acts as a double-edged sword, with both beneficial and detrimental effects on the health of the organism, and considered to be an example of evolutionary antagonistic pleiotropy. Activation of the p53/p21$^{WAF1/CIP1}$ and p16^{INK4A}/pRB tumor suppressor pathways play a central role in regulating senescence. Several other pathways have recently been implicated in mediating senescence and the senescent phenotype. Herein we review the molecular mechanisms that underlie cellular senescence and the senescence associated growth arrest with a particular focus on why cells stop dividing, the stability of the growth arrest, the hypersecretory phenotype and how the different pathways are all integrated.

Keywords: cellular senescence, cell cycle arrest, senescence associated secretory phenotype (SASP), DNA damage response (DDR), DREAM complex

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INTRODUCTION

Cellular senescence, a seminal discovery of Hayflick and Moorhead (1961) is a process that globally regulates cell fate and can be considered a hallmark of aging (Hayflick and Moorhead, 1961; López-Otín et al., 2013). Hayflick demonstrated that upon serial passaging, normal human diploid fibroblast cell strains cease to divide *in vitro* after a fixed number (40–60) of population doublings, the Hayflick limit (Hayflick and Moorhead, 1961).

Senescence is triggered by developmental signals or different kinds of stress. Depending on the cell type and intensity and nature of the stress, cells may respond by inducing repair, cell death

or senescence (Surova and Zhivotovsky, 2013; Galluzzi et al., 2018; Sapieha and Mallette, 2018). Cells can undergo senescence in response to various intrinsic and extrinsic stimuli, including progressive telomere shortening, changes in telomeric structure, mitogenic signals, oncogenic activation, radiation, oxidative and genotoxic stress, epigenetic changes, chromatin disorganization, perturbed proteostasis, mitochondrial dysfunction, inflammation, and/or tissue damage signals, irradiation, or chemotherapeutic agents, nutrient deprivation (Di Micco et al., 2006; Kuilman et al., 2010; Passos et al., 2010; Pazolli et al., 2012; García-Prat et al., 2016; Mikuła-Pietrasik et al., 2020).

These different types of stress signals give rise to different types of senescence such as telomere dependent replicative senescence, programmed senescence or non-telomeric stress-induced premature senescence including oncogene-induced senescence (OIS), unresolved DNA damage induced senescence, epigenetically induced senescence and mitochondrial dysfunction associated senescence (Toussaint et al., 2002; Debacq-Chainiaux et al., 2016). An extensive study by Petrova et al. (2016) identified more than 50 small chemical compounds that can induce premature senescence and senescence-like states. Recent studies have demonstrated that treatment with some anticancer agents, chemotherapeutic drugs or ionizing radiation provoke "therapy-induced senescence (TIS)" in tumor cells (Di Micco et al., 2006; Ewald et al., 2010; Dörr et al., 2013; Toso et al., 2014; Petrova et al., 2016; Dabrowska et al., 2018; Saleh et al., 2018, 2019; Mikuła-Pietrasik et al., 2020).

Senescence is now considered to be a highly dynamic, multi-step process, during which the properties of senescent cells continuously evolve and diversify in a context dependent manner (Van Deursen, 2014; Boisvert et al., 2018). It is associated with multiple cellular, molecular changes and distinct phenotypic alterations including a stable and generally irreversible proliferation arrest unresponsive to mitogenic stimuli. Senescent cells remain viable with alterations in metabolic activity and are usually resistant to apoptosis (Wang, 1995; Hampel et al., 2004; Marcotte et al., 2004; Ryu et al., 2007; Sanders et al., 2013; Childs et al., 2014; Zhu et al., 2015). They undergo dramatic gene expression changes along with chromatin remodeling and engagement of a persistent DNA damage response (DDR) (Rodier et al., 2009; Wang et al., 2009; Sulli et al., 2012; Chandra et al., 2015). One characteristic feature of senescent cells is increased lysosomal activity (Kurz et al., 2000; Lee et al., 2006), macromolecular damage (Gorgoulis et al., 2019), and a temporal cascade in the development of the complex senescence-associated secretory phenotype (SASP) (Coppé et al., 2008, 2010a; Saleh et al., 2018). Senescent cells can also develop morphological and structural changes, including an enlarged, flattened, multinucleated morphology with enlarged vacuoles (Campisi and D'Adda Di Fagagna, 2007), altered composition of the plasma membrane and a remarkable nuclear enlargement (Kuilman et al., 2010; Salama et al., 2014; Frescas et al., 2017; Hernandez-Segura et al., 2018; Ramos et al., 2020). These complex changes to the cell serve to implement various aspects of senescence such as growth arrest and the development of SASP secretome.

Initially senescence was thought to be a tissue culture artifact. However, multiple subsequent studies have demonstrated the importance of senescence in different physiological and pathological processes (Burton and Krizhanovsky, 2014). Senescence plays key physiological roles in normal development (Muñoz-Espín et al., 2013; Storer et al., 2013), maintaining tissue homeostasis, tissue remodeling and repair (Yun et al., 2015), wound healing (Ramakrishna et al., 2012; Demaria et al., 2014), secretion of insulin by pancreatic beta cells (Helman et al., 2016), and limits tumor progression by ensuring that potentially dysfunctional, damaged or transformed cells do not perpetuate their genomes to the next generation (Collado et al., 2007; Hanahan and Weinberg, 2011; Kang et al., 2015; Childs et al., 2017; Maciejowski and De Lange, 2017; Faget et al., 2019; Wang et al., 2020).

Senescent cells have been found to accumulate exponentially with increasing chronological age in multiple tissues (Muñoz-Espín and Serrano, 2014; Hudgins et al., 2018). The early work of Hayflick and Moorhead (1961) for the first time hinted toward a relationship between senescence and aging, but subsequent discoveries have demonstrated the presence of senescent cells *in vivo* and an increase in their number with age supporting the hypothesis that senescence itself can drive aging and is one of its key hallmarks (Hayflick and Moorhead, 1961; Hayflick, 1965; López-Otín et al., 2013).

Cellular senescence also has deleterious effects as it can hinder tissue repair and regeneration and contribute to tissue and organismal aging due to the accumulation of senescent cells and depletion of stem/progenitor cell compartments and secretion of SASP (Coppé et al., 2010a; Campisi et al., 2011). Senescent cells have been observed in several age-related diseases such as atherosclerosis, diabetes, lung disease, and many others (Muñoz-Espín and Serrano, 2014; Chandrasekaran et al., 2017; McHugh and Gil, 2018). Although senescence is associated with aging, cells can undergo senescence irrespective of organismal age due to different signals apart from telomere shortening. In accordance with this the use of transgenic mouse models have allowed the detection of senescent cells in different age related pathologies and enabled the development of genetic or pharmacological strategies to demonstrate that selective elimination of senescent cells can prevent or delay age-related tissue dysfunction to extend life span and improve health span (Baker et al., 2011; Xu et al., 2015a; Baker et al., 2016; Hashimoto et al., 2016; Zhao et al., 2018).

Cellular senescence is a cellular program which acts as a double-edged sword with both beneficial and detrimental effects on the health of the organism, and thereby considered to be an example of evolutionary antagonistic pleiotropy (Williams, 1957; Kirkwood and Austad, 2000; Campisi, 2003; Giaimo and D'Adda di Fagagna, 2012; Ohtani et al., 2012; Schosserer et al., 2017).

Taken together, senescence is both a physiologically fundamental and pathologically relevant program, with its role depending on the context and the specific situation. Here, we review the different mechanisms controlling cellular senescence with a special focus on cell cycle arrest and SASP. We detail the complexity of the mechanisms involved in SASP regulation, focus on the key mediators, characteristic hallmarks and the different pathways involved in manifesting cellular senescence

as well as the cell cycle arrest and its key regulators along with the role of the DREAM complex and its associated components. The significance of cellular senescence in different contexts such as its role *in vivo*, in cancer and aging are also discussed. At the end we discuss the translational relevance and suitability for identifying and characterizing senescent cells *in vivo* to explore potential future avenues for exploiting the benefits and preventing the detrimental aspects of senescent cells such as suppressing the SASP or selectively eliminating senescent cells to increase health span.

SENESCENCE MEDIATED CELL CYCLE ARREST

The cell cycle is a sequence of coordinated events which lead to cell division, critical for both development and viability of multicellular organisms. A stable cell cycle arrest which marks an inability of the cell to continue dividing is an indispensable and one of the defining features of senescent cells. Cell cycle arrest can be an alarm response instigated by aberrant proliferation or deleterious stress stimuli to prevent the propagation of dysfunctional cells.

Cellular senescence is different from another form of growth arrest known as quiescence, in that senescence occurs in G1 and possibly G2 phase of the cell cycle (Di Leonardo et al., 1994) as opposed to quiescence which happens in G0. Another crucial difference is that quiescent cells can resume proliferation in response to appropriate signals such as stimulation by growth factors or mitogenic signals whereas senescent cells cannot (Campisi and D'Adda Di Fagagna, 2007; Calcinotto et al., 2019; Gorgoulis et al., 2019; Mohamad Kamal et al., 2020). This is beautifully explained by Blagosklonny's theory of 'hyperfunction' which states that aging is a quasi-program, that occurs as a consequence of processes occurring during development and growth in early life (Blagosklonny, 2013). For example, during growth arrest, the nutrient sensing pathways like mTOR (mechanistic target of Rapamycin) remain active but now as opposed to cell proliferation and growth, this initiates cellular senescence. Therefore, the choice between senescence and quiescence is governed to a certain extent by the mTOR pathway. Cells with persistent activation of mTOR undergo a stable senescent growth arrest, whereas cells undergo quiescence when mTOR is inhibited (Korotchkina et al., 2010; Blagosklonny, 2012). Apoptosis is a programmed cell death in which the remains of a dead apoptotic cell are removed by engulfment by another cell, whereas in senescence, the senescent cell is not immediately eliminated and remains metabolically active despite being in an arrested state. Senescence is also different from terminally differentiated cells which have also irreversibly withdrawn from the cell cycle wherein undifferentiated precursor cells are converted into specialized effector cells (Baumann, 2016; Hinze and Boucrot, 2018; Mohamad Kamal et al., 2020). All together distinct signaling pathways are involved in terminal differentiation such as Notch, Wingless and Hedgehog (Gorgoulis et al., 2019). Terminally differentiated cells can also undergo cellular senescence showing that senescence does not depend on an active cell cycle (Jurk et al., 2012; von Zglinicki et al., 2020). Although the cell cycle arrest in cellular senescence is believed to be irreversible, studies have recently shown that senescent cells can under certain circumstances re-enter the cell cycle such as in tumor cells (Galanos et al., 2016; Patel et al., 2016; Milanovic et al., 2018; Saleh et al., 2019) or be reprogrammed into induced pluripotent stem cells (Banito and Gil, 2010; Lapasset et al., 2011).

Cell cycle arrest in senescence is largely mediated via activation of either one or both p53/p21$^{WAF1/CIP1}$ and p16^{INK4A}/pRB tumor suppressor pathways (Rovillain et al., 2011; Kobashigawa et al., 2019; Liu and Wan, 2019). Both these pathways are complex as they involve many upstream regulators and downstream effectors along with varying side branches (Chau and Wang, 2003; Levine and Oren, 2009). Both pathways are also interlinked with extensive crosstalk (Martín-Caballero et al., 2001; Zhang et al., 2006; Yamakoshi et al., 2009). They maintain the senescence state mainly by inducing widespread changes in gene expression as p53 and pRB are key transcriptional regulators; p21$^{WAF1/CIP1}$ acts downstream of p53 whereas p16^{INK4A} acts upstream of pRB. They are the crucial components of each pathway as they are cyclin-dependent kinase inhibitors (CDKIs) and act as negative regulators of cell cycle progression. Prolonged overexpression of any of these four critical components (p53, pRB, p16^{INK4A}, p21$^{WAF1/CIP1}$) is sufficient to induce senescence (McConnell et al., 1998). **Figure 1** summarizes the different signals and pathways involved in mediating senescence mediated cell cycle arrest.

p53/p21$^{WAF1/CIP1}$ Pathway

p53/p21$^{WAF1/CIP1}$ is activated in response to DNA damage caused by telomere attrition, oxidative or oncogenic stress. Constitutive DNA damage response (DDR) signaling leads to chronic activation of p53 which induces cellular senescence. Inactivation of p53 mediated signaling by a variety of approaches can disrupt the onset of cellular senescence (Shay et al., 1991; Beauséjour et al., 2003).

p53, famously known as the 'Guardian of the genome' plays a key role in manifestation of cellular senescence via several different mechanisms (Lane, 1992; Kastenhuber and Lowe, 2017). Activation of p53 is dependent on various post translational modifications such as phosphorylation, methylation, acetylation, sumoylation, ubiquitination, and neddylation (Kruse and Gu, 2009). Increased Ser-15 phosphorylation by Ataxia Telengectasia Mutated (ATM) kinase results in p53 stabilization and was found to be the only common change between replicative senescence and DNA damage induced senescence (Webley et al., 2000). Activated p53 regulates expression of a set of anti-proliferative genes (Olivier et al., 2010; Kastenhuber and Lowe, 2017).

As p53 performs different functions within a cell, it is regulated at multiple different levels by different factors. MDM2, an E3 ubiquitin ligase regulates the levels of p53 in conjunction with MDM4. Interaction of p53 with FOXO4 during cellular senescence plays a crucial role in regulating its transcriptional activity and localization (Baar et al., 2017). The signaling mediated by FOXO and its target protein 4E-BP regulates aging in Drosophila by removing damaged proteins

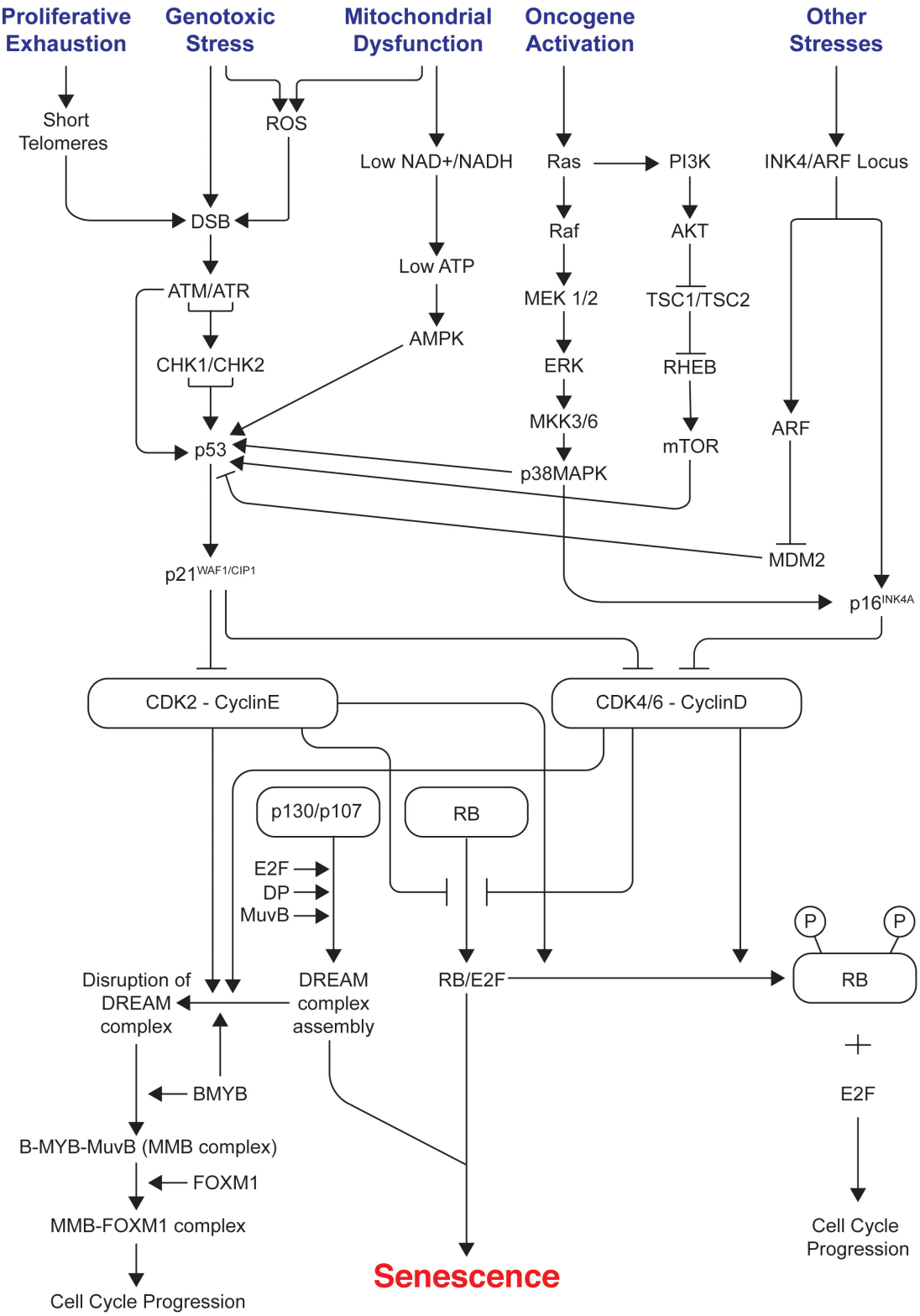

FIGURE 1 | Signals and pathways involved in mediating senescence mediated cell cycle arrest. The figure shows the different intrinsic and extrinsic stimuli capable of inducing cellular senescence. Key pathways involved in manifesting cell cycle arrest in senescence such as p53/p21$^{WAF1/CIP1}$ and p16^{INK4A}/RB tumor suppressor pathways, DDR, AMPK, p38/MAPK, PI3K/AKT/mTOR are illustrated. It indicates how different pathways are interconnected and how the assembly of repressive DREAM complex triggers senescence and its disruption leads to cell cycle progression. ROS, reactive oxygen species; DDR, DNA damage response; DREAM, dimerization partner (DP), RB-like, E2F, and MuvB core complex.

thereby delaying muscle function decay and extending life span (Demontis and Perrimon, 2010).

$p21^{WAF1/CIP1}$, a 21 KDa protein encoded by the CDKN1A gene, is a member of the Cip/Kip family of CDKIs in addition to p27 and p57. It is capable of inactivating all CDKs, thereby inhibiting cell cycle progression (Wade Harper et al., 1993). It inhibits the kinase activity of cyclin-CDK complexes by interacting with cyclins through the two cyclin binding motifs (Cy1 and Cy2). This leads to inhibition of phosphorylation of the RB family of proteins and subsequent association with E2Fs and formation of the DREAM complex thereby leading to a cell cycle arrest (Chen et al., 1996; Whittaker et al., 2017; Wiedemeyer, 2018). $p21^{WAF1/CIP1}$ plays a dual conflicting role in cell cycle progression depending on its level of expression (Al Bitar and Gali-Muhtasib, 2019). High levels of $p21^{WAF1/CIP1}$ inhibit the kinase activity of cyclinD/CDK4,6 complexes leading to inhibition of cell cycle progression whereas low levels of $p21^{WAF1/CIP1}$ act as an assembly factor for cyclinD/CDK4,6 complex and promote its activation resulting in cell cycle progression (Labaer et al., 1997; Welcker et al., 1998; Cheng et al., 1999; Deng et al., 2018). $p21^{WAF1/CIP1}$ was the first identified transcriptional target for p53 (El-Deiry et al., 1993). As it is known to interact with and inactivate various cyclin/CDK complexes, it is capable of inducing cell cycle arrest at any stage of the cell cycle as opposed to the INK4 family of CDKIs which specifically bind and inactivate CDK4 and CDK6, thereby inducing a cell cycle arrest only during G0/G1 phase (Wade Harper et al., 1993; Pavletich, 1999; Sherr, 2000). $p21^{WAF1/CIP1}$ can also be activated by p53 independent mechanisms by other stimulators such as nuclear receptors including androgen, vitamin D and retinoid receptors. Members of the Krüppel-like factor (KLF) transcription factor (TF) family can activate the *CDKN1A* gene by cooperating with p300-CREBBP (Aliouat-Denis et al., 2005; Abbas and Dutta, 2009).

Induction of $p21^{WAF1/CIP1}$ is crucial for initiation of senescence mediated growth arrest by different stimuli (Noda et al., 1994; Hernandez-Segura et al., 2017). Upregulation of $p21^{WAF1/CIP1}$ plays a key role in developmental senescence as mice lacking it show defects in embryonic senescence, apical ectodermal ridge maintenance and patterning as well as other developmental defects. Developmental senescence is a transient programmed cellular senescence that occurs during mammalian embryonic development (Muñoz-Espín et al., 2013; Storer et al., 2013). However, expression of $p21^{WAF1/CIP1}$ does not persist in senescent cells as it is mainly required for induction of senescence (Stein et al., 1999; Sharpless and Sherr, 2015; He and Sharpless, 2017; Song et al., 2020). In contrast $p16^{INK4A}$ is required to maintain the senescent state.

In addition to the transcriptional control of $p21^{WAF1/CIP1}$ by p53 dependent and independent mechanisms, it is also regulated at the post translational level. Newly synthesized $p21^{WAF1/CIP1}$ is stabilized by WISp39, a Hsp90 binding tetratricopeptide repeat protein, that prevents its proteasome mediated degradation (Jascur et al., 2005). Additional post-translational modifications such as phosphorylation can modulate binding partners or change the subcellular location, which has the potential to alter its function by blocking its ability to act as a CDKI (Child and Mann, 2006). $p21^{WAF1/CIP1}$, when present within the nucleus, inhibits cell cycle progression, whereas upon phosphorylation it gets transported to the cytoplasm where it functions as an anti-apoptotic protein (Ping et al., 2006). Therefore, $p21^{WAF1/CIP1}$ plays multiple roles within the cell by regulating different processes (Karimian et al., 2016; Georgakilas et al., 2017).

Transient stress leads to induction of p53 which activates DNA repair and leads to quiescence (Vousden and Prives, 2009; Kasteri et al., 2018). Cells can resume proliferation upon resolution of the stress (Childs et al., 2015). Additional signals and persistent stress can lead to sustained expression of p53 and activation of $p16^{INK4A}$ contributing to a long lasting cell cycle arrest (Sharpless and DePinho, 2006; Salama et al., 2014; Kruiswijk et al., 2015). Cell fate is determined by different factors which is further complicated by the context dependent role of $p16^{INK4A}$ and $p21^{WAF1/CIP1}$.

p53 is known to indirectly downregulate expression of many factors required for cell cycle progression. Essentially all the genes are downregulated indirectly by p53 as only about 3% of them are directly bound by it (Fischer et al., 2014). Repression by p53 involves direct activation of $p21^{WAF1/CIP1}$ leading to formation of different repressive complexes such as RB/E2F and DREAM (Fischer et al., 2014, 2016a,b; Fischer and Müller, 2017).

$p16^{INK4A}$/pRB Pathway

The RB family of pocket proteins is one of the main targets of cyclin-CDK complexes and their best-known function is binding to and inactivating E2F complexes leading to repression of E2F target gene transcription. There are three members of the RB pocket protein family: RB1 (pRB), RBL1 (p107), and RBL2 (p130). These proteins share a common bipartite pocket region comprising a LXCXE motif, which allows them to interact directly with other proteins (Dyson, 1998).

pRB when dephosphorylated binds to E2Fs thereby forming a repressive RB-E2F complex. These repressive complexes bind to the promoter regions of E2F target genes and inhibit the transcription of genes required for cell cycle progression (Fischer and Müller, 2017). To enhance transcription repression, they recruit factors such as histone deacetylases (HDACs) and the histone methyltransferase SUV39H1. At the restriction point this inhibition is removed by hyperphosphorylation of RB by cyclinE-CDK2 which leads to release of E2Fs, thereby promoting transcription of S phase genes and hence progression of the cell cycle (Zhang et al., 2000). It has been suggested that crosstalk between RB and mitogenic AKT signaling pathways play a key role in the quiescence to senescence switch by regulating overlapping functions of Forkhead transcription factors, FOXO3a and FOXM1 (Eijkelenboom and Burgering, 2013; Lam et al., 2013; Imai et al., 2014). Moreover Argonaute (AGO2), microRNA (let-7) and RB1 interact in the nucleus to repress certain E2F target genes such as CDC2 and CDCA8 during senescence (Benhamed et al., 2012).

INK4/ARF locus encodes three tumor suppressors namely $p16^{INK4A}$ and $p14^{ARF}$ encoded by CDKN2A gene and $p15^{INK4B}$ by CDKN2B gene (Sharpless, 2005; Gil and Peters, 2006). Similarly, to $p21^{WAF1/CIP1}$, $p15^{INK4B}$, and $p16^{INK4A}$ are CDKIs

and affect cell cycle progression by binding to and inhibiting CDK4/6. In contrast, $p14^{ARF}$ establishes cross talk between the p53 and pRB pathways by regulating the stability of p53 by binding to and inhibiting MDM2, responsible for its proteasome-mediated degradation (Gil and Peters, 2006; Kim and Sharpless, 2006). Expression of ARF is regulated by p53 via a negative feedback loop (Kotake et al., 2011).

$p16^{INK4A}$ is a 16 KDa protein that directly binds to CDK4/6 and blocks the formation of cyclinD-CDK4/6 complexes, thereby preventing phosphorylation of RB and promoting expression of E2F target genes (Serrano et al., 1993). This crucial role is evident from the fact that loss of the $p16^{INK4A}$ gene or inherited mutations within it have been frequently related to several human cancers particularly malignant melanoma (Gil and Peters, 2006; Kim and Sharpless, 2006; Li et al., 2011). This suggests that inactivation or loss of $p16^{INK4A}$ leads to bypass of senescence, thereby promoting cancer.

Epigenetically induced senescence mostly acts by inducing $p16^{INK4A}$ expression as opposed to DNA damage-induced senescence which relies mainly on $p21^{WAF1/CIP1}$ (Petrova et al., 2016). Since epigenetic modifiers are capable of maintaining the senescent state without inducing any cell stress, epigenetically induced senescence has been characterized as 'causeless' which makes it similar to the senescence observed during development or upon aging as opposed to DNA damage-induced senescence which occurs prematurely due to induction of different forms of stress (Petrova et al., 2016).

Replicative senescence is also linked to derepression of the CDKN2A locus. In young tissues, the CDKN2A locus is normally expressed at a very low undetectable level whereas it becomes derepressed leading to a high-level of expression upon aging (Krishnamurthy et al., 2004). The molecular mechanisms underlying this derepression are not completely understood but have been associated with loss of polycomb group of proteins but independent of p53 (Jacobs et al., 1999; Bracken et al., 2007).

Polycomb proteins are a group of conserved proteins required to maintain stable repression of specific target genes by histone modification (Gould, 1997; Van Lohuizen, 1998). Polycomb protein complexes such as PRC1/PRC2 silence the INK4/ARF locus (Martin et al., 2014). Therefore, $p16^{INK4A}$ mediated senescence can be induced via disruption of PRC1/PRC2 complex components such as CBX7, BMI1 or EZH2 followed by decrease in levels of H3K27me3 (Jacobs et al., 1999; Bracken et al., 2003, 2007; Gil et al., 2004). Epigenetic regulation of the INK4/ARF locus is not limited to polycomb proteins as other epigenetic regulators such as ZRF, MLL1 or JMJD3 are also involved in its regulation (Gil and Peters, 2006; Agger et al., 2009; Barradas et al., 2009; Kotake et al., 2009; Ribeiro et al., 2013). Polycomb group members such as CBX7, EED, EZH2 and SUZ12 are downregulated by microRNAs miR-26b, miR-181a, miR-210, and miR-424, which leads to $p16^{INK4A}$ activation and hence senescence induction (Overhoff et al., 2014).

The epigenetic alterations occurring during senescence are quite diverse and cell type and context dependent. Alterations in DNA methylation are observed during replicative senescence whereas cells undergoing oncogene induced senescence do not show any such alterations in DNA methylation (Cruickshanks et al., 2013; Cheng et al., 2017; Xie et al., 2018).

Cell cycle progression can also be affected by TGF-β as it maintains RB in a hypophosphorylated state, thereby inducing cell cycle arrest in lung epithelial cells in G1 (Laiho et al., 1990). In addition to $p16^{INK4A}$, $p15^{INK4B}$ has been shown to an effector of TGF-β meditated cell cycle arrest by inhibiting CDK4/6 (Hannon and Beach, 1994).

DREAM Complex Mediated Cell Cycle Arrest

Sadasivam and DeCaprio (2013) described the DREAM complex as the master coordinator of cell cycle-dependent gene expression. DREAM is a multi-subunit complex formed by the assembly of p130 and p107 (RB family of pocket proteins) with Dimerization partner (DP), E2F4-5 and a Multivulval class B (MuvB) core complex which represses most if not all gene expression in quiescence (Litovchick et al., 2007). The MuvB core complex comprises LIN9, LIN37, LIN52, LIN54, and RBBP4 (Sadasivam et al., 2012), originally identified in *Caenorhabditis elegans* (Fay and Yochem, 2007).

During G0 all cell cycle dependent gene expression is repressed by binding of the DREAM complex. It has been shown that in mammalian cells, phosphorylation of p130 leads to dissociation of the DREAM complex resulting in the MuvB core complex recruiting B-MYB to activate late S-phase genes, and FOXM1, in G2 phase, to activate mitotic gene expression (Litovchick et al., 2007; Schmit et al., 2007; Sadasivam et al., 2012). Since the DREAM complex binds to cell cycle genes homology region (CHR) promoter elements in addition to E2F binding sites (Schmit et al., 2009; Müller and Engeland, 2010; Müller et al., 2012), it has the potential to regulate a larger set of genes than RB and perform distinct regulatory functions apart from RB/E2F complexes (Müller et al., 2012; Guiley et al., 2015; Fischer and Müller, 2017). Assembly of the DREAM complex also requires phosphorylation of the LIN52 component of the MuvB core complex at Serine-28 (Litovchick et al., 2011). Even though the role of the DREAM complex in cellular senescence is not fully understood, it has been shown that disorganization of DREAM complex by ectopic expression of a non-phosphorylatable LIN52 leads to suppression of Ras-induced senescence (Litovchick et al., 2011; Iness et al., 2019).

Initially, the detailed mechanism by which p53 mediates transcriptional repression of a plethora of genes was not understood, however this changed after the availability of genome-wide ChIP data on p53 binding sites and the discovery of the mammalian DREAM complex along with its target genes (Litovchick et al., 2007; Schmit et al., 2007). This has led to the observation that p53 induction leads to the formation of the repressive DREAM complex and the identification of the p53-DREAM pathway (Quaas et al., 2012). The discovery of $p21^{WAF1/CIP1}$-DREAM-E2F/CHR pathway has provided a clearer explanation of how p53 downregulates a plethora of genes by activating $p21^{WAF1/CIP1}$. It has also demonstrated the role of the p53-DREAM pathway in halting cell cycle progression in response to a number of stress signals including

DNA damage as it leads to activation and stabilization of p53 (Horn and Vousden, 2007).

The key step in the p53-DREAM pathway is the upregulation of $p21^{WAF1/CIP1}$ via direct binding of p53 to sites present in the $p21^{WAF1/CIP1}$ promoter (Quaas et al., 2012). As $p21^{WAF1/CIP1}$ is a CDKI it blocks phosphorylation of the pRB related pocket proteins, p107 and p130 as well as RB. In the unphosphorylated state p107 and p130 proteins bind to the MuvB core complex promoting the assembly of the repressive DREAM complex. Therefore, activation of p53 can shift the equilibrium from the activating MMB-FOXM1 complex to the repressive DREAM complex in a $p21^{WAF1/CIP1}$ dependent manner (Quaas et al., 2012). During this stage, the DREAM complex shows parallel regulation along with pRB mediated regulation because lack of phosphorylation of pRB leads to the formation of repressive RB/E2F complexes (Dyson, 2016).

A recent study using meta-analyses of genome-wide studies has identified a catalog of more than 250 high confidence target genes of the p53-DREAM pathway (Engeland, 2017). This pathway controls genes important for cell functions spanning from the start (G1 phase) to the end of the cell cycle (M phase). Hence, p53 employs its master coordinator functions via the DREAM complex mediated mainly by $p21^{WAF1/CIP1}$. Defects in the p53-pathway contribute to a loss of checkpoint control not only at the G1/S transition but at all checkpoints up to completion of the cell cycle. Activator E2F1-3 proteins bind to E2F elements for maximum expression of the genes involved in S phase whereas MMB-FOXM1 complex binds to CHR promoter elements to upregulate genes expressed in late G2 and M phase of cell cycle. The identification and detailed understanding of the DREAM complex has provided a clearer explanation for the precisely timed regulation of the G2/M cell cycle genes in addition to expression of late S phase genes by B-MYB-MuvB (MMB). This complements the well-established regulation of G1/S cell cycle genes by RB mediated repression of E2F TFs. In some senescent cells, senescence associated heterochromatin foci (SAHF) formation by pRB dependent reorganization of chromatin leads to silencing of E2F target genes (Narita et al., 2003). The stability of the cell cycle arrest during senescence is enforced by ROS production, secretion of cytokines and the heterochromatinization of E2F target genes. Derepression of retrotransposons and ribosome biogenesis defects have recently been discovered to be features of cell cycle arrest observed in senescent cells (Lessard et al., 2018; De Cecco et al., 2019).

There are striking chromatin alterations in senescent cells (Adams, 2007). Along with DDR (D'Adda Di Fagagna, 2008) and formation of PML bodies (Ferbeyre et al., 2000), SAHFs are the most prominent morphological change in chromatin (Narita et al., 2003). SAHF foci can be readily detected by DNA dyes such as DAPI and are characterized by enrichment of heterochromatin-associated repressive histone marks such as H3K9Me2, H3K9Me3 and chromatin reorganizing proteins such as heterochromatin protein (HP1), histone repressor A (HIRA) and anti-silencing function-1a (ASF1a), high mobility group A (HMGA) proteins, increased nuclear pore density and loss of linker histone H1 (Funayama et al., 2006; Chandra et al., 2012; De Cecco et al., 2013; Sadaie et al., 2013; Swanson et al., 2013; Salama et al., 2014; Chandra, 2016; Criscione et al., 2016; Boumendil et al., 2019; Chan and Narita, 2019). Since SAHFs are not seen in all senescent cells, it seems that they are cell type and stimulus dependent (Kennedy et al., 2010; Di Micco et al., 2011; Aird and Zhang, 2013; Criscione et al., 2016; Zirkel et al., 2018). Downregulation of Lamin B1, a key component of the nuclear lamina is a key feature of senescent cells, is known to trigger global and local chromatin changes impacting gene expression and promoting SAHF formation during senescence (Freund et al., 2012; Sadaie et al., 2013; Shah et al., 2013; Chandra et al., 2015).

Along with different mechanisms controlling cellular senescence, non-coding RNAs especially micro RNAs (miRNAs) have been demonstrated to play a key role in mediating cellular senescence alone or in conjunction with other effectors. Multiple studies have demonstrated that different miRNAs modulate the levels of key senescence effectors such as p53 (Hu et al., 2010; Burns et al., 2011; Xiao et al., 2011), $p21^{WAF1/CIP1}$ (Borgdorff et al., 2010), $p16^{INK4A}$ (Lal et al., 2008; Overhoff et al., 2014; Philipot et al., 2014), and SIRT1 (Suh, 2018; Baker et al., 2019; Barnes et al., 2019). miR 124, miR-34a/b/c, and miR-29a/b/c are upregulated in response to p53 activation and facilitate cellular senescence by downregulating survival and cell proliferation factors (Hermeking, 2010; Boon et al., 2013; Hu et al., 2014; Xu S. et al., 2019). A recent mRNA microarray and gene co-expression network analysis has revealed that most of the mRNAs that were downregulated by the activity of miRNA's were involved in regulation of cell cycle progression (Xu S. et al., 2019). Fascinatingly, Ccna2 mRNA emerged as a common target of miR-29 and miR-124, which act as antagonists of $p21^{WAF1/CIP1}$. This study showed that Ccna2 silencing significantly induced senescence whereas ectopic expression of exogeneous Ccna2 reversed the effect of miR-29 and miR-124, thereby substantially delaying cellular senescence and enhancing cell viability (Xu S. et al., 2019). This highlighted the important effect of Ccna2 in cellular senescence and identified a novel senescence regulator p53/miRNAs/Ccna2 pathway which acts independently of the canonical p53/$p21^{WAF1/CIP1}$ pathway as the p53 responsive miRNAs were found to be significantly upregulated during senescence in $p21^{WAF1/CIP1}$ deficient cells (Xu S. et al., 2019). Recently long non-coding RNAs (lncRNAs) which are more than 200 nucleotides long and capable of binding to DNA, RNA or proteins have been demonstrated to play a role in regulating senescence (Kim C. et al., 2017; Hu et al., 2018).

THE SENESCENCE ASSOCIATED SECRETORY PHENOTYPE

Although senescent cells are in a growth arrested state, they remain metabolically active. Senescence does not only affect the events inside the cell but has the potential to affect the surroundings and communicate with neighboring cells by secreting a complex mixture of secreted factors which can alter the behavior of nearby non-senescent cells (Sun et al., 2018; Lopes-Paciencia et al., 2019; Mohamad Kamal et al., 2020). Cells undergoing senescence demonstrate significant changes in their

secretome and exhibit a hyper secretory phenotype called the Senescence Associated Secretory Phenotype (SASP) (Coppé et al., 2010a) or Senescence-Messaging Secretome (SMS) (Kuilman and Peeper, 2009), one of the key hallmarks of senescence (Gorgoulis et al., 2019).

The main components of SASP include a plethora of soluble signaling factors, such as, pro-inflammatory cytokines, chemokines, growth modulators, angiogenic factors, proteases, bioactive lipids, extracellular matrix components, and matrix metalloproteinases (MMPs) (Coppé et al., 2010a; Freund et al., 2010; Acosta et al., 2013; Lopes-Paciencia et al., 2019). Even though multiple studies have identified the SASP components in different cell types, the exact composition of SASP remains elusive and is the topic of ongoing research. IGFBP3, IGFBP4, and IGFBP7 are key players of SASP that are suggested to be involved in mediating senescence by paracrine signaling (Wajapeyee et al., 2008; Severino et al., 2013; Özcan et al., 2016). The ability of IGFBP3 to induce senescence is regulated by tissue-type plasminogen activator inhibitor-1 (PAI-1) system (Elzi et al., 2012). PAI-1 is a critical downstream target of p53 involved in inducing replicative senescence via PI(3)K-PKB-GSK3β-cyclin D1 pathway (Kortlever et al., 2006). SASP plays a key role in mediating several of the pathophysiological effects of senescent cells and is therefore closely linked to its beneficial as well as deleterious effects (Rodier and Campisi, 2011). The SASP composition and strength varies substantially, depending on the inducer of senescence, duration of senescence, environment and cell type (Coppé et al., 2008, 2011; Maciel-Barón et al., 2016). The observed outcomes are both context dependent and cell type specific. However, NF-κB dependent pro-inflammatory factors are the key components of SASP with IL-6 and IL-8 being the most conserved and robustly expressed cytokines (Hardy et al., 2005; Davalos et al., 2010; Freund et al., 2010; Soto-Gamez and Demaria, 2017).

DNA damage, dysfunctional telomeres, genomic damage, epigenomic perturbation, mitogenic proliferative signals, oxidative stress or other senescence-inducing stimuli, all leading to prolonged DDR express SASP to different extents (Acosta et al., 2008; Kuilman et al., 2008; Rodier et al., 2009; Coppé et al., 2010b; Pazolli et al., 2012). In contrast, SASP is not detectable in cells where senescence is induced by ectopically expressing $p21^{WAF1/CIP1}$ or $p16^{INK4A}$, despite displaying other key senescence markers (Coppé et al., 2011). Therefore, DNA damage is an essential driver of SASP. However, a study by Freund et al. (2011), identified a novel canonical DNA damage response signaling independent mechanism that regulates SASP via p38MAPK (Freund et al., 2011). It was demonstrated that p38MAPK induced SASP mainly by inducing NF-κB activity (Freund et al., 2011). Similarly, induction of senescence by mitochondrial dysfunction presents a distinct secretory phenotype (Wiley et al., 2016).

Senescence associated secretory phenotype factors can reinforce and spread senescence by exerting their effects in both autocrine and paracrine fashion. Factors like IL-1A and IL-6 act in a cell-autonomous manner to reinforce the senescent state whereas many other SASP factors act by exerting non-cell-autonomous effects which enables alteration of the behavior of neighboring cells including manifestation of senescence in healthy, proliferation competent cells (Acosta et al., 2008, 2013; Nelson et al., 2012). This type of non-cell autonomous stable growth arrest is referred to as paracrine senescence. It was first demonstrated as a senescence bystander effect wherein senescent cells were capable of inducing DDR in neighboring cells (Nelson et al., 2012). This study suggested the involvement of reactive oxygen species (ROS) in paracrine senescence as it was observed that senescence occurred via gap junction-mediated cell to cell contact enabling transfer of ROS (Nelson et al., 2012, 2018). Autocrine and paracrine senescence along with immunosurveillance explain the accumulation of senescent cells observed upon aging and its detrimental effects.

Senescence associated secretory phenotype proteins can be secreted into the extracellular environment in a variety of ways. Many members are produced as soluble proteins which can be directly secreted, whereas others are initially expressed as transmembrane proteins that require ectodomain shedding for secretion (Stow and Murray, 2013). Enzymes like ADAM17 have been reported to be upregulated in OIS and cancer and are responsible for regulating the ectodomain shedding of many cell membrane-bound SASP factors (Effenberger et al., 2014; Morancho et al., 2015). Additionally, small exosome-like extracellular vesicles have recently emerged as key components of the senescent cell secretome to enable more distal functions, such as enhancing cancer cell proliferation (Takasugi et al., 2017), an intriguing topic requiring further investigation. SASP has been described as a temporally regulated dynamic program that can be divided into an initial rapid DDR-associated phase followed by an early self-amplification phase eventually leading to a late 'mature' phase (Malaquin et al., 2016).

Mechanisms Involved in the Dynamic Regulation of the Senescence Associated Secretory Phenotype

Multiple different nuclear and cytoplasmic factors such as DNA damage, cytoplasmic chromatin fragments (CCFs), transposable elements, and toll like receptors (TLR) have been shown to trigger SASP. Different pathways such as p38MAPK (Freund et al., 2011), JAK2/STAT3 (Hubackova et al., 2010; Xu et al., 2015b), inflammasome (Acosta et al., 2013), mTOR (Herranz et al., 2015; Laberge et al., 2015), phosphoinositide-3-kinase (PI3K) pathway (Bent et al., 2016; Zhang et al., 2018), HSP90 (Di Martino et al., 2018), non-coding RNAs (Bhaumik et al., 2009; Yap et al., 2010; Puvvula et al., 2014; Panda et al., 2017; Baker et al., 2019; Barnes et al., 2019), GATA4/p62-mediated autophagy (Kang et al., 2015), macroH2A1 and ATM (Chen et al., 2015) are all involved in the development and regulation of SASP. It is dynamically and temporally, regulated at multiple different levels such as chromatin modification, transcription, secretion, mRNA stability and translation. **Figure 2** shows the different mechanisms involved in SASP regulation.

Most of the cascades involved in inducing and dynamically regulating SASP ultimately converge on the activation of two transcription factors, NF-κB and CEBPβ which are found to be enriched in the chromatin fractions of senescent cells

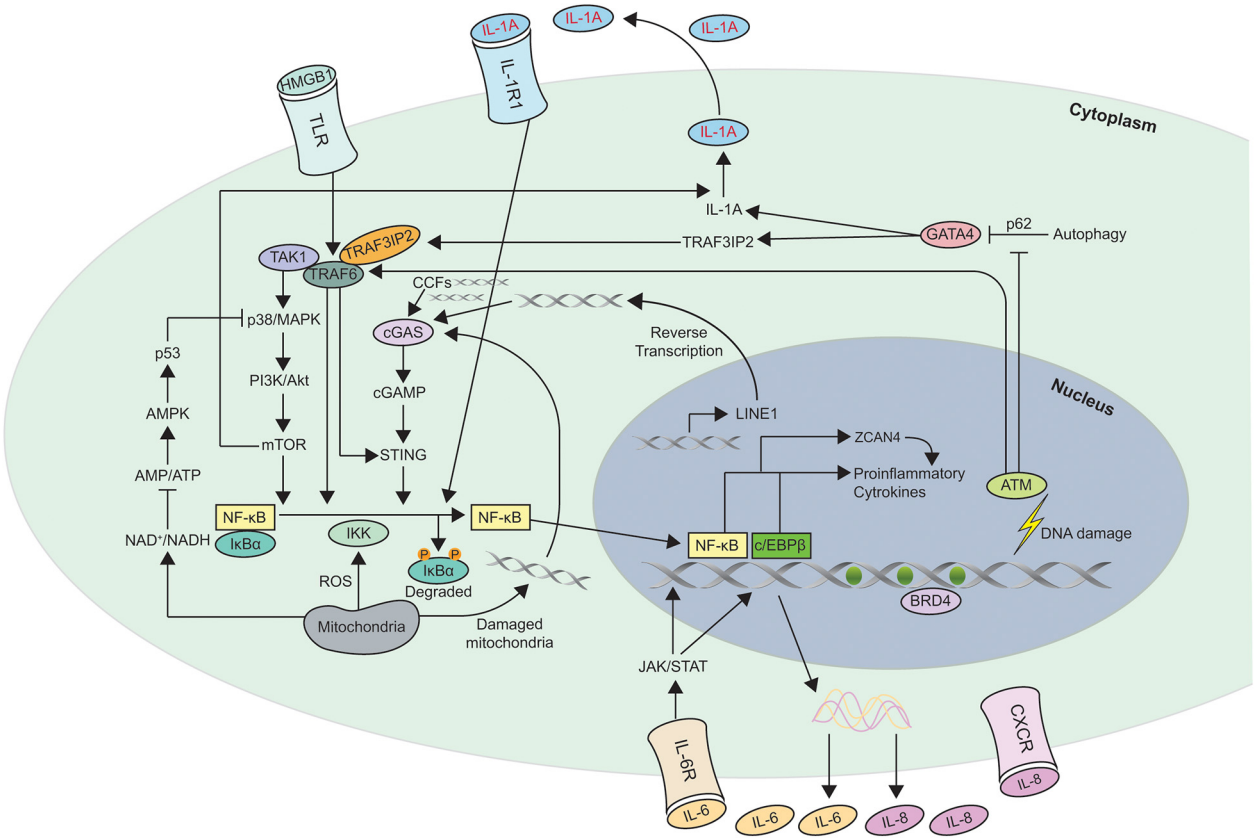

FIGURE 2 | Schematic of the different mechanisms involved in Senescence Associated Secretory Phenotype (SASP) regulation. This figure shows the different pathways involved in regulating SASP. Most of the pathways converge to activate the transcription factors NF-κB and c/EBPβ in senescent cells. The autocrine feed forward signaling of different pro-inflammatory cytokines such as IL-1A, IL-6, and IL-8 is illustrated. ZCAN4 promotes the expression of inflammatory cytokines via NF-κB. TAK1 activates p38/MAPK, a kinase that subsequently engages PI3K/Akt/mTOR pathway. mTOR is capable of activating the NF-κB signaling directly as well as indirectly via IL-1A. GATA-4 links autophagy and DNA damage response to SASP via IL-1A and TRAF3IP2. NAD^+, ROS, and DNA from damaged mitochondria are also involved in regulating SASP. Increase in transcription of LINE-1, a retrotransposable element in senescent cells facilitates accumulation of cDNA in the cytoplasm which leads to the activation of cGAS/STING pathway. In addition to LINE-1, CCFs, and DNA from damaged mitochondria are recognized by cGAS to generate cGAMP which subsequently activates STING to induce expression of SASP factors. The triggers for SASP activation can originate within the cell such as DNA damage, CCFs, cytosolic DNA or act on membrane receptors such as HMGB1, IL-1A, IL-6, and IL-8. Degradation of the inhibitor IκBα which sequesters NF-κB in cytosol, leads to nuclear translocation of NF-κB leading to expression of SASP genes. Recruitment of the chromatin reader BRD4 to newly activated super-enhancers adjacent to key SASP genes is needed for the SASP and downstream paracrine signaling. CCFs, cytoplasmic chromatin fragments; ROS, reactive oxygen species.

(Acosta et al., 2008; Kuilman et al., 2008; Chien et al., 2011; Ohanna et al., 2011; Huggins et al., 2013). NF-κB and CEBPβ cooperatively control the transcription of key regulators of the inflammatory SASP proteins such as IL-1A, IL-6, and IL-8 which in turn positively regulate NF-κB and CEBPβ activity in an autocrine feed forward manner to enhance SASP signaling (Acosta et al., 2008; Kuilman et al., 2008; Orjalo et al., 2009; Rodier et al., 2009; Huggins et al., 2013; Zhang et al., 2018). IL-1A is a master regulator of SASP as ectopic expression of IL-1A can partially reproduce the inflammatory SASP characterized by expression of IL-1B, IL-6, IL-8, and CCL2 (Orjalo et al., 2009; Acosta et al., 2013). Cells which develop SASP can also transmit the phenotype to surrounding cells in a non-cell autonomous fashion via a complex secretory program orchestrated by the inflammasome; a multiprotein complex comprising caspase 1 and several adapter molecules (Schroder and Tschopp, 2010; Strowig et al., 2012), mainly by IL-1A and TGF-β mediating signaling both in cell culture and *in vivo* models of oncogene induced senescence (Acosta et al., 2013). Therefore, different components of SASP can reinforce the senescent state by amplifying or transmitting SASP via autocrine or paracrine signaling pathways (Kortlever et al., 2006; Acosta et al., 2008; Kuilman et al., 2008; Wajapeyee et al., 2008; Zhang et al., 2018). Moreover knock down of different SASP genes prevents senescence thereby highlighting the key role played by autocrine signaling mechanisms in regulating SASP and the senescent state (Acosta et al., 2008; Kuilman et al., 2008; Wajapeyee et al., 2008).

Senescence associated secretory phenotype gene expression can also be regulated by epigenetic changes. Persistent DNA damage leads to proteasome mediated degradation of G9a and GLP, two major histone H3K9 dimethyl transferases. This causes a global decrease in H3K9 dimethylation, an epigenetic mark for

gene silencing and leads to induction of IL-6 and IL-8 (Takahashi et al., 2012). Other epigenetic regulators such as BRD4 (Tasdemir et al., 2016), MLL1 (Capell et al., 2016), HMGB2 (Guerrero and Gil, 2016), histone variant macroH2A1 (Chen et al., 2015), and GATA4 (Kang et al., 2015) are also involved in regulating SASP. In normal cells, GATA-4 bound to autophagy regulator, p62, is degraded by selective autophagy whereas induction of senescence in DNA damaged cells leads to suppression of autophagy and hence stabilization of GATA-4. The stabilized GATA-4 enhances SASP via TRAF3IP2 and IL-1A mediated NF-κB activation and establishes GATA4 as a separate branch of the senescence regulatory pathway independent of p53 and $p16^{INK4A}$ for inducing SASP (Kang et al., 2015).

mTOR signaling has been demonstrated to be involved in regulating mammalian lifespan (Harrison et al., 2009). The exact mechanisms by which mTOR modulates aging are not clear but recent advances suggest a role in mediating SASP and cellular senescence. An mTOR dependent mechanism for regulating SASP at the post-transcriptional level has also been identified. Inhibition of mTOR by rapamycin suppresses the secretion of inflammatory cytokines by senescent cells (Herranz et al., 2015; Laberge et al., 2015). It also differentially regulates translation of IL-1A, the master regulator of SASP that subsequently engages IL-6/IL-8 (Orjalo et al., 2009; Laberge et al., 2015) and MAP kinase-activated protein kinase 2 (MAPKAP2) through 4EBP1, a translation repressor protein (Herranz et al., 2015). MAPKAP2 is known to phosphorylate and inhibit zinc finger protein 36L1 (ZFP36L1), an mRNA binding protein which binds to AU rich elements in the 5′-end of transcripts of proinflammatory SASP components and target them for degradation (Herranz et al., 2015) thereby enabling mTOR to indirectly regulate SASP by regulating mRNA stability. Hence, mTOR interacts with the p38MAPK signaling pathway as MAPKAP2 is a downstream target of p38MAPK. In cells undergoing OIS, spatial integration of mTOR and autophagy which couples protein synthesis and degradation boosts the production and secretion of SASP components in a distinct cellular compartment at the trans site of the Golgi apparatus, the TOR-autophagy spatial coupling compartment (TASCC) (Narita et al., 2011; Young et al., 2013; Herranz et al., 2018).

Last year yet another mechanism of SASP regulation demonstrating a novel role of NAD+ metabolism involving HMGA-NAMPT-NAD^+ signaling axis in regulating SASP was identified (Nacarelli et al., 2019). HMGA proteins modify chromatin structures to regulate senescence (Narita et al., 2006). Nicotinamide phosphoribosyl transferase (NAMPT) catalyzes the rate limiting step in the NAD salvage pathway from nicotinamide (NAM) (Garten et al., 2015; Verdin, 2015). It was found that in OIS, HMGA1 plays an instrumental role in upregulating NAMPT through an enhancer element which in turn promotes inflammatory SASP in response to an increased NAD^+/NADH ratio (Nacarelli et al., 2019). In accordance with this, inhibition of HMGA1 and NAMPT suppresses OIS initiation showing that HMGA-NAMPT-NAD^+ signaling promotes proinflammatory SASP through the NAD^+ mediated suppression of AMPK. AMPK suppresses p53 mediated inhibition of p38MAPK to enhance NF-κB activity to promote expression of proinflammatory SASP proteins. Thus HMGA-NAMPT-NAD^+ mediated expression of proinflammatory SASP is independent of CEBPβ activity. Enhanced glycolysis and mitochondrial respiration have been shown to promote NAD^+ dependent proinflammatory SASP. Taken together, the ratio of NAD^+/NADH regulated by NAMPT which acts downstream of HMGA1 governs the strength of proinflammatory SASP. This suggests that an increase in the ratio of NAD^+/NADH is capable of converting a low proinflammatory SASP into a high proinflammatory SASP.

Recently TLR, an innate immune receptor which recognizes pathogen-associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs), was shown to trigger induction of SASP (Davalos et al., 2013; Kawasaki and Kawai, 2014; Loo et al., 2017, 2020; Hari et al., 2019). New key SASP components, acute-phase serum amyloids A1 and A2 (A-SAAs), which act as senescence associated DAMPs and induce SASP through TLR2 after oncogenic stress have been identified (Hari et al., 2019). Lipoteichoic acid (LTA), a component of the cell wall of gram positive gut microbiota is recognized by TLR2 and induces expression of SASP components creating a tumor promoting micro environment that promotes development of obesity associated hepatocellular carcinoma (Loo et al., 2017). In accordance with this, HMGB1 secreted by senescent fibroblasts is recognized by TLR4, followed by increase in SASP secretion (Davalos et al., 2013). These findings establish the critical role played by innate immune sensing mechanisms in regulating senescence.

Recent studies have revealed that cytoplasmic chromatin fragments and transposable elements can stimulate cyclic GMP–AMP synthase linked to stimulator of interferon genes (cGAS-STING) pathway and regulate SASP both *in vitro* and *in vivo* where senescence was induced by different stimuli (Dou et al., 2017; Glück et al., 2017; Yang et al., 2017; Li and Chen, 2018). The mechanisms implicated in the accumulation of cytoplasmic DNA in senescent cells include compromised nuclear integrity due to loss of the nuclear lamina protein, Lamin B1 (Dou et al., 2017) and downregulation of cytoplasmic DNAases such as DNAase 2 and TREX1 (Takahashi et al., 2018) frequently observed in senescent cells. De Cecco et al. (2019) reported derepression of long-interspersed element-1 (L1 or LINE-1), the only human retrotransposable element capable of autonomous retrotransposition, in senescent cells. This activation of L1 which is mediated by TREX1, RB1 and FOXA1 leads to accumulation of cDNA in the cytoplasm as L1 possesses high reverse transcriptase activity. The accumulated cDNA has been demonstrated to trigger cGAS/STING signaling pathway, leading to production of SASP factors (De Cecco et al., 2019).

cGAS a 522 amino acid protein, is a cytosolic DNA sensor that activates innate immunity upon sensing aberrant double stranded (ds) DNA molecules irrespective of the source. In the presence of ATP and GTP, cGAS catalyzes the production of 2′3′ cyclic GMP-AMP (cGAMP) which stimulates the adaptor protein, STING. STING recruits TANK-binding kinase 1 (TBK1) and IK-B kinase (IKK) (Ablasser et al., 2013; Diner et al., 2013; Gao et al., 2013; Sun et al., 2013, 2018; Wu et al., 2013; Zhang et al., 2013; Ablasser and Gulen, 2016; Chen et al., 2016). TBK1 phosphorylates

the transcription factor IRF3, leading to its translocation from cytosol to the nucleus where it activates the transcription of type-I interferons such as IFN-β (Sun et al., 2013, 2018; Loo et al., 2020). IKK activates transcription factor NF-κB to induce expression of pro-inflammatory cytokines such as IL-6 and IL-8 (Barber, 2015; Sun et al., 2018; Loo et al., 2020). Hence, the two key downstream pathways activated downstream of cGAS-STING involve activation of type-I interferon and NF-κB.

Recently, a novel role of cGAMP as a soluble extracellular immunotransmitter produced and secreted by malignant cells has been identified. Using a genome wide CRISPR interference screen, SLC19A1, was identified as the first known major importer of cGAMP, which is taken up by host cells to activate intracellular STING pathway to elicit an antitumor immune response. This suggests a highly likely possibility that senescent cells secrete cGAMP via SLC19A1 to promote a paracrine innate immune response (Luteijn et al., 2019; Ritchie et al., 2019). Interestingly, cGAS/STING pathway was recently shown to regulate the induction of TLR2 and A-SAAs in oncogene induced senescence, suggesting that TLR2 signaling occurs downstream of cGAS/STING and is mainly regulated by NF-κB (Hari et al., 2019).

Loss of cGAS compromised senescence due to reduced SASP in different *in vivo* models and also accelerated the spontaneous immortalisation of mouse embryonic fibroblasts thereby highlighting the crucial role of the cGAS/STING pathway in tumor suppression due to immune-mediated clearance of premalignant cells (Dou et al., 2017; Glück et al., 2017; Umbreit and Pellman, 2017; Yang et al., 2017). Identification of the cGAS-STING pathway has shown that nuclear genomic DNA not only acts as a stable nuclear entity that encodes genetic information but can also serve to act as a 'danger-signal' when in the cytoplasm and alarm the immune system by inducing the proinflammatory SASP pathway.

Most of the regulatory mechanisms reviewed here have detailed the control of the expression of pro-inflammatory arm of SASP which is shown to be highly conserved among different forms of cellular senescence such as replicative, irradiation-induced, and OIS. However, the pro-inflammatory arm is not the only subset of SASP as its composition is highly variable and heterogeneous (Wiley et al., 2017). **Table 1** details the SASP factors involved in different senescence contexts. This suggests the possibility that different SASP factors are regulated by different mechanisms. Recent findings have indicated dynamic signaling by NOTCH1, a transmembrane receptor, as a crucial regulator of SASP composition which governs the transition between the inflammatory secretome and TGF-β enriched secretome. In OIS, NOTCH1 activity correlates with the expression of TGF-β1 and TGF-β3 factors and inversely corelates with expression of typical inflammatory cytokines such as IL1A, IL-6, and IL-8. This NOTCH1 mediated suppression of inflammatory cytokines is manifested primarily by the repression of c/EBPβ mediated transcription and not NF-κB. In accordance with this, NOTCH1 inhibition has been shown to facilitate the upregulation of proinflammatory SASP components (Hoare et al., 2016; Ito et al., 2017). This highlights the novel role of NOTCH1 as a temporospatial controller of

TABLE 1 | Selected list of SASP factors involved in different senescence contexts.

Senescence	SASP factors
Replicative senescence	Angiogenin, bFGF, COX-2, CXCR2, Eotaxin-3, Fas, FGF-7, Fibronectin, GM-CSF, GROα,β,γ, HCC-4, HGF, ICAM-1, IFN-1, IGFBP1, IGFBP2, IGFBP3, IGFBP4, IGFBP5, IGFBP6, IL-1A, IL-1B, IL-6, IL-7, IL-8, IL-11, IL-15, IL-13, Leptin, MCP-1, MCP-2, MCP-4, MIF, MIP-1α, MIP-3α, MMP-1, MMP-2, MMP-3, MMP-10, Osteoprotegerin, PAI-1, PAI-2, PGE-2, PIGF, SCF, sgp130, sTNF RI, sTNF RII, TGFβ, TIMP-2, tPA, TRAIL-R3, uPA, uPAR, WNT2.
DNA-damage-induced senescence	Acrp30, Amphiregulin, Angiogenin, bFGF, BTC, CTACK, EGF-R, ENA-78, Eotaxin-3, Fas, FGF-7, GCP-2, GDNF, GITR, GM-CSF, GROα,β,γ, HCC-4, HGF, I-309, ICAM-1, IGFBP1, IGFBP2, IGFBP3, IGFBP4, IGFBP5, IGFBP6, IL-1A, IL-1B, IL-6, IL-6R, IL-7, IL-8, IL-11, IL-13, IL-15, IL-1R1, IL-2R-α, I-TAC, Leptin, MCP-1, MCP-2, MCP-4, MIF, MIP-1α, MIP-3α, MMP-1, MMP-2, MMP-3, MMP-10, MMP-12, MMP-13, MMP-14, MSP-a, Oncostatin M, Osteoprotegerin, PDGF-BB, PIGF, RANTES, SCF, SDF-1, sgp130, sTNF RI, sTNF RII, Thrombopoietin, TIMP-1, TIMP-2, tPA, TRAIL-R3, uPA, uPAR, VEGF
Oncogene-induced senescence	Angiogenin, AREG, A-SAA, bFGF, BLC, CCL1, CCL2, CCL7, CCL20, COX2, CXCR2, CXCL5, CXCL6, ENA-78, Eotaxin-3, GCP-2, G-CSF, GITR, GMCSF, GROα,β,γ, HCC-4, HGF, I-309, ICAM-1, IFN-1, IFN-γ, IGFBP-4, IGFBP-6, IGFBP7, IL-1A, IL-1B, IL-6, IL-6R, IL-7, IL-8, IL-13, I-TAC, LIF, MCP-1, MCP-2, MCP-4, MIF, MIP-1α, MIP-3α, MMP1, MMP3, MMP10, NAP-2, Oncostatin M, Osteoprotegerin, PAI-1, PGE-2, PIGF, SDF-1, sgp130, sTNF RI, t-PA, TIMP-1, TIMP-2, uPAR, VEGF
Therapy-induced senescence	AREG, CXCL8, IL1A, IL-1B, IL-6, MMP2, MMP3, PAI-1, SPINK1, t-PA, WNT16B
Mitochondrial dysfunctional associated senescence	Lacks IL-1-dependent factors but includes IL-10, CCL-27, TNF-α,
SASP factors involved in development	CD-44, CSF-1, FGF, IGFBP-5, WNT5A
SASP factors involved in wound healing	CCL-2, CCL-5, CCN1, CCN2, PAI-1, PDGF-AA, VEGF

Data are based on Kuilman and Peeper (2009), Coppé et al. (2010a), Freund et al. (2010), Wiley et al. (2016), and Lopes-Paciencia et al. (2019).

AREG, amphiregulin; A-SAA, acute-phase serum amyloids A1 and A2; COX2, cyclooxygenase 2; CXCR, CXC chemokine receptor; bFGF, basic fibroblast growth factor; EGF, endothelial growth factor; GMCSF, granulocyte-macrophage colony stimulating factor; GRO, growth-related oncogene; HGF, hepatocyte growth factor; ICAM, intercellular adhesion molecule; IFN, interferon; IGFBP, insulin-like growth factor binding protein; IL, interleukin; MCP, membrane cofactor protein; MMP, matrix metalloproteinase; PAI, plasminogen activator inhibitor; PDGF, platelet-derived growth factor; PGE2, prostaglandin E2; PIGF, placental growth factor; SCF, stem cell factor; SDF, stromal cell derived factor; sTNFR, soluble tumor necrosis factor receptor; TGFβ, transforming growth factor β; TIMP, tissue inhibitor of metalloproteinases; TNF, tumor necrosis factor; t-PA, tissue-type plasminogen activator; TRAIL, tumor necrosis factor related apoptosis-inducing ligand; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; VEGF, vascular endothelial growth factor.

SASP composition dictating the functional balance between two distinct secretomes; the pro-inflammatory and the TGF-β enriched immunosuppressive secretome. Another cause of senescence growth arrest, mitochondrial dysfunction, different from senescence due to genotoxic stress has recently been identified (Wiley et al., 2016). Mitochondria normally oxidize NADH to NAD^+ and mitochondrial dysfunction decreases the NAD^+/NADH ratio mostly in the cytosol, leading to activation of 5′ adenosine monophosphate activated protein kinase (AMPK), resulting in p53 activation and mitochondrial dysfunction associated senescence (miDAS) (Wiley et al., 2016). The key feature of miDAS is the distinct secretory phenotype, different from canonical SASP caused by genotoxic stress. This SASP lacks the canonical IL-1 mediated inflammatory components but comprises interleukin (IL)-10, tumor necrosis factor alpha (TNF-α) and chemokine (C-C motif) ligand 27 (CCL27) (Wiley et al., 2016).

Acute stress-associated phenotype (ASAP), characterized by expression of IL-6 and Timp-1, represents an early phase of cellular response observed immediately after exposure to cytotoxic agents. In contrast, in most cells, SASP develops gradually over a course of 5–10 days after senescence markers are detected (Gilbert and Hemann, 2010, 2011; Sun et al., 2018). ASAP occurs in the context of PI3K/Akt/mTOR signaling suppression independently of DDR and mTOR signaling, further distinguishing it from canonical SASP (Bent et al., 2016). DNA damaged human stromal cells, transition from transient ASAP to chronic SASP during acute DDR; this is mediated by expression of Zscan4 enhanced by the ATM-TRAF6-TAK1 axis (Zhang et al., 2018). Interestingly following DNA damage, TAK1, a crucial kinase involved in ASAP, eventually activates PI3K/Akt/mTOR and p38MAPK pathways to sustain persistent SASP signaling (Zhang et al., 2018). Therefore, the heterogeneous nature of SASP is due to the involvement of multiple different signaling molecules required for manifesting SASP in a stressed setting. Senescent cells can communicate with the surroundings through juxtacrine NOTCH/JAG1 signaling (Hoare et al., 2016; Ito et al., 2017) or ROS secretion (Kuilman et al., 2010; Nelson et al., 2012, 2018) or cargo transfer by formation of cytoplasmic bridges (Biran et al., 2015) or release of extracellular vesicles such exosomes (Lehmann et al., 2008; Takasugi et al., 2017).

Functions of the SASP

Collectively, the SASP secretome is the characteristic of senescent cells that confers most of its biological effects both the beneficial as well as deleterious effects, and therefore is the key regulator of normal physiology and pathology associated with cellular senescence. The composition of SASP is heterogenous; the functions are also quite diverse and depend on the genetic context of the cells exposed to SASP and the neighboring environment (**Table 1**). The effect of autocrine and paracrine signaling of different SASP factors in a specific context are pleiotropic which explains the paradoxical roles for cellular senescence. For example, IL-6 and IL-8 are two main SASP components which have been shown to play both positive and negative roles in different biological processes such as wound healing, tissue repair and tumor progression (Coppé et al., 2010a; Demaria et al., 2014).

Findings so far suggest that the SASP might have originated to help damaged senescent cells to communicate with neighboring cells about their compromised state and initiate tissue repair and regeneration by stimulating the nearby progenitor cells, or to stimulate the immune system to promote their immune clearance (Xue et al., 2007; Kang et al., 2011; Iannello et al., 2013; Tasdemir et al., 2016; von Kobbe, 2018; Mohamad Kamal et al., 2020). Senescence may also have evolved as an exaptation of developmental senescence or as a viral defense mechanism. SASP components control a multitude of functions and play key beneficial physiological roles such as: accelerated wound healing by secreting factors such as PDGF-AA and CCN1 (Jun and Lau, 2010; Demaria et al., 2014), promoting stemness and tissue plasticity in response to damage to maintain tissue homeostasis (Ritschka et al., 2017; Taguchi and Yamada, 2017), embryonic development (Muñoz-Espín et al., 2013; Storer et al., 2013), fibrotic scar degradation (Krizhanovsky et al., 2008; Lujambio et al., 2013), and tumor suppression (Lujambio et al., 2013; Rao and Jackson, 2016). SASP mediated autocrine and paracrine signaling that reinforces senescence contribute to tumor suppressive functions of SASP by limiting the proliferation of cells at risk followed by immune clearance of the premalignant cells (Acosta et al., 2008, 2013; Kuilman et al., 2008; Wajapeyee et al., 2008; Kang et al., 2011; Hubackova et al., 2012; Nelson et al., 2012; Toso et al., 2014). However, the factors that determine the balance between repair, regeneration and senescence, in response to damage, require further examination.

Recent discoveries suggest that the deleterious effects of SASP overshadow its beneficial properties. Inflammatory SASP components and accumulation of immature immunosuppressive myeloid cells in solid tumors promote tumorigenesis by driving cell migration, growth, invasion, angiogenesis and eventually metastasis (Krtolica et al., 2001; Coppé et al., 2006; Yoshimoto et al., 2013; Di Mitri et al., 2014; Eggert et al., 2016; Demaria et al., 2017; Kim Y.H. et al., 2017; Chen et al., 2018). This demonstrates the multifaceted interaction between SASP, immune cells and cancer, in accordance with Eggert et al. (2016), where in the initial stages, SASP mediated recruitment of immature myeloid cells (iMC) which when differentiated into macrophages clear the pre-malignant senescence cells to prevent cancer initiation. On the other hand, in later stages the tumor cells block the maturation of the accumulated iMC which eventually promote the growth of established hepatocellular carcinoma by inhibiting NK cell functions (Eggert et al., 2016). SASP also mediates the harmful effects of senescent cells which accumulate upon chemotherapy treatment as their chronic presence promotes local and systemic inflammation. Elimination of therapy-induced senescent cells can prevent cancer recurrence (Demaria et al., 2017). Recently, another SASP factor amphiregulin (AREG) has been shown to drive cancer resistance via EFGR pathway and augment malignancy (Xu Q. et al., 2019). Multiple studies have shown that most of the age-related pathologies stem from low level chronic inflammation referred to as inflammaging or sterile inflammation which can also result in premature aging (Franceschi et al., 2007; Chung et al., 2009; Franceschi and Campisi, 2014; Jurk et al., 2014). Therefore, SASP mediated autocrine and paracrine signaling may explain how a relatively small number of senescent

cells can bring about durable, local and systemic effects *in vivo*, which promote chronic diseases and age-associated functional decline (Allavena et al., 2008; Coppé et al., 2010a; Lecot et al., 2016; Contrepois et al., 2017). Taken together, SASP components have the potential to alter different cellular processes within the microenvironment wherein a chronic SASP causes negative outcomes whereas a short-lived transient SASP is beneficial.

Senescence associated secretory phenotype is nonspecific, context dependent and highly heterogeneous which can be regulated at multiple different levels. However, the difficulty in the identification of a general regulatory mechanism restricts its utility as an unequivocal marker for senescence as no unique form of SASP is known to exist (Coppé et al., 2010a; Hernandez-Segura et al., 2017; Sun et al., 2018). Nevertheless studying the composition of SASP can be very helpful in defining different senescence programs and their context and potentially be used to target SASP for therapeutic purposes (Lecot et al., 2016). For example, the presence of different MMPs and growth factors like VEGF and PDGF-A indicate the involvement of senescent cells in and tissue repair and wound healing (Jun and Lau, 2011; Demaria et al., 2014) whereas, age-related or therapy-induced senescent cells are mainly linked with secretion of inflammatory factors (Baker et al., 2016; Demaria et al., 2017). Analysis of individual cells following induction of cellular senescence by single cell RNA sequencing has revealed surprisingly significant cell to cell variation in SASP gene expression (Wiley et al., 2017). Therefore, it is possible that single cell profiling may allow us to understand which particular SASP component drives a particular function in a specific context *in vivo*.

THE DNA DAMAGE RESPONSE (DDR)

Different intrinsic (telomere attrition, hyperproliferation, oxidative damage) and extrinsic (γ-irradiation, ultraviolet radiations, chemotherapeutic drugs) stimuli lead to persistent DDR signaling which results in irreparable DNA damage and induce cellular senescence (D'Adda Di Fagagna, 2008; Fumagalli et al., 2012). It has been observed that a single unresolved DNA DSB is capable of inducing senescence (Di Leonardo et al., 1994). DDR machinery in human fibroblasts senses an uncapped, double stranded chromosome free end exposed due to progressive telomere shortening in replicative senescence to initiate a DDR (D'Adda Di Fagagna et al., 2003). Human somatic cells lack the catalytic subunit of telomerase at a level sufficient to maintain telomeres after repeated cell division which results in shortening of telomeres due to the end replication problem (Shay and Wright, 2019). During oncogene induced senescence, oncogene activation initially triggers a hyperproliferative phase which induces cellular senescence. The mitotic signals increase the usage of origins of replication resulting in stalled replication forks and accumulation of genomic damage that eventually activates the DDR (Bartkova et al., 2006; Di Micco et al., 2006; Halazonetis et al., 2008; Gorgoulis and Halazonetis, 2010). Both DDR and ARF tumor suppressor mechanisms are involved in mediating oncogene induced senescence (OIS); DDR is more sensitive and requires less oncogenic load than ARF (Evangelou et al., 2013; Gorgoulis et al., 2018). DDR in replicative senescence is dependent on the telomere length whereas in OIS it is not due to telomere length, even though telomere dysfunction is associated with OIS (D'Adda Di Fagagna et al., 2003; Suram et al., 2012). Both telomeric and non-telomeric DNA damage have been shown to play equivalent roles in triggering senescence (Nakamura et al., 2008). Prolonged DDR signaling caused directly or indirectly by DNA DSBs can enforce senescence growth arrest mostly regulated via the p53/p21$^{WAF1/CIP1}$ pathway (Fumagalli et al., 2012).

DNA damage such as single-strand and double-strand breaks activates DDR, which is a classical, evolutionarily conserved, robust response to damaged DNA. Normally cells are able to deal with DNA damage, but cells must undergo either apoptosis or senescence if the damage is irreparable, to prevent progression of damaged cells. The choice between apoptosis and senescence depends on the extent and the duration of the DNA damage signaling. It has now been suggested that prominent short-term DNA damage activates apoptosis whereas prolonged mild DNA damage induces cellular senescence (Petrova et al., 2016).

Irrespective of the stimuli driving the DDR, classical DDR mainly involves the p53/p21$^{WAF1/CIP1}$ tumor suppressor pathway. Multiple different DNA damage sensors such as replication protein A (RPA) (Zou and Elledge, 2003) and the RAD9-RAD1-HUS1 (9-1-1) (Weiss et al., 2002) complex detect exposed single-stranded breaks and MRE11-RAD50-NBS1 (MRN) (Stracker et al., 2004; Moreno-Herrero et al., 2005) complex detect DNA double-stranded breaks and recruit the upstream protein kinases ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and RAD3-related (ATR) to the site of damage (D'Adda Di Fagagna, 2008). Although both ATM and ATR are activated upon DNA damage, they have distinct DNA specificities; ATM gets activated predominantly by DSBs whereas ATR in addition to double strand breaks responds to a broad spectrum of DNA damage such as genotoxic stress caused by DNA replication stress initiated by oncogenes (Maréchal and Zou, 2013). Once at the site of damage ATM and ATR amplify the DDR signal by phosphorylating other DNA damage mediator proteins, such as histone H2AX to form γ-H2AX which aids in the assembly of other specific DNA repair complexes, forming nuclear foci that are stable sites of dynamic accumulation of different DDR proteins (Lukas et al., 2003). Dynamic changes in histone modification such as histone methylation are also critical for regulating DNA double-strand break (DSB) repair by activating ATM kinase which also contributes to the formation of transient repressive chromatin structures which serve to stabilize the damaged chromatin and promote assembly of DSB-signaling proteins (Ayrapetov et al., 2014).

CHK1 and CHK2 are the downstream diffusible kinases which act far from the site of DNA damage and propagate the damage signal by phosphorylating the final effector substrates such as p53. Phosphorylation of p53 on Serine-20 by CHK2 leads to a reduction in binding affinity of the E3 ubiquitin ligase MDM2 to p53, leading to an increase in p53 levels. p53 is also phosphorylated at Serine-15 directly by ATM (Chehab et al., 1999). CHK1 negatively regulates CDC25, a dual-specificity protein phosphatase which promotes the G2 to M transition,

by phosphorylating Serine-216 leading to a G2 growth arrest (Peng, 1997). Phosphorylated p53 upregulates the expression of $p21^{WAF1/CIP1}$, a potent universal CDKI leading to cell cycle arrest (Sulli et al., 2012).

In addition to the role of DDR in manifesting senescence associated cell cycle arrest, DDR signaling also mediates SASP by inducing NF-κB activation. The genotoxic stress sensor ATM and PARP-1 stimulate NF-κB transcriptional activity (Stilmann et al., 2009). Chemotherapeutic drugs or oxidative stress induced DNA damage engage PARP-1/ATM/ NF-κB signaling cascade to induce senescence in melanoma and non-melanoma cells (Ohanna et al., 2011). These senescent cells develop a PARP-1 and NF-κB associated secretome (PNAS) containing chemokine CCL2 along with other SASP factors thereby augmenting the invasiveness of melanoma cells which might have escaped senescence. Blocking PARP-1, ATM, or NF-κB in melanoma cells prevents the secretion of chemokine CCL2 thereby restricting the deleterious pro-invasive properties of the inflammatory SASP mediated by PNAS (Ohanna et al., 2011).

As most of the senescence-inducing stimuli eventually impinge directly or indirectly on DNA, persistent DDR signaling is a characteristic feature of many senescent cells. DDR associated features such as DNA damage foci which can be detected by immunostaining of γ-H2AX; DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS) (Rodier et al., 2011) and telomere-dysfunction induced foci (TIF) (Herbig et al., 2006) or phosphorylated p53, can be used as markers for cellular senescence. However, despite this, DDR markers have a limited potential for identifying senescent cells *in vivo* since DDR independent mechanisms are also capable of inducing senescence via $p53/p21^{WAF1/CIP1}$ pathway (Alimonti et al., 2010; Freund et al., 2011; Muñoz-Espín et al., 2013; Storer et al., 2013; Salama et al., 2014). DDR can also be activated by other DNA-damaging stimuli which do not lead to the development of the senescent state but are rather involved in physiological non-pathological settings or are in the process of responding to a transient repairable DNA damage.

DISCUSSION

Here we have reviewed the different mechanistic pathways as well as the various mediators which underlie the finite proliferative of normal somatic cells and how entry into senescence leading to a stable cell cycle arrest and secretion of the SASP proteins is regulated. Although bypassing senescence and acquiring a limitless replicative potential is a key event required for malignant transformation, the underlying signaling pathways and the basis for the stability of the growth arrest are poorly understood (Hanahan and Weinberg, 2011). A greater understanding is therefore essential if we are to prevent tissue dysfunction without increasing the risk of developing cancer. There is also abundant room for further progress in better understanding the mechanisms underlying the short-lived, transient senescence which benefits tissue development, regeneration and repair as this is less well-characterized in comparison to the deleterious effects of stable senescence.

One of the key stumbling blocks in the field of senescence is the lack of a single, universal, robust, biomarker that allows identification of senescent cells with high sensitivity and specificity and is capable of differentiating them from terminally differentiated, quiescent, and other non-dividing cells. Growth arrest is a key feature which can be readily demonstrated *in vitro* using colony-formation assays or by BrdU/EdU-incorporation assays that measure DNA synthesis (Cavanagh et al., 2011; Crane and Bhattacharya, 2013; Mead and Lefebvre, 2014; Adan et al., 2016; Bhaskara, 2016). However, DNA synthesis measurement is not totally specific since DNA repair may still be active. Measuring the expression levels of CDKIs $p16^{INK4A}$ and $p21^{WAF1/CIP1}$ are key to detecting cell cycle arrest but are not expressed persistently particularly $p21^{WAF1/CIP1}$ by senescent cells (Herbig et al., 2004; Da Silva-Álvarez et al., 2019). Accumulation of high levels of $p16^{INK4A}$ is required to maintain the senescent state by preventing RB inactivation enabling it to be extensively used as a marker for senescence in most normal untransformed cells and tissues (Hara et al., 1996; Sharpless and Sherr, 2015; Wiley et al., 2017). However, $p16^{INK4A}$ is also expressed in non-senescent cells and cells that are transiently arrested, and senescence can also occur independently of $p16^{INK4A}$ coupled with the lack of specific antibodies limits its use as a biomarker for senescence (Sharpless and Sherr, 2015; Herranz et al., 2018).

Due to the heterogeneous and dynamic nature of senescence, there is currently no single totally reliable biomarker (Carnero, 2013). Recently a multi-marker, three-step workflow which allows accurate detection of senescent cells has been proposed (Gorgoulis et al., 2019). The first step includes assessing senescence-associated-beta-galactosidase (SA-β-gal) activity and/or lipofuscin accumulation (GL-13 or SBB). The second step examines frequently observed markers of senescent cells including transcriptional signatures linked to the cell-cycle arrest and SASP such as increased expression of the cyclin-dependent kinase inhibitors and a subset of SASP genes, along with decreased expression of proliferation markers such as cyclins, CCNA2 and CCNE2 and LMNB1. The third step consists of identification of factors that are anticipated to be altered in the specific context. Single-cell transcriptome and proteome profiling of tissues along with development of sophisticated high-throughput methods and machine learning tools will be key to understanding the nature of senescent cells and may aid in identifying potential therapeutic approaches (Vougas et al., 2019). To help with the identification of genes associated with senescence a novel database SeneQuest[1] has been established (Gorgoulis et al., 2019).

A recent study by Martínez-Zamudio et al. (2020), revealed links between enhancer chromatin, transcription factor recruitment, and senescence competence. They demonstrated that a hierarchical transcription factor network defines the senescence transcriptional program and identified activator protein 1 (AP-1) as a master regulator that drives the transcriptional program of senescent cells thereby revealing

[1]http://Senequest.net

promising pathways with therapeutic implications for modulation of senescence *in vivo*.

Accumulating evidence has demonstrated that both anti-senescence and pro-senescence therapies could be beneficial depending on the context (Myrianthopoulos et al., 2019; van Deursen, 2019). Pro-senescence therapies help limit damage by restraining proliferation and fibrosis during carcinogenesis and active tissue repair whereas anti-senescence agents enable elimination of accumulated senescent cells to restore tissue function, and potentially aid organ rejuvenation (McHugh and Gil, 2018; Gorgoulis et al., 2019). It has been found that cells which escape from senescence post-chemotherapy re-enter the cell cycle, are highly aggressive, chemo-resistant, and exhibit stem cell characteristics and can contribute to cancer recurrence (Milanovic et al., 2018; Saleh et al., 2019). Since several therapeutic modalities trigger senescence in tumors, it is important to decipher the mechanisms involved in the escape from senescence as a more detailed understanding may allow the development of better therapies and also help to reduce the off-target effects contributing to unwanted toxicity.

A thorough understanding of SASP regulation is required to exploit it for therapeutic purposes. There is a growing need for further research to investigate how the different signaling pathways regulating SASP such as p38MAPK, mTOR, GATA4, TAK1, cGAS/cGAMP/STING are interconnected and how SASP manifests the age-related pathologies. Inhibition of SASP without perturbing the stable growth arrest would allow reduction of the deleterious effects while maintaining tissue homeostasis and other physiological roles. However, targeting SASP for therapeutic purposes has to be undertaken with great care since it has both beneficial and deleterious roles due to the plethora of components.

Identification of key SASP factors secreted by senescent cells in aged tissues and residual tumors in the post-treatment period might have potential as biomarkers for real-time medical surveillance. The advent of powerful genetic and pharmacological tools to dissect the relationship between accumulated senescent cells and aging should improve our understanding of how accumulated senescent cells lead to age associated decline. The detailed kinetics of accumulation of senescent cells during the lifetime of an organism, remain to be established. It is important to note that despite the link between senescent cells and organismal aging, senescence and aging should not be considered synonymous as cells can undergo senescence due to a multitude of stimuli in addition to telomere shortening irrespective of organismal age. Identification of factors which control or determine the balance between senescence, regeneration and repair require investigation.

A greater in-depth understanding of the underlying mechanisms which regulate senescence will provide promising translational opportunities to develop new therapeutic approaches which minimize the detrimental consequences of senescence. Targeting senescence using senolytics to selectively eliminate senescent cells or modulate SASP using small molecules or antibodies will not only aid in treatment of senesce related diseases but may contribute toward improving the health span of individuals.

AUTHOR CONTRIBUTIONS

RK and PJ have contributed equally in the preparation of manuscript. Both authors contributed to the article and approved the submitted version.

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Interphase Chromosomes in Replicative Senescence: Chromosome Positioning as a Senescence Biomarker and the Lack of Nuclear Motor-Driven Chromosome Repositioning in Senescent Cells

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This study demonstrates, and confirms, that chromosome territory positioning is altered in primary senescent human dermal fibroblasts (HDFs). The chromosome territory positioning pattern is very similar to that found in HDFs made quiescent either by serum starvation or confluence; but not completely. A few chromosomes are found in different locations. One chromosome in particular stands out, chromosome 10, which is located in an intermediate location in young proliferating HDFs, but is found at the nuclear periphery in quiescent cells and in an opposing location of the nuclear interior in senescent HDFs. We have previously demonstrated that individual chromosome territories can be actively and rapidly relocated, with 15 min, after removal of serum from the culture media. These chromosome relocations require nuclear motor activity through the presence of nuclear myosin 1β (NM1β). We now also demonstrate rapid chromosome movement in HDFs after heat-shock at 42°C. Others have shown that heat shock genes are actively relocated using nuclear motor protein activity via actin or NM1β (Khanna et al., 2014; Pradhan et al., 2020). However, this current study reveals, that in senescent HDFs, chromosomes can no longer be relocated to expected nuclear locations upon these two types of stimuli. This coincides with a entirely different organisation and distribution of NM1β within senescent HDFs.

Keywords: replicative senescence (RS), genome organisation, nuclear motors, chromatin dynamics, chromosome territories, nuclear myosin 1β, chromosome 10

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INTRODUCTION

Senescence is described as a gradual accumulation of non-dividing cells throughout the reproductive life span of culture (Hayflick and Moorhead, 1961; Kill et al., 1994; Ben-Porath and Weinberg, 2004), it is a major obstacle to continuous propagation of cells, and thus is often regarded as a tumour suppressing mechanism (Kill, 1998; Campisi, 2001, 2003a,b). Various studies showing a functional link between increasing number of senescent cells (Dimri et al., 1995; Li et al., 1997; Pawelec et al., 1999) and decreasing activity of stem cells (Collado et al., 2007) with the age of tissue or organism, suggested a link between cellular senescence and organismal ageing (Hayflick and Moorhead, 1961; Campisi, 2003b; Smith and Kipling, 2004; Collado et al., 2007). More recently, organismal ageing has been directly proven to be caused by the accumulation of senescent cells within an organisms' body (Chang et al., 2016; Folgueras et al., 2018), adding a burden to tissues by secreting a plethora of antagonistic and deleterious molecules (Gorgoulis et al., 2019) through the Secretory Associated Senescence Pathway (SASP), inducing senescence in nearby cells (Acosta et al., 2013), termed paracrine senescence (Hernandez-Segura et al., 2018).

Senescence can be caused by various different stimuli, and the different types of senescent cells may even have different roles within the body (Bridger and Foster, 2021). In replicative senescence (RS), cells reach senescence through serial division, and are permanently arrested although metabolically active (Hayflick and Moorhead, 1961). RS cells display telomere shortening (Harley et al., 1990; Allsopp et al., 1995; Blackburn, 2001; Cawthon et al., 2003; Masutomi et al., 2003; Ben-Porath and Weinberg, 2004; Ogami et al., 2004; Davis and Kipling, 2005; Canela et al., 2007), with accumulation of DNA damage through an inability to repair it (Chen et al., 2020), de-repression of $p16^{INK4a}$ loci (Zindy et al., 1997; Chkhotua et al., 2003; Krishnamurthy et al., 2004; Ressler et al., 2006) and alterations in Rb/p13 or $p53/p21^{CIP1}$ pathways, both inducing senescence in different ways (Chen et al., 2020). Oxidative stress-induced premature senescence (SIPS) is elicited through external or internal metabolic oxidative agents, causing severe or irreparable DNA damage (te Poele et al., 2002; d'Adda di Fagagna et al., 2003; Parrinello et al., 2003; Bartkova et al., 2006; Di Micco et al., 2006). Oncogene-induced senescence (OIS) comes about via the activation of oncogenes such as Ras or the inactivation of tumour suppressor genes (Prieur and Peeper, 2008). Senescence can also be induced by mitochondrial dysfunction (Wiley et al., 2016), chemotherapy drugs, inhibition of histone methyl transferases or histone deactylases (Petrova et al., 2016).

Cellular senescence is known to be a mechanism to avoid tumourigenesis but it also has regulatory roles in embryogenesis and wound healing (Coppé et al., 2010; Muñoz-Espín et al., 2013, Storer et al., 2013; Graziano and Gonzalo, 2017). Senescence is such an important mechanism it is evolutionary conserved, cells from mammals, birds, reptiles, flies, and yeast undergo growth arrest and exhibit senescent phenotypes after repeated doublings (Stanulis-Praeger, 1987; Shiels et al., 1999; Lanza et al., 2000).

Replicative senescent cells exhibit an altered behaviour and phenotype as compared to their proliferating counterparts. Senescent fibroblasts possess a larger, flatter morphology (Bowman et al., 1975; Sherwood et al., 1988), with an enlarged nucleus (Mehta et al., 2007; Mitsui and Schneider, 1976), increased adhesion to the extra cellular matrix, fewer cell–cell contacts (Campisi, 2000; Narita et al., 2003; Ben-Porath and Weinberg, 2004) and increased aneuploidy (Benn, 1976; Sherwood et al., 1988; Mukherjee et al., 1995). Moreover, in recent years many studies have demonstrated alterations to the genome organisation of senescent cells (Bridger and Foster, 2021). Exit from the cell cycle into senescence is also accompanied by changes in chromatin modifications (Rai and Adams, 2013) i.e., methylation and acetylation (Wilson and Jones, 1983; Singhal et al., 1987; Imai and Kitano, 1998; Lander et al., 2001; Wagner et al., 2001; So et al., 2006; Dimauro and David, 2009; Grandinetti et al., 2009) with CpG islands being globally demethylated (Cheng et al., 2017) and other specific CpG islands being hypermethylated (Cruickshanks et al., 2013). Core histones are decreased (Lee et al., 2020). Other chromatin remodelling alterations include specific reduction in H3K4me3, H3K9me3, H4K20me3, H3K27me3, H3K36me3, (Di Micco et al., 2011; Salama et al., 2014, Sen et al., 2016; Cheng et al., 2017), deactylation of H4K16 (Contrepois et al., 2012), and H3K56, with increased levels of H3K9ac and H3K27ac associated with specific gene promoters (Gorgoulis et al., 2019; Zhang et al., 2020; Yi and Kim, 2020). With respect to heterochromatin, there is evidence of increased heterochromatisation (Kreiling et al., 2011), with the formation of specific senescence associated heterochromatin foci (SAHF; Chandra and Narita, 2013). But SAHFs are not found in all types senescent cells (Sati et al., 2020). They are a marker of OIS and are created through changes in nuclear pore density at the nuclear edge (Boumendil et al., 2019) and lamin B1 reduction, altering the positioning of genomic regions at the nuclear periphery (Sadaie et al., 2013). SAHFs contain regions of condensed chromatin associated with late replicating, gene-poor regions of the genome. Their function is not clear but they do represent an alteration to genome organisation and regulation (Sati et al., 2020).

In proliferating cells gene-poor regions of the genome are attached to the nuclear envelope through interactions with B-type lamins and other cell specific nuclear envelope proteins (de Las Heras et al., 2017). These specific regions have been termed Lamina Associated Domains (LADs) and have been selected by using an exogenous construct containing the gene for lamin B1 combined with a bacterial enzyme DNA adenine methyltransferase that specifically methylates any associated DNA so that it can be isolated and sequenced (van Steensel and Belmont, 2017). LADs comprise about one third of the genome and so are highly significant in organising the genome within cell nuclei and have some overlap with nucleolar associated domains (NADs; Németh et al., 2010; van Koningsbruggen et al., 2010). NADs remain very similar in replicative senescent embryonic fibroblasts (Dillinger et al., 2017). However, some LADs are released from the nuclear edge with the loss of lamin B receptor (Arai et al., 2019)

and lamin B1 in senescence (Shimi et al., 2011; Freund et al., 2012; Hutchison, 2012; Lukášová et al., 2017, 2018). Indeed, chromosome 18 is less attached to the nucleoskeleton than chromosomes 1, 13, 17 in replicative senescent cells, as is *CTNNA1* gene compared to *CNDD1* (cyclin D1) (Godwin et al., 2021). The LADs in OIS cells are different to proliferating and quiescent cells (Lenain et al., 2017). Chromosome conformation capture has also identified global changes in genome organisation in OIS cells (Chandra et al., 2015; Criscione et al., 2016; Zirkel et al., 2018). However, HiC chromosome conformation capture experiments unequivocally demonstrated that OIS is not comparable to RS (Sati et al., 2020). The interactions between topologically associated domains (TADs) A (active) and B (inactive) compartments are different in the two types of senescence, with OIS and RS both having increased long-range interactions of genomic regions, but with RS cells displaying more A to B interactions, indicating decreased genome compaction (Sati et al., 2020).

Since the genome is highly organised within the nuclei of proliferating cells (Croft et al., 1999) it came as no surprise to find non-random genome organisation through whole chromosome positioning analysis in non-proliferating cells, serum starved quiescent and replicative senescent cells. Bridger et al. (2000) demonstrated that the gene-poor chromosome 18, located at the nuclear periphery in proliferating human dermal fibroblast (HDF) skin cells, was found deep within nuclei, attached to the nucleoskeleton in non-proliferating cells. It was not clear if chromosome positioning would be different in senescent cells when compared to a cell's other pathway to leave the cell cycle, quiescence. However, when opening up the panel of chromosomes studied, it became obvious some chromosomes do not move at all when entering G0, some move to the nuclear periphery and some to the nuclear interior (Bridger et al., 2000; Meaburn et al., 2007; Mehta et al., 2007, 2010; Gillespie et al., 2015; Belak et al., 2020). Many of these chromosomal locations positions have been confirmed by chromosome conformation capture data (Das et al., 2020). This change in location of the chromosomes would place the chromosomes into a different nuclear compartment, exposing them to a alternative nuclear environment and interactive anchorage points. It is strongly supported in the literature that chromosome and gene spatial positioning is a further epigenetic mechanism for regulating gene expression (Sivakumar et al., 2019). Thus understanding how the genome, chromosomes and genes behave in senescent cells will be an important step in revealing the important differences in young proliferating cells and replicative senescent cells.

By using our FISH chromosome mapping assay, we have demonstrated in young proliferating HDFs whole chromosomes move to new locations within cell nuclei when an external stimulus, such as being placed in low serum for 15 min (Mehta et al., 2010). This is rapid relocalisation requires nuclear myosin 1β (NMIβ), presumably within a nuclear motor complex with actin, using energy (Mehta et al., 2008; Bridger, 2011). Interestingly, both chromosomes 18 and 13 move rapidly to the nuclear interior, in similar locations to where they are located in senescent HDF (Bridger et al., 2000; Meaburn et al., 2007). Further, positioning of all human chromosomes in quiescent HDFs determined that the organisation of chromosome territories in interphase nuclei still remains radial as it is in proliferating cells, but the territories of some chromosomes such as 1, 6, 8, 10, 11, 12, 13, 15, 18, and 20 re-localise and alter positions as the cells enter a state of quiescence (Mehta et al., 2010). Chromosomes are also relocated to areas of DNA repair foci via nuclear motors containing nuclear myosin 1β (Mehta et al., 2013; Kulashreshtha et al., 2016).

To assess whether global spatial repositioning of individual whole chromosomes occurs in replicative senescence of HDFs we have further employed the individual whole chromosome positioning assay (Clements et al., 2016) and in combination with previous studies revealed the nuclear positions of all human chromosomes in senescent primary HDFs. Here, we identify that some chromosomes are found in different nuclear locations, compared to proliferating HDF. More interestingly, we have demonstrated that this was not simply due to exiting the cell cycle since there were clear differences in the spatial positioning of chromosome territories between cells made quiescent and cells that have become senescent by serial passage. Most notable of these is chromosome 10. As a further demonstration of the senescence-specific nature of this positioning, no chromosome movement was apparent for chromosome 10 after placing senescent cultures into low serum. This was not surprising since NMIβ, which is known to be involved in whole chromosome movement, had an altered distribution to proliferating cells, forming aggregates.

This study here, in combination with other studies, delineates the positioning patterns of the chromosomes within senescent HDFs and reveals that territories of chromosome 10, sit in opposing locations in senescent HDFs as compared to the same cell line made quiescent by serum starvation. Thus, the positioning of chromosome 10 could be considered a new biomarker to delineate between the two non-proliferating cell statuses i.e., senescence and quiescence, and easy to establish as a robust, but quick assay to differentiate between quiescent and senescent cells.

MATERIALS AND METHODS

Cell Culture

Human dermal fibroblasts (2DD, Bridger et al., 1993 and 1HD, Bridger et al., 1998) were grown in Dulbecco's Modified Eagles Medium supplemented with 10% newborn calf serum (NCS). The cells were passaged twice weekly so that they never became contact inhibited and were used at a high passage number >30, where the majority (95%+) of cells were negative for the proliferation marker pKi-67 (Kill et al., 1994). To make cells quiescent HDF were placed in 10% NCS for 48 h and this was washed out and the cells placed in 0.5% NCS for 7 days. For cultures to be deemed proliferating >65% of the cells had to be Ki67+ and were never passaged beyond passage 15. Quiescent cultures were generated by treating cells with 0.5% NCS/DMEM for 7 days. In order

to subject the cells to a heat-shock they were incubated at 42°C for 1 h.

Two-Dimensional Fluorescence *in situ* Hybridisation

Harvested HDFs were initially allowed to swell in 0.075M KCl and then fixed in ice-cold 3:1 (v/v). methanol:acetic acid. The suspension was placed onto glass microscope slides and aged for two days at room temperature. The fixed cells were dehydrated by subjecting them to an ethanol series (100, 90, and 70%, 5 min each). For denaturing, the slides were placed in 70% formamide, 2X SSC, pH 7.0, at 70°C for 2 min. After denaturation, the slides were immediately plunged in ice-cold 70% ethanol for 5 min and then taken through the ethanol series and air-dried.

Directly labelled total human chromosome DNA probes (Appligene Oncor) were denatured by incubating at 70°C for 10 min followed by 30 min reannealing at 37°C. Hybridisation of probe to sample took place over 18 h in a humidified chamber. The slides were washed three times for 5 min each in 50% formamide, 2X SSC, pH 7.0 at 45°C and then with 0.1X SSC prewarmed at 60°C.

Three-Dimensional Fluorescence *in situ* Hybridisation

For 3D-FISH, cells were grown for 2 days on sterile glass "SuperfrostTM" slides at 37°C, 5% CO2 at a starting density of 1×10^5 cells/slide. Then washed in 1× PBS and fixed in 4% paraformaldehyde (w/v). Cells were permeabilised with 0.5% Triton-X100 (v/v) and 0.5% saponin (w/v) in 1X PBS solution for 20 min at room temperature and then rinsed. The slides were incubated then in a solution of 20% glycerol for at least 30 min at room temperature prior to being snap-frozen in liquid nitrogen for 15–30 s before being stored at −80°C. Chromosome painting probes were denatured at 75°C for 10 min and then allowed to re-anneal at 37°C for 10 min. The freeze–thaw process in liquid nitrogen, as described before was repeated for further 4–5 times with soaking the slides in 20% glycerol between each freeze–thaw. Excess glycerol was washed from the slides using three changes of 1X PBS for 10 min each, followed by depurination in 0.1N HCl for 5 min at room temperature. Excess acid was washed away with 2X SSC for 15 min with three changes of the buffer and then slides were incubated in 50% formamide, 2X SSC, pH 7.0 solution overnight. The slides were denatured by incubation in denaturation buffer A (70% formamide, 2X SSC, pH 7.0) pre-warmed at 73°C for precisely 3 min. The slides were then rapidly transferred to denaturation buffer B (50% formamide, 2X SSC, pH7.0) pre-warmed at 73°C for 1 min. The slide was immediately presented to the probe on the coverslip and incubated in a humidified chamber at 37°C for 2 days and washed as for 2D-FISH.

Indirect Immunofluorescence

The FISH slides were incubated with anti-Ki-67 antibody (1:1500 dilution, Novacastra) for 1 h at 37°C. After washing in phosphate buffered saline, the slides were incubated in the swine anti-rabbit-TRITC secondary antibody (1:30 dilution, DAKO) for 1 h at 37°C. After washing in 4X SSC, the slides were mounted and counterstained with 4, 6-diamidino-2-phenylindole (DAPI) in Vectashield mounting medium (Vecta Laboratories).

For nuclear myosin 1β staining, cells were grown on 13 mm glass coverslips and fixed in ice-cold methanol:acetone (1:1) on ice for 10 min. Dual staining experiments were performed with mouse anti-pKi-67 and rabbit anti-nuclear myosin 1β (Sigma), diluted to 1:1,500 and 1:50 in PBS/1%NCS (v/v), respectively for 1 h at room temperature. After washing, secondary antibodies were employed: Swine anti-rabbit conjugated to TRITC (DAKO) and donkey anti-mouse (Jackson's laboratories) conjugated to FITC were diluted 1:30 and 1:70 in PBS/1%NCS (v/v), respectively and left for 1 h at room temperature in the dark. Slides were mounted and counterstained in Vectashield containing DAPI.

Microscopy and Image Capture

After 2D FISH, interphase nuclei were examined and imaged using a Leica fluorescent microscope with a 100× oil immersion lens (Leica). Random pKi-67 negative nuclei were imaged. Grey-scale images of these nuclei were captured from the microscope using Photometrics cooled charged-coupled device (CCD) camera. These images were pseudocoloured and merged using Digital Scientific software, the Quips Pathvysion, Smart Capture VP V1.4

The images of 3D nuclei, prepared by 3D FISH, were captured using a Nikon confocal laser scanning microscope (TE2000-S) equipped with a 60X/1.49 Nikon Apo oil immersion objective. The microscope was controlled by Nikon confocal microscope C1 (EZ – C1) software version 3.00. Stacks of optical sections with an axial distance of 0.2 μm were collected from random nuclei. Stacks of 8-bit grey-scale 2D images were obtained with a pixel dwell of 4.56 and 8 averages were taken for each optical image.

Image Analysis

2D-FISH

Fifty nuclei for each chromosome were analysed using a bespoke erosion analysis script in IPLab as described in Croft et al. (1999), a gift from Prof Wendy Bickmore, MRC Human Genetics Unit. The script divides nuclei into five shells of equal area and measures the pixel intensity of the DAPI signal and the chromosome probe in each of the five shells. The probe signal is normalised by dividing the percentage of the probe signal by the percentage of DAPI signal in each shell. Histograms were plotted and standard error bars representing ± standard error of the mean (SEM) are shown. Statistical analyses were performed using the two tailed Student's *t*-tests. The necessary controls have been performed whereby a new researcher will repeat chromosomes already delineate to be sure that the results are reproducible and consistent between the data sets.

3D-FISH

The positioning of chromosomes in relation to the nuclear periphery was assessed by measurements obtained using Imaris Software (Bitplane scientific solutions), whereby the distance between the geometric centre of each chromosome territory and the nearest nuclear edge was measured. Measurements for at

least 20 nuclei were performed for each chromosome. Frequency distribution curves were plotted with the distance between the centre of chromosome territory and the nearest nuclear periphery on the *x*-axis and the frequency on the *y*-axis. Statistical analyses were performed using the two tailed Student's *t*-tests.

RNA Extraction

RNA was extracted from samples using the MP24 fastprep (MP Biomedical) system, following the manufactures protocol. superRNAsin (Ambion) was added to each sample prior to snap freezing and storage at −80°C. $N = 4$ biological replicates for each of proliferative, quiescent and replicative senescent RNA samples was analysed to monitor changes in transcript abundance. Paired immuno-fluorescence analysis of cells using Ki-67 was performed to determine the status of each culture.

Microarrays

Microarray analysis was carried out using Op Human ReadyArray HS1200 slides (Microarrays Inc.), with the 3DNA Array 900 labelling kit (Genisphere). One microgram of RNA derived from proliferative, quiescent, or replicative senescent 2DD cultures was resuspended in 5 μl H_2O and 1 μl of RT primer was added with the correct dendrimer target sequence for labelling of the samples on the array. The mixture was heated to 80°C for 5 min to denature, placed on ice for 2 min, and the following reagents were added to each reaction: 2 μl of first-strand buffer, 1 μl of 0.1 M DTT, 0.5 μl of SUPERase-In (provided with the 3DNA 900 kit), 0.5 μl of dNTP mix (provided with the 3DNA 900 kit), and 0.5 μl of SuperScript III (Invitrogen). The reaction was incubated for 2 h at 42°C and stopped by adding 1 μl of 1 M NaOH/100 mM EDTA and incubating at 65°C for 10 min to denature the cDNA/RNA hybrids and degrade the template RNA. The reverse transcription reaction was then neutralised by adding 1.2 μl of 2 M Tris-HCl pH 7.5. 1 μl of H_2O was then added to each cDNA sample, the samples were mixed, and then 1 μl of sample was then assessed using the Qubit® single-stranded DNA assay on a Qubit® 1.0 Fluorometer, to check that a sufficient quantity of cDNA was present.

Samples were then mixed to form the hybridisation mix for the microarray slides. About 12.7 μl of each cDNA were mixed with 40 μl of 2× SDS-based hybridisation buffer and 14.6 μl of H_2O, to a final volume of 80 μl. The mixture was heated to 80°C for 10 min in order to denature secondary structures, and then it was cooled to 60°C in preparation for addition to the slide.

The array slides were pre-hybridised at 65°C for 20 min with 3.5 × SSC, 0.1% SDS and 10 mg/ml BSA solution in a volume of 50 ml. The slides were washed in MilliQ water for 1 min, in isopropanol for 1 min, and dried using a Microarray High Speed centrifuge (Arrayit Corporation). The slide was then pre-scanned for the second time with the GenPix 5.1 scanner to check it was clean and undamaged, before the hybridisation was set up. The microarray slide was then placed into a clean SlideBooster (Advalytix) on a layer of 45 μl AS100 AdvaSon coupling solution (Beckman Coulter), with 60 μl more in the thumb hole at the base of the slide. The wells of the slide booster were each filled with 500 μl of AdvaHum AM102 humidifying solution (Beckman Coulter), and a 24 mm × 60 mm LifterSlip was placed on top of the microarray slide. The assembly was then pre-warmed to 55°C, and when it reached temperature, the hybridisation solution was pipetted underneath the LifterSlip. The microarrays were then hybridised for 16 h. The slides were then washed in 2 × SSC, 0.2% SDS at 55°C for 10 min, followed by a wash in 2 × SSC at room temperature for 10 min, followed by a wash in 0.2 × SSC at room temperature with orbital rotation of 150 rpm for 10 min. The slides were then dried using a Microarray High Speed centrifuge (Arrayit Corporation). For each slide, 2.5 μl of the Cy3 capture reagent was mixed with 2.5 μl of the Cy5 capture reagent, with 40 μl of 2 × SDS-based hybridisation buffer and 35 μl of H_2O, to a final volume of 80 μl, to make the second hybridisation mix. This was heated at 80°C for 10 min, and then cooled to 55°C in preparation for addition to the slide. The SlideBooster was assembled as before, and pre-warmed to 50°C. When it was warm, the second hybrisation mix was added, and the arrays were incubated for 4 h. Array slides were washed in 2 × SSC, 0.2% SDS at 55°C for 10 min, followed by a wash in 2 × SSC at room temperature for 10 min, followed by a wash in 0.2 × SSC at room temperature for 10 min. The slides were then dried using a Microarray High Speed centrifuge (Arrayit Corporation).

RNA-Seq

Two RNA-seq replicates were used for each sample type, as recommended by the ENCODE Consortium's Standards, Guidelines and Best Practices for RNA-Seq.[1] RNA was isolated using the FastPrep-24 instrument (MP Biomedicals) according to the manufacturer's instructions. RNA integrity was determined using the Bioanalyzer (Agilent Technologies) with RNA having an RNA integrity number above 9.0 used for further analysis. For sequencing library synthesis, polyadenylated RNAs were purified using oligo dT-beads (Invitrogen) with random hexamers, and used as primers for the cDNA library construction prior to paired-end sequencing. Sequencing was performed using Illumina GxII platform. All sequencing reactions resulted in the generation of 50 bp paired-end reads. RNA-seq reads were subjected to quality control using the standard Illumina pipeline. Raw sequence reads were mapped against the GRCh37 assembly reference genome using the following command to TOPHAT 2. No trimming of reads was performed prior to mapping. The BAM files produced by TOPHAT 2 were then imported into SEQMONK.[2] The feature probe generator function in SEQMONK was used to generate probes based on mRNA annotations from ENSEMBL. The number of reads that mapped to each probe was then quantitated, and normalised using the widely used RPKM method. A constant value of 0.05 was added to each value in order to prevent cases of division by zero when calculating FC values. To note gold standard senescent biomarker genes $p16^{INK4a}$ and $p21^{CIP1}$ are both upregulated in the senescent cells used, as well 39 other genes associated with senescence CellAge: The Database of Cell Senescence Genes.[3]

[1]http://genome.ucsc.edu/ENCODE/protocols/dataStandards/ENCODE_RNAseq_Standards_V1.0.pdf

[2]http://www.bioinformatics.babraham.ac.uk/projects/seqmonk

[3]https://genomics.senescence.info/cells/

RESULTS

Positions of Human Chromosomes in Senescent Human Dermal Fibroblasts

In this study, we now complete the radial mapping of all human chromosomes in RS HDF nuclei using whole chromosome painting probes and FISH, followed by analysis of interphase position through a bespoke erosion script for 2D positioning and 3D analysis of the reconstruction of optical sections. To achieve this HDFs were harvested and fixed for standard 2D-FISH and hybridised with whole chromosome painting probes for chromosomes 1–12, 14–17, 20–22, and Y. The positions of the other chromosomes has been completed previously in other studies (see **Supplementary Table 1**). Senescent cells within late passage primary cultures, which had been grown in 10% serum and were not permitted to reach confluency at any stage of their passaging, were identified by the lack of the proliferative marker pKi-67 (Clements et al., 2016; **Figure 1**).

As has been executed previously for HDF (Croft et al., 1999; Bridger et al., 2000; Boyle et al., 2001; Meaburn et al., 2005, 2007; Mehta et al., 2010; Bikkul et al., 2018), in order to position the chromosome territories images of 50+ random nuclei were captured (**Figure 1**) and individual chromosome positions assessed by using an erosion analysis script which measures the intensity of fluorescent signal in five concentric shells of equal area, made by eroding the nuclear outline from the edge to the nuclear interior. The position of whole chromosomes is revealed by normalisation through dividing the signal of the chromosome within a shell with the measured signal for the amount of DNA stained by DAPI (Croft et al., 1999; Clements et al., 2016), and the data plotted as histograms (**Figure 2**). The shape of the graph indicates where the chromosomes are located and so we assign a category to each shape of graph. With a skew towards shells 1 and 2 the chromosome is said to be peripheral, with a skew towards shells 4 and 5 the chromosome is said to be interior and where the histogram peaks in shell 3 the chromosome is said to be intermediate in nuclear location (**Figure 2** and **Supplementary Table 1**).

Since this experiment completes the mapping of all the chromosomes by 2D-FISH in young proliferating HDF (Croft et al., 1999; Meaburn et al., 2007; Mehta et al., 2010), quiescent (Bridger et al., 2000; Mehta et al., 2010) and senescent HDF (Bridger et al., 2000; Meaburn et al., 2007; this study); we are now in a position to note changes in chromosome location between these cell cycle statuses. This reveals that there are major nuclear location changes for whole chromosome territories in senescent cells, as compared to previously published data for young proliferating HDF using the same cells and methods. The categorisation of all the chromosome positions in HDFs in **Supplementary Table 1** has been collated from a number of papers, from two different laboratories but, importantly, using the same methodology and analysis script. Furthermore, many of the chromosome positions are confirmed using 3D-FISH, confocal imaging and 3D measurement and analyses. The position of many chromosomes are unaffected by entrance in to senescence. However, chromosomes 1, 5, 6, 10, 12, 15, and 16 occupy differential locations in senescent cells to that of young proliferating HDF cells (**Supplementary Table 1**). These are in addition to chromosome 18 and 13 that have been shown previously to be relocated in senescent HDFs (Bridger et al., 2000; Meaburn et al., 2007; **Supplementary Table 1**). Chromosomes 1, 5 and 6 relocate from an intermediate location to a peripheral location in senescent cells, whereas 12, 13, and 18 move from a peripheral location to the nuclear interior. Chromosome 16 relocates from an interior location to an intermediate location and chromosome 10 relocates from an intermediate location to an interior location (**Supplementary Table 1**).

Since we know that chromosomes reorganise when cells exit the cell cycle in quiescence (Bridger et al., 2000; Mehta et al., 2010), we also wanted to determine if the repositioning events we detect are common to cells that have just exited the cell cycle or are specific to senescence. When the comparison between positioning categories was made between young HDF made quiescent by 7 days serum-starvation and the senescent cells, there are also some differences, with chromosomes 5, 8, 10, 11, 15, 16, and 20 having chromosomes in different spatial categories (**Supplementary Table 1**), e.g., for chromosome 5 the territories are in an intermediate location in quiescent HDF but towards the nuclear periphery in senescent cells. However, there are two chromosomes, chromosomes 10 and 15, which are located in different nuclear compartments in proliferating, quiescent and senescent cells (**Supplementary Table 1**). These repositioning events do not represent a general reorganisation of the genome since there are also chromosomes that do not change their location category at all; the peripheral chromosomes are 2, 3, 4, 7, 9, and X and the interior chromosomes are 14, 17, 19, 21, 22, and Y.

When the categorised chromosome positions are plotted against chromosome size in Mb (**Figure 3**) it is very noticeable that in quiescent and senescent cells the distribution of chromosome territories adheres much more to a size-distribution than in proliferating cells. Thus, it appears in non-proliferating cells chromosome territories are positioned more according to their size with larger chromosomes at the nuclear periphery and smaller chromosomes within the nuclear interior.

Chromosome 10 Occupies Differential Locations in Young Proliferating, Young Quiescent and Old Senescent Human Dermal Fibroblasts

The most interesting chromosome with respect to the difference between the non-proliferative states was chromosome 10; which occupies an intermediate position in young proliferating cells (**Figures 4A,D**), but a peripheral location in when placed in low serum for 15 minutes - 7 days (**Figures 4B,E**) and localises at the nuclear interior in senescent cells (**Figures 4C,F**). These positions were confirmed both in 2D (**Figure 4G**) and 3D analyses (**Figure 4H**). The normalised percentage chromosome signal is greatest in shells 1 and 2 for quiescent HDF, in shells 2 and 3 for proliferating HDF and in shells 3 and 4 for senescent cells. In 3D analyses, optical sections of 20 nuclei were collected on the confocal microscope and reconstructed using Imaris software.

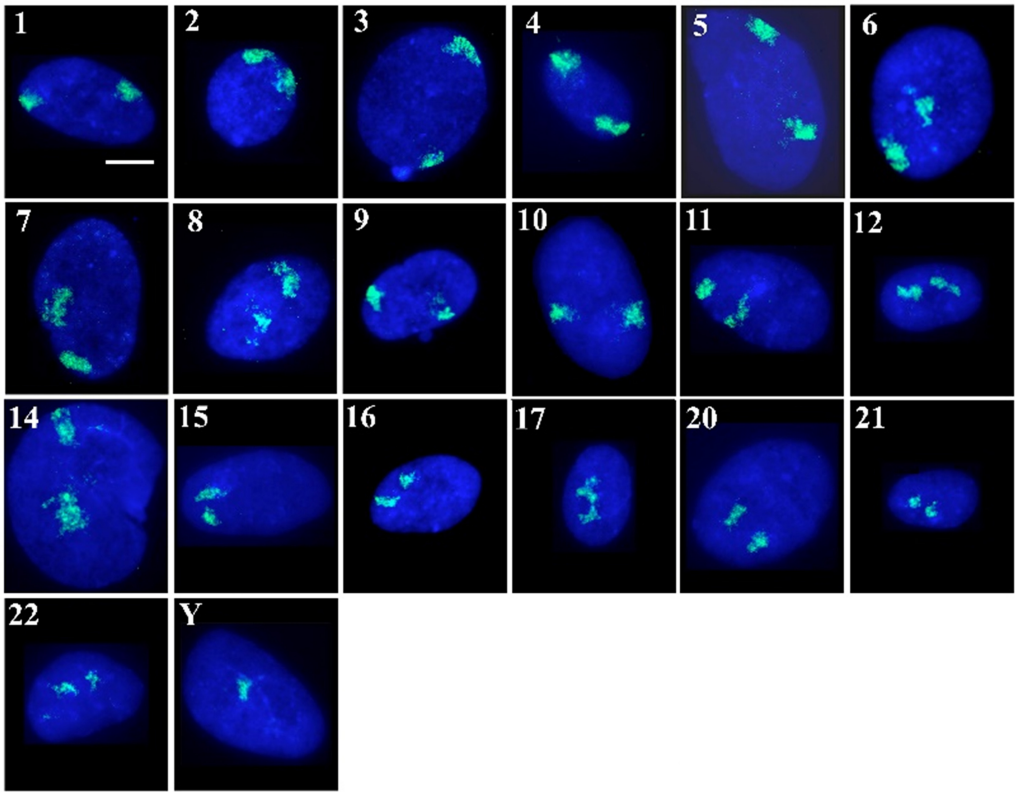

FIGURE 1 | Human chromosome territories in normal senescent human dermal fibroblast nuclei: Representative images displaying the spatial arrangement of human chromosome territories (in green) in senescent interphase nuclei of fibroblasts, stained with DAPI (blue). The numbers/letters by the side of each nucleus indicates the chromosome hybridised to by fluorescence *in situ* hybridisation. All the cells were grown in 10% new born calf serum and were found to be negative for the proliferation marker pKi-67. Scale bar = 10 μM.

The geometric centre of the chromosome territory in 3D was determined and a measurement made to the nearest nuclear periphery, as delineated by DAPI staining. The measurements were binned in 0.5 μm increments and a frequency distribution created for the measurements of chromosome 10 in young proliferating, young quiescent and late passage senescent HDF. In the frequency distribution, the peak for chromosome 10 in quiescence cells is the closest to the nuclear periphery, followed by proliferating cells and then with the peak for the senescence measurements being the furthest away from the nuclear periphery.

This large difference in the nuclear localisation of chromosome 10 provides a novel and robust new biomarker for differentiating between quiescent and senescent cells.

Differences in Expression of Genes located on Chromosome 10 in Proliferating, Quiescent and Senescent HDFs

The differential locations of chromosome 10 territories in non-proliferating cells provide an excellent model system in which to study more detailed aspects of chromosome behaviour and the importance of spatial positioning to regulate function. Using this model, we extracted total RNA from proliferating, quiescent and replicative senescent fibroblasts to determine what effect the relocalisation of chromosome 10 has on transcript abundance from this chromosome using a microarray analysis. Our data demonstrate that 33 genes increase transcripts and 39 genes have a significant decrease in transcripts when senescent cells are compared to proliferative cells (**Figure 5**). Interestingly, only four genes with increased transcript levels were found in both quiescence and senescent cells, whereas 15 genes decreased transcript levels in both compared to proliferative cells (**Figure 5**). This demonstrates that the repositioning of the chromosome 10 into the nuclear interior senescence does not mean that genes will be repressed. Although only 72 genes were identified to have significantly changed transcript abundance from chromosome 10 during senescence we were able to identify specific pathways that were enriched for. We identified that there are changes in transcript abundance related to cell cycle control and steroid hormone biosynthesis, however these pathways were also enriched for in quiescent cells as well, further suggesting that the change in chromosome location was unrelated to gene expression changes. This is supported by RNA-seq data that also show up and down-regulation of genes on chromosome 10 when it is either at

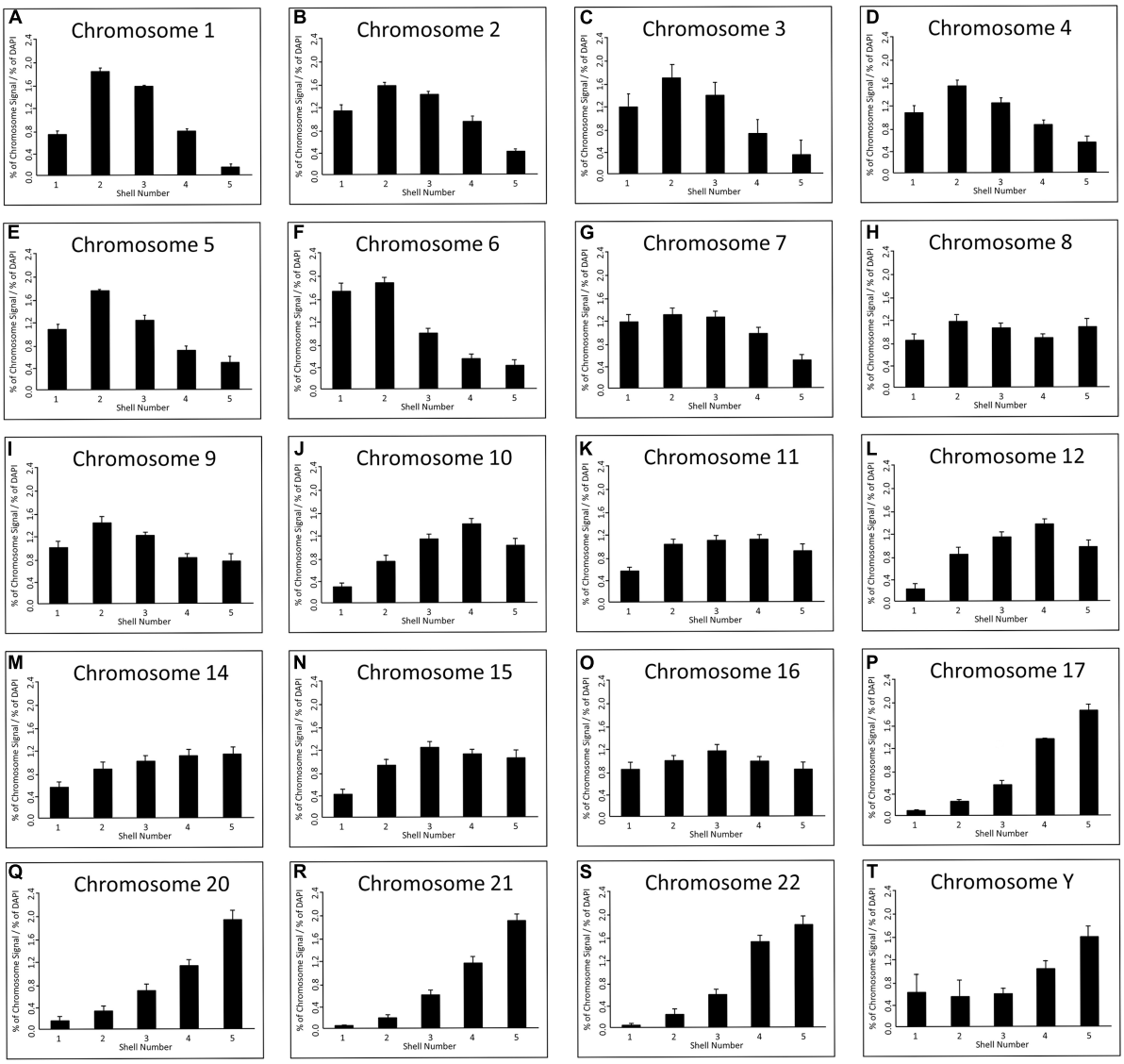

FIGURE 2 | Spatial distribution of human chromosome territories in normal senescent fibroblast nuclei: Digital images (>50 nuclei) for each chromosome were analysed by a simple erosion analysis script (Croft et al., 1999; Clements et al., 2016). The script divides the cell nuclei into five shells of equal area and measures the % of signal intensity from both chromosome signal and the DNA (DAPI). The % of chromosome signal is normalised by division of the % of DAPI in each of the eroded shells (*y*-axis); and the shell numbers of 1–5 are on the *x*-axis. The error bars represent the standard error of mean (SEM). Shells 1 and 2 denote the nuclear periphery and shells 4 and 5 the nuclear interior. chromosomes as indicated above each graph in senescent nuclei as visualised by FISH and specific probes and erosion analysis. **(A)** Chromosome 1, **(B)** Chromosome 2, **(C)** Chromosome 3, **(D)** Chromosome 4, **(E)** Chromosome 5, **(F)** Chromosome 6, **(G)** Chromosome 7, **(H)** Chromosome 8, **(I)** Chromosome 9, **(J)** Chromosome 10, **(K)** Chromosome 11, **(L)** Chromosome 12, **(M)** Chromosome 14, **(N)** Chromosome 15, **(O)** Chromosome 16, **(P)** Chromosome 17, **(Q)** Chromosome 20, **(R)** Chromosome 21, **(S)** Chromosome 22, **(T)** Chromosome Y.

the nuclear periphery or interior in non-proliferating cells (**Supplementary Table 1**).

Chromosomes in Senescent Cells Cannot Be Induced to Relocate After a Stimulus

We have demonstrated that specific chromosomes can be actively repositioned rapidly upon a stimulus in young proliferating cells via nuclear motors comprising nuclear myosin 1β (Mehta et al., 2010). We sought to investigate whether chromosomes can be induced to actively relocate in cells that have become senescent. Thus, we placed late passage cultures into low serum to induce chromosome repositioning. We analysed the nuclear positions of both chromosomes 10 and X using a standard 2D-FISH assay (**Figures 6A–H**). We found that in senescent HDFs chromosome 10 territories did not relocate to the nuclear periphery (**Figure 6C**), where they are found in young quiescent HDF (**Figure 6B**) but remained within the nuclear interior (**Figure 6C**). When compared to senescent HDF grown in

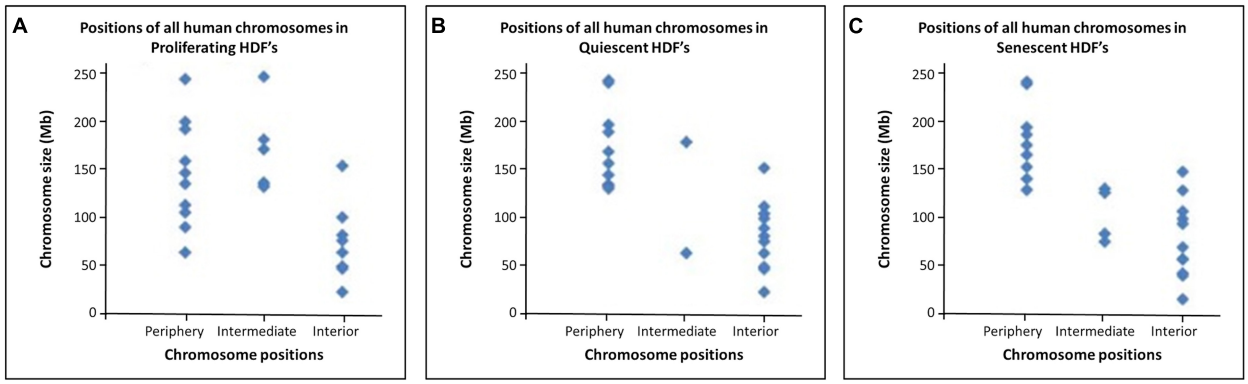

FIGURE 3 | Relationship between chromosome size and nuclear location within proliferating, quiescent and senescent HDFs: The size (Mb) of each chromosome falling within a positioning category (nuclear periphery, at an intermediate location, or in the nuclear interior) in proliferating, quiescent and senescent cells are displayed in panels **(A–C)**, respectively.

10% serum in the senescent cells placed in low serum there was a significant shift ever more towards the nuclear interior (**Figure 6D**). As expected, the X chromosome territories did not change their position at the nuclear periphery (**Figures 6E–H**). To investigate a further stimulus we subjected senescent HDFs to a 42°C heat-shock for 1 h with continuous 5% CO_2, fixed cells for 2D-FISH and analysed the nuclear location of chromosome 11, the chromosome containing a number of heat shock genes. In young proliferating cells (positive for Ki67) significantly relocate chromosome 11 to a new nuclear location, more towards the nuclear interior than the intermediate location in cells after heat shock (**Figure 6I**). However, there is no movement of chromosome 11 at all in senescent cells when responding to heat-shock (**Figure 6J**), this correlates with heat shock gene transcription failing in senescent cells (Sabath et al., 2020).

Differential Nuclear Myosin Iβ Distribution in Normal Proliferating, Quiescent, Senescent HDFs

We have previously demonstrated that NM1β is required for whole chromosome movement when HDF are placed in low serum (Mehta et al., 2010) and have shown its distribution is considerably altered in quiescent (Mehta et al., 2010) and in Hutchinson-Gilford Progeria Syndrome (HGPS) HDFs (Mehta et al., 2011). Therefore, we questioned if the distribution of NM1β was also affected in senescent HDFs, which could explain the lack of chromosome repositioning in senescent cells, post-stimulus. In proliferating HDFs, NMIβ is found distributed throughout the nucleoplasm, along the nuclear envelope and within the nucleoli (Mehta et al., 2010; **Figures 7A–C**). When HDFs enter quiescence this distribution of NMIβ is lost and NM1β becomes accumulated in large aggregates throughout the nucleoplasm (**Figures 7D–F**). In the senescent HDFs, the distribution of NM1β was not as it is in proliferating HDF but was more similar to quiescent cells with large aggregates and some nucleoplasmic stain (**Figures 7G–I**). More specifically, NMIβ positive cells were analysed for the distribution pattern of NMIβ, and we classified the different distribution patterns (**Figure 7J**). The fraction of cells in each category was scored in over 200–500 cells in three independent experiments and correlated with the presence of pKi-67 in passage 11 (young passage) cells, or with the absence of pKi-67 in passage 43 (late passage) cells, and in serum starved passage 11 cells (quiescence) (**Figure 7J** and **Supplementary Table 2**). Proliferating HDF displayed 87% with a proliferating distribution of NMIβ of a nucleoplasmic, nuclear rim and nucleolar distribution, whereas this dropped to 0.3% and 2.5% in quiescent and senescent cells respectively (**Supplementary Table 2**). The largest fraction (72%) of NM1β pattern in the quiescent cells was the aggregates-only pattern with 25% displaying NM1β only at the nucleolus. Interestingly, the late passage cultures had 81% of their Ki67 negative cells displaying aggregates of NM1β. Thus, the lack of relocation of chromosomes in response to stimuli in senescent cells is correlated with an altered distribution of NM1β into aggregates.

DISCUSSION

Using the Bickmore and Perry analysis method of localising chromosome territories in 2D fixed and flattened nuclei, and employing the original analysis script to radially position normalised chromosome signal (Croft et al., 1999; Clements et al., 2016), all human chromosomes in young proliferating HDF (Croft et al., 1999; Boyle et al., 2001; Meaburn et al., 2007) and quiescent HDF (Bridger et al., 2000; Mehta et al., 2010) have been mapped. Many of these chromosomal locations have been confirmed by 3D-FISH and analysis of confocal laser scanning microscopy optical images. The study presented here completes the nuclear positioning of all human chromosomes in normal replicative senescent primary HDF using the same analysis methods. Comparisons of the distribution of chromosomes in replicative senescent nuclei is similar to quiescent nuclei in that there is a definite influence of chromosome size in positioning, with smaller chromosomes towards the nuclear interior and larger chromosomes towards the nuclear periphery (**Supplementary Table 1**; Sun et al., 2000; Cremer et al., 2001, Bolzer et al., 2005). However, we reveal that chromosome

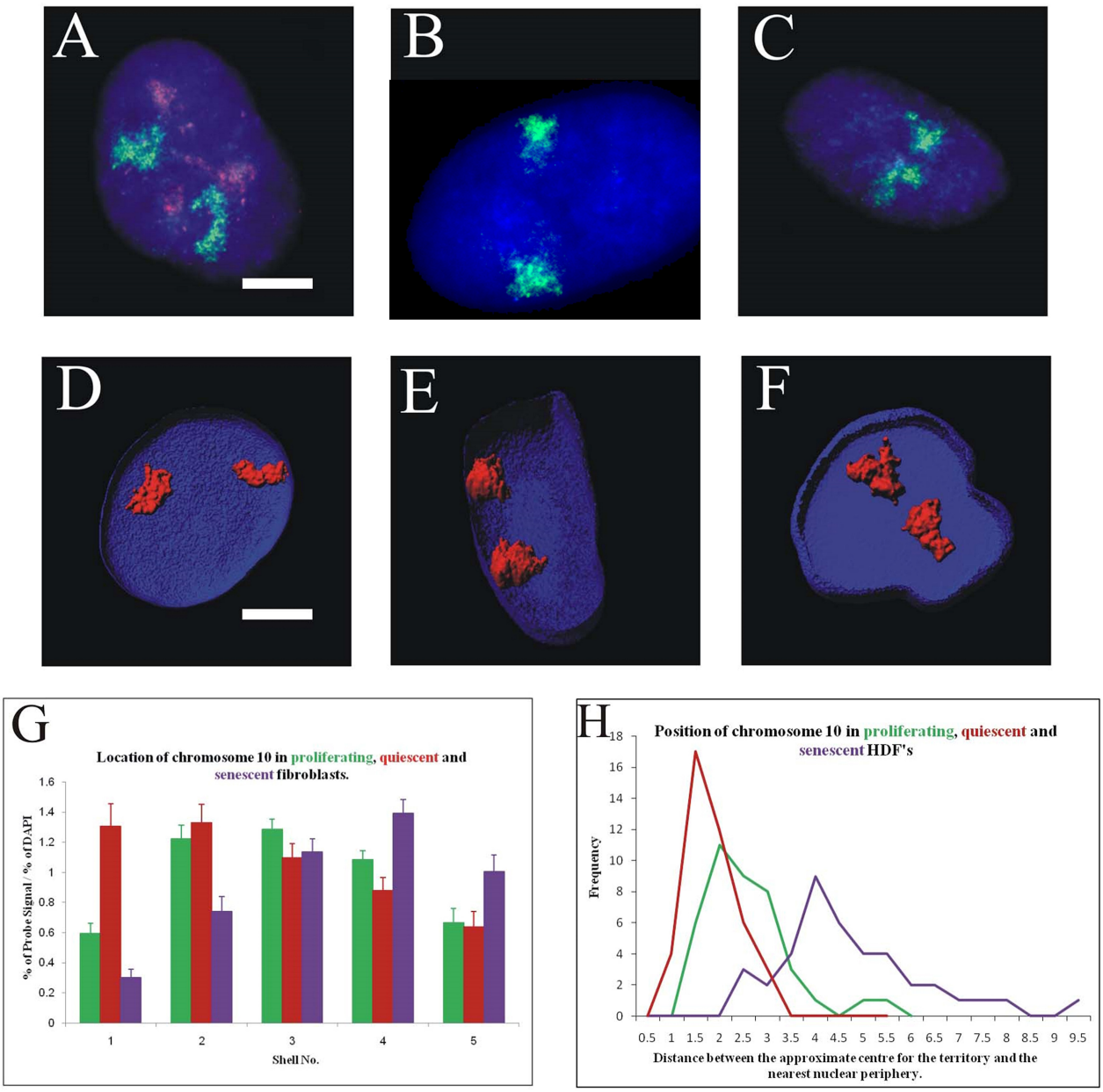

FIGURE 4 | Differential location of chromosome 10 territories in proliferating, quiescent and senescent cell nuclei: Panels **(A–C)** represent cell nuclei that have been subjected to 2D-FISH, displaying chromosome territories (green) and the proliferation marker pKi-67 (red). Panels **(D–F)** display 3D reconstructions of cell nuclei that have been prepared for 3D-FISH and optical imaged using a confocal laser scanning microscope. Chromosome territories are in red the nuclei delineated in blue (DAPI). Scale bar = 10 μm. Panel **(G)** displays comparative histograms of the position of chromosome 10 in proliferating (green), quiescent (red), and senescent (purple) nuclei, as determined by 2D FISH and erosion script analysis. Error bars represent standard error of the mean (SEM). Panel **(H)** displays comparative frequency distributions of measurements for the position of chromosome 10 in proliferating (green), quiescent (red), and senescent (purple) in 3D preserved nuclei. Measurements have been made from the geometric centre of each chromosome territory to the nearest edge in 3D. Unpaired, unequal variance, two-tailed Student's *t*-test at 95% confidence interval ($p < 0.05$) has been performed.

positioning is not entirely equivalent in quiescent and replicative senescent nuclei and that there are specific differences between the two non-proliferating statuses. Most notably, there is one chromosome that is found in opposing nuclear locations in these two types of non-proliferating HDFs. This is human chromosome 10, whose territories are located at the nuclear periphery in quiescent cells and in the nuclear interior in replicative senescent cells. This disparate positioning must be regulated and our hypothesis is that the plethora of genes concerned with proliferation on chromosome 10 (see Deloukas et al., 2004) would need to be regulated differently in the two arrested situations, since one situation is irreversible and the other

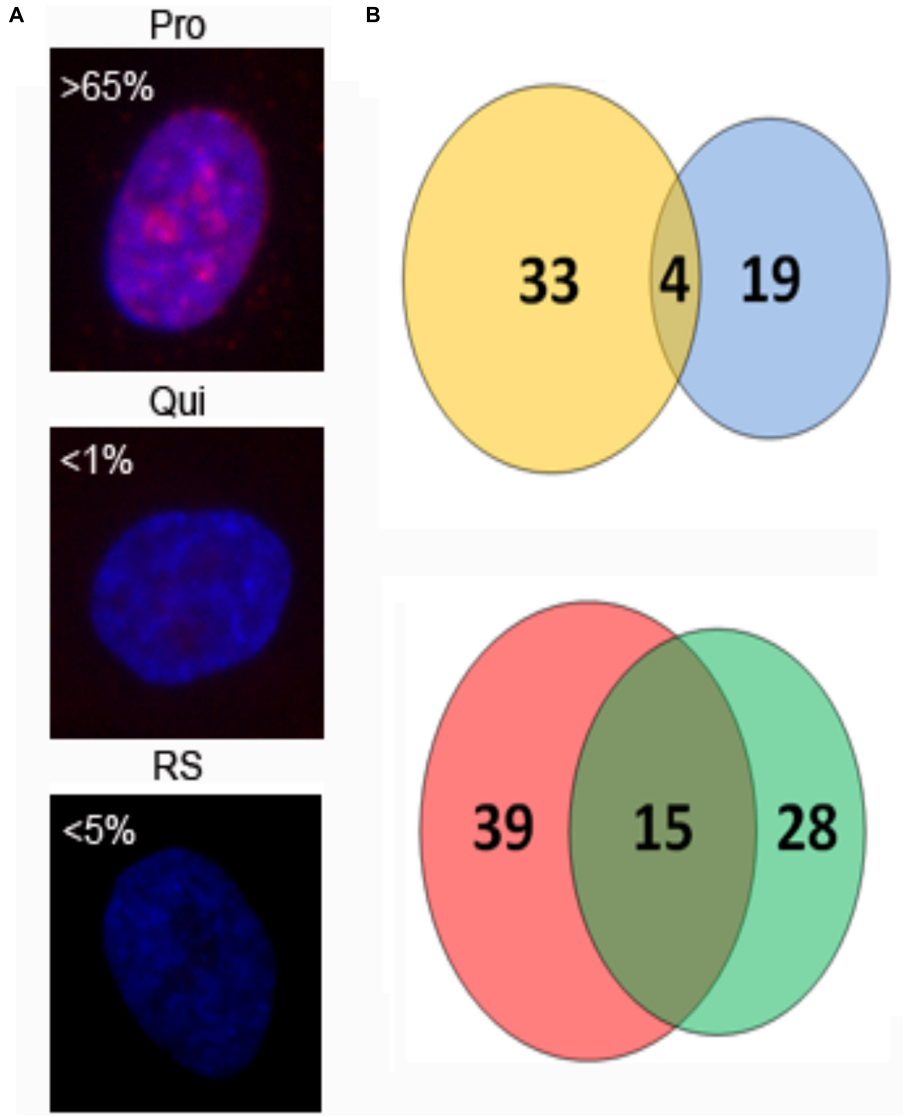

FIGURE 5 | Number of chromosome 10-associated genes with changed transcript abundance in quiescent and replicative senescent fibroblasts. 2DD fibroblast cultures were immuno-labelled for the proliferative marker Ki67 [panel **(A)**, red] to show growth status. Chromatin is counterstained in DAPI (blue). Percent Ki67+ cells are indicated and show the presence of Ki67 in proliferating (Pro), quiescent (Qui), and replicative senescent (RS) cultures. RNA was extracted from parallel cultures and used in microarray analysis to identify genes that had increased **(B, top panel)** or decreased **(B, lower panel)** transcript abundance from chromosome 10 as 2DD cells become quiescent or replicative senescent. Thirty three genes increased transcript levels in replicative senescent samples (yellow circle) and 19 in quiescent samples (blue circle) with 4 genes in common between the data sets. Thirty nine genes exhibited significantly decreased transcript levels in replicative senescent samples (red circle), 28 in quiescent samples (green circle) with 15 of these common between the data sets.

reversible, with caveats. However, we have demonstrated here that there is expression from chromosome 10 in both quiescent and replicative senescent cells (**Supplementary Table 1**) – these can be the same genes or different genes. It is no surprise that downregulation of proliferation genes in both non-proliferating situations has occurred but it would be interesting to analyse the method of silencing for genes such as *CDK1* and *SIRT1* on chromosome 10. The reorganisation of chromosome 10 as well as other chromosome such as 18 and 13, may represent the gain or loss of specific long-range chromatin interactions that influence whether fibroblasts proliferate, quiesce or become senescent. Indeed there are large areas of heterochromatin surrounding nucleoli with which association may elicit a silencing effect on chromatin. Therefore, specific genes on chromosome 10 may become irreversibly silenced in senescent cells by relocation to more internal positions. When analysing specific gene expression from chromosome 10 we found some genes become down-regulated in senescent cells that are up-regulated in quiescent HDF (**Figure 5** and **Supplementary Table 1**), further indicating the nuclear edge is not exclusively an area of down-regulation. Interestingly, cells made senescence through stress i.e., stress induced premature senescence (SIPS) seem to

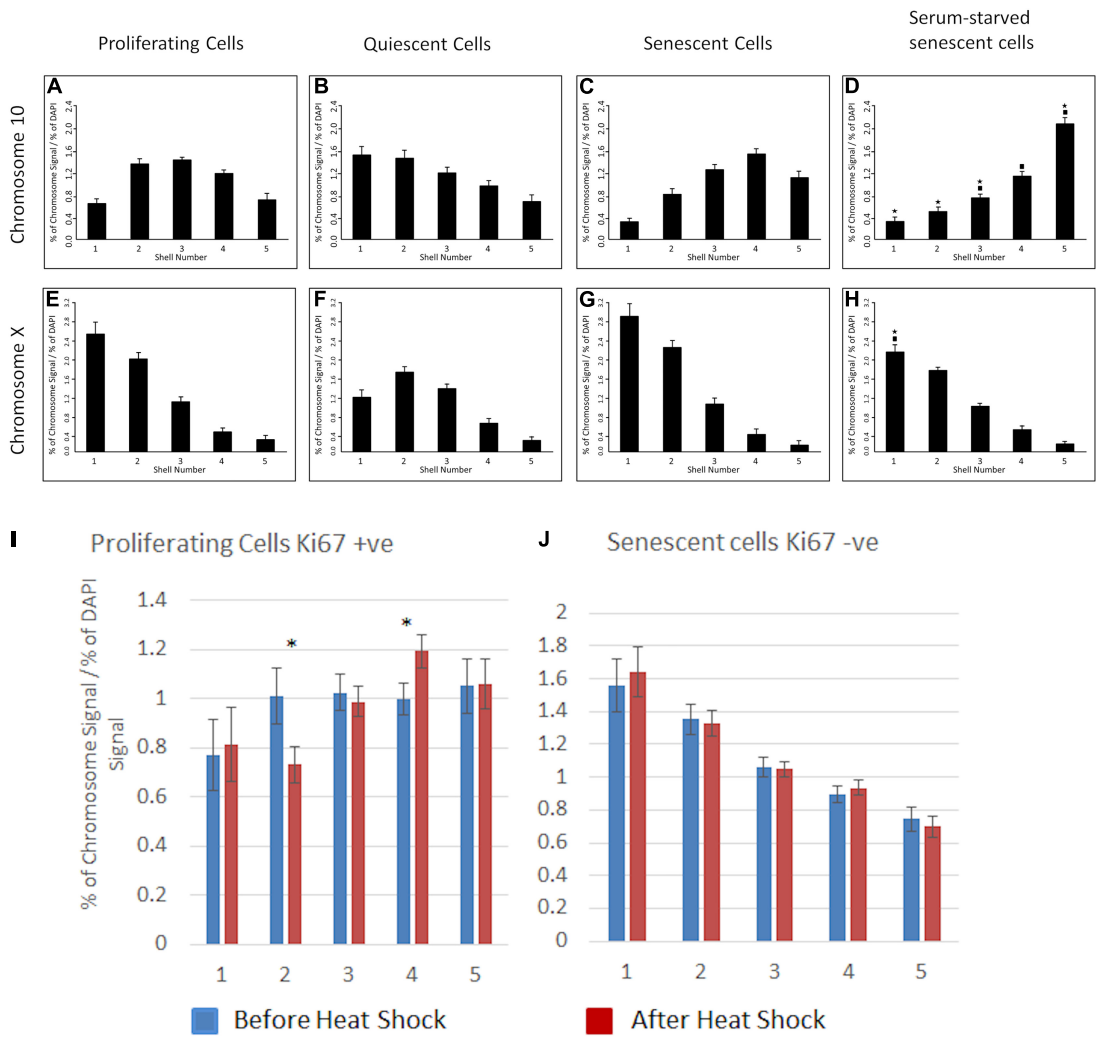

FIGURE 6 | Chromosomes 10, X or 11 do not relocate to new nuclear locations in senescent nuclei upon serum removal or heat shock. For senescent cells HDFs were grown in 10% NCS until the culture became mostly comprised of non-confluent senescent cells as determined by the absence of anti-pKi-67. Young proliferating cells were collected from early passage cultures where anti-Ki67 staining was in over 65% of cells. The cultures were serum-starved by incubation with 0.5% NCS for 7 days (chromosome 10 and X) or subjected to a heat-shock (42°C, 1 h, chromosome 11). Positions of chromosomes 10 and X were determined using 2D-FISH erosion analysis and anti-Ki67 staining to differentiate between proliferating and senescent cells. Chromosome 10 **(A–D)**, chromosome X **(E–H)**, chromosome 11 **(I,J)**. Panels **(A–H)**: The asterisks indicate statistical difference ($p < 0.05$), as assessed by Student's *t*-test, to the normal quiescent cells. The filled-in squares indicate statistical difference ($p < 0.05$) to normal senescent cells grown in 10% serum. In panels **(I,J)** asterisks indicate statistical difference ($p < 0.05$), as assessed by Student's *t*-test for before and after heat-shock.

display chromosome 10 at the nuclear periphery, where it is located in quiescent HDFs (data not shown). This is similar to the nuclear position of chromosome 10 revealed by analysis of HiC data for OIS in WI38-hTERT fibroblasts (Das et al., 2020). This suggests that different types of senescence may have different positioning patterns for specific chromosomes. Understanding these differences may give us a greater insight into the mechanisms that control genome positioning patterns and health of the cells.

We have shown that movements of whole chromosomes require NMIβ to be present in the correct distribution (Mehta et al., 2010; Bridger and Mehta, 2011; Mehta et al., 2011). Here we show that senescent cells do not have the ability to relocate chromosome 10 to the nuclear periphery upon serum removal nor chromosome 11 towards the nuclear interior upon a heat-shock. We have demonstrated that both non-random movements occur in young proliferating HDFs (Mehta et al., 2010; **Figure 6**). This strongly implies that the chromosome movement mechanism may not be functional in senescent cells and this finding correlates with the senescent nuclei containing aggregated NMIβ, rather than dispersed NM1β throughout the nucleoplasm, as it is in proliferating cells. Furthermore, our RNA-seq studies also reveal the gene *MYO1C*, encoding NM1β, to be down-regulated in the senescent HDFs (**Supplementary Figure 1**). Contrary to this, *MYO1C* is not found as a gene upregulated nor associated with senescence in

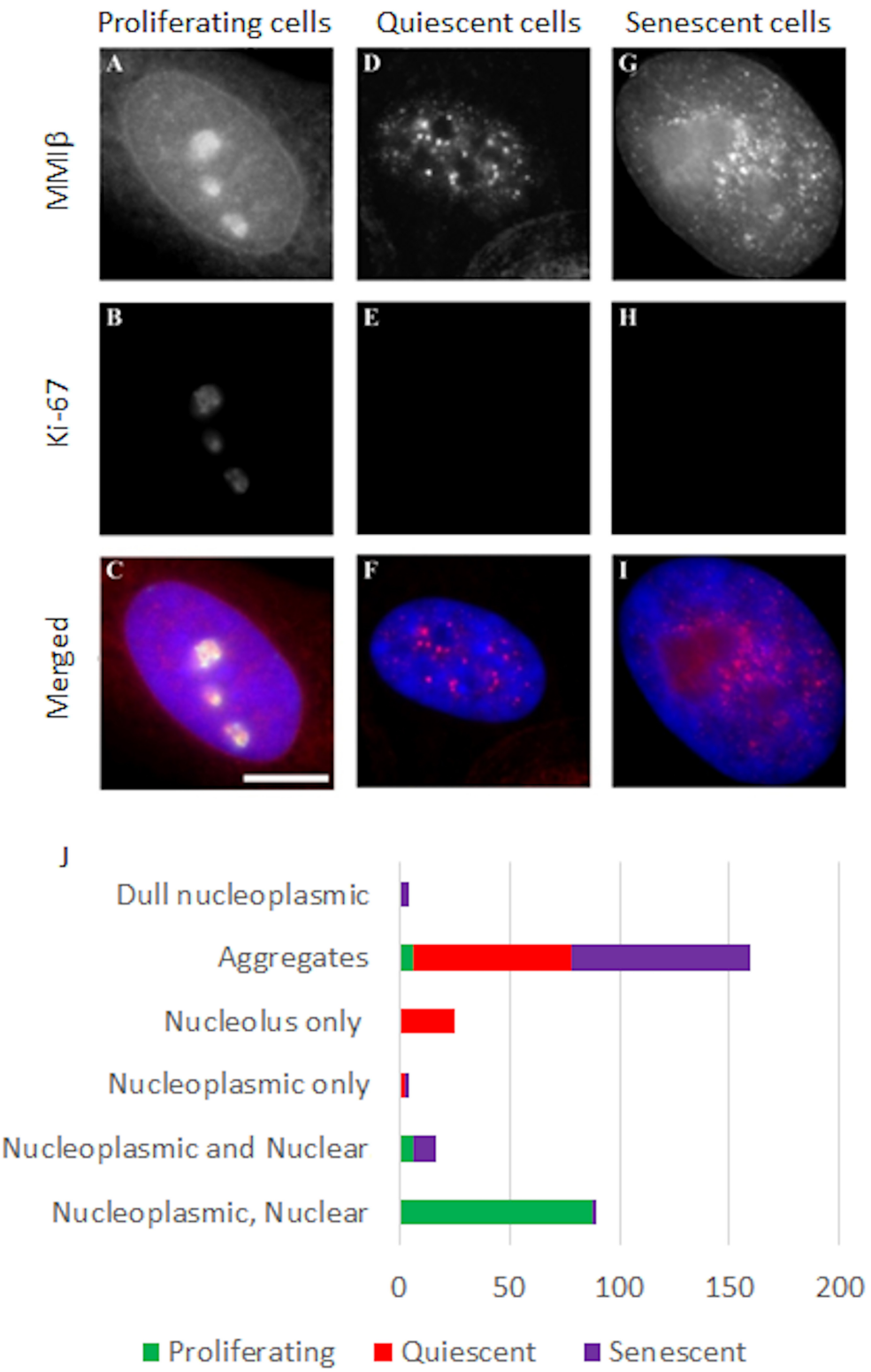

FIGURE 7 | Representative images displaying the distribution of NMIβ in proliferating, quiescent and senescent HDFs. Young, serum starved young and senescent HDF were grown on glass coverslips, fixed with methanol:acetone and co-stained for nuclear myosin 1β [red, **(A,D,G)**] and pKi-67 [green, **(B,E,H)**]. DNA is delineated by DAPI and merged images are displayed in panels **(C,F,I)**. Scale bar = 5μm. **(J)** displays the fractions of the different patterns of nuclear myosin 1β -nucleoplasmic, nuclear rim and nucleolar; nucleoplasmic and nuclear rim, nucleoplasmic only, nucleolus only, aggregates and dull nucleoplasmic. Error with respect to standard deviation can be found in **Supplementary Table 2**.

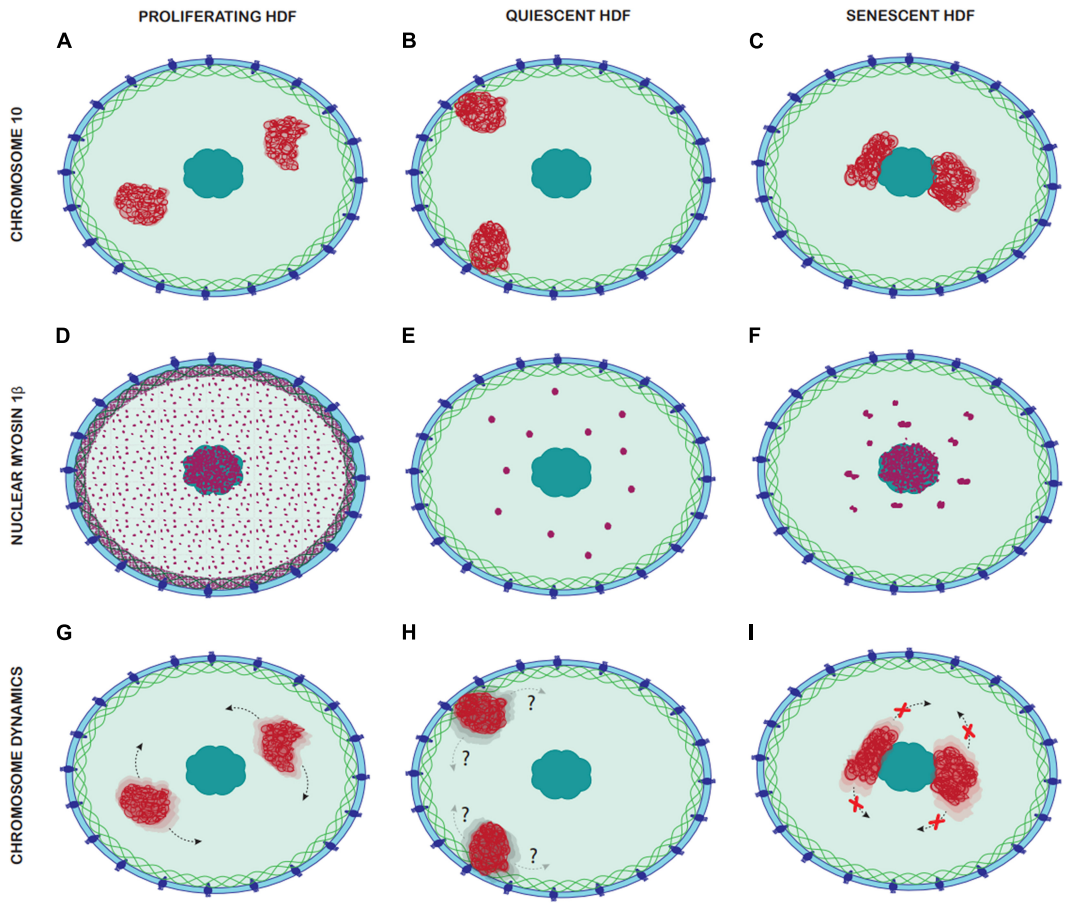

FIGURE 8 | Comparison of Chromosome 10 location, nuclear myosin 1β distribution and chromosome dynamics in proliferating, quiescent, and senescent HDF. Panels **(A–C)** represent the different locations of chromosome 10 in proliferating, quiescent and senescent HDF, with chromosome 10 territories represented in red. **(A)** In proliferating HDF chromosome 10 occupies and intermediate nuclear position. **(B)** In quiescent HDF chromosomes 10 occupies a peripheral nuclear position. **(C)** In senescent HDF chromosome 10 occupies an interior nuclear position. Panels **(D–F)** represent the distribution of nuclear myosin 1β in the nucleus of proliferating, quiescent, and senescent HDF, with nuclear myosin 1β represented in purple. **(D)** In proliferating HDF, there is a dense accumulation of nuclear myosin 1β in the nuclear lamina and nucleoli, and it is also distributed homogenously through the nucleoplasm. **(E)** In quiescent HDF, nuclear myosin 1β accumulates in large spherical aggregates through the nucleoplasm. **(F)** In senescent HDF, nuclear myosin 1β accumulates in large non-spherical aggregates through the nucleoplasm but is also densely accumulated in the nucleoli. Panels **(G–I)** represent the chromosome dynamics in proliferating, quiescent and senescent HDF, with chromosome 10 territories represented in red. **(G)** In proliferative HDF, chromosome 10 can be repositioned rapidly upon a stimulus via nuclear motors. **(H)** Chromosome dynamics in quiescent HDF remains unknown. **(I)** In senescent HDF, chromosome 10 cannot be repositioned upon stimuli and thus remains in the same nuclear location.

the databases genAGE,[4] HCSGD (Dong et al., 2017), CellAge[5] and on the reverse does not come up as a gene that could be used as a reference gene in qPCR due to it not changing its expression in senescent compared to proliferating cells (González-Bermúdez et al., 2019; Hernandez-Segura et al., 2019).

With further work, the nuclear position of chromosome 10 could be a reliable marker to differentiate between quiescent and replicative senescent cells, since there are presently a range of issues with biomarkers to differentiate decisively and easily between the two non-proliferating states (Hernandez-Segura et al., 2018), a number of markers is combined to be more certain (Gorgoulis et al., 2019) and even then it is not so easy to differentiate between different types of senescence.

Thus, the spatial organisation of chromosomes within interphase nuclei not only differs between various cell types (Meaburn and Misteli, 2007; Bridger et al., 2014; Sivakumar et al., 2019), but also is distinct as cells traverse from a proliferating to a non-proliferating state in their life span; thus stressing the role of this differential organisation in genome function. In addition to this, differences in organisation of NMIβ between proliferating and non-proliferating cells also suggest a plausible role of nuclear motors in chromosomal organisation within the cell nucleus (**Figure 8**).

Although, nuclear motor proteins have a number of roles in genome function (Venit et al., 2020), it appears that at least one of the roles NM1β plays in young proliferating cells, whereby

[4]https://genomics.senescence.info/genes/
[5]https://genomics.senescence.info/cells/

chromosomes and genes respond to stimuli to be relocated to new non-random active locations is not functional in old cells. The nuclear distribution of NM1β is considerably different in senescent cells when compared to young proliferating cells, so a further possible biomarker candidate? Since it is highly probable that the nuclear myosins are involved in chromosome repositioning use F-actin it is pertinent to note the accumulation of G-actin in senescent cells (Kwak et al., 2004). It is highly possible that the inability to move chromatin around upon response to a stimulus in cells is in part involved in the mechanisms to prevent re-entry of senescent cells into the proliferative cell cycle. Indeed, others have predicted that once changes to genome organisation occur in senescent cells they are metastable (Chiang et al., 2019).

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI GEO; GSE164446.

AUTHOR CONTRIBUTIONS

IM: experimentation and design, some writing, and figures. KR: data for heat shock – 2 graphs. RP: **Figure 8**. MF: data for a chromosome position. KM: experimental design and some writing. IK: senescent cells and figures. CE: RNA Seq and analysis. JB: experimental design, supervision, data analysis, writing, and figures. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.640200/full#supplementary-material

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REVIEW
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Simple Detection Methods for Senescent Cells: Opportunities and Challenges

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Cellular senescence, the irreversible growth arrest of cells from conditional renewal populations combined with a radical shift in their phenotype, is a hallmark of ageing in some mammalian species. In the light of this, interest in the detection of senescent cells in different tissues and different species is increasing. However much of the prior work in this area is heavily slanted towards studies conducted in humans and rodents; and in these species most studies concern primary fibroblasts or cancer cell lines rendered senescent through exposure to a variety of stressors. Complex techniques are now available for the detailed analysis of senescence in these systems. But, rather than focussing on these methods this review instead examines techniques for the simple and reproducible detection of senescent cells. Intended primary for the non-specialist who wishes to quickly detect senescent cells in tissues or species which may lack a significant evidence base on the phenomenon it emphasises the power of the original techniques used to demonstrate the senescence of cells, their interrelationship with other markers and their potential to inform on the senescent state in new species and archival specimens.

Keywords: ageing, senescence, lipofuscin, labelling index, detection

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WHAT IS A SENESCENT CELL?

Historical Background

The initiation of the first true cultures of metazoan cells by Alexis Carrel (Carrel, 1913) is a landmark in the history of biology. Carrel was primarily interested in understanding the regulatory factors controlling wound healing. In attempting to do so he established cell culture as a valuable experimental tool for the study of cell morphology and function. Gradual refinement of tissue culture techniques have allowed the culture of progressively more complex cell types and the development of model systems for the study of many fundamental biological processes.

Initially however, studying ageing *in vitro* appeared impossible. Although it had been proposed that exhaustion of the growth capacity of somatic cells was responsible for organismal ageing (Wesimann, 1889), Carrel's best known experiments appeared to falsify this hypothesis. Carrel initiated cultures of chick fibroblasts which his co-workers claimed to have cultured continuously for over 30 years, much longer than the lifespan of the intact organism (Ebeling, 1942). It followed that the ageing process had to operate at the level of the tissue or organism not the single metazoan cell, which seemed as immortal as its bacterial counterpart. As tissue culture gained in popularity through the 1950s the failure to produce cultures of "immortal" cells was attributed to poor experimental technique. This was not innately implausible given the extraordinary difficulties then involved in carrying out tissue culture experiments that today would be considered trivial. This "poor culture

technique" argument has reappeared regularly as cytologists have pushed the boundaries of tissue culture to the technological limits of their day (Perillo et al., 1989) and will doubtless reoccur in the future, sometimes justifiably.

However, in the early 1960's (Hayflick and Moorehead, 1961; Hayflick, 1965) Hayflick demonstrated that normal human fibroblasts would only proliferate for a finite number of passages in culture. Latent infectious agents, composition of the medium and depletion of key metabolites were all shown not to be responsible for this failure to grow. Hayflick also observed that fibroblast cultures initiated from embryos grew substantially better than those derived from adults. Combined with the demonstration that normal somatic cells displayed limited lifespans during serial transplantation experiments these observations, led Hayflick to formulate *a cellular theory of ageing* (Hayflick, 1965; Hayflick, 1979). The original tenets of this hypothesis were that a finite lifespan is an intrinsic property of normal human cells, that cell growth *in vitro* is somehow related to human ageing and that cultured primary fibroblasts were a useful model system in which to study some aspects of *in vivo* ageing. Hayflick termed this failure to grow "senescence" a term which, for good or ill, has stuck.

Since Hayflick's original discovery many different cell types have been shown to behave analogously to human fibroblast populations *in vitro*. Senescent cells have also been shown by a variety of methods to be present in many different tissues *in vivo*. This finally silenced the criticism, not uncommon among gerontologists until the 2000s, that senescence was simply a "tissue culture artefact" unworthy of study. Rather, senescence appears to be a ubiquitous characteristic of cells derived from regenerative somatic tissue and a primary causal mechanism of ageing and age-related disease.

However, as the list of cell types showing senescence has grown specialists in different branches of biology have found themselves grappling with questions once restricted to cytologists working predominantly on fibroblasts. How can senescent cells be simply and reproducibly detected both *in vitro* and *in vivo*? and to what extent can the same techniques be used in different cell types and different species? To address these questions this article will review the different markers available for the detection of senescent cells with an emphasis on the phenotypic aspects of senescence that originally validated them and how these in turn may inform on the senescent state in other contexts.

Terminology

Clarity is essential when discussing the biology and growth dynamics of cells *in vitro* because the literature uses overlapping, sometimes contradictory, terms for the same cell populations (Hayflick, 1990). Without prejudice to other classification systems, this review will follow the usage of Shall (1987) in which cell cultures can be considered to fall into three types with respect to growth capacity: *Primary* cells are derived from normal tissue and display a limited lifespan in culture (although in some fields only the initial explant population is referred to as "primary"). "Primaries" are sometimes referred to as mortal cell cultures or as *cell strains*-the term originally used by Hayflick and favored by this author [although some researchers restrict use of it to subpopulations selected from a culture by cloning, e.g., Freshney (2005)]. *Continuous cell lines* are by definition composed of cells with an unlimited growth potential (mortal cell populations sometimes being termed *finite* cell lines). In the Shall typography *continuous lines* fall into two broad categories; *immortal cells* are non-tumorigenic, possess unlimited proliferative capacity and at that time were largely derived from rodents (e.g., 3T3 cells). By contrast, *transformed cells* represented the vast majority of cell lines produced from humans (e.g., HeLa or HepG2 cells). Transformed cell lines have the unlimited lifespan of continuous cell lines but form tumors in nude mice and show a number of other characteristics, such as growth in soft agar, which reflect this tumorigenicity *in vitro*. The underlying molecular mechanisms controlling growth and giving rise to this typography have become progressively clearer over the years (e.g., Shay et al., 1993; Zou et al., 2009).

Only cell strains spontaneously become senescent in culture, although senescence can be induced in cell lines through a variety of methods (typically, but not exclusively, involving DNA damage). Senescence is distinct from *quiescence*, a reversible growth arrested state which can be induced by the removal of serum from the medium of primary or immortal cells or through contact inhibition. In systems in which it is experimentally possible readily to separate the two states (e.g., epidermal keratinocytes grown in low calcium medium) senescence is distinct from terminal differentiation (Norsgaard et al., 1996).

"Senescence" is also regularly used in two distinct ways which can occasion further confusion. Senescence of the *entire culture*, originally called the "Phase III phenomenon" (Hayflick, 1979) is failure of the culture to proliferate under conditions which had previously allowed sustained cell growth. This growth is generally measured as Population Doublings (PDs) calculated as the number of times the cell population doubles in number during the course of culture **Eq. 1**.

$$PD = \frac{\log_{10}(\text{Numbers cells harvested}) - \log_{10}(\text{Number cells seeded})}{\log_{10}2} \quad (1)$$

Primary cell populations typically go through 30–60 cumulative population doublings (CPD) by which time they are overwhelmingly composed of *senescent cells* (the second sense of the word as used by cytogerontologists). It is worth reiterating that a "senescent" culture is not necessarily free of growth competent cells, although they will be in a minority. This occurs because all that is required for the population to cease to expand and enter an apparently static phase is for the rate of production of newborn cells to be less than or equal to the population death rate (Kalashnik et al., 2000). The highly variable survival times of senescent populations from different cell types reported in the literature (days or weeks in the case of senescent HUVECs compared with months or years in the case of dermal fibroblasts) results in part from this dynamic.

The canonical feature of a senescent cell is its failure to divide in response to a conventional mitotic stimulus but this is also accompanied by radical alterations in cell physiology. Together these constitute the core phenotypes of cellular senescence from

which the validity of all histological markers ultimately derive. Because it is possible to separate the altered phenotype from the cessation of growth in some contexts (e.g., the serial passage of adrenocortical cell strains) the failure of growth and the altered phenotype are considered separately below.

THE CORE PHENOTYPES OF SENESCENT CELLS

The Failure to Grow

Hayflick and Moorehead (1961) working with 25 independent strains of human fetal fibroblasts made the central finding that primary cultures cease to expand, and defined three distinct stages of growth *in vitro*. Phase I, the explant culture, was considered to terminate with the formation of the first confluent sheet of cells. Phase II was characterised by vigorous growth requiring repeated subculture. Phase III was a decline phase in which, after approximately 50 CPD, the cells showed cessation of mitosis, the accumulation of cell debris and degeneration of the culture (Hayflick, 1965). These three stages can be observed in a wide variety of different cell types but patience is required since 50 CPD takes roughly a year of continuous passage for fibroblasts (although fibroblast cultures with both greater and lesser proliferative capacities are common).

Hayflick's original model carried two implicit assumptions. Firstly, that the decline in proliferative ability seen during "Phase III" resulted from cell death. Secondly, that the cultures studied were homogeneous populations of cells which were either all growing (in Phase I and II) or all non-growing (Phase III). Simple cell death as a cause of Phase III was quickly excluded by the demonstration that RNA synthesis (measured by incorporation of tritiated uridine) occurred in all population phases (Macieira-Coelho et al., 1966).

Although proliferative homogeneity was disproved a little later it is still widely assumed to be a feature of normal cell populations-perhaps because Hayflick's original figure showing the three phase model is so regularly reproduced in tissue culture manuals. But Smith and Hayflick (1974) and Smith and Whitney (1980) demonstrated, by isolating and then culturing hundreds of individual WI38 and WI26 clones at different mass culture population doubling levels, that primary fibroblast cultures contain mixtures of clones with very variable growth potentials. Re-cloning a clone with a high divisional capacity produced sub-clones with a range of division potentials that gradually shifted towards smaller clones as the culture aged. Later studies examined the replicative capacity of thousands of isolated glial cells using the Pontén mini-cloning technique (Pontén et al., 1983). This assay is based on the use of custom tissue culture plates. These are created by the deposition of arrays of some hundreds of circular "islands" of palladium (typical areas 45,800 μm^2 or 18,000 μm^2), onto the central area of tissue culture plates that no longer allow cell adhesion (e.g., by being pretreated with agarose) whilst at the same time a ring of palladium is seeded around the outside (the overall effect is somewhat akin to islands of palladium in a lake or inland sea). In contrast to the clone isolation experiments of Smith and Hayflick (1974) or Smith and Whitney (1980) Pontén mini-cloning makes it possible to control for growth artefacts arising from low cell densities by simultaneously plating cells at normal densities on the ring of palladium forming the lake "shore" and cells at clonal densities on the islands (achieved by using a metal spacer to separate the low and high density subpopulations until they have attached). The fraction of non-dividing glial cells in these studies (n = 1760 clones) increases steadily from the earliest to the final passage (~35 population doublings) in a very similar pattern to dermal fibroblasts and represented an early demonstration that senescence could be observed in cells from multiple different tissues.

Labelling studies complemented these single cell analyses. Cristofalo and Sharf (1973) carried out an analysis of the divisional kinetics of embryonic fibroblasts. This required 72 h 3H-thymidine pulse-labeling experiments at every passage throughout the culture lifespan. The fraction of cells which entered S phase was then estimated by autoradiography. They observed that unlabelled (senescent) cells were present in very young cultures, that a few labelled cells were present even in "senescent" cultures and that the fraction of unlabeled cells increased smoothly with serial passage. Additional experiments using WI38 fibroblasts demonstrated that although overall cell cycle length (T_c) increased approximately two fold (from ~19 to ~30 h) with serial passage this was insufficient to explain the decline in the labelling index (Grove and Cristofalo, 1977). Thus the fraction of growing cells in the culture declines as the CPD level increases and fibroblast cultures are mixtures of label-excluding, senescent cells and their growing counterparts, the proportions of which alter as the cultures age. If these basics are neglected when studying a primary cell population then the results of any assay that generates an average value from the culture (in practice anything from Western blotting to next generation sequencing) become fundamentally insecure.

It is often unappreciated that such kinetic analyses underpin virtually all the more "sophisticated" techniques sometimes recommended for the identification of senescent cells today (González-Gualda et al. 2021). Although labelling studies have become progressively easier to perform through the replacement of 3H-thymidine first by 5-bromo-2′-deoxyuridine (allowing label incorporating cells to be detected by immunocytochemistry rather than autoradiography) and subsequently by 5-ethynyl-2′-deoxyuridine (which simplifies the technique still further through the use of a copper (I) catalyzed "click-chemistry" reaction between the 5-ethynyl group and an azide conjugated dye such as Alexa-594 or Pacific Blue) the basic principles and problems inherent to kinetic analyses are much the same today as they were in the 1970s.

The major problem associated with labelling is the need to ensure that the proportion of cells that take up the label accurately reflects the true culture growth fraction. A short labelling time (e.g., 30–90 min) underestimates this because serially passaged cultures are asynchronous and cells in G_1, G_2, and M cannot incorporate label. Since the minimum S phase time is 6–8 h in cultures of MRC5 and WI38 fibroblasts regardless of CPD

(Griffiths, 1984) roughly 70% of division competent WI38 cells will not be in S phase at any one time (T_c = 19–30 h). Long labels of the type used by Cristofalo and Sharf (1973) avoid this problem but overestimate the growth fraction. This is because growth fractions are calculated as the ratio of nuclei that have incorporated label over the total scored (typically 400 positive or 1,000 total nuclei giving the 95% confidence interval). With 72 h labels, cells in S phase when the label is introduced will go through G_2 and M phase during the labelling period and will thus be scored as two separate positive nuclei; a process that could be repeated twice with typical T_c values.

A long label should thus be seen as providing a minimum value for the number of senescent cells present in a population (< 5% label incorporating nuclei on a 72 h label is often used as the definition of a fully senescent culture) whilst short labels are useful for determining the decline in a growth fraction during serial passage, particularly if comparing rates between multiple cell populations (Faragher et al. 1993). In theory, T_c–T_s is the perfect labelling time but in practice almost never attempted whereas labelling times of 24 or 48 h are the hardest to interpret but occur in the literature with monotonous regularity (perhaps because they are simply easier to work into routine laboratory schedules). Minor problems with analogue labelling include the frustrating feature that media which contain high levels of thymidine (such as Ham's F12 or some specialist media with "trade secret" compositions) can cause short labels to fail. BrdU labelling is also incompatible with the simple terminal transferase dUTP nick end labeling (TUNEL) apoptosis assay due to photolysis of any DNA that has incorporated BrdU. Ironically such labelled cells make excellent positive controls in a TUNEL assay.

Given that advantages and disadvantages are inherent in the use of thymidine analogues alternative methods based on the detection of proteins varying in amount and/or conformation through the cell cycle have also been employed to study senescence. Of these antibodies against Topoisomerase II and proliferating cell nuclear antigen (PCNA) have both been used in primary fibroblast cultures (Kill et al., 1994) but have similar problems to the use of thymidine analogues in accurately capturing the total culture growth fraction (PCNA detection is restricted to S phase and Topoisomerase II to G_2/M). The most robust endogenous marker is probably Ki67, a multifunctional protein that is highly expressed in cycling cells but typically absent from cells in G_0 (Gerdes et al., 1984). Although Ki-67 levels can vary in the first G_1 following cell cycle re-entry after this the protein remains immunologically detectable in all cell cycle phases rendering it particularly useful in distinguishing senescent and growing cells within cultures. Using it Thomas et al. (1997) compared the rates of loss of the growth fraction in human dermal fibroblasts and peritoneal mesothelial cells which have very similar replicative lifespans *in vitro*. Mesothelial cells started out with a higher initial growth fraction than fibroblasts but lost it significantly faster (−2.2% Ki67 positive per PD compared with −0.89% for 2DD dermal fibroblasts). These findings illustrate the value of determining the kinetics of entry into senescence for the cell type studied; extrapolating from the fibroblast literature should be done with caution.

A feature common to all label exclusion methods is their inability to distinguish between quiescent and senescent cells. This is a major impediment *in vivo*, almost a fatal flaw, because the overwhelming majority of cells are quiescent most of the time. But, even here the technique can sometimes be used to advantage. Wolf and co-workers (Li et al. 1997) examined the effects of age and long-term caloric restriction on the accumulation of senescent cells in the murine lens (which has the advantage that cell proliferation is limited to a defined zone). Animals from 4 to 45 months of age were subject to one of two complementary procedures; either a two week infusion of 2 μg/g body weight per hour BrdU by osmotic minipump followed by histochemical detection of labelled cells in the proliferative zone and equator of the lens or simple sacrifice and isolation of lens epithelial cells which were then subjected to a colony size analysis of the type pioneered by Smith (Smith and Hayflick, 1974). Compared to their young counterparts old animals showed a highly significant ($p < 0.001$) increase in the number of label excluding cells present in the lens *in vivo*. These changes were mirrored *in vitro* where ~40% of lens cells from 33 month old mice were senescent compared to ~20% of cells from their 6 month old counterparts. The conclusion that the label excluding lens cells *in vivo* are the same population as the senescent lens cells *in vitro* seems inescapable and in both arms of the study calorie restriction significantly reduced their accumulation.

Mechanisms of Senescence: Pathway Components as Specific Markers

In parallel with the study of the kinetics of senescence in primary populations researchers attempted to unravel the mechanistic basis of the process in individual cells. Early cell fusion experiments demonstrated that the phenotype of senescence was common between fibroblast strains from different donors, that it was dominant over growth in synkaryon fusions between growing and senescent cells (Littlefield, 1973) and that senescent nuclei or cytoplasm inhibited DNA synthesis in nuclei derived from young cells when partnered with senescent ones in heterokaryon experiments (Norwood et al. 1990). This work was complemented by the isolation of pairs of daughter cells generated by single mitotic events followed by determination of their proliferative potential. In a significant proportion of these pairs large proliferative differences between the daughters were observed indicating that human fibroblast senescence was controlled by the unequal partitioning of some controlling molecule which, although its nature at that time was unknown, was present at a concentration of less than 100 copies per cell (Jones et al., 1985). Taken together these results were consistent with a model in which senescence was controlled in the nucleus by a few elements and effected by one or more proteins which inhibited the transition of cells from G_1-S phase.

As proposed by Olovnikov (1973), the finite elements regulating human fibroblast senescence eventually proved to be telomeres (which progressively shortened with cell division due to the absence of the repair enzyme telomerase). The primary effector protein, initially isolated by expression screening as

senescent cell-derived inhibitor (sdi) was the now well-known cyclin dependent kinase inhibitor $p21^{waf}$ (Noda et al. 1994). This core mechanistic model was validated by the simultaneous demonstrations that the reintroduction of telomerase into a range of human cell types prevented senescence (Bodnar et al. 1998; Wyllie et al. 2000) and that microinjection of blocking antibodies against $p21^{waf}$ (Ma et al. 1999) or its major transcription factor p53 (Gire and Wynford-Thomas, 1998) rescued human fibroblasts from senescence. The observation that senescence in human fibroblasts is associated with activation of the DNA double-strand regulated ataxia-telangiectasia mutated (ATM) signalling pathway leading to the focal accumulation of the repair protein 53BP1 and phosphorylated histone H2AX (γ-H2AX) at telomeres (d'Adda di Fagagna et al. 2003; Gire et al. 2004) extended these studies and raised the possibility that the detection of components of this senescence mechanism could be used specifically to identify senescent cells *in vivo*.

This approach is attractive in principle. Techniques based on the measurement of telomere length distributions within a population have been used as evidence of cell turnover *in vivo* and, by inference, the presence of senescent cells in the populations sampled. Using this approach Allsopp et al (1995) demonstrated that human peripheral blood lymphocytes show an average reduction of 2 Kbp of telomere length over 50 years *in vivo* whereas brain tissue mean telomere length remains constant over the same period. But these methods are complicated by the fact that telomere shortening is not the sole, or even the predominant, mechanism by which mammalian cells enter senescence. Even in humans the telomere-dependent pathway is only used by a subset of cell types. Additional problems of interpretation have emerged as these techniques have grown in popularity.

Sedivy and co-workers (Herbig et al. 2006; Jeyapalan et al. 2007) using a baboon model were among the first to use this approach at the single cell level. Importantly, baboons had recently been shown to display age-associated telomere shortening in leukocytes that closely paralleled that seen in humans (Baerlocher et al. 2003) giving some confidence that telomere-dependent senescence was shared between the two species. An initial study of 30 baboons (age range 5–30 years, 15 males and five females) showed an age-related exponential increase in 53BP foci and γ-H2AX co-localisation with telomeres (so-called telomere dysfunction–induced foci or TIFs). 200–600 fibroblasts were scored from each biopsy with approximately a quarter of the cells positive for these markers, and thus presumably senescent, in the oldest animals. Less than 5% of cells were positive in the youngest members of the cohort.

However, a further study combining the analysis of baboon fibroblasts grown to senescence *in vitro* with the analysis of skin and muscle biopsies from animals of different ages revealed marked differences in the staining pattern of senescent cells *in vitro* and presumptively senescent cells *in vivo* (Jeyapalan et al. 2007). The panel of potential markers used included not only TIF detection but staining for ATM, HIRA, p53, and the cyclin dependent kinase inhibitors p21 and p16. The last was included because it was known to increase markedly in multiple strains of fibroblasts (WI38, IMR-90, MRC5, and NHDF) once senescence was established (Alcorta et al. 1996). HIRA staining was absent from muscle biopsies regardless of donor age but increased from ~20% of fibroblasts in young skin biopsies to over 70% in those derived from 30-year-old baboons. However, the increase in HIRA staining occurred linearly, not exponentially, with age suggesting a distinct aetiology from that giving rise to TIFs. Whilst senescent baboon fibroblasts *in vitro* were uniformly p21 positive, with multiple γH2AX foci associated with ATM, 53BP1 and telomeres (over 80% of γH2AX foci colocalized with telomeric sequences) only 10% of these cells were p16 positive. But in skin biopsies fibroblasts stained for 53BP1 usually contained only a single focus (70% of 53BP1 positive cells in young animals and 65% in very old animals). *In vivo* p21 staining in dermal fibroblasts was largely absent but p16 staining was abundant. Such differences between senescent cells *in vitro* and *in vivo* should caution against simplistic "if it stains for *x* it's a senescent cell" styles of thinking about any specific marker detection assay.

The most intellectually parsimonious hypothesis consistent with the phenotype of senescent baboon fibroblasts *in vivo* is that researchers are visualising a subset of cells that have been senescent for weeks or months. Stein et al. (1999) reported that whilst p21 progressively accumulates in IMR90 human fibroblasts serially passaged to senescence and is present exclusively in newly senescent cells it subsequently disappears. In contrast $p16^{Ink4a}$ is initially absent but increases after IMR90s enter senescence and remains elevated for at least 2 months. Thus, $p16^{INK4a}$ is an excellent potential pathway biomarker for senescent cells in situations where label exclusion or markers of increased cell size (q.v.) cannot readily be used. However some exceptions have been reported (Frescas et al. 2017a).

A particularly important study in this respect is that of Liu et al. (2009) who measured p16 levels in peripheral blood T lymphocytes from human donors (n = 170) aged from 18 to 80. $p16^{INK4a}$ mRNA expression measured using Taqman quantitative RT-PCR showed a highly significant exponential relationship with donor chronological age (Log_2[p16] gives $R^2 = 0.4$, $p < 0.0001$ *vs* donor age). Although a similar relationship was observed at the protein level multiple technical difficulties militated against using this measure. Perhaps predictably $p16^{INK4a}$ levels increased more rapidly with age in smokers compared to non-smokers but provocatively the authors found a blunting of the age-p16 relationship with exercise intensity and duration. The positive association between levels of IL6 (a marker of frailty) and p16 could have indicated an element of "reverse causation" (frail people cannot exercise) however since it is now clear from rodent data that exercise facilitates the clearance of senescent cells by the immune system this can probably be discounted (Schafer et al. 2016).

Even though the measurement of telomere length has been significantly simplified in recent years by the introduction of quantitative-PCR (Montpetit et al. 2014) measurement of $p16^{INK4a}$ message probably offers advantages over this technique for the detection of senescent cells because $p16^{INK4a}$ appears regardless of whether the arrest mechanism is telomere dependent or independent and the message shows a greater

dynamic range than mean telomere length (~10 fold over 60 years compared to ~2-fold for telomere length over the same period). Nonetheless any quantitative PCR assay remains a relatively costly system to invest in and optimize which begs the question-are there other aspects of the senescent cell phenotype that could be exploited to develop detection systems of equal value but greater ease of use particularly for small scale studies?

The Altered Phenotype

Cytological Changes as Markers of Senescence

Senescent cultures of fibroblasts can be clearly distinguished from their growing counterparts by simple light microscopy because the cells are unmistakably bigger. Schneider and Mitsui (1976) found that the modal volume of cultured WI38 fibroblasts increased by approximately 40% from early passage to senescence (from 1930 ± 20 μm^3 to 2,655 ± 234 μm^3). Even larger increases occurred in both RNA (110%) and protein content (~80%). Sherwood et al. (1988) studied IMR90 human fibroblasts using multiparameter flow cytometry and found that mean cell size shifted approximately two-fold between passages 28 and 53. Consistent with earlier work (Cristofalo and Sharf, 1973; Smith and Whitney, 1980) a few such large cells were present in early passage cultures. However senescent cultures were dominated by these large cells whilst their cycling counterparts, even in late-passage cultures, remained relatively small. Similar patterns emerge with other adherent cell types undergoing senescence (e.g., mesenchymal stem cells see Adewoye et al. 2020). Thus, in principle it is possible to identify some types of senescent cells simply by this increase in size coupled with non-invasive markers such as autofluorescence (Poot et al. 1985; Bertolo et al. 2019). But not all cell types show this alteration in cell size at senescence, T cells being the best-known exception (Perillo et al., 1989). Thus, size alone cannot be used as a marker of senescence unless there is prior evidence that hypertrophy accompanies growth arrest.

In senescent fibroblasts this increase in size and protein content is accompanied by a range of ultrastructural changes particularly increased nuclear size, nuclear abnormalities, larger autophagic vacuoles and increased numbers of lysosomes (Lipetz and Cristofalo, 1972; for review see; Stanulis-Praeger, 1987). This latter change is of particular importance for the detection of senescence. Whilst Cristofalo and Kabakjian (1975) were probably the first researchers to report increased activities for the lysosomal enzymes acid phosphatase and β glucuronidase as WI38 cells entered senescence. Campisi and co-workers (Dimri et al., 1995) working with seven different strains of human fibroblasts (including WI-38) modified a histochemical assay for lysosomal β-galactosidase activity to develop probably the most widely used technique for the detection of senescent cells. This colorimetric assay is based on cleavage of the soluble, colourless lactose analogue X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to form an insoluble blue precipitate (5,5′-dibromo-4,4′-dichloro-indigo). By shifting the pH of the reaction buffer away from the β-galactosidase optimum of pH4 to pH6 the researchers rendered the enzyme less efficient and ensured that only cells with lots of lysosomal β-galactosidase could cleave enough X-gal to generate visible precipitates. Since this effectively limits detectable precipitate to large cells (more formally cells with a high lysosomal mass) which are usually senescent, it is perhaps unsurprising that Dimri et al. (1995) reported a high correlation between cells that were "senescence-associated β-galactosidase" (SAβ-gal) positive and cells that were label excluding on 3H-thymidine long labels.

The original SAβ-gal staining technique is simple and robust, rendering it suitable for use in a variety of settings. Given the range of lysosomal enzymes and substrates available for them this approach is also applicable to a wide range of other enzymes. We found some years ago (Dropcova and Faragher, unpublished observations) that when the optimum pH for the enzyme is shifted both dipeptidyl peptidase-4 (CD26) and amino peptidase M show similar "senescence associated" staining patterns to β-galactosidase (unfortunately the coupling chemistry produces a "red on yellow" staining pattern that is harder to score than SAβ-gal). Recently, Hildebrand et al. (2013) showed that the lysosomal enzyme α-fucosidase, is upregulated in cells made senescent through a range of different techniques. Enzyme activity can be visualised using 5-bromo-4-chloro-3-indolyl-α-L-fucopyranoside (X-fuc) in an incubation buffer similar to that for SAβ-gal (but shifted to pH 5.0 not pH6.0). This gives a blue stain similar to X-gal but with the advantage that α-fucosidase induction is somewhat stronger at senescence, particularly in murine cells rendered senescent by drug treatment.

Although the original SAβ-gal histochemical techniques use a paraformaldehyde fixation step a range of fluorescent substrates are also available for β-galactosidase (Kurz et al. 2000; Okamoto et al. 2006; Filho et al. 2018) allowing the visualisation of SAβ-gal in live cells (provided the lysosomes are temporarily alkalinised for example by treatment with bafilomycin A_1). One substrate (5-dodecanoylaminofluorescein di-β-D-galactopyranoside) has been successfully used for flow cytometry (Kurz et al. 2000; Noppe et al. 2009) opening up the possibility of flow sorting viable senescent cells. Solution phase variations on these techniques are also available for another style of quantitative read out (Cho and Hwang, 2011).

Users of these techniques should be aware of two potential complications. Firstly, there can be significant delay between entry into senescence and detectable SAβ-gal staining. This was first reported by Thomas et al. (1997) in AGO7086A human mesothelial cells dual stained for Ki67 and SAβ-gal although it was not observed in HUVECs or RPE cells analyzed using identical techniques (Rawes et al., 1997; Kalashnik et al. 2000). Secondly, an increase in the amount of β-galactosidase at senescence is necessary for visualisation. Kurz et al. (2000) using HUVEC showed a 15-fold increase in the amount of enzyme from early passage to senescence; an increase that was paralleled by the increase in lysosomal mass. This does not require an increase in cell size explaining why it is possible, though rarely attempted, to identify senescent T cells by SAβ-gal staining (Gerland et al. 2004; Yang et al. 2018) even though their nucleocytoplasmic ratio is unchanged. However, senescent cells with very short post-mitotic survival times or unaltered lysosome contents will be effectively invisible.

Whilst Stein et al. (1999) had noted that the increase in $p16^{INK4}$ roughly parallels the increase in cell size and SAβ-gal in senescing human fibroblasts a more comprehensive temporal analysis was undertaken by Cho and Hwang (2011). Using MCF-7 breast cancer cells rendered senescent by exposure to adriamycin the researchers measured cell cycle-related proteins (including p21 and p53 levels), SAβ-gal activity, cell volume and autofluorescence among other markers. Consistent with earlier studies the levels of p21 and p53 increased within the first 24 h following exposure to the drug whilst cell size increased only for the first 2 days. In contrast, autofluorescence increased 5-fold above baseline levels over 8 days in a roughly linear fashion whilst the number of SAβ-gal positive cells increased in a sigmoidal fashion before stabilising after 6 days. Nonetheless, SAβ-gal activity continued to increase beyond this from an eightfold increase over baseline at day 6 post treatment to a maximum 14-fold increase by day 8. Differences of this scale are in principle detectable simply by immunocytochemical staining for the enzyme itself although this has rarely been attempted (Joselow et al. 2017).

Thus, although senescent cells from any tissue are potentially detectable by variants of catalytic histochemistry for lysosomal enzyme activity, timing is central to the absolute value of the readout (raising issues of standardisation) and to be visualised cells must 1) have a substantial increase in lysosomal mass irrespective of hypertrophy and 2) have spent sufficient time senescent for enzyme activity to have built up to detectable levels. It should also be borne in mind that any other alterations in cell physiology that meet these criteria will generate a false positive signal. Most famously this can occur in primary fibroblast cultures held confluent or immortal cultures at high cell densities (Severino et al. 2000) and illustrates an important quirk of the routine employment of the technique.

As it stands, the research literature is heavily slanted towards reports of the detection of SAβ-gal *in vitro* rather than *in vivo*. This is ironic because the key advantage of the technique is that it gives researchers the capacity to detect senescent cells in tissue samples. When first introduced SAβ-gal was effectively the only assay which allowed senescent cells to be distinguished from their quiescent counterparts in this context. Ironically, in some publications (e.g., Xia et al. 2020) SAβ-gal has been used *in vitro* to check if a cell type of interest is senescent whilst its senescence *in vivo* is established using another method (e.g., $p16^{INK4a}$ staining). It is questionable whether using SAβ-gal staining like this is adding much value to the study.

Unfortunately, reviews of SAβ-gal staining also tend to gloss over the key question of how such stained sections should be scored. This is regrettable, particularly for those using it for the first time because no real consensus has yet emerged around scoring (which raises issues of inter study comparability). A few examples illustrate the range of approaches that have been adopted.

Originally, Dimri et al. (1995) relied on blind scoring of 20 dermal sections from human donors of varying age by a specialist pathologist with the staining frequency presented as a simple scale from minus (indicating no staining) through to "+ + +" (positive cells in all sections of dermis, multiple clusters in all sections of epidermis). Similarly, Paradis et al. (2001) used independent assessment by two pathologists to gauge the localization and number of positive cells in 57 biopsies from normal and abnormal human livers. SAβ-gal staining was classified simply as either "absent" (no or < 10% SAβ-gal positive cells visible) or "present" (> 10% SAβ-gal positive cells visible). Simultaneous review of sections was adopted if the pathologists differed in their opinion.

Kim et al. (2008) took a much more quantitative approach to scoring SAβ-gal in nucleus pulposus chondrocytes within intervertebral disc sections from 25 patients. Here every chondrocyte on the whole section was counted (under × 200 magnification with Nuclear Fast Red counter-stain) and the SAβ-gal positive fraction presented as a percentage. A similarly rigorous histomorphometric approach was used by Gruber et al. (2007) in 57 human disc samples giving an overall incidence of 29.9% (SD ± 24.8, range from 0 to 92%.) positive cells.

Berkenkamp et al. (2014) studied the frequency of senescence in mouse renal epithelial cells *in vivo* using a semi-quantitative approach in which the frequency of SAβ-gal positive cells in 10 random fields of view within representative kidney sections were scored. This was sufficient to yield a statistically significant difference ($p < 0.05$) between young (3–5 months) or old (18 + month) animals.

In contrast Melk et al. (2003) studying cell senescence in Fischer 344 rat kidneys *in vivo* quantified SAβ-gal staining simply by imaging kidney sections using Image-Pro Plus Software. A set of slides without the eosin counterstain the group normally employed were photographed, average staining density for the whole section was calculated and the mean staining density of two independent experiments, in arbitrary units, was used as the basis for further calculations. Even this simple approach proved adequate for the demonstration of a significant difference in SAβ-gal staining levels between young (9 months) and old (24 months) rats (0.008 ± 0.003 *vs* 0.020 ± 0.007 arbitrary units respectively, $p < 0.005$).

Metabolic Changes as Markers of Senescence

Alterations in the amounts and activity of lysosomal enzymes are a subset of the changes that occur in cellular metabolism with senescence. Increased lysosomal size results, at least in part, from dysregulated proteostasis, a key hallmark of ageing. Misfolded proteins are degraded less effectively and form aggregates which, when internalised by lysosomes, contribute to the highly cross-linked and complex materials known collectively as lipofuscin or ceroid (Yin, 1996; Moreno-García et al. 2018). In classical histology these terms are distinct referring to material accumulating within post mitotic or mitotic cell types respectively but colloquially the "ceroid" within senescent cells is often simply called "lipofuscin". This highly heterogenous "junk" material is responsible for the increased autofluorescence of senescent cells and its presence gives another visualisation option. In essence, rather that staining for the elevated activity of lysosomal breakdown enzymes, simply stain for the "junk" they are trying to break down instead.

This approach can be particularly useful if trying to visualize senescent cells in tissue samples fixed in ways that inactivate lysosomal enzymes (e.g., formalin-fixed paraffin-embedded blocks). As noted earlier immediately following the onset of senescence different markers build up to detectable levels at different rates (Cho et al., 2011) but since the majority of senescent cells *in vivo* will not usually be immediate entrants this is probably not a serious flaw under normal circumstances. Melk et al. (2003) correlated lipofuscin levels (graded independently by two observers) with SAβ-gal staining in the tubular epithelium of their rat samples and found a highly significant association ($p < 0.001$) in both young and old animals.

Melk et al. (2003) used a classic histological stain (based on the periodic acid-Schiff reaction) to visualie lipofuscin. Recently Georgakopoulou et al. (2013) used another (Sudan Black B counterstained with Nuclear Fast Red) to co-localise lipofuscin and SA-β-gal in senescent cells. Building on the success of this, but conscious of the difficulties of interpretation of traditional lipofuscin staining, the group undertook the *de novo* synthesis of a series of Sudan Black B analogues coupled to biotin which they validated in a range of test systems against both proliferation markers (Ki67, BrdU) and SAβ-gal (Evangelou et al. 2017). This approach allowed the ready visualization of lipofuscin using a peroxidase conjugated anti-biotin antibody and diaminobenzidine (DAB) visualization (although any of a range of anti-biotin detection systems, such as those based on avidin would also probably have worked). In γ-irradiated human fibroblasts *in vitro* one such analogue (GL13) was used to visualize the kinetics of lipofuscin accumulation giving a timescale broadly similar to that observed by Cho et al. (2011) for autofluorescence. However, all enzyme-based visualisation systems produce staining of variable intensity, so the absolute numbers of senescent cells visualized by this type of lipofuscin detection may differ significantly between researchers depending on simple variables such as the incubation time.

Similarly, Masaldan et al. (2018) building on prior work demonstrating the accumulation of iron within senescing IMR90 fibroblasts and HUVECs (Killilea et al. 2003) studied iron accumulation in mouse embryonic fibroblasts rendered senescent by sublethal γ-irradiation, serial passage or oncogene activation. After 10 days of growth arrest all these types of senescent rodent cells accumulated extremely large amounts of intracellular iron (~15–20 fold compared to growth competent controls). Human fibroblasts and prostate epithelial cells rendered senescent by irradiation or serial passage also accumulated lesser quantities of iron after longer periods of growth arrest (~3.3 to 8.4-fold 21 days post senescence depending upon the cell type and mechanism by which senescence was induced). This accumulation of iron in senescent rodent cells results from the upregulation of the transferrin receptor and a tenfold elevation of ferritin. This in turn was shown to result from impaired lysosome-mediated degradation of this key iron storage protein. Practically, this significant difference in iron handling between the senescent and growing states allowed the group to visualize senescent cells in mouse liver by staining with a rabbit polyclonal antibody against ferritin (in combination with an HRP labelled secondary antibody, DAB visualization and a haematoxylin counterstain). Serial liver sections from young and old animals were stained for SA-β-gal and ferritin respectively and scored by observing four independent fields of view. These findings open up a wide range of potential staining strategies for senescent cells since techniques for the visualization of iron in histological samples are well developed and encompass everything from classical stains such as Pearl's Prussian blue through to quantum dots and can be used in formalin-fixed paraffin-embedded material (Meguro et al., 2007; Duan et al.,2018; van Duijn et al. 2013).

Advanced Glycation End products (AGEs) are often grouped with lipofuscin but unlike the latter can form both extra and intracellularly. They are a heterogeneous group of compounds typically formed by the nonenzymatic (Maillard) reactions of glucose with proteins or lipids. To date limited work has been done to address whether the visualization of AGEs can be used to detect senescent cells but initial studies by Sell et al. (1998) suggest that this should be possible. The authors looked at the accumulation of the AGE pentosidine in strains of reticular and papillary fibroblasts derived from a single donor and passaged to senescence as well as peripheral blood T lymphocytes from 27 donors of varying ages (17–97 years) and states of health. Levels of pentosidine were quantified by HPLC and increased approximately three-fold in both types of fibroblast over the culture replicative lifespan ($p < 0.0007$) whilst T lymphocytes showed a highly significant increase in pentosidine level with donor age ($p < 0.0003$). In related work, Kueper et al (2007) demonstrated that the intermediate filament protein vimentin aggregates in human dermal fibroblasts *in vitro* due to its modification by pentosidine and carboxymethyllysine (as well as other AGES such as pyrraline and carboxyethyllysine). More recently Frescas et al. (2017b) conducted a deliberate screen for senescence-specific markers by immunising mice with mouse lung fibroblasts rendered senescent using bleomycin. Human fibroblasts rendered senescent by IR were used as a secondary screen alongside untreated controls. One of the IgMs generated by this approach (9H4) showed approximately a two-fold greater level of staining on senescent cells in this screen. Subsequent Western blotting showed that 9H4 recognises a modified form of vimentin that is presented at the cell surface and then secreted (possibly facilitating the clearance of senescent cells by the innate immune system). The secreted vimentin was modified by a malondialdehyde adduct on cysteine 328. This is provocative because malondialdehyde is an end product of lipid peroxidation that subsequently gives rise to immunologically detectable AGEs (Sajithlal and Gowri Chandrakasan, 1999). Since antibodies to pentosidine and other AGEs such as carboxymethyl lysine are commercially available it would seem likely that senescent cells could be identified histochemically using this type of approach.

Vimentin is not the first example of a modified form of a normal protein found at the surface of senescent cells. Porter et al. (1990), Porter et al. (1992) had previously reported the generation of three monoclonal antibodies (SEN-1, SEN-2, and SEN-3) which recognized epitopes on fibronectin only exposed when human fibroblasts become senescent. These antibodies (tragically lost in a laboratory accident shortly thereafter Jim Smith-personal

communication) could detect senescent human fibroblasts, keratinocytes and mammary epithelial cells *in vitro* and *in vivo* in a species-specific manner. If the secretion of modified proteins is a means of signalling for immune clearance then it is likely that there will be significant variations between cell types, species and perhaps individuals as is seen for other components of the Senescence Associated Secretory Phenotype (SASP).

Changes in cellular components as a consequence of dysregulated proteostasis should be conceptually distinguished from those which arise from the transcriptional or post transcriptional regearing that accompanies entry into senescence. These latter changes offer a great many potential markers for the senescent state but can vary widely in the same cell type depending on the inducing stimulus. Nelson et al. (2014) used a microarray-based approach (based on the Affymetric Human Genome U133 Plus 2.0 GeneChip) to compare the transcriptomes of IMR90 fibroblasts rendered senescent either by serial passage or by retroviral infection with H-RAS-V12 (oncogene induced senescence). Compared to proliferation competent controls 5,424 genes were differentially expressed at replicative senescence and 3,188 in OIS. However, there was only moderate overlap between the two states (~33% of those genes altered by serial passage were altered by OIS but ~56% of these was also altered by classical replicative senescence). Interestingly although both types of senescence were associated with $p16^{INK4a}$ upregulation the cluster of genes associated with this pathway showed significant differences between the two states (69 out of 118 transcripts commonly downregulated and only 6 out of 31 transcripts commonly upregulated). This has important implications for the use of specific pathway components, as opposed to end points, as pan-specific markers of senescence between different states and tissues.

FUTURE CHALLENGES IN SENESCENT CELL DETECTION

It has been clear for decades that senescence occurs in fibroblast cultures from many mammalian species (Röhme, 1981) but the cell senescence literature remains heavily slanted towards humans and rodents. This is unfortunate because there are several other species which serve as excellent models for human ageing changes or age-related diseases. Given the evidence for the key role played by senescence in ageing the routine detection of senescent cells in these models will become increasingly important but the relevant literature base is currently weak or non-existent.

This is a potential problem because comparative studies of rodent and human fibroblasts show that extrapolation of senescence markers other than label exclusion from one species into another should be done with caution unless there are prior data available. The three models below have been selected to illustrate both the potential gains and the current issues involved in broadening the detection of senescent cells beyond humans and mice.

In the pig, which provides a good model for cardiovascular and intravertebral disc ageing in humans as well as a new model for human progeria (Dorado et al. 2019) there is a small but coherent evidence base for senescent cell detection. Senescent cells can be visualized by the SAβ-gal assay post formaldehyde-fixation (Shi et al. 2018) and serially passaged foetal porcine fibroblasts enter telomere-dependent senescence in a manner that closely resembles that seen in humans. This means that telomere shortening, TIF detection, $p16^{INK4a}$ and $p21^{WAF}$ can all potentially be used as markers for the senescent state in porcine tissue *in vivo* (Fukuda et al. 2012; Ji et al. 2012; Donai et al. 2014; Shi et al. 2018). However, Oh et al. (2007) demonstrated that it is possible to produce immortal clones of fibroblasts from both mini-pigs and three-way crossbred meat animals simply by culturing the cells using the 3T3 passage technique. This is not possible using human fibroblasts (McCormick and Maher, 1988) and suggests either that the finding is artefactual or that the molecular pathways regulating entry into senescence are not identical in the two species.

The ageing horse is a particularly valuable model for the types of age-related tendon injuries seen in older humans due to the many similarities between the human Achilles and the equine superficial digital flexor tendon in structure, matrix composition and function. Cultures of human tenocytes from aged and functionally degenerate Achilles show upregulation of $p16^{INK4a}$ protein and a five-fold increase in the SAβGal positive fraction compared to healthy controls (Kohler et al. 2013) a finding that renders the detection of senescent cells in the equine tendon an important goal but studies on senescent cells in equines are limited. Foetal horse kidney cells can be immortalised with SV40 large T antigen (Maeda et al. 2007) indicating p53 and pRb dependent checkpoints and adult equine fibroblasts can be induced to proliferate continuously by the stabilisation of telomere length (Vidale et al. 2012) suggesting equines show telomere dependent senescence. SA-β-gal, $p16^{INK4a}$ protein and γH2AX foci are all detectable in equine chondrocytes following γ irradiation (Copp et al. 2021) suggesting that these would also be useful markers. But as with most other animal models the kinetics of accumulation of these markers post entry into senescence remain unstudied. The type of studies carried out by Cho et al. (2011) are sorely needed.

Selective breeding has created hundreds of dog breeds which vary widely in lifespan presenting an exceptional opportunity to identify pathways associated with ageing. Unfortunately, whilst there is evidence for most of the known hallmarks of ageing in canines (Sándor and Kubinyi, 2019) research on the presence and phenotype of senescent cells in dogs is fragmentary. Whilst breed-specific telomere length has been shown to be a strong predictor of average life span and pathology (Fick et al. 2012) and SA-β-gal has been used to identify senescent canine fibroblasts *in vitro* there are clearly important differences between human and canine senescence. As with pigs, You et al. (2004) were able to isolate spontaneously immortalized clones from cultures of dog embryonic fibroblasts *in vitro*. These were shown to have mutations in either p53 or $p16^{INK4a}$ suggesting that these pathways control senescence in canine fibroblasts but appear to be "leaky" compared to humans. A single study suggests that the proinflammatory phenotype of senescent canine dermal fibroblasts may also differ significantly from those of humans

and rodents (Jimenez et al. 2020). Given the potential value of the model for biogerontology systematic characterisation of canine senescence at the cellular level would clearly be extremely valuable.

Two issues are likely to arise as the detection of senescent cells in different species becomes more commonplace. Firstly, there is a distinct possibility that the relative importance of senescence as an ageing mechanism may differ markedly between them as a result of evolutionary history. Ageing exists as a result of the declining force of natural selection with age and results from two non-exclusive modes of evolutionary gene action. Senescence is thought to have arisen through one of these, antagonistic pleiotropy. This is the selection for alleles or processes that enhance the reproductive success of organisms early in life but which have deleterious effects in the later life course. But, ageing also occurs as a consequence of the inability of natural selection to remove late acting deleterious alleles; a mode of gene action known as mutation accumulation. A wealth of experimental data shows that the relative contributions of antagonistic pleiotropy and mutation accumulation to the evolution of ageing vary widely between species.

One reason for this variation may be that selectively neutral deleterious alleles show frequencies that are influenced by genetic drift and the rate of drift is heavily influenced in turn by effective population size. Unlike most species, humans have undergone both significant genetic bottlenecks and have shifted their survivorship curve from a Type-II population to a Type-I population. This significantly increases drift and thus may well alter the relative importance of cellular senescence as an ageing mechanism between species (Overall and Faragher 2019). Put crudely, humans are evolutionary outliers.

Secondly, different species have very different lifespans which foregrounds the relative extent to which markers of chronological and biological ageing are uncoupled between them. Evidence is emerging from the study of human senescence that some markers better reflect absolute chronological time than cell replication frequency. Maier and colleagues (Waaijer et al. 2012) measured the fraction of senescent cells (*via* $p16^{INK4a}$ immunostaining) in the dermis and epidermis of a selected sub-group of subjects from the Leiden Longevity Study who were biologically younger than age-and environmentally matched controls (n = 89 per group). The levels of senescent cells correlated closely with the pathological status of the donors whilst age and environment matched controls showed higher levels of senescent cells than the biologically young group. The authors concluded that $p16^{INK4a}$ is a marker of biological time. Further studies using the same experimental design (Waaijer et al. 2016) investigated the relationship between markers of DNA damage (micronuclei, p53BP1 damage foci and telomere associated foci) and health status (n = 40 in each group). There p53BP1 and telomere associated damage foci, but not micronuclei, increased significantly with chronological age but, unlike $p16^{INK4a}$ there was no association between these markers of DNA damage and the health status of the subjects examined. The authors concluded that p53BP1 and telomere associated damage foci effectively measured human chronological age. Studies of this type are likely to be necessary in other species to properly assess the contribution that cell senescence makes to their overall ageing. However, forewarned is at least forearmed and the range of simple detection techniques available suggests that the gaps in our current understanding can be closed much faster in novel systems than was the case when senescence markers were first systematically applied to human systems in the 1970s and 1980s.

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The author confirms being the sole contributor of this work and has approved it for publication.

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MINI REVIEW
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Chondrocyte Aging: The Molecular Determinants and Therapeutic Opportunities

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Osteoarthritis (OA) is a joint degenerative disease that is an exceedingly common problem associated with aging. Aging is the principal risk factor for OA, but damage-related physiopathology of articular chondrocytes probably drives the mechanisms of joint degeneration by a progressive decline in the homeostatic and regenerative capacity of cells. Cellular aging is the manifestation of a complex interplay of cellular and molecular pathways underpinned by transcriptional, translational, and epigenetic mechanisms and niche factors, and unraveling this complexity will improve our understanding of underlying molecular changes that affect the ability of the articular cartilage to maintain or regenerate itself. This insight is imperative for developing new cell and drug therapies for OA disease that will target the specific causes of age-related functional decline. This review explores the key age-related changes within articular chondrocytes and discusses the molecular mechanisms that are commonly perturbed as cartilage ages and degenerates. Current efforts and emerging potential therapies in treating OA that are being employed to halt or decelerate the aging processes are also discussed.

Keywords: senescence, osteoarthritis, chondroprotection, degeneration, regenerative medicine

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INTRODUCTION

Articular cartilage is a highly specialized connective tissue found at the ends of the articulating bones that allows transmission of forces and provides a smooth surface for low-friction movement of weight-bearing joints. Articular cartilage is composed of a dense extracellular matrix (ECM) with a sparse distribution of highly specialized cells called chondrocytes. It is avascular,

Abbreviations: ATP, adenosine triphosphate; AGEs, advanced glycation end products; AMPK, AMP-activated protein kinase; ACI, autologous chondrocyte implantation; DNMT1, DNA (cytosine-5)-methyltransferase 1; ECM, extracellular matrix; EVs, extracellular vesicles; ER, endoplasmic reticulum; FOXO3, forkhead family O subclass transcription factor 3; *IL-1*β, interleukin-1 beta; LDL, mtDNA, low-density lipoproteins; mitochondrial DNA; MRC, mitochondrial respiratory chain; TFAM, mitochondrial transcription factor A; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-κB; NO, nitric oxide; OA, osteoarthritis; PGC-1α, peroxisome proliferator-activated receptor g coactivator 1α; PTEN, phosphatase and tensin homolog deleted on chromosome 10; ROS, reactive oxygen species; RAGE, receptor for advanced glycation end-products; SASP, senescence associated secretory phenotype; Sirt1, Sirtuin 1; UPS, ubiquitin-proteosome system.

aneural, and alymphatic in nature; hence, it has a poor self-repair capacity, and therefore damage to cartilage in weight-bearing joints is at a higher risk of progressing into more serious joint conditions such as osteoarthritis (OA).

Aging is also a major risk factor for the development of OA. Age-related changes in articular cartilage predispose individuals to develop OA; additional factors including biomechanical, genetic, or systemic metabolic factors can accelerate the progression of the condition (Del Carlo and Loeser, 2003). It is generally accepted that OA is a multifactorial disease and, this supersedes a more simplistic notion of it being a disease of "wear and tear." Newer mechanisms attributed to the disease include cell senescence and the senescence-related secretory cell phenotype, chondrocytes' reduced reactivity to growth factors, mitochondrial dysfunction, oxidative stress, and abnormal accumulation of advanced glycation end products (AGEs). It is the cumulative effect of the mechanical load and associated cell dysfunction over the years that result in cartilage breakdown and the macroscopic clinical evidence of "wear and tear." Hence, OA is regarded as a naturally occurring irreversible phenomenon, rather than a specific, potentially treatable disease (Loeser, 2011); however, in light of new studies, this paradigm is being challenged, and new thinking may lead to novel medical approaches for the prevention, reversal, or treatment of OA. Therefore, this mini-review highlights the molecular mechanisms underlying aging of chondrocytes and degeneration of cartilage.

MOLECULAR AND CELLULAR REGULATION

Proteostasis (Autophagy, UPS, and Protein Folding Mechanism)

One hallmark of aging is loss of proteostasis caused by dysfunctional ubiquitin-proteasome system (UPS), protein folding, and autophagy. Age-related decreases in proteostatic activity impact cellular differentiation and viability and inflammatory processes in disease. Indeed, impaired proteasomal function in human osteoarthritic chondrocytes can contribute to decreased levels of sox9 and aggrecan, factors that are crucial for chondrocyte function and maintenance (Serrano et al., 2018). Immunoproteasomes reflects a persistent antistress mechanism in aging tissue. Activation of forkhead transcription factor (FoxO) in response to reduced IGF-1 signaling enhances longevity (Löw, 2011). These findings indicate that IGF-I or insulin can reduce protein degradation rapidly by suppressing autophagy *via* mTOR activation and independently Akt suppressing FoxO transcription, which also inhibits proteasomal degradation through the reduction of transcription of ubiquitin ligases atrogin-1 and MuRF1 (Vellai et al., 2009). It is noteworthy that age-related decline in expression of molecular chaperones induces endoplasmic reticulum (ER) stress and cellular apoptosis in articular cartilage (Tan et al., 2020), which suggests that loss of proteostasis induces ER stress in aged articular chondrocytes. Autophagy is one arm of the proteostasis network that coordinates proteome and organelle quality control and degradation as well as the regulation of energy and nutrient supply, thereby maintaining cell survival and normal biosynthetic function in virtually all cell types. Autophagy is necessary for lifespan extension in several organisms, and multiple autophagy-related proteins are directly modulated by longevity pathways. Autophagy is a protective and homeostatic mechanism in normal cartilage especially in modulating cellular responses to stress. If autophagic pathways are compromised, cells may undergo apoptosis, leading eventually to cartilage degeneration (Caramés et al., 2010). Autophagy-related protein-7 is an essential regulator of autophagosome assembly; when depleted in chondrocytes, they accumulate large numbers of glycogen granules, hardly proliferate, and died specifically in the proliferative zone without any ER-stress signal (Horigome et al., 2020). Suppression of autophagy in prechondrogenic cells leads to defective chondrogenesis, through a lack of glycogenolytic supply of glucose to avascular prechondrocytes. Enhanced autophagy is reported to affect intracellular metabolic activity, i.e., by regulating the metabolism of nutrients, protein, and lipid and can delay the progression of OA (Luo et al., 2019). Intriguingly, key bioenergy sensors such as the AMP-activated protein kinase (AMPK) signaling pathway and Sirtuin 1 (Sirt1) also have roles in the regulation of autophagy, senescence, and aging (Ong and Ramasamy, 2018).

Conversely, cellular regulators of autophagy such as Sirt1, forkhead family O subclass transcription factor 3 (FoxO3), mammalian target of rapamycin (mTOR), nuclear factor-κB (NF-κB), and p53 have pivotal roles in energy metabolism, gene and protein expression, and aging. Sirtuin, known for its roles in stress resistance and longevity, and FoxO3, a major modulator of cellular metabolism, proliferation, and oxidant stress resistance, enhance autophagy, whereas mTOR, and NF-κB repress autophagy following stress and inflammation, respectively (Lotz and Caramés, 2011). The loss of autophagy in articular cartilage under mechanical or inflammatory stress is associated with aging-related cell death and increasing OA severity (Goldring and Berenbaum, 2015). Experimental evidence suggests that autophagy plays both a cytoprotective and death-promoting role in the pathogenesis of OA (Chang et al., 2013). Autophagy is activated as an adaptive response to hypoxic conditions; it also plays a cytoprotective role under various types of stress including disease treatment with DNA-damaging reagents, ER stress, nutrient and energy deprivation, as well as radiation. Overall, the age-dependent decline in autophagic activity contributes to the accumulation of damaged macromolecules and susceptibility to aging-related OA (Caramés et al., 2010). In contrast to autophagy-induced cell survival, the occurrence of OA autophagy in OA chondrocytes may be over-induced to the extent that the essential cellular constituents for cell survival are degraded leading to autophagic cell death. The mechanisms by which autophagy regulates the pathogenesis of OA have yet to be fully unraveled (Chang et al., 2013); however, it seems understanding its role holds immense potential in targeting the process to modulate aging of chondrocytes and eventually may be a promising therapy for treating OA.

Mitochondrial Dysfunction

Mitochondrial oxidative phosphorylation accounts for up to 25% of the total steady-state adenosine triphosphate (ATP) production in cartilage; however, in OA chondrocytes, mitochondrial functions including mitochondrial respiratory chain (MRC) activity and ATP synthesis are altered. Intriguingly, proteomic analysis of healthy and OA cartilage reveals 26% of the deregulated protein signature is related to respiratory chain function (Ruiz-Romero et al., 2009). The accumulation of mitochondrial DNA (mtDNA) deletions and point mutations or the indirect effects of nitric oxide (NO), proinflammatory cytokines, prostaglandins, and reactive oxygen species (ROS) on MRC function and ATP synthesis could lead to chondrocyte dysfunction. For example, deficiency of mitochondrial superoxide dismutase 2 and increases ROS in chondrocytes leads to mitochondrial dysfunction (Gavriilidis et al., 2013).

Thus when ROS generation exceeds the antioxidant activity threshold of chondrocytes, oxidative stress impairs MRC protein complexes resulting in reduced ATP production, deprivation of energy reserve, impaired matrix synthetic function, and reduced chondrocyte viability. Importantly, mitochondrial dysfunction affects several pathways that are critically involved in OA pathology, including oxidative stress generation, chondrocyte apoptosis, cytokine-induced chondrocyte inflammation, and matrix catabolism, as well as ECM calcification (Blanco et al., 2011). MRC dysfunction perturbs the homeostatic balance of healthy cartilage by inducing the production of proinflammatory stimuli and matrix metalloproteinases promoting catabolic glycosaminoglycan release, while simultaneously suppressing the synthesis of proteoglycans thereby exacerbating cartilage degeneration. Certain mtDNA haplogroups predispose people to OA; mtDNA haplogroup U is associated with an increase in radiologic severity of knee in OA, conversely mtDNA haplogroup J safeguards against hip and knee in OA (Blanco et al., 2011; Goldring and Berenbaum, 2015). AMPK and Sirt1 work together to maintain biological homeostasis through suppressing oxidative stress, NF-κB activation, and deregulation of several inflammatory and catabolic responses. NF-κB activation of inflammatory and catabolic responses is suppressed by deacetylating the p65 NF-κB subunit and priming it for proteasomal degradation, resulting in enhancing autophagy *via* repair of dysfunctional mitochondria. However, deficiency of AMPK activation in OA and aging chondrocytes could lead to reduced expression of Sirt1 and peroxisome proliferator-activated receptor g coactivator 1α (PGC-1α), thereby contributing to chondrocyte mitochondrial dysfunction. New evidence has demonstrated that pharmacologic activation of AMPK inhibits inflammation-induced catabolic activities, upregulates expression of antioxidant enzymes and prevents excessive mitochondrial ROS production. Importantly, activation of the AMPK/Sirt1/PGC-1α signaling pathway reverses impaired mitochondrial biogenesis capacity in human OA chondrocytes via mitochondrial transcription factor A (TFAM) mediation. The concept of therapeutic activation of chosen components of the AMPK/Sirt1/PGC-1α pathway is yet to be validated in an *in vivo* animal model of OA and in human OA (Wang et al., 2015). In addition to Sirt1, a recent study has highlighted the role of Sirt3-mediated mitochondrial homeostasis in OA. Sirt3, which is mainly located in mitochondria, can exert its deacetylation activity to regulate mitochondrial function, regeneration, and dynamics (He et al., 2020). Mitochondrial dysfunction-induced chondrocyte phenotypic inflammatory and matrix degradation responses also occur *via* ROS-mediated activation of c-Jun N-terminal kinase (JNK)-mitogen-activated protein kinase (MAPK)/cFos-AP1 pathways in chondrocytes of osteoarthritic and aged cartilage (Ansari et al., 2020). Although ROS generation in cells is inevitable, in human chondrocytes autophagy activation protects against mitochondrial dysfunction caused by accumulated ROS damage. Taken together, an intimate and highly coordinated link between bioenergy systems and chondrocyte aging or OA is now evident. This link is regulated through a balanced redox system, protective mechanisms such as autophagy, and apoptosis-survival/longevity pathways such as JNK-MAPK/cFos-AP1 and AMPK/Sirtuins pathways (López de Figueroa et al., 2015).

Oxidative Stress

Oxidative stress ensuing from an imbalance of ROS synthesis and antioxidant defense is a result of increased ROS synthesis or decreased level of antioxidant and can be measured in chondrocytes by, for example, an increased level of nitrotyrosine, a measure of ROS-induced oxidative damage to proteins (Loeser et al., 2002; Del Carlo and Loeser, 2003; Hui et al., 2016), and an increased ratio of oxidized glutathione to reduced glutathione with age (Del Carlo and Loeser, 2003). *In vitro* studies suggest that when under oxidative stress cellular antioxidant enzymes are inactivated *via* nitration of catalytically active tyrosine residues; Tyr106 and Tyr104 (Savvides et al., 2001). Furthermore, peroxynitrite (ONOO-), a potent oxidant formed from the reaction of NO with superoxide, is probably responsible for the inactivation of thiol-related antioxidant enzymes (Del Carlo and Loeser, 2003). With an imbalanced redox status, the susceptibility of chondrocytes to oxidant-mediated cell death increases, and albeit indirectly, predisposes the older individuals to develop OA (Del Carlo and Loeser, 2003). In response to inflammatory mediators, mechanical stress, and partial oxygen pressure (pO_2), chondrocytes can produce an abnormal level of ROS that exceed their antioxidant capability leading to a disturbance of redox homeostasis. Overproduction of ROS oxidizes membrane phospholipids, intracellular and extracellular components, nucleic acids, and transcription factors, leading to impaired biological activity and cell death. ROS or secondary byproducts of oxidative stress likely lead to oxidation of collagens and proteoglycans by covalently modifying the primary structure of the proteins. ROS induces oxidative cleavage of collagens and proteoglycan by breaking the amino acid bonds or amino acid side chains. Additionally, oxidative posttranslational modifications induce the unfolding of collagens and proteoglycans employing steric hindrance or by altering hydrogen bonds and electrostatic interactions. Ultimately, crosslinking within the proteins or between neighboring proteins give rise to alterations in protein secondary and tertiary structure, the spatial orientation of collagen fibers and bundles, as well

as surface charge and tension of proteins, which all impair the biomechanical properties of ECM. Initially, oxidative stress causes posttranslational modification of ECM proteins, and following the second set of signals, including AGE- and ROS-induced inflammation and catabolic pathways, act in conjunction to promote degeneration of articular cartilage, leading to the OA phenotype (Hardin et al., 2015). Stress-induced chondrocyte apoptosis is mediated *via* PI3K/Akt and caspase pathways at the very early stages of cellular stress (Lee et al., 2020). Indeed, extensive oxidative stress decreases the synthesis of collagen and proteoglycan *via* regulation of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which negatively regulates PI3K/Akt and ERK/MAPK pathways endogenously, and these pathways are essential for the synthesis of ECM proteins. Interestingly, a recent study has highlighted that the prolonged activation of Akt signaling caused an accumulation of ROS and triggered chondrocyte senescence as well as senescence-associated secretory phenotype (SASP) in PTEN-deficient mice (Xie et al., 2019). On the other hand, chronic administration of the antioxidant *N*-acetylcysteine suppresses chondrocyte senescence (Xie et al., 2019), suggesting the vital role of an antioxidant in mitigating aging of chondrocytes and OA progression. Collectively, degradation products and cellular content embodying oxidized molecules could aggravate synovial inflammation and create a vicious cycle, established by further degradation of products and newly synthesized ROS (Henrotin et al., 2003). Newly discovered evidence shows that a progressive loss of cartilage ECM and cellularity with advancing age is associated with elevated levels of oxidative stress, apoptosis, MMP13 expression and activity, as well as a decline in autophagy. All these age-related changes partially explain the significant predisposition of aged joints to degeneration and development of OA. In addition to basal mitochondrial ROS synthesis, ROS are also synthesized by the activated receptor for advanced glycation end-products (RAGE) through activation of NAD(P)H oxidase. ROS directly damage proteins, activate p38 MAPK-induced apoptosis and NF-κB-induced cartilage breakdown *via* upregulation of ADAMTS-5 and MMP13, suggesting the importance of oxidative damage and the IL-1 pathway in initiating the age-related changes that lead to the development of OA. In support of this mechanism, a recent study has revealed the involvement of double-stranded RNA-dependent protein kinase R (PKR) in regulating p38 MAPK and p53-dependent destruction of Akt, resulted in aberrant mitochondrial biogenesis and increased oxidative stress in chondrocytes (Ma et al., 2019). Predictions by computational modeling, show the inhibitory effect of blocking the IL-1 pathway on MMP13 production and inhibition of ALK1-mediated MMP-13 synthesis in the amelioration of cartilage degeneration of aged cartilage. These latter studies establish a firm evidential basis for therapeutic interventions (Hui et al., 2016).

Telomere Shortening and Telomerase Dysfunction

In human articular chondrocytes, the average rate of telomere shortening is approximately 40 base pairs/year (Martin and Buckwalter, 2001) Telomeres maintain chromosome stability by preventing chromosomal end fusion, and in embryonic stem cells telomeres are enzymatically renewed through the activity of telomerase (Kuszel et al., 2015). Most somatic cells lack detectable telomerase activity and so are susceptible to telomere shortening (Greider, 1998). Cellular stress can reactivate inactive telomerase gene expression, leading to telomere extension and the reacquisition of genomic stability; unregulated reactivation of telomerase can also lead to malignant transformation (Mollano et al., 2002). An analysis of telomeres in equine articular chondrocytes has shown that telomerase activity decreases with advancing age and telomerase activity is present in prepubescent horses but not postpubescent horses, implying that telomerase-positive chondrocytes from prepubescent donors are superior for cartilage repair approaches. Furthermore, it was found that while anabolic stimuli do not affect prepubescent telomerase activity, catabolic stimuli diminishes it (Wilson et al., 2014). In general, chondrocyte chromosomal telomere shortening is positively associated with biological aging and pathogenesis of OA (Martin and Buckwalter, 2001; Tamayo et al., 2011). In addition to the mean telomere length of cells, critically short telomeres appear to have a disproportionate influence on cell viability and fate (Kuszel et al., 2015). In 2012, Harbo et al. (2013) documented that the mean telomere length and the appearance of ultra-short telomere (below 1,500 basepairs) correlate with OA severity, proximity to lesions, and senescence level. The direct relationship between ultra-short telomeres and biological aging has, however, yet to be fully elucidated. A gradual reduction of mean telomere length reflects replicative senescence whereas the presence of ultra-short telomere is suggestive of stress-induced senescence. Therefore, ultra-short telomeres are potential biomarkers of oxidative damage and their presence is indicative of cellular senescence (Maria Harbo et al., 2012). OA is believed to be an accelerated local aging disease associated with premature articular cartilage senescence, and, shortened telomeres and increased chromosomal aberrations in chondrocytes can contribute to locally advanced senescence (Fragkiadaki et al., 2020). Generalized increases in genomic instability lead to an accelerated systemic senescent phenotype, as shown by the increased numerical chromosomal aberrations in peripheral blood leukocytes from OA individuals that possibly enhance the age-related degenerative joint disease (Tamayo et al., 2011). Telomeres shortening by oxidative stress may be clinically important in the early diagnosis and prognosis of OA, and understanding its relationship with other metabolic factors holds a great promise in developing therapeutic targeting of chondrocytes and related disorders.

Chondrocyte Senescence

Human articular chondrocytes can become senescent with advancing age especially following trauma and decreased cellular homeostasis of critical cellular pathways such as autophagy (Martin and Buckwalter, 2001). Two different mechanisms of senescence are suggested in chondrocytes: replicative senescence and stress-induced premature senescence (Rim et al., 2020). Upregulation of expression of inflammatory cytokines and cell cycle arrest-related genes such as interleukin-1 beta (*IL-1*β),

p16, *p21*, *p53*, and *p38* MAPK induces senescence directly (Vinatier et al., 2018), while downregulation of chondrocyte phenotypic maintenance genes such as *SOX9*, *BMP-2*, *IGF-1*, and *TGF*-β induces senescence indirectly. *In vivo* research is required to support these concepts and only then can articular cartilage regeneration strategies be developed to overcome the current impediments to tissue repair (Ashraf et al., 2016). Various types of cell-intrinsic and cell-extrinsic stress stimuli activate cellular senescence program orchestrated by the interplay of various cellular signaling cascades which eventually activate cell cycle arrest/senescence regulators, either p53 or p16 or both (van Deursen, 2014). Cell senescence *via* activation of p53-p21-pRb pathway can be reversed by inactivation of p53 or oncogenic Ras; p53-inactivated cells resume extensive proliferation culminating in crisis, whereas oncogenic Ras resumes limited cell proliferation. Once cells fully engage the p16-pRb pathway, subsequent inactivation of p53 and pRb, as well as silencing of p16, stimulates DNA synthesis (S phase) which lead to failure in activation of proliferation, indicating permanent cell cycle arrest. This evidence suggests that p16 is essential for establishing the irreversibility of senescence (Beauséjour et al., 2003). Senescence induction is regulated by many signaling pathways including p38MAPK/NF-κB pathways and Akt signaling that hamper the integrity of articular cartilage. In general, Akt can transduce both proanabolic and procatabolic signaling in response to diverse stimuli during cartilage repair (Greene and Loeser, 2015) exemplified by *PTEN*-deficient articular chondrocytes that exhibit high levels of senescence inducers $p16^{Ink4a}$ and p53, senescence-associated β-galactosidase activity, and typical features of a SASP (Xie et al., 2019).

As discussed earlier, telomeres shorten with the chronological age of chondrocyte donors and telomere changes are associated with senescence-like phenotypic drift (Martin and Buckwalter, 2001; Musumeci et al., 2015). Owing to the postmitotic nature of articular cartilage where chondrocyte renewal is virtually absent, stress-induced shortening of telomere is more likely than replicative shortening of telomeres. Stimuli including excessive mechanical loading, inflammation, and persistent oxidative stress cause an increased level of ROS which leads to DNA, protein, lipid and organelle damage. DNA damage induces telomere shortening that impacts the Hayflick limit, i.e., the ability of cells to re-enter the cell cycle for further rounds of cell division ultimately leads to cellular senescence and that propagation of senescence leads to cell death (Musumeci et al., 2015). Senescent chondrocytes arrest in the G1 phase of the cell cycle secret SASP, in which accumulation of the SASP-expressing cells contributes to tissue senescence by impairing the ECM attributed to the increased production of degradative enzymes, MMPs. Moreover, aging and/or OA-related decline in the anabolic and proliferative response to growth factors as well as the loss of cellularity support the concept that chondrocyte senescence contributes to the progression of cartilage degeneration (Musumeci et al., 2015). Apart from biological aging, *in vitro* serial expansion (four passages) of chondrocytes in monolayer culture reported to turn on the senescence- and dedifferentiation-mediated genes, leading to the loss of cartilage regeneration ability (Ashraf et al., 2016). Taking all into account, the association between aging/trauma, senescence, and phenotypic changes reduce the number of healthy and functioning chondrocytes, hence promoting cartilage degeneration and eventually lead to osteoarthritic pathophysiology.

Reduced Growth Factor Response

In articular cartilage, several growth factors are known to modulate signaling pathways involved in the stimulation of cellular quiescence, growth, division, and differentiation, hence regulating the development and homeostasis of cartilage. It is executed *via* multiple modes including the level of receptors, the concentration of growth factor ligands and growth factors. TGF-β signals *via* the ALK5 receptor and maintains young chondrocytes in a quiescent state however, the level of ALK5 receptor declines with age leading to an increased ratio of ALK1–ALK5. Despite the protective role of TGF-β under normal physiological condition, enhanced signaling *via* ALK1 in aged chondrocytes leads to an upregulation of MMP-13, thereby initiating homeostatic imbalance and cartilage breakdown (Hui et al., 2016). Insulin growth factor 1 (IGF-1) has been shown to have anabolic effects in cartilage under normal circumstances, and decreased levels of IGF-1 also play a critical role in switching the balance toward catabolic metabolism during the development of OA (Wei et al., 2017). Under conditions of oxidative stress, IGF-1 does not promote chondrocyte survival (Del Carlo and Loeser, 2003). Given that chondrocyte responsiveness to growth factor stimulation decreases with age (Loeser et al., 2002), the effect of increased oxidative stress in decreasing the survival-promoting capacity of IGF-1 is amplified (Del Carlo and Loeser, 2003). Excessive levels of ROS have been found to inhibit activation of the IRS-1/PI3K/Akt signaling pathway, which normally promotes matrix synthesis, while at the same time ROS activates the ERK MAP kinase which suppresses aggrecan, type II collagen, and Sox-9 expression. Sustained activation of ERK is associated with cell senescence, and a study using rat chondrosarcoma cells has shown that sustained ERK activation, mediated by FGFR3, promoted the expression of markers that are involved in the senescent phenotype. Extracellular ROS also contribute to the inhibition of the Akt pathway through oxidized low-density lipoproteins (LDL). Oxidized LDL binding to LOX-1 has been found to induce chondrocyte senescence which was associated with reduced levels of Akt phosphorylation after IGF-1 stimulation. Oxidative stress induced by oxidized LDL has also been associated with the promotion of the hypertrophic chondrocyte phenotype which has been described in OA cartilage (Loeser, 2011). It is intriguing to understand how aging intertwines with the expression of growth factor receptors which is implicated in survival and the response level of the cells to stimulatory and inhibitory signals to modulate their activities. Current efforts in this line is crucial in paving a path for new improved interventions not only to treat aging associated cartilage conditions but also to provide prevention strategies for healthy aging, and this is discussed in the later section on therapeutic opportunities.

Epigenetics

It is proposed that genetic factors determine the 20–30% of the variation in human lifespan whereas non-genetic factors, stochastic events, and environment determine the remaining 70–80% of the variation. Stochastic events and environmental factors lead to epigenetic modifications, and these are a major contributor to the aging phenotype. During aging, mammalian cells undergo extensive epigenetic changes, resulting in global DNA hypomethylation and promoter hypermethylation (Muñoz-Najar and Sedivy, 2011). It has been suggested that the global DNA hypomethylation during aging is likely the outcome of the passive demethylation of heterochromatic DNA caused by a progressive loss of DNA (cytosine-5)-methyltransferase 1 (DNMT1) function and/or erroneous targeting of this enzyme by other cofactors. Genomic DNA hypomethylation possibly leads to an overexpression of *de novo* factors. DNA methylase DNMT3b could lead to DNA methylation, resulting in aberrant hypermethylation of promoter CpG islands of many genes that are initially unmethylated. The role of epigenetics in linking aging and OA is still an emerging and promising field. Promoter hypermethylation of estrogen receptor and insulin-like growth factor II (IGF2) during aging predispose elderly to sporadic colorectal tumorigenesis (Muñoz-Najar and Sedivy, 2011), similarly, promoter hypermethylation of estrogen receptor and IGF2 may cause a deficiency of estrogen and IGF2 in maintaining articular cartilage, thereby accelerating cartilage turnover and predispose the elderly (and in particular females) to develop OA. However, there is no experimental evidence supporting a direct relationship between hypermethylation of these genes during aging and OA. On the other hand, epigenetic mechanisms could mediate aberrant gene expression of transcription factors, cytokines, ECM degradative enzymes, and ECM proteins in articular chondrocytes, thus triggering the onset of OA. It has been documented that DNA methylation and histone acetylation can mediate the downregulated expression of SOX9 in advanced OA. The question whether epigenetically modified expression of SOX9 in articular cartilage is the cause or the result of OA has yet to be answered (M. Zhang and Wang, 2015). Upregulation of microRNAs miRNA-199a-3p and miRNA-193b with age may be involved in the chondrocyte senescence by downregulating anabolic factors such as SOX9, aggrecan, and collagen type II. Conversely, downregulation of miRNA-320c expression with age may be involved in the juvenile-like phenotypic properties of chondrocytes by downregulating catabolic factor ADAMTS-4. These findings suggest that miRNA-199a-3p, miRNA-193b, and miRNA-320c could be functional markers of cartilage degeneration and evaluation of donor tissues for cartilage grafting (Ukai et al., 2012).

Metabolism

Macroanatomically, chronic metabolic and oxidative stress lead to a thinning of the collagen and proteoglycan layers as well as disorganization of collagen fiber orientation. Microanatomically, superficial, transitional, and radial zones of cartilage exhibit a loss of chondrocytes and ECM proteins (Hardin et al., 2015). Subchondral ischemia resulting from hypertension associates with OA compromising nutrient exchange into articular cartilage, hence prompting bone remodeling. Ectopic lipid deposition in cartilage induced by dyslipidemia might initiate the development of OA, impaired by deregulated cellular lipid metabolism in joint tissues. Hyperglycemia accelerates oxidative stress and AGE product formation which are implicated in cartilage degeneration, whereas low-grade systemic inflammation contributes to a degenerative internal cartilage niche that leads to OA progression OA. Obesity-related metabolic factors, particularly altered levels of adipokines lead to the expression of various proinflammatory factors and degradative enzymes, leading to the inhibition of cartilage matrix production, simultaneously spur remodeling of subchondral bone (Zhuo et al., 2012).

Modulation in ECM Components

The half-life of type II collagen on average is approximately 100 years (Verzijl et al., 2000) and aggrecan 3.5 years (Maroudas et al., 1998). In contrast to the whole aggrecan protein, the aggrecan G1 domain with the role of hyaluronic acid binding has a lower turnover rate of approximately 25 years. Aggrecan turnover gives rise to proportionately more G1 domain fragments occupying hyaluronic acid, therefore, inferior aggrecan structures are generated upon aging (Hardin et al., 2015). Age-related changes not only occur in chondrocytes but also in the cartilage matrix, thereby contributing to OA development. MRI studies show that knee cartilage thinning occurs during aging, particularly on the femoral side of the joint and on the patellae, suggesting a gradual loss of cartilage matrix with age. Thinning can be due to the loss of chondrocytes and/or reduced growth factor responsiveness but also in part to something as simple as reduced water content. Excessive collagen cross-linking, visualized by yellowing of tissue through glycation, increases cartilage stiffness and brittleness, thereby increasing susceptibility to fatigue failure. Increased levels of AGEs in cartilage is correlated with declining anabolic activity (Loeser, 2011).

In surveying the numerous molecular pathways underlying the degenerative process in OA, their relationship to senescence/aging or longevity of chondrocytes, only serves to highlight their intricacy and interdependency. Hence, understanding the complexity of these pathways and the discovery of tools targeting them that are relevant to depressing inflammation, oxidative stress, and senescence in aging chondrocytes may be important in combating, treating, or reversing chronic diseases like OA.

THERAPEUTIC OPPORTUNITIES AND FUTURE PERSPECTIVE

Degeneration of cartilage is considered to be a multifactorial dysregulation of cellular systems, where the cellular processes are interlinked and regulate each other. Fundamentally, aging and degeneration of the cartilage that leads to OA is attributed by increased inflammation and decreased regenerative potential of cartilage. Ideally, therapeutics that could restore impaired function of chondrocytes and reverse/delay cartilage aging

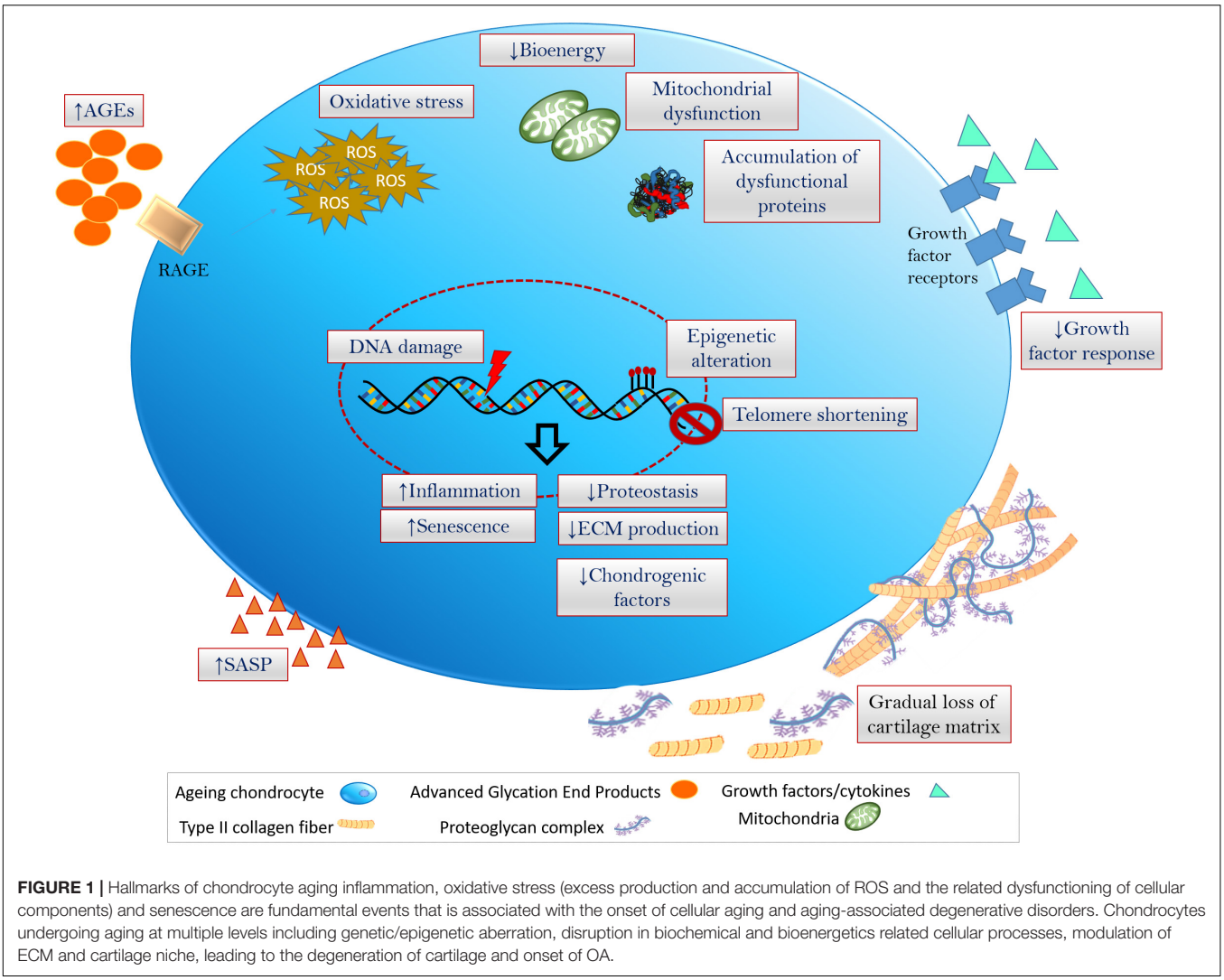

FIGURE 1 | Hallmarks of chondrocyte aging inflammation, oxidative stress (excess production and accumulation of ROS and the related dysfunctioning of cellular components) and senescence are fundamental events that is associated with the onset of cellular aging and aging-associated degenerative disorders. Chondrocytes undergoing aging at multiple levels including genetic/epigenetic aberration, disruption in biochemical and bioenergetics related cellular processes, modulation of ECM and cartilage niche, leading to the degeneration of cartilage and onset of OA.

will involve the modulation of the latter two elements. We, in this review, propose the treatments to achieve this could be pharmacological interventions or/and cell-based therapy that offer suppression of excessive inflammation and support regenerative capabilities.

Pharmacological Treatments

Collectively, understanding molecular determinants of chondrocyte aging and OA has paved a way in identifying potential pharmacological treatments that can regulate these deregulated pathways, hence reversing or delaying the degeneration of cartilage. Treatments including apoptosis-inducing reagents in a model of genetic apoptosis inhibition, arsenic trioxide, hypoxia, mETC inhibitors, and a short mitochondrial form of $p19^{ARF}$ have been reported to induce autophagic cell death (Yongqiang Chen et al., 2010). Despite the promising outcomes by mTOR inhibitors in the treatment of cancer and other diseases, rapamycin (mTOR inhibitor) and its rapalogs have not been tested in OA in the clinical setting. mTOR has a negative feedback inhibition on the activity of PI3K/Akt pathway, and the inhibition of mTOR leads to increased activity of the PI3K/Akt/NF-kB pathway, which may increase MMP secretion by chondrocytes. Therefore, by simultaneously targeting the PI3K/Akt/NF-kB pathway, dual inhibition of PI3K and mTOR can be considered a potential therapeutic approach for OA (Pal et al., 2015). Pharmacological upregulation of autophagy by rapamycin reduces the severity of experimental OA, synovitis, ADAMTS-5, and IL-1β expression, thus enhancing chondrocyte survival and preventing glycosaminoglycan loss. Though these results are encouraging, the potent antiproliferative and immunosuppressive effects of rapamycin pose an additional challenge in translating such strategies for human applications. Nutrient supplementation with non-immunosuppressive compounds such as spermidine, polyamines, or ω-6 polyunsaturated fatty acid or treatment with activators of the UPS could be considered, yet the safety, specificity, and efficacy need to be experimentally and clinically validated. Glucosamine is a safe and widely used dietary supplement that has the potential in enhancing cartilage health in human, and also acts as an effective activator of autophagy. Glucosamine activates autophagy *in vitro* and *in vivo via* the Akt/Fox03/mTOR signaling pathways, raising the feasibility that

glucosamine can be utilized to maintain cellular homeostasis and joint health although such treatment needs to be continual and long term to be beneficial (Goldring and Berenbaum, 2015). Disease and pain control with glucocorticoid therapy in OA has been employed extensively for decades, acting by diffusing across cellular membranes, binding their cognate nuclear receptors and, interrupting the inflammatory and immune pathways at a number of levels. A recent study demonstrated that dexamethasone, a synthetic glucocorticoid, increases the intracellular ROS levels, autophagy markers, and expression of FoxO3. In response to the increased ROS level, autophagy is induced as a defense mechanism *via* ROS/Akt/FoxO3 pathway which subsequently protects human chondrocytes from ROS-induced apoptosis. Of note, long-term administration of dexamethasone increasing ROS level could upregulate the expression of ADAMTSs and MMPs *via* ROS-dependent manner, thereby contributing to advancing cartilage degeneration (Shen et al., 2015). There have been many studies that have pursued the targetting of these metabolic pathways in order to stabilise or reverse OA disease especially using natural products, nutrients, pharmacological agents and biologics such as cell-free and cell-based regenerative strategies, and a selection of these are captured in **Table 1**.

The use of ROS scavengers is probably the simplest strategy to prevent stress-induced senescence; antioxidants, ascorbic acid (vitamin C), *N*-acetylcysteine, sodium pyruvate, sodium selenite, and Trolox (*see also* **Table 1**) improve mesenchymal stem cell cellular "health," increase cell yield, and maintain the differentiation potential of cells (Turinetto et al., 2016). RNA interference of $p16^{Ink4a}$ in OA cell cultures can restore their anabolic metabolic responsiveness to growth factors, similar to younger fetal chondrocytes, but the effect appears to require continual treatment to suppress $p16^{Ink4a}$ expression (Zhou, 2004). To prove the causal role of senescent cells in chronic disease, Baker et al. (2011) developed an *in vivo* transgene model to selectively remove $p16^{Ink4a}$ +ve senescent cells by apoptosis in aged and prematurely aging hypomorphic $BubR1^{H/H}$ mice which then showed delayed onset of sarcopenia, prevention of adipose loss and cataract formation. Baar et al. (2017) used cell-penetrating peptides that target FOXO4 interactions with p53 localized at DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS) to sensitize senescent cells to p53-dependent apoptosis. FOXO4-p53-interfering peptides can be used to induce apoptosis in senescent cells *in vitro* and *in vivo*, in the latter context reducing fragility and renal failure in fast aging $Xpd^{TTD/TTD}$ and naturally aging mice. Similarly, dasatinib and quercertin also function in combination to eliminate senescent cells and reduce frailty in aged mice through inhibition of Src kinase and antiapoptotic Bcl-xL (Xu et al., 2018). The use of dasatinib/quercertin to treat OA has been mooted (McCulloch et al., 2017), but their use *in vitro* to prepare cells for ACI is possibly a more targeted application. Despite convincing data pointing to the causal role of senescence in chronic conditions, in light of the evidence demonstrating senescent cell participation in promoting wound healing (Demaria et al., 2014), stem cell priming and plasticity (Ritschka et al., 2017), and limb regeneration (Yun et al., 2015), aspects of their physiological function has to be appraised. In cartilage, the accumulation of senescent cells in adult tissues may help to retain cellularity in a tissue that otherwise could be depopulated below a threshold level for maintenance of the ECM.

Evaluation of recent studies suggests the secretory profile of senescent cells constitutes a transient signal to initiate repair processes, while persistent activation of a proinflammatory secretome is the basis of chronic wounding and disease (Kowald et al., 2020). Again, these are reasons why senolytic interventions may be best suited to generating a stable, progenitor population for cell therapy rather than treating OA joints.

Regenerative Therapies

Cell-Based Therapies

Pharmacological treatments to regulate pathways leading to accelerated aging of chondrocytes in cartilage can potentially reduce the severity of OA in patients with established disease affecting the whole joint. This approach is less amenable for younger patients who at first presentation are symptomatic with localized cartilage lesions in their joints. These lesions can be repaired using a variety of cell therapies that overcome an inherent barrier to cellular migration facing chondrocytes embedded within a dense ECM at the wound edges (Hunziker, 2002). For example, mesenchymal stem cells (MSC) in the marrow beneath the overlying subchondral bony plate can be released into the lesion by drilling or puncturing through the plate. However, microfracture produces transient fibrocartilaginous repair tissue that is functionally suboptimal.

Another, no less-invasive procedure, autologous chondrocyte implantation (ACI), requires two surgeries, the first to remove a cartilage biopsy, from which cells are cultivated, that are then transplanted in a second procedure into the debrided lesion under a periosteal flap (Brittberg et al., 1994). The latter procedure produces more hyaline cartilage than microfracture. The inherent disadvantage of ACI is the number of cells isolated and expanded from the biopsy limits the size of the lesion that can be repaired because the ability of cells to efficiently redifferentiate reduces markedly upon >5 passages in culture (Schulze-Tanzil et al., 2002). Also, replicative senescence within the expanded cell population may also further limit their repair potential. To overcome these limits, repair strategies have progressed to use allogeneic mesodermal (and tissue-specific) progenitors and adult stem cells/MSCs; as allogeneic transplanted cells differentiate and become embedded in a supportive ECM, they are effectively immune privileged.

As mentioned earlier, mature chondrocytes have limited ability to repair cartilage defects due to an inherent inability to migrate through the ECM, one approach to repair cartilage defects is by introducing a new cell population to stimulate repair and produce structural repair of lesions. Hence, MSCs are considered to be an excellent compatible cellular source that are easily expanded in culture, and following seeding in an artificial matrix, can be implanted into a cartilage defect and retain the capacity to undergo chondrogenesis and generate hyaline cartilage.

TABLE 1 | Chondroprotective therapeutics and the underlying mechanism of action targeting aging and degeneration associated determinants.

Categories of therapeutic strategies	Therapeutics	Therapeutic mechanism	References
Natural products/nutrients and their derivatives	Ascorbic acid	• Protection for human chondrocytes against oxidative stress	Chang et al., 2015
	Baicalin	• Prevented the apoptosis of endplate chondrocytes by inhibiting the oxidative stress • Inhibited endoplasmic reticulum stress • Protects human OA chondrocytes against IL-1β-induced apoptosis • Protects the degradation of ECM through activating autophagy via miR-766-3p/AIFM1 axis	Pan et al., 2017; Cao et al., 2018; Li et al., 2020
	Curcumin	• Protected the mitochondrial function, hence prevented cartilage degeneration • improves age-related and surgically induced osteoarthritis by promoting autophagy • Inhibited apoptosis of chondrocytes through activation ERK1/2 signaling Pathways induced autophagy • Inhibited the PERK-eIF2α-CHOP pathway through promoting SIRT1 expression in oxidative stress	Li et al., 2017; Feng et al., 2019; Nicoliche et al., 2020
	Delphinidin (a primary plant pigment, and also an antioxidant)	• Cytoprotects chondrocytes against oxidative stress through activation of autophagy	Lee et al., 2020
	Diosmin	• Chondroprotective effect via modulating oxidative stress	Yi–Ru Chen et al., 2019
	Polyphenols derived by olive extracts (e.g., Oleuropein)	Targeted Cx43 and senescence	Varela-Eirín et al., 2020
	Resveratrol	• Exerted anabolic, anti-catabolic, anti-inflammatory and chondroprotective effects • Delays cartilage degeneration autophagy via AMPK/mTOR pathway	Im et al., 2012; Qin et al., 2017
	Vitamin D	• Activated autophagy via mediating the AMPK–mTOR signaling pathway in chondrocytes, to reduce osteoarthritis	Kong et al., 2020
Pharmacological agents (biological factors/Drugs)	Irisin, a cleaved form of fibronectin type III domain containing 5 (FNDC5)	• Modulated Oxidative Stress • Regulated mitochondrial Integrity • Regulated autophagy	Wang et al., 2020
	Fenofibrate	• Senotherapeutic molecules with pro-autophagic activity	Nogueira-Recalde et al., 2019
	Navitoclax (ABT263)	• A specific inhibitor of the BCL-2 and BCL-xL proteins • Reduced inflammation • Senolytic drug	Yang et al., 2020
	Peroxiredoxin II (Prx II)	• Anti-oxidative stress and anti-aging effects • Reduced oxidative stress and cell senescence in chondrocytes by activating the p16-CDK4/6-pRb-E2F signaling pathway	Shao et al., 2020
	Rapamycin	• A specific inhibitor of the mTOR signaling pathway • Enhanced expression of autophagy regulators and prevents chondrocyte death.	Caramés et al., 2012; Pal et al., 2015; Bao et al., 2020
Biologics (cell-based)	Articular cartilage progenitors	• Resistance to telomere erosion through the expression of telomerase • Tissue replacement therapies	Dowthwaite, 2004; Williams et al., 2010; McCarthy et al., 2012; Jiang et al., 2016
	Adult stem cells (tissue-specific and mesenchymal stem cells)	• Reduced catabolic effect • Reduced inflammation—via their indirect regenerative effects (secretomes and EVs) • Immunomodulatory effect • Anti-apoptosis and anti-fibrosis • Tissue replacement therapies	Samuel et al., 2018; Samuel et al., 2019
	Embryonic stem cells/induced pluripotent stem cells	• Tissue replacement therapies	Chang et al., 2020; Gardner et al., 2019
Biologics (cell-free)	Platelet-rich plasma	• Reduced inflammation • Regulates cell chemotaxis • Improved angiogenesis • Enhanced cell proliferation and cell differentiation • Enhanced ECM production, hence matrix deposition	Moussa et al., 2017; Garbin and Olver, 2020

(Continued)

TABLE 1 | Continued

Categories of therapeutic strategies	Therapeutics	Therapeutic mechanism	References
	Extracellular vesicles/exosomes	• Improved cartilage thickness • Increased matrix deposition • Better subchondral bone integrity • Reduced synovial cell apoptosis • Reduced MMPs	Wang et al., 2017; Khatab et al., 2018; Zhang et al., 2019; Jin et al., 2020

Additionally MSCs can also be used to produce paracrine factors to induce cartilage repair, either alone or implanted in combination with autologous articular chondrocytes (Sariset al., 2021).

In vitro expanded tissue-specific articular cartilage progenitors exhibit resistance to telomere erosion through the expression of telomerase, and, in contrast to bone marrow-derived MSCs, they preferentially differentiate to form hyaline cartilage rather than calcified cartilage or bone (Dowthwaite, 2004; Williams et al., 2010; McCarthy et al., 2012). Cell kinetic and telomeric analysis of articular chondroprogenitors from normal and OA human cartilage show approximately 50% of OA progenitors undergo accelerated senescence following culture expansion (Fellows et al., 2017). Zhou (2004) discovered OA chondrocytes show a higher trend of $p16^{Ink4a}$ expression than age-matched controls and fetal cartilage, and, that this pattern of expression extends to cells following isolation and culture expansion. These data argue for the isolation of chondroprogenitors from younger non-diseased donors for culture expansion (Adkisson et al., 2010). Senolytic or senostatic molecules can be used to maintain a healthy viable progenitor population during an *in vitro* cell culture expansion phase, removing cells that would otherwise compromise repair through "bystander" effects upon transplantation.

Cell-Free-Based Regenerative Therapies

Platelet-Rich Plasma-Based Therapy

In recent years, much effort has also been directed to study the therapeutic value of naturally occurring biomolecule pools such as platelet-rich plasma (PRP) for cartilage regeneration. The presence of many important growth factors in PRP may enhance the anabolic signal for regeneration, and thus may offer therapeutic benefit to patients with OA (Marmotti et al., 2015). Applying a similar theoretical framework, PRP may also enhance cellular expansion and chondrogenesis of the MSCs, thus may synergistically improve cartilage repair. The reproducible positive effect of PRP on chondrocyte and MSC proliferation and chondrogenic differentiation indicates that the adjunct of use of PRP may be advantageous to promote cellular expansion *in vitro* for the enhancement of cell-based therapy.

The regenerative capacity of PRP is mainly attributed to its broad biomolecular composition, including chemokines, cytokines, small molecules, adhesive proteins, proteases, antiproteases, exosome-derived microRNAs, receptor ligands, and growth factors, all of which are essential components for the initiation and maintenance of the tissue healing response (Garbin and Olver, 2020). This includes regulation of cell chemotaxis, angiogenesis, cell proliferation, cell differentiation, and ECM production (Foster et al., 2009; Gobbi et al., 2012; De La Mata, 2013). Multiple clinical studies have shown that intra-articular injection of PRP significantly ameliorates OA symptoms (Kon et al., 2010; Filardo et al., 2011; Gobbi et al., 2012; Patel et al., 2013; Raeissadat et al., 2013). Intriguingly, the use of PRP has been shown in immature bovine cartilage explants to induce articular cartilage maturation including reorganization of the ECM into a more adult-like state and this may underly some of the efficacy noted for PRP injections especially in younger patients (Zhang et al., 2017).

Extracellular Vesicles as an Emerging Therapeutic Approach

The relevance of extracellular vesicles (EVs) in regulating the development of age-related conditions is based on the notion that EVs are one of the known mechanisms responsible for the maintenance of cellular homeostasis (Baixauli et al., 2014; Desdín-Micó and Mittelbrunn, 2017). Loss of EV regulatory influence contributes to the deregulation of processes essential for cellular integrity and signaling pathways involved in cellular metabolism and growth (Yafei Wang et al., 2017). Consequently, this leads to the development of cellular events such as oxidative stress, protein aggregation, mitochondrial dysfunction, and inflammation (Klaips et al., 2018), all of which are contributing factors of aging, as discussed earlier. Intriguingly, stem cells, as somatic cells, release a large number of EVs (Drago et al., 2013; Katsuda et al., 2013; Tetta et al., 2013). The role of EVs in mediating tissue repair by stem cells from which they are derived has been consistently demonstrated (Camussi et al., 2010; Ratajczak, 2011; Panagiotou et al., 2016).

Preclinical studies especially *in vivo* studies revealed injection of secretomes or EVs derived from multiple cellular sources improves cartilage thickness, matrix deposition, and subchondral bone integrity, reduced synovial cell apoptosis, and reduced MMPs in animal injury models (Yafei Wang et al., 2017; Khatab et al., 2018; Zhang et al., 2019; Jin et al., 2020).

While these *in vivo* studies directly injected EVs intra-articularly, their study designs were highly varied. Moving forward, clinical studies for EV transplantation requires deliberate consideration on the standardization of EV preparation protocol, dose, and injection times, thus allowing more reproducible and comparable datasets to be used to progress treatments for cartilage degeneration in OA.

CONCLUSION

In summary, cellular homeostasis of chondrocytes and cartilage is maintained through the molecular sensors regulating complex yet interlinked cellular events including bioenergetical homeostasis, survival, the balance of oxidative and antioxidative production, genetic integrity, mechanobiology, and intercellular communications within the tissue niche. When deregulation occurs in tissue and cellular homeostasis of cartilage, it leads to degenerative disorders. In this review, we have highlighted the hallmarks of chondrocyte aging and degeneration of cartilage in light of their key molecular determinants and their underlying mechanisms. Recent efforts in developing therapeutics that target deregulated cellular homeostasis are captured too. Moving forward, novel approaches for activating the deregulated survival pathways and restore the balance of homeostasis through naturally occurring nutrients and natural products or pharmacological interventions or even innovative strategies using biologics for slowing down or reversing aging of cartilage should be further investigated. This new generation of treatment strategies can potentially make a significant impact on improving the lives of patients suffering from many aging associated chronic diseases like OA.

AUTHOR CONTRIBUTIONS

TSR conceptualized the scope of the manuscript. TSR, YMY, and IMK drafted the manuscript. All authors approved the final manuscript.

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REVIEW
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Geroprotectors and Skeletal Health: Beyond the Headlines

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Osteoporosis and osteoarthritis are the most common age-related diseases of the musculoskeletal system. They are responsible for high level of healthcare use and are often associated with comorbidities. Mechanisms of ageing such as senescence, inflammation and autophagy are common drivers for both diseases and molecules targeting those mechanisms (geroprotectors) have potential to prevent both diseases and their co-morbidities. However, studies to test the efficacy of geroprotectors on bone and joints are scant. The limited studies available show promising results to prevent and reverse Osteoporosis-like disease. In contrast, the effects on the development of Osteoarthritis-like disease in ageing mice has been disappointing thus far. Here we review the literature and report novel data on the effect of geroprotectors for Osteoporosis and Osteoarthritis, we challenge the notion that extension of lifespan correlates with extension of healthspan in all tissues and we highlight the need for more thorough studies to test the effects of geroprotectors on skeletal health in ageing organisms.

Keywords: aging, senescence, osteoarthritis, osteoporosis, geroprotectors, mouse models

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INTRODUCTION

In the United Kingdom, musculoskeletal disorders are responsible for approximately one third of General Practitioner consultations and for a NHS budget of nearly £5 billions/annum (Executive, 2015). In addition, an estimated 8.9 million working days were lost in 2020 for musculoskeletal disorders, accounting for 34% of all working days lost due to ill-health in the United Kingdom alone (Health and Safety Executive, 2020). As a whole, musculoskeletal disorders cause more functional limitations in the adult population in the western world than any other group of disorders (Woolf and Pfleger, 2003).

The two most frequent musculoskeletal diseases are osteoarthritis (OA) and osteoporosis (OP). OA, the most common age-related joint pathology, is characterized by cartilage degradation and inflammation in the joint, thickening of the bone plate, changes in subchondral bone and formation of osteophytes (Lotz and Caramés, 2011). Symptomatic knee OA occurs in 10% of men and 13% of women aged 60 years or older (Zhang and Jordan, 2010). The number of people affected with symptomatic OA is likely to increase due to the aging of the population and the obesity epidemic, an important risk factor driving OA (Raud et al., 2020). Patients with OA have higher levels of comorbidity compared to those of similar age without OA (Kadam et al., 2004). OA is significantly associated with other musculoskeletal diseases such as other arthropathies, synovial and tendon disorders and non-musculoskeletal comorbidities such as gastritis, intestinal diverticula and

ischemic heart disease (Kadam et al., 2004). There is no effective cure for OA. Current management of OA is limited to symptoms' alleviation (Bijlsma et al., 2011) followed by joint replacement when pharmacological management of the pain is no longer effective.

OP is characterised by significant bone loss and increased risk of fractures. Osteoporosis is defined by the World Health Organization when the mineral bone density of a person is 2.5 standard deviations below the young normal mean. It is most frequent in post-menopausal women and it affects around one in five men and one in two women over the age of 50 (Poole and Compston, 2006). Although present drugs reduce the risk of fractures, the number needed to treat (i.e., the number of patients that need to be treated for one to benefit compared with a control in a clinical trial) to prevent a fracture is >50 over 1–3 year period (Crandall et al., 2014), suggesting the need to find new more effective interventions for OP. Furthermore, 92% of patients affected with osteoporosis present other age-related comorbidities that can include cardiovascular, neurological and gastric conditions (Salive, 2013).

The frequent association of both OP and OA with co-morbidities often results in problems of polypharmacy, including increased adverse events and reduced efficacy of their treatments due to drug-drug interaction or disease-drug interactions (Van Der Heide et al., 2018). Up until now, research has focused predominantly on the identification of drugs for the maintenance of function of single tissues (i.e., bone or cartilage) or the identification of treatments for individual musculoskeletal diseases. However, as both conditions are associated with high level of comorbidities and polypharmacy, this approach is ineffective (Tinetti et al., 2012) and approaches which target clusters of diseases would be an advantage.

Osteoarthritis and Ageing

The onset of OA is characterised by alteration in the extracellular matrix (ECM) produced by chondrocytes, which stimulates their increased proliferative response, in an attempt to restore articular cartilage (Goldring, 2000). This leads to formation of chondrocyte clusters and increased synthesis of irregular matrix components such as proteoglycans and collagen (Rothwell and Bentley, 1973). With advancing age and OA progression chondrocytes show hallmarks of ageing, such as mitochondrial dysfunction and increased oxidative stress, senescence and inflammation (Loeser et al., 2002; Grishko et al., 2009; Goldring and Otero, 2011; Loeser et al., 2016) as well as aging associated changes in autophagy (Caramés et al., 2010). This results in a reduced ability to produce ECM and an increase in catabolic processes largely mediated by proinflammatory cytokines and mediators such as metalloproteinases (Burrage et al., 2006). In turn, the ECM becomes more vulnerable to damage, leading to the onset of OA, increased cartilage degradation and disease progression. The importance of ageing in driving the disease is highlighted by the fact that aged mice show signs of cartilage degradation and develop the full osteoarthritis phenotype faster and more aggressively than young mice after destabilization of the medial meniscus or following injury (Huang et al., 2017). Therefore, targeting mechanisms of ageing may offer new opportunities for treatment for OA.

Osteoporosis and Ageing

The adult skeleton is continuously remodelled by osteoclasts, which resorb bone, osteoblasts, which form new bone and osteocytes. Osteocytes derives from osteoblasts and are contained in the bone matrix. Through secreted factors they coordinate the activity of osteoclasts and osteoblasts in response to physical and hormonal stimuli. Osteocytes, Osteoblasts and their precursors secrete RANK-L which binds to RANK (receptor activator of nuclear factor κ-B) receptor on osteoclasts precursors, initiating their proliferation and differentiation to mature osteoclasts able to resorb bone. Osteoclast activation is inhibited by another protein known as osteoprotegerin (OPG) produced by osteocytes and osteoblasts, which acts as a decoy RANK-L and therefore competes with RANK-L for receptors. Although RANK-L and macrophage colony stimulating factor (M-CSF) are essential for osteoclastogenesis, additional cytokines such as TNF-alpha and IL-1 are likely to contribute to the regulation of osteoclast formation both in physiological and pathological condition such as oestrogen deficiency in postmenopausal women. Features of bone ageing include reduction in bone mass and bone mineral content, changes in bone shape and structure with loss of trabecular bone, thinning of cortical bone and increased porosity, enlargement of the medullary cavity, higher levels of bone marrow fat, and increase in bone turnover (Pignolo et al., 2019). At the cellular level, there is an increase in osteoclast resorption and a decrease in osteoblast bone formation, leading to a reduction in bone density and increased risk of fracture. Increased age has long been associated with reduced bone mass, which is largely thought to be due to hormonal deficiency, mainly oestrogen due to menopause. However, age-associated bone loss occurs even in individuals with normal levels of sex steroids (Riggs et al., 2008) and there is a close association between the effects of loss of oestrogen and dysregulation of mechanisms driving ageing. Similarly, to OA dysregulation of mechanisms of ageing such as inflammation, autophagy, increased oxidative stress and senescence have also been associated with OP (Farr and Khosla, 2019; Yin et al., 2019). Some of these mechanisms have been shown to be deficient in presence of decreased oestrogen. In an ovariectomised rat model, a significant reduction in levels of autophagy in osteocytes correlated with an increase in oxidative stress and bone loss (Yang et al., 2014). In addition, ovariectomy (OVX) resulted in significant acceleration of the epigenetic clock, the DNA methylation changes occurring with age (Stubbs et al., 2017) suggesting a close link between oestrogen deficiency and ageing, the two main drivers of OP. Therefore, ways to target mechanisms of ageing may offer new opportunities for the development of improved treatment in OP.

GEROPROTECTORS

Recent work has shown that it is possible to prevent or even reverse the dysregulation of oxidative stress, autophagy and the

occurrence of senescence using a new class of drugs called geroprotectors. Geroprotectors are drugs that delay or reverse ageing processes and in doing so target the major risk factors for age-related diseases. They promise to promote health span of more than one organ system at the same time in animal models (Figueira et al., 2016; Bellantuono, 2018). Studies in model organisms or retrospective studies in patients show that they can ameliorate tissue dysfunction and reduce the onset and severity of many diseases [reviewed in (Morsli and Bellantuono, 2021)]. Over 200 compounds have been classified as geroprotectors, each reported to slow ageing and/or extend lifespan in a variety of organisms (geroprotectors.org).

Such drugs could have distinct advantages over present treatments in OP and offer new opportunities for OA due to the fact that they may be able to prevent both OP and OA and their co-morbidities. However, the effects of geroprotectors on skeletal health have received little attention compared to other organ systems with the assumption that these drugs will work equally well for all tissues. Here we review the evidence available to address whether geroprotectors have potential for the care of skeletal age-related diseases and their co-morbidities. We focus on drugs with a good safety profile, which have been shown to target ageing pathways, extend the lifespan and healthspan in animal models and have some evidence of improving health in humans by demonstrating protection from multiple-age-related diseases (Partridge et al., 2020) and for which there are well designed studies in animal models of OP and OA or clinical data available.

Among the most studied geroprotector is Rapamycin. It is an inhibitor of the mTOR signalling pathway, a nutrient sensing pathway closely associated with ageing and longevity (Johnson et al., 2013; Saxton and Sabatini, 2017). mTOR is a highly conserved biological pathway, encompassing two distinct complexes mTORC1 and mTORC2. The two complexes differ in composition and function, with mTORC1 having Raptor and mTORC2 containing Rictor (Saxton and Sabatini, 2017). mTORC1 is acutely inhibited by Rapamycin, whereas mTORC2 requires chronic exposure to be affected (Li et al., 2014). It targets multiple mechanisms of ageing including autophagy, oxidative stress, DNA repair (Li et al., 2014; Figueira et al., 2016). More recently Rapamycin has also been shown to inhibit the Senescence Associated Secretory Phenotype (SASP) (Wang et al., 2017), composed of pro-inflammatory and tissue remodelling factors and secreted by senescent cells. Already in clinical use as an immunosuppressor, Rapamycin has been tested extensively in animal models of ageing and age-related diseases [reviewed in (Morsli and Bellantuono, 2021)]. An analogue of Rapamycin, RAD001, has been tested in clinical trials at a substantially lower dose to elicit geroprotective effects to delay immunosenescence with excellent tolerability (Mannick et al., 2014; Mannick et al., 2018).

Metformin, used in the treatment of type 2 diabetes (T2D) (Bosi, 2009; Aroda et al., 2017), has also been extensively investigated for its additional mechanisms of action related to ageing (Valencia et al., 2017; Kulkarni et al., 2020). This includes inhibition of inflammation, reduction in DNA damage and inhibition of SASP (Algire et al., 2012; Moiseeva et al., 2013; Ashabi et al., 2015). There are multiple evidence in animal models and retrospective human studies that metformin has positive effects on multiple age-related diseases [reviewed in (Morsli and Bellantuono, 2021)]. Indeed a clinical trial, using Metformin to extend survival and reduce the incidence of multiple diseases (the TAME study) has obtained FDA approval (Barzilai et al., 2016).

Less studied but interesting in the context of OA is Acarbose (ACA), an intestinal α-glucosidase inhibitor, FDA-approved to treat diabetes and acts by inhibiting digestion of complex carbohydrates and reducing postprandial hyperglycaemia (Dinicolantonio et al., 2015). The mechanisms by which ACA leads to lifespan extension are not well understood. It is considered a calorie restriction mimetic and it has been reported to improve parameters of health, including reduced incidence of lung tumours in males mice, reduced liver degeneration in both sexes and glomerulosclerosis in female mice (Harrison et al., 2019), improved neuromuscular function in females, balance/coordination and grip strength in both sexes (Herrera et al., 2020). Age-related cardiac hypertrophy was seen only in male mice, and this male-specific ageing effect was attenuated by ACA (Herrera et al., 2020).

Similarly, less known but tested in the context of OA is 17α-estradiol (17α-E2), a naturally occurring enantiomer of 17β-estradiol (17β-E2), yet appears to be non-feminizing due to minimal activation of classical oestrogen receptors, ERα and ERβ (Stout et al., 2016). It has been shown to extend lifespan in male mice (Harrison et al., 2014), ameliorate age-associated metabolic and inflammatory dysfunction (Stout et al., 2017) and improve male glucose tolerance across much of adult life (Garratt et al., 2017). When administered in later life it maintains body weight, with larger muscle mass and fibres, increased grip strength and coordination (Garratt et al., 2019). The metabolic improvements are sex-specific and influenced by gonadal hormones (Garratt et al., 2017). Little is known on the molecular basis of 17α-E2 on lifespan and healthspan. The metabolic improvements appear to be associated with enhanced hepatic mTORC2 signalling, increased AKT activity and phosphorylation of FOXO1 (Garratt et al., 2017), increased AMPKα and reduced mTOR complex 1 activity in visceral adipose tissue (Stout et al., 2017). These latter changes were not found in liver or quadriceps muscle (Stout et al., 2017).

Of interest, particularly in the context of OA, is Glucosamine (GluN), an amino-monosaccharide derived principally from chitin, a compound found in the exoskeleton of marine invertebrate. It is a component of glycoproteins, proteoglycans and glycosaminoglycans. The main compounds containing GluN are glucosamine hydrochloride, glucosamine sulphate, N-Acetylglucosamine (GlcNAc). Those compounds have different phamarcokinetic and pharmacodynamics and seem to act through different mechanisms, which may account in part for the heterogeneity of response observed in the different studies. For example glucosamine sulphate requires a stabiliser in the form of salt and is therefore less pure than glucosamine hydrochloride, necessitating higher dosage (Owens et al., 2004). Glucosamine (sulphate/hydrochloride) have been shown to extend lifespan in *C. Elegans* and in ageing mice by mimicking similar effects to a low carbohydrate diet. Indeed, it has been

shown to activate AMPK, which in turn promotes mitochondrial biogenesis, increase aminoacid transport and inhibits glycolysis (Weimer et al., 2014). GlcNAc has also been seen to promote lifespan extension in *C. Elegans* but by promoting proteasome activity and autophagy (Denzel et al., 2014). Treatment with GlcNAc showed an increase in mobility function in models of Parkinson's and Alzhemer's disease, suggesting effects on healthspan as well (Denzel et al., 2014). In addition, examination of abitual use of glucosamine supplements in a retrospective study showed a lower incidence of coronary heart disease and stroke (Ma et al., 2019), and a reduction in mortality due to cancer, respiratory, digestive and cardiovascular diseases (Li Z.-H. et al., 2020). However, glucosamine users were also more active and often took additional supplements compared to the control group, making it difficult to assess whether these effects were due to GluN uptake *per se*. GluN has been shown to have a good safety profile and may have promise as geroprotector (Zeng et al., 2015) but well controlled prospective preclinical and clinical studies with long duration are required to assess whether any of the GluN compounds improves health span safely.

Spermidine is also emerging for its geroprotective properties. It is a naturally occurring polyamine, and its concentration has been shown to decline with age in both males and females rat tissues and in erythrocytes (Jänne et al., 1964). Administration of spermidine extends lifespan in aged cells, *C. Elegans*, Drosophila (Eisenberg et al., 2009) and mice (Eisenberg et al., 2016) and it has been shown to improve some parameters of health. It increase B cell function in aged mice and humans (Zhang et al., 2019), cardiac function and arterial stiffness in aged mice (Eisenberg et al., 2016) and synapse ageing in drosophila (Maglione et al., 2019). Dhal salt sensitive rats fed high salt diet receiving spermidine showed reduced high blood pressure, delayed progression of heart failure and renal abnormalities seen in presence of hypertension (Eisenberg et al., 2016). In addition, administration of spermidine reduced the severity of liver lesions in a mouse model of liver cirrhosis (Yue et al., 2017) and of retinal ganglion cell death in a mouse model of optic nerve injury (Noro et al., 2015). In a community-based cohort study participants taking spermidine showed significant lower all-cause mortality (Kiechl et al., 2018). These effects seem to be the result of increased autophagy, mitophagy and mitochondrial biogenesis (Eisenberg et al., 2016). However, changes in other mechanisms associated with ageing have also been seen in presence of spermidine supplementation such as decreased histon H3 acetylation with possible consequences for gene expression (Eisenberg et al., 2009). Safety profile will require appropriate assessment in prospective studies as the results in animal models have been obtained using a wide range of doses, some of which may show toxicity when translating into humans.

Senolytics [reviewed in (Kirkland and Tchkonia, 2020)] are among the most promising geroprotectors due to their intermittent mode of administration to periodically eliminate newly formed senescent cells. This has the potential to reduce side effects and be more cost-effective. Senescent cells are characterised by irreversible cell cycle arrest and the presence of SASP. They accumulate with age and they have been shown to be causal to multiple age-associated tissue dysfunction or age-related diseases in animal models (Jeyapalan et al., 2007; Wang et al., 2009). Senolytics work by targeting specific survival pathway and selectively inducing senescent cell death (Kirkland et al., 2017). The senolytics Dasatinib, a tyrosine kinase inhibitor, and Navitoclax, a BCL2 family inhibitor, are among those most studied. They both have anti-neoplastic activity and side effects include increased risk of bleeding, immunosuppression and increased risk of infections (Wilson et al., 2010; Futosi et al., 2012; Rodriguez et al., 2012). Dasatinib has little or no senolytic activity on its own but has senolytic activity only when given in combination with Quercetin (Zhu et al., 2015). Quercetin and Fisetin (another senolytic) are flavonoids found in plants and fruits and sold as food supplements for their antioxidant activities with a good safety profile. The dose required to elicit senolytic activity is higher than that which is recommended as a food supplement and appropriate long-term safety studies are required. Several clinical trials with these drugs are ongoing.

Geroprotectors to Target OA

In vitro studies on the effects of geroprotectors are limited. Rapamycin has been found to modulate chondrocytes' survival, cell death and senescence [reviewed in (Pal et al., 2015)]. In addition, it has been shown to increase expression of autophagy genes in human and mouse chondrocytes (Zhang et al., 2015) and in bovine cartilage explants (Caramés et al., 2012b). This increase was associated with an increased expression of two major proteins of the cartilage matrix, aggrecan and type II collagene, and a decreased expression of metalloproteinases and chemokines (Zhang et al., 2015). Similarly, metformin has been shown to increase chondrocyte survival and delay senescence by increasing AMPK and reducing TORC1 signalling (Feng et al., 2020) resulting in attenuated aggrecanase activity and proteoglycan breakdown in chondrocytes grown in presence of inflammatory cytokines (Li H. et al., 2020). Senolytics Navitoclax, Fisetin and UBX0101 showed a reduction in markers of inflammation, an increase in markers of matrix deposition (e.g., glycosaminoglycans) (Jeon et al., 2017; Zheng et al., 2017; Yang et al., 2020) and an improvement in chondrocytes proliferation (Jeon et al., 2017) in human OA chondrocytes. Glucosamine has been shown to have anabolic effects by inducing the production of hyaluronic acid in human chondrocytes and synovial cells and regulate expression of inflammatory cytokines. A summary of the key *in vitro* studies is here (Henrotin et al., 2012). Spermidine has only recently been studied in the context of OA. *In vitro* it shows chondro-protective effects from oxidative damage and cell death, and anti-inflammatory properties (Silvestri et al., 2018; D'adamo et al., 2020).

In vivo most studies testing geroprotectors to delay or reverse the onset of OA are mainly performed in rodent models at young age following induction of OA by destabilization of the meniscus (**Table 1**). Administration of Rapamycin for 10 weeks at 1 mg/kg/day after transection of the medial meniscal tibial ligament and the medial collateral ligament in 8 weeks old mice decreased cartilage degradation (Caramés et al., 2012a). Similarly, intra-

TABLE 1 | Summary of studies testing geroprotectors to attenuate OA in experimental models and patients.

Geroprotector	Model	Age at the start of the experiment	Key findings (compared to controls)	Reference
Rapamycin 1 mg/kg/day i.p. Starting at the time of MMTL + MCL	Mice C57Bl/6—Transection of MMTL and MCL	8 weeks	↓Cartilage loss	Caramés et al. (2012a)
Rapamycin (10 µl of 10 µM solution) 2×/week intra-articular injection starting at the time of DMM	Mice C57Bl/6—DMM	10 weeks	↓Cartilage loss	Takayama et al. (2014)
Rapamycin (100 ng^{-1} µg) intra-articular gelatin hydrogel starting at the time of DMM	Mice C57Bl/6—DMM	8 weeks	↓Cartilage loss	Matsuzaki et al. (2014)
Rapamycin14 mg/kg/day	Mice UM-HET	Assessed at natural death	No difference in OA score	Ewart et al. (2020)
Acarbose 1,000 mg/kg/day	Mice UM-HET	Assessed at natural death	No difference in OA score	Ewart et al. (2020)
17-α-estradiol 14.4 mg/kg/day	Mice UM-HET	Assessed at natural death	No difference in OA score	Ewart et al. (2020)
Metformin Intra-gastric 200 mg/kg/day starting 3 days post-DMM Intra-articular 0.1 mmol/kg twice/week post-DMM	Mice C57Bl/6—DMM	8–10 weeks	↓Cartilage loss. ↑paw withdrawal threshold ↓weight-bearing asymmetry	Li et al. (2020a)
Metformin 205 mg/kg/day in drinking water 2 weeks prior to DMM 2 weeks post-DMM	Mice C57Bl/6—DMM	10 weeks	↓Cartilage loss ↓Synovitis ↓Osteophytes No effect on subchondral bone mass ↑paw withdrawal threshold ↑spontanous activity	Li et al. (2020b)
Metformin 51.7 mg/kg/day in drinking water 1 month post-PMM	Rhesus macaques—PMM	8.5–11.5 years	↓Cartilage loss ↓subchondral bone mass ↑standing and walking time	Li et al. (2020b)
Fisetin 20 mg/kg/day gavage immediately after DMM	Mice C57Bl/6—DMM	10 weeks	↓Cartilage loss ↓subchondral bone mass ↓Synovitis	Zheng et al. (2017)
Navitoclax 0.25, 1, 5 µM intra-articular injection at the time of DMM twice/week for 2 weeks	SD Rat—DMM	4–6 weeks	↓Cartilage loss ↓subchondral bone mass ↓osteophytes	Yang et al. (2020)
UBX0101 intra-articular (10 µl of 0.2–5 mM) 14 days post-ACLT 42 days post-ACLT	Mice C57Bl/6—ACLT	10 weeks	↓Cartilage loss ↓subchondral bone mass ↓Pain ↓osteophytes (only with d14 post-ACLT treatment)	Jeon et al. (2017)
UBX0101 intra-articular (10 µl of 1 mM) 2 weeks post-ACLT	Mice C57Bl/6—ACLT	19 months	↓Pain No change in cartilage loss, subchondral bone mass	Jeon et al. (2017)
UBX0101 intra-articular	Phase II clinical trial—OA patients	N/A	No difference in pain	Inc, (2020)
Glucosamine Sulphate oral 250 mg/kg/day starting at 5 weeks post ACLT for 10 days	Wistar Rats ACLT	N/A	↓Pain ↓Cartilage loss	Wen et al. (2010)
Glucosamine Hydrochloride oral (approx. 1,000 mg/kg/day) for 8 weeks	Wistar Rats ACLT	10 weeks	↓Cartilage loss ↓Bone erosion	Naito et al. (2010)
Glucosamine	Systematic review and meta-analysis of clinical trials	Approx. 40–70 years old (when available)	No difference observed on pain scores	(Liu et al., 2018) (Runhaar et al., 2017) (Zhu et al., 2018) (Wandel et al., 2010)

(Continued on following page)

TABLE 1 | (*Continued*) Summary of studies testing geroprotectors to attenuate OA in experimental models and patients.

Geroprotector	Model	Age at the start of the experiment	Key findings (compared to controls)	Reference
Glucosamine	Systematic review and meta-analysis of clinical trials	N/A	↓VAS pain No effect on WOMAC	(Ogata et al., 2018) (Simental-Mendía et al., 2018)
Spermidine 0.3–3–6 mM/day for 4–8 weeks	C57BL6 + ACLT	12 weeks	↓Cartilage loss ↓Osteophytes (@3–6 mM) ↓inflammation ↓MMP13 ↑Aggecan/collagenII	Chen et al. (2020)

MMTL, medial meniscotibial ligament; MCL, medial collateral ligament; DMM, destabilization of the medial meniscus, PMM, partial medial meniscectomy; ACLT, anterior cruciate ligament transection, i.p, Intra-peritoneal; SD, Sprague-Dawley; VAS, Visual Analogue Scale; WOMAC, Western Ontario and McMaster Universities Osteoarthritis Index.

articular administration of rapamycin twice a week for 8 weeks in 10 weeks old mice following destabilization of the medial meniscus (DMM) or injection of hydrogel containing rapamycin in the joint in 8 weeks old DMM mice showed a reduction of OARSI score (Matsuzaki et al., 2014; Takayama et al., 2014). In all these studies rapamycin was given immediately after the DMM and prior to the establishment of damage in young animals. A similar experimental design has been used to test other geroprotectors such as Fisetin, Metformin, Spermidine or Navitoclax with similar results (Zheng et al., 2017; Li J. et al., 2020; Chen et al., 2020; Yang et al., 2020). However, the administration of Metformin (4 mg/day in drinking water, until the animals were sacrificed), was also administered 2 weeks after DMM surgery and caused partial but significant reduction in cartilage degradation, suggesting that Metformin may be beneficial even when given at very early stages of damage (Li J. et al., 2020). This has been confirmed in adult Rhesus Macaques where Metformin was administered 1 month after surgery and significantly alleviated cartilage degradation and subchondral bone thickening with a reduction in pain-related behaviour and improvement in the duration of standing and walking (Li J. et al., 2020). Similarly, testing of glucosamine in young rats showed attenuation of cartilage degradation following transection of the anterior cruciate ligament using either GluN sulphate (Wen et al., 2010) or hydrochloride (Naito et al., 2010) even when given 5 weeks post-induction of OA at high dose (Naito et al., 2010).

The effects of geroprotectors become less effective when administered in older mice or in young mice when the disease is already well established. Intra-articular treatment with a new senolytic UBX0101 prevented OA disease in young mice. However, administered when OA was established only led to improvement in some aspects of the disease such as cartilage structure and pain, but no rescue of subchondral bone remodelling and osteophyte formation (Jeon et al., 2017). When the disease was triggered in 19-month-old mice administration of the same molecule in advance stages of the disease showed no improvement in cartilage structure despite evidence of senescent cell clearance from the articular cartilage (Jeon et al., 2017). This was associated with a lack of increased expression of prochondrogenic genes, suggesting that there may be an age-related decline in the proliferative capacities of articular chondrocytes and ability to produce matrix with age, impacting further on responses. Similarly a phase I clinical trial with UBX0101 in patients with moderate to severe OA did not show any improvement in pain score used as primary outcome (Inc, 2020).

Similarly little or no response was observed by our group and others when the effect of Acarbose, 17-α-estradiol and Rapamycin were assessed in aged UM-HET mice. UM-HET3 are produced by a cross between (BALB/cByJ × C57BL/6J) F1 mothers and (C3H/HeJ × DBA/2J) F1 fathers and have been used at the National Institute on Ageing Intervention Testing Programme (NIA-ITP) (Nadon et al., 2017).

All three compounds have been shown to extend lifespan in these mice, although with some sex differences (Harrison et al., 2009; Miller et al., 2011; Harrison et al., 2014; Miller et al., 2014; Strong et al., 2016; Harrison et al., 2019). Similarly to what reported in the study by (Ewart et al., 2020) our unpublished data showed a significant increase in cartilage degradation with age in these mice in both males and females (**Figures 1**, **2** and **Supplementary Material**). However, none of the treatments showed any significant improvement in cartilage degradation measured by OARSI scores in both males and females (**Figures 3A–C** and **Supplementary Material**) assessed at 12 and 22 months of age. Treatment started at 4 months of age with the exception of 17-α-E2, which started at 10 months of age when the oestrus cycle starts reducing to avoid a potential interference with sexual development (Nelson et al., 1982). Drugs were used at the same concentrations shown to elicit lifespan extension (1,000, 14, and 14.4 mg per kg of diet for acarbose, rapamycin and 17-α-E2, respectively). These data are in line with what reported by Ewart et al. (2020) when testing the effect of acarbose and 17-α-E2estradiol in the same mice using a different experimental design. In the latter, mice were collected at the time of death, whereas in our experiments the mice were culled at fixed time points, reducing some of the variability that may have arisen with the previous experimental design. It is still possible that our study was underpowered. However, even if this was the case, it indicates that any effect is very small. It is possible that these drugs may work in aged mice only in situation of challenge (e.g., following DMM surgery) and therefore they should be tested in mice following induction of OA disease in older age.

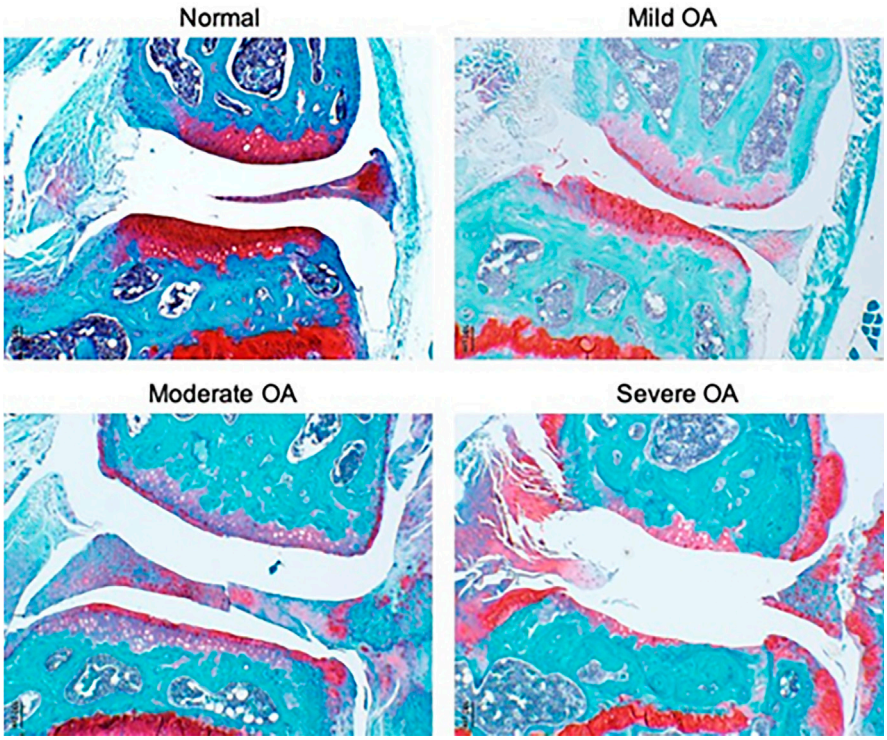

FIGURE 1 | Representative images of joint pathology in UM-HET3 mice. Normal includes joints with an OARSI score of 0–.5, mild 1–2, moderate 3–4 and severe 5–6.

No studies are available to test the effects of Glucosamine in aged animals. However, there is an abundance of studies in patients affected by OA with very contrasting results as highlighted by meta-analysis and systematic reviews (Wandel et al., 2010; Runhaar et al., 2017; Liu et al., 2018; Ogata et al., 2018; Zhu et al., 2018). The discrepancy seems to lie in whether the trials contained biases (e.g., trials led by industry were more likely to show positive results) (Vlad et al., 2007) or the sample size (trials with over 100 patients seem to show no effect of glucosamine on pain) (Wandel et al., 2010; Runhaar et al., 2017). In addition, the formulation of glucosamine produced by Rottapharm seemed to be the most effective (Towheed et al., 2005; Vlad et al., 2007; Runhaar et al., 2017). However, it is unclear whether this is due to the formulation or to the fact that trials with the Rottapharm formulation targeted patients at early stages of disease. Whilst recent meta-analysis concluded that studies have shown modest or no efficacy of glucosamine on pain or other parameters of OA questions remain whether the treatment should be tested for longer, with a higher dose (Mccarty et al., 2019) and whether patients should be stratified based on severity of the disease and/or age, a factor that is never considered in the analysis.

Overall, these studies highlight that reducing the severity of OA in older organisms may be challenging and not sufficient on its own. These studies challenge the notion that extension of lifespan can be considered an indirect measure of health span for all tissues. They highlight the need for a comprehensive assessment of the effects of each drugs in all tissues including the skeletal tissues with natural ageing and in models of disease.

Geroprotectors to Target OP

In vitro studies are primarily focused on the effects of rapamycin on osteoaclasts and osteoblasts. Based on studies using mouse, rabbit and human cells Rapamycin has been shown to reduce osteoclasts' formation, survival and activity (Glantschnig et al., 2003; Kneissel et al., 2004; Browne et al., 2017). Effects on osteoblasts' proliferation, survival and differentiation are inconclusive with differences in reports depending on the dose used and the species from which the cells were derived and whether they were primary or cell lines (Kneissel et al., 2004; Singha et al., 2008; Xian et al., 2012; Huang et al., 2015; Browne et al., 2017; Wu et al., 2019). This is particularly true for its effects on differentiation. For example the analogue of rapamycin Everolimus showed no effect on the osteoblast marker Alkaline phosphatase (ALP) when hMSC were induced to differentiate to the osteoblastic lineage for 7 days at 1 nM but showed a reduction in ALP expression at higher concentration of 10 and 100 nM (Browne et al., 2017). In contrast Runx2 and Osteocalcin, two other markers of osteoblasts differentiation were increased at 1 and 10 nM in the same human osteoblasts cultures but were decreased in murine cultures (Browne et al., 2017). There are many reasons for these discrepancies such as osteoblasts differentiation may proceed at different rates in

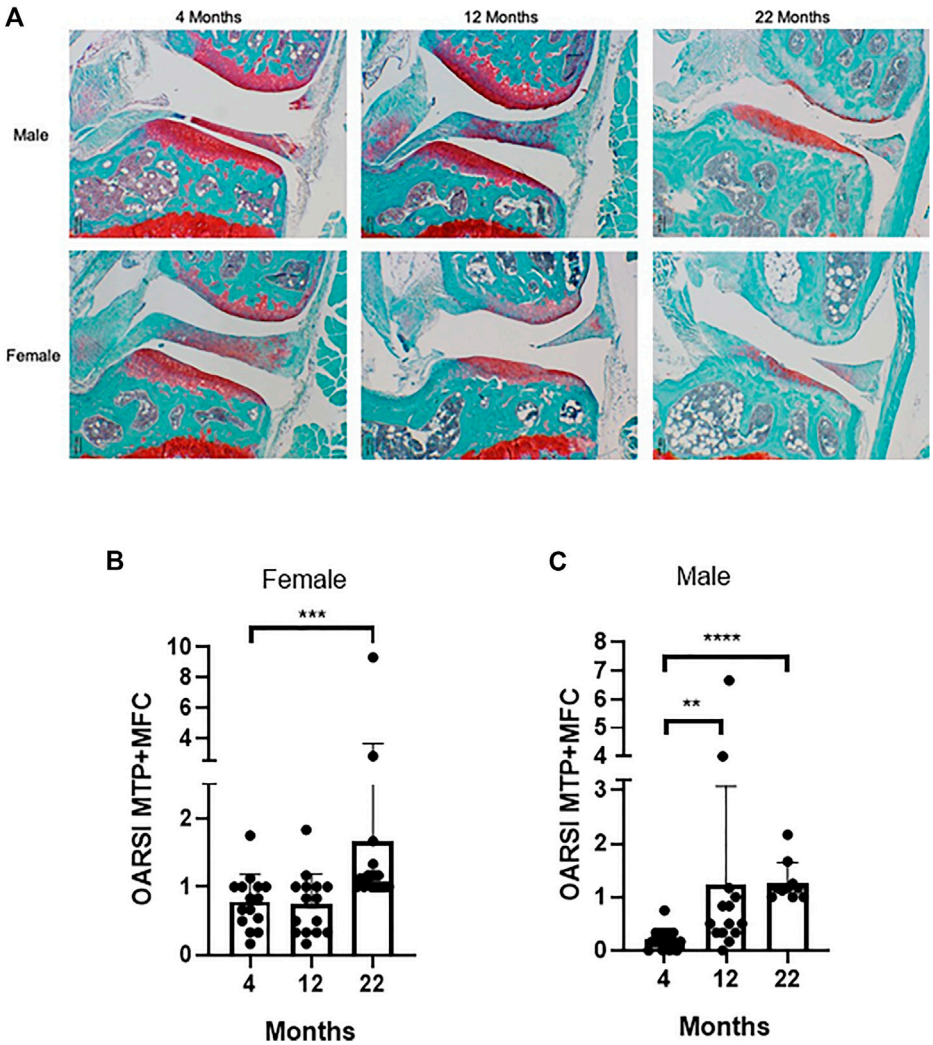

FIGURE 2 | UM-HET3 mice develop joint pathology with age **(A)** Representative examples of joint pathology in male and female UM-HET3 mice at different ages; **(B)** Cartilage changes in female mice at 4 months (n = 16), 12 months (n = 15) and 22 months (n = 18); **(C)** Cartilage changes in male mice at 4 (n = 17), 12 (n = 14) and (n = 10) 22 months. Values are the mean ± SD of OARSI score for the medial tibia plateau (MTP) plus the medial femoral condyle (MFC). Data were analysed by Kruskal-Wallis test and Dunn's multiple comparisons test, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$.

human, mouse cultures and cell lines and markers of osteoblasts differentiation are dynamic, i.e., they can be upregulated and downregulated at different rates over the period of observation. Assessments of markers over multiple time points may be required to shed some light.

In vitro studies with other geroprotectors are very scant. Fisetin has been shown to inhibit osteoclasts' formation and differentiation but effects on osteoblasts were not reported (Léotoing et al., 2013). Navitoclax reduced senescent cell burden but it also negatively impacted on the number of bone progenitors and osteoblasts in culture inducing apoptosis (Sharma et al., 2020). Spermidine reduced osteoclasts differentiation but did not affect their survival and had no effect on survival and differentiation of osteoblasts (Yamamoto et al., 2012). N-Acetyl glucosamine increased osteoblasts differentiation and mineralization and attenuated the negative effects of hydrogen peroxide on survival and proliferation of osteoblast (Jiang et al., 2018).

Most *in vivo* studies (summarised in **Table 2**) have used Rapamycin or one of its derivatives, Everolimus to test their effects on bone loss. Rapamycin and Everolimus have been shown to delay bone loss in mice in situations of challenge, i.e., in models of ovariectomy, iron load, cancer bone disease or ageing (Kneissel et al., 2004; Luo et al., 2016; Browne et al., 2017; Wu et al., 2019). These effects are primarily the result of inhibition of osteoclasts formation and activity with the exception of the study utilising the iron load model where no difference has been observed in the number of osteoclasts but an increase in ALP+ osteoblasts has been reported. A study in 24 months old rats receiving Rapamycin at 1 mg/kg/day for 12 weeks showed positive effects on osteoblasts activity with an increase in serum osteocalcin and mineral apposition rates (Luo et al., 2016).

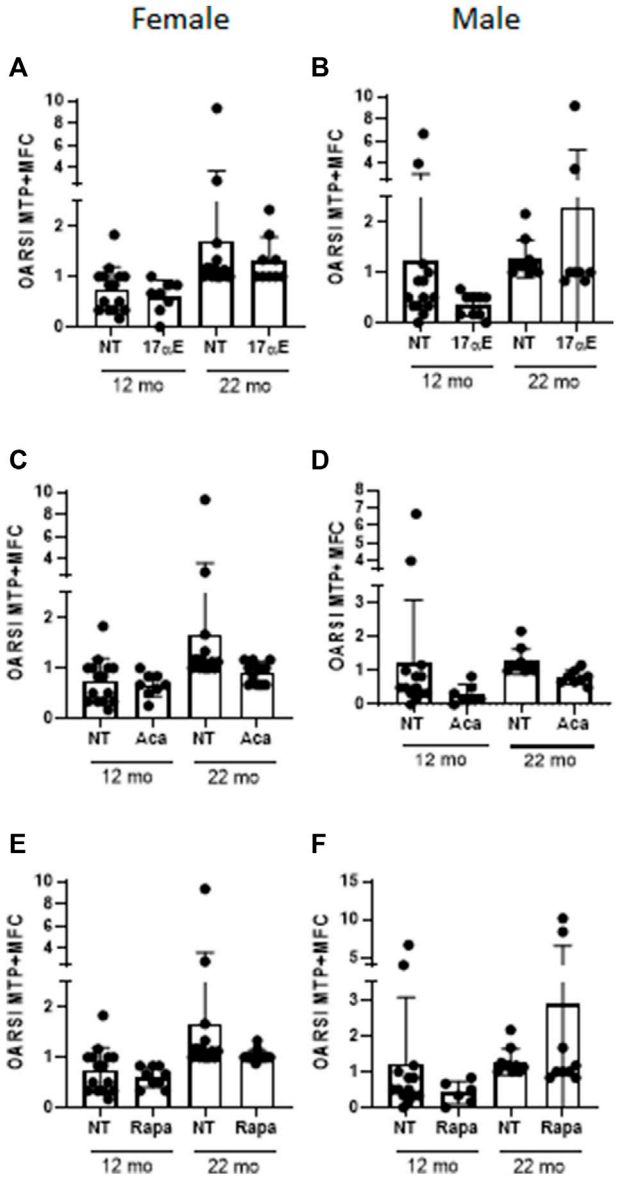

FIGURE 3 | No effect on joint pathology in UMHET3 mice following treatment with 17-α-Estradiol, Acarbose and Rapamycin **(A)** Cartilage changes in female mice at 12 months (NT n = 15; 17 αE n = 8) and 22 months (NT n = 18; 17 αE n = 9) following treatment with 17-α-Estradiol (17 αE); **(B)** Cartilage changes in male mice at 12 months (NT n = 14; 17 αE n = 9) and 22 months (NT n = 10, 17 αE n = 8) following treatment with 17 αE; **(C)** Cartilage changes in female mice at 12 months (NT n = 15; ACA n = 8) and 22 months (NT n = 18; ACA n = 12) following treatment with Acarbose (ACA); **(D)** Cartilage changes in male mice at 12 months NT n = 14; ACA n = 7) and 22 months (NT n = 14; ACA n = 8) following treatment with ACA; **(E)** Cartilage changes in female mice at 12 months (NT n = 15; Rapa n = 8) and 22 months (NT n = 18; Rapa n = 12) following treatment with Rapamycin (Rapa); **(F)** Cartilage changes in male mice at 12 months (NT n = 14; Rapa n = 6) and 22 months (NT n = 10; Rapa n = 9) following treatment with Rapamycin (Rapa). Values are the mean ± SD of OARSI score for the medial tibia plateau (MTP) plus the medial femoral condyle (MFC). Data were analysed by Kruskal-Wallis test and Dunn's multiple comparisons test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. NT, not treated.

The discrepancy in recording positive effects on osteoblasts' activity may be due to the short length of administration of Rapamycin in some of the studies (4–8 weeks). Whilst osteoclasts have shorter lifespan *in vivo* (2 weeks) osteoblasts turnover takes approximately 3 months (Manolagas, 2000) and therefore it is possible that only those studies assessing osteoblastogenesis for longer periods of time were able to detect an effect.

Of interest is the fact that most studies report outcomes only in trabecular bone and do not assess cortical bone, despite both being important to confer bone strength. Kneissel et al. (2004) (Kneissel et al., 2004) reported a partial protection in trabecular bone but not in cortical bone following treatment of 9 months old rats with Everolimus for 4–8 weeks at the dose of 3 mg/kg/day. These data suggest that the effects may be limited to the trabecular bone, the more metabolic active part of the bone.

TABLE 2 | Summary of *in vivo* testing of geroprotectors to attenuate bone loss in experimental models.

Geroprotector	Model	Age at the start of the experiment	Key findings (compared to controls)	Reference
Rapamycin 1 mg/kg weight/day, i.p. 12 weeks	SD rats	24 months	↑BMD ↑Trabecular BV/TV and number, thickness ↑MAR ↓N Oc and serum Tracp 5b ↑serum OCN	Luo et al. (2016)
Everolimus 0.5 mg/kg/day 1.5 mg/kg/day 3.0 mg/kg/day Gavage 4–8 weeks treatment	Wistar Rats—OVX	9 months	Attenuated cancellous bone loss ↓trabecular number No effect on cortical bone ↓N Oc No difference in cancellous bone formation rates	Kneissel et al. (2004)
Everolimus i.p 2 days post tumor injection or OVX 1 mg/kg/day for 4 weeks	Mice NMRI nude + MDA-MB-231 Mice C57BL/6 + OVX	6 weeks 9 weeks	OVX model ↑BMD ↑Trabecular BV/TV ↓N Oc Nude tumour model ↓N tumor lesions ↑BMD ↑Trabecular BV/TV ↓N Oc	Browne et al. (2017)
Rapamycin 3 mg/kg/day, i.p. for 2 months	Mice Hepcidin knockout C57Bl/6 + OVX	8 weeks	↑BMD ↑Trabecular BV/TV No difference in cortical bone ↑ N ALP + Ob No diff in N Oc	Wu et al. (2019)
Dasatinib (5 mg/kg)and Quercetin (50 mg/kg)monthly for 4 months by gavage	Mice C57Bl/6	20 months	Vertebrae ↑Trabecular BV/TV, number and thickness ↓N Oc No difference in Ob numbers, BFR, MAR Femur ↑cortical thickness ↑strength ↓endocortical N Oc ↑endocortical N Ob	Farr et al. (2017)
Fisetin 5 mg/kg/day or 50 mg/kg/day for 1 week by gavage prior to OVX followed by 5 mg/kg/day 25 mg/kg/day for 4 weeks by gavage 5 mg/kg/day 25 mg/kg/day 50 mg/kg/day for 3 weeks by gavage	Mice C57Bl/6 + OVX C57Bl/6 + LPS	8 weeks	↑BMD ↑serum OCN ↑BMD	Léotoing et al. (2013)
Navitoclax 50 mg/kg/day for 2 weeks by gavage	Mice C57Bl/6	24 months	↑Trabecular BV/TV	Sharma et al. (2020)
N-Acetyl Glucosamine 100 mg/kg/day 250 mg/kg/day for 12 weeks	Sprague-Dawley Rats	12 weeks	↑BV/TV ↑Trabecular bone area	Jiang et al. (2018)
Spermidine 0.3–3 mM/day drinking water	C57BL6 mice + OVX	8 weeks	↑BV/TV ↓N Oc	Yamamoto et al. (2012)

SD, Sprague-Dawley; i.p, intra-peritoneal; BMD, Bone mineral density; MAR, Mineral apposition rates; BFR, Bone Formation Rates; Oc, Osteoclasts; OCN, osteocalcin; Ob, Osteoblasts; ALP, Alkaline phosphatase; LPS, lypopolysaccharide.

Long-term studies with Rapamycin and its derivatives are required to assess its effect on osteoblastogenesis and whether both cortical and trabecular bone benefit from the intervention when exposed for prolonged periods. Careful consideration needs to be given to the dose and time of administration and the type of mTOR inhibitor as prolonged administration of Rapamycin may have side effects. Intermittent dosing has been proposed to avoid adverse events (Arriola Apelo et al., 2016). However, regimen of Rapamycin 2 mg/kg once every 5 days has been shown to inhibit mTORC1 complex but loss of glucose tolerance persisted in the same way than what was observed when given daily (Houde et al., 2010). In humans no major side effects have been seen with weekly dosing of Everolimus and this was enough to improve immune responses (Mannick et al., 2014). However, Everolimus administered at a weekly dose did not produce any difference on bone parameters (Kneissel et al., 2004), suggesting that daily dose may be required to detect effects. However, Everolimus may still be preferable to Rapamycin. Indeed when given daily it had reduced impact on glucose tolerance compared to daily Rapamycin despite being equally efficacious in inhibiting proteins of the TORC1 complex (Arriola Apelo et al., 2016).

The effect with senolytics has shown mixed results. Pharmacologic clearance of senescence cells in aged mice (20 months) treated with Dasatinib and Quercetin (DQ) for 4 months by single monthly administration showed improvement of both the trabecular and cortical bone in femur and vertebrae (Farr et al., 2017). DQ suppressed resorption by reducing osteoclast numbers and improved osteoblast numbers on the cortical bone surface but not on the trabecular bone surface (Farr et al., 2017).

When Fisetin was given to 8 weeks old mice, 1 week before OVX, an increase in trabecular bone volume fraction, thickness and number were observed 4 weeks after OVX (Léotoing et al., 2013). A similar effect was also reported when using a model of inflammation-induced bone loss by Lypopolysaccharide injection (Léotoing et al., 2013). However, it is unlikely that these effects are due to Fisetin's senolytic activity. Very low levels of senescent cells have been reported in mice before 8 months of age (Farr et al., 2017). Studies in aged mice are required to determine whether Fisetin has senolytic effects and prevent bone loss observed with age.

Detrimental effects to trabecular bone were reported in aged male and female C57BL/6 mice (24 months old), when they were treated with the senolytic drug Navitoclax once daily for 2 weeks with signs of apoptosis on bone cells (Sharma et al., 2020). The same dose was used in the study by Chang et al. (Chang et al., 2016) to eliminate senescent cells. Indeed it showed improved proliferation and regeneration ability of hematopoietic stem cells (HSC), compatible with a reversal of HSC to a more youthful phenotype (Chang et al., 2016). However, Navitoclax was administered only for 7 days in the study by Chang et al. (Chang et al., 2016) as opposed to 14 days in the study by (Sharma et al., 2020). This may account for the toxicity observed. The toxicity of Navitoclax is well known and therefore improved regimen should be tested, particularly with the new galacto-conjugated Navitoclax, where the drug can be preferentially activated by SA-β-gal activity primarily in senescent cells (González-Gualda et al., 2020).

Studies on spermidine and Glucosamine are still in their infancy and limited to young mice. Spermidine was administered at 0.3–3 mM/day orally to 8 weeks old ovariectomised C57BL6 mice and analysed 28 days after OVX. Analysis of vertebral bone showed an increase in BV/TV associated with a decreased in the number of osteoclasts and no effects on osteoblasts (Yamamoto et al., 2012). N-Acethyl Glucosamine was administered at 250 mg/kg and 100 mg/kg/day to 12 weeks old ovarectomised Sprague-Dawley rats for 12 weeks. An increase in bone mineral density and trabecular bone area was observed. This was associated with signs of increased osteoblasts differentiation and mineralizations (Yamamoto et al., 2012). Effects on osteoclasts were no reported. Although these studies are promising, more in depth studies in aged mice are required to assess whether these agents hold promise for attenuating bone loss with age.

CONCLUSION

Geroprotectors potentially have additional benefits to treat OA and OP and their co-morbidities. However, few studies focus on skeletal health despite their burden of disease. Only one study with the combination of senolytics DQ shows signs of improvement in a model of bone loss and no improvement has been demonstrated so far in aged models of OA. These studies highlight that extension of lifespan cannot be considered a surrogate marker for extension of health span in all tissues and thorough studies in aged models of OP and OA are required to assess the real benefit of geroprotectors to improve skeletal health.

AUTHOR CONTRIBUTIONS

AR, MB, and JG performed the experiments, analysed the data, reviewed and approved the manuscript. ED designed the study, reviewed and approved the manuscript. MN wrote and approved the manuscript. IB designed the experiments, analysed the data, wrote and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.682045/full#supplementary-material

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