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Oral Senescence: From Molecular Biology to Clinical Research

Eric Kenneth Parkinson[*†] *and Stephen Stewart Prime*[*†]

Centre for Immunobiology and Regenerative Medicine, Barts and the London School of Medicine and Dentistry, Blizard Institute, London, United Kingdom

Cellular senescence is an irreversible cell cycle arrest occurring following multiple rounds of cell division (replicative senescence) or in response to cellular stresses such as ionizing radiation, signaling imbalances and oxidative damage (stress-induced premature senescence). Even very small numbers of senescent cells can be deleterious and there is evidence that senescent cells are instrumental in a number of oral pathologies including cancer, oral sub mucous fibrosis and the side effects of cancer therapy. In addition, senescent cells are present and possibly important in periodontal disease and other chronic inflammatory conditions of the oral cavity. However, senescence is a double-edged sword because although it operates as a suppressor of malignancy in pre-malignant epithelia, senescent cells in the neoplastic environment promote tumor growth and progression. Many of the effects of senescent cells are dependent on the secretion of an array of diverse therapeutically targetable proteins known as the senescence-associated secretory phenotype. However, as senescence may have beneficial roles in wound repair, preventing fibrosis and stem cell activation the clinical exploitation of senescent cells is not straightforward. Here, we discuss biological mechanisms of senescence and we review the current approaches to target senescent cells therapeutically, including senostatics and senolytics which are entering clinical trials.

Keywords: senescence, oral, cancer, fibrosis, telomerase, inflammation, SASP, metabolism

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***Correspondence:**
Eric Kenneth Parkinson
e.k.parkinson@qmul.ac.uk
Stephen Stewart Prime
stephensprime@gmail.com

†These authors have contributed equally to this work

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INTRODUCTION

There have been several reviews on the subject of senescent cells in human disease (1, 2). Classical cellular senescence is defined as an irreversible cell cycle arrest that is distinct from quiescence, terminal differentiation and apoptosis. This form of senescence occurs following multiple rounds of cell division [replicative senescence (3)] or following a wide range of cellular stresses such as ionizing radiation, signaling imbalances or oxidative damage [stress-induced premature senesce or SIPS; (4)]. More recently, the definition of senescence has been broadened to include developmental senescence, oncogene-induced senescence (OIS) and cancer therapy-induced senescence where an irreversible cell cycle arrest is either unstable (4) or may not occur at all (5). OIS does not occur in young healthy cells (6–8) with low levels of $p16^{INK4A}$ and not all oncogenes induce senescence (9), so cells need to be damaged before OIS operates and even then, only when expressed at a sufficient level (10). Senescence is a double-edged sword in that it operates as a suppressor in the early stages of malignancy (11) but also, has the capacity to promote tumor development (12) and tumor progression (11, 13) once the tumor has developed *via* molecules of the senescence-associated secretory phenotype (SASP)

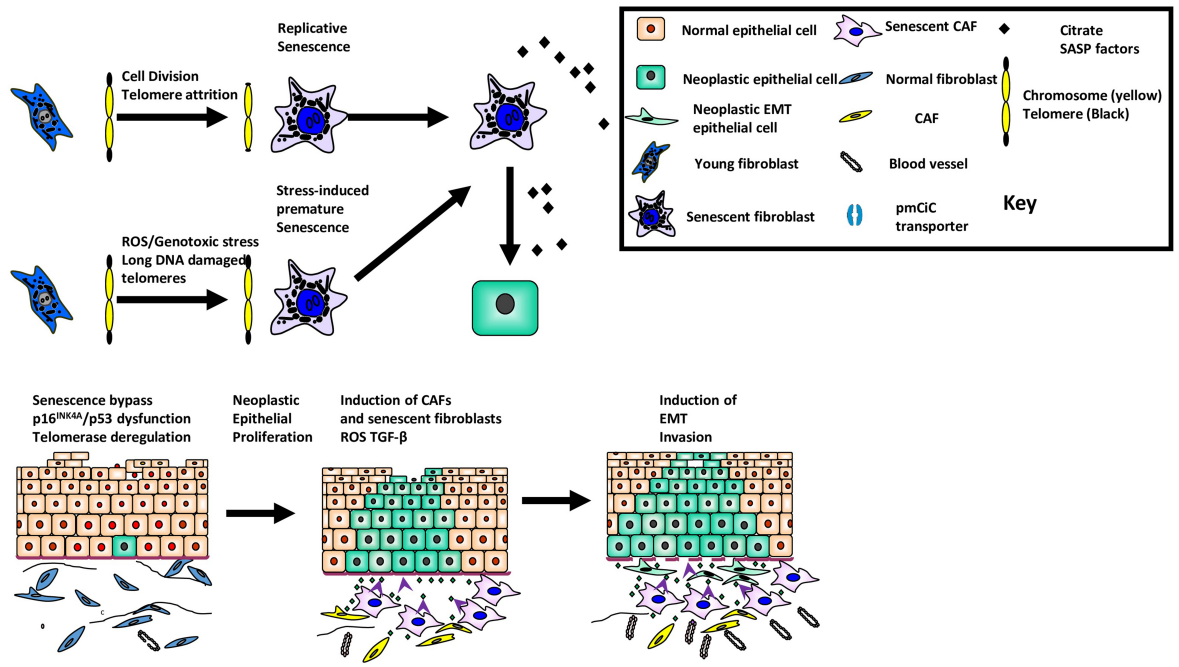

FIGURE 1 | The role of cellular senescence in oral cancer development and progression. The figure summarizes the role of senescence as a suppressor of malignancy in keratinocytes and its paradoxical role as a promoter of tumor progression in the developing tumor environment.

such as interleukins, matrix metalloproteinases (MMPs), vascular endothelial growth factor (13) and certain metabolites (14) (summarized in **Figure 1**). Even small numbers of senescent cells can be deleterious (15) and there is evidence that senescent cells are instrumental in age-related pathologies (16), in response to cancer therapy (17) and in chronic inflammatory conditions (18).

THE SASP AND THE EXTRACELLULAR SENESCENCE METABOLOME (ESM)

During the establishment of senescence, an array of proteins (13) and metabolites (19) accumulate in the extracellular milieu and collectively are referred to as the senescence-associated secretory phenotype [SASP; (13)] or the extracellular senescence metabolome [ESM; (19)], respectively.

More specifically, the SASP consists of a large number of cytokines, chemokines and immunomodulatory molecules, growth factors, shed surface molecules and survival factors, together with promoters of angiogenesis, fibrosis and tissue re-modeling (13). The mechanisms of senescence and the constituents of the SASP vary between cell types which makes the SASP cell type- and mechanism-specific. More recent data indicate that the SASP is highly heterogeneous and varies temporally even within populations of the same cell type (20). Some of the cytokines of the SASP increase to detectable levels in the plasma of older humans and have been proposed to be biomarkers of age-related conditions such as frailty (21). This sterile inflammation is known as inflammaging (22) and is thought to be due to the accumulation of senescent cells which display inappropriate levels of cytoplasmic chromatin; it is mediated by the cyclic GMP–AMP synthase (cGAS)–stimulator of the interferon gene (STING) (cGAS-STING) pathway that induces inflammatory cytokines (23).

Recently, the oral keratinocyte SASP has been examined in more detail as part of a comprehensive survey of SIPS (24). Many of the transcripts and proteins of the SASP are consistent between fibroblasts and mortal pre-neoplastic keratinocytes. However, senescence-specific exosomes induce the interferon pathway in neighboring monocytes *via* the cGAS-STING pathway (25) and numerous SASP factors (particular prostaglandins) and metabolites are dependent on the keratinocyte senescence program (26, 27). Although there is no evidence for senescence in human pre-malignant lesions *in vivo*, it has been described in other human pre-malignant conditions (28) and $p16^{INK4A}$ is upregulated in high-grade pre-malignant lesions (29). Current thinking indicates that the SASP may be released in two waves which are dependent on different pathways and which modulate the immune system in different ways with the common goal being the promotion of neoplasia (30).

The ESM metabolites (citrate, C-mannosyl tryptophan, urate, and eicosapentaenoate), are amongst the 22 metabolites most significantly elevated with chronological age and aging traits [loss of lung function and bone mineral density (31)]; the function of most of these metabolites in age-related diseases, however, is unclear. A role for C-mannosyl tryptophan in apoptosis has been predicted based on bioinformatics and, more recently, a role for citrate has been suggested in the pathobiology of type 2 diabetes (32), memory (33), heart rate (34), blood pressure (34), and cancer (35, 36).

ORAL KERATINOCYTE SNESCENCE

Whilst the senescence regulators telomerase, p53 and pRB/$p16^{INK4A}$ are common to both keratinocytes and fibroblasts, their exact roles are not quite the same. Mechanistically, following telomere dysfunction, disabling p53 in both fibroblasts (37) and keratinocytes (38) extends the proliferative lifespan of fibroblasts but not keratinocytes, whereas knockdown of $p16^{INK4A}$ has no effect on its own. However, the combined knockdown of p53 and $p16^{INK4A}$ extends the proliferative lifespan of fibroblasts further and induces a phenomenon resembling crisis in both cell types (37, 38). In serum-free culture systems, $p16^{INK4A}$, but not $p14^{ARF}$, accumulates following proliferative exhaustion in the absence of telomere attrition (39). $p16^{INK4A}$ accumulation in keratinocytes is also associated with the expression of laminin gamma 5 delta 2, a hyper-motile phenotype seen in both carcinoma-*in-situ*, and in experimental wounding *in vitro* (29). This form of senescence is bypassed by plating keratinocytes on collagen type 1, disabling $p16^{INK4A}$ or p53 and is delayed by inhibition of the transforming growth factor beta (TGF-β) pathway (40). Epidermal keratinocytes do undergo a phenotype resembling senescence in aging humans (41) and this appears to be mediated by a paracrine mechanism associated with senescent melanocytes that induce telomere-associated DNA damage foci (TAFs) in neighboring suprabasal keratinocytes. However, there is no evidence of $p16^{INK4A}$ accumulation in aged keratinocytes *in situ* and it is not clear whether TAFs persist in the basal layer of squamous epithelia because telomerase should gradually resolve the TAFs (4, 42).

SENESCENCE AS A SUPPRESSOR OF ORAL SQUAMOUS CELL CARCINOMA DEVELOPMENT

RAS and *RAF* oncogenes induce senescence when over-expressed (10). Senescence is observed in pre-malignant lesions of several cancer types (43, 44) and the elimination of senescent cells in mice precipitates tumor progression (11). Whilst *RAS* and *RAF* mutations are rare in oral cancer (45, 46), $p16^{INK4A}$ accumulates in oral carcinoma *in situ* (29) which is suggestive of senescence. Both SIPS and classical senescence are mediated by the pRB/$p16^{INK4A}$ and $p14^{ARF}$/p53 pathways (1) and upon their dysfunction in oral cancer, telomere crisis and genetic instability ensue (38, 47) followed by telomerase deregulation and cellular immortality (38, 47–49). The dysfunction of $p16^{INK4A}$, p53, and telomerase in both human papillomavirus (HPV) positive and negative head and neck squamous cell carcinomas (HNSCC) is nearly ubiquitous (50). Further, deregulation of telomerase by activating mutations of the promoter of the catalytic component of the enzyme TERT is a common event in HPV negative SCCs (51), but this is not enough to account for the increase in telomerase in 90% of HNSCC (52) and further genetic alterations are required (53).

FIBROBLAST SENESCENCE IN THE ORAL CANCER ENVIRONMENT

The tumor microenvironment consists of neoplastic epithelial cells and non-neoplastic stromal cells. The predominant stromal cell-type are fibroblasts and when these cells become "activated," the term cancer associated fibroblast (CAF) is used. Fibroblast activation occurs *de novo* by reactive oxygen species (ROS) derived from the epithelial cancer cells (**Figure 1**) and by constituents of the SASP [IL-1β, IL-6, osteopontin (54)]. CAFs are heterogeneous and express a variety of different proteins, the significance of which is unclear. Current thinking indicates that fibroblast activation is context-dependent, plastic and likely to fall along a continuum rather than into discrete subsets (55). These observations may account for recent observations where CAFs with pro-tumorigenic and anti-tumorigenic phenotypes have been described. Stromal features are predictive of mortality (56) and ECM deposition and organization correlate with poor prognosis (57) in oral squamous cell carcinoma (OSCC).

There is a plethora of data showing that CAFs possess the ability to influence the hallmarks of cancer (54). In OSCC, for example, CAFs promote invasion and epithelial dis-cohesion, induce epithelial-mesenchymal transition and cause resistance to apoptosis (30, 58, 59). CAFs regulate fibroblast activation in an autocrine manner, remodel the ECM in a paracrine fashion which leads to a pro-fibrogenic phenotype and also, maintain persistent chronic inflammation. Further, CAFs show up-regulation of glycolysis and down-regulation of oxidative phosphorylation [reverse Warburg effect (60)] which creates a favorable hypoxic environment for epithelial tumor development and progression.

CAFs undergo senescence through secondary senescence induction from other senescent cells although at present, it is unclear whether this leads to functional heterogeneity. Whilst senescent fibroblasts are likely to contribute to the tumor-promoting environment, they probably do so in concert with non-senescent CAFs in a complementary manner. It is unknown whether stromal cells help keratinocytes to escape cellular senescence and this warrants further investigation.

The role of senescence in fibrosis is complex. In the short term, senescent cells can promote wound repair (61) and can also ameliorate fibrosis of the skin *in vivo* (62). Further, senescent cells are targeted by both the innate and the adaptive immune systems (11) and disabling either senescence or the immune system promotes fibrosis (63). Paradoxically, persistent inflammation, a property of many oral diseases, can induce senescence (64) and if senescent cells evade the immune system and persist, they can increase fibrosis. Damaged senescent cells avoid immune

recognition through matrix metalloproteinase-dependent shedding of NKG2D ligands reinforced *via* paracrine suppression of NKG2D receptor-mediated immunosurveillance (65).

SENESCENCE IN CANCER THERAPY AND THE POOR HEALTH OF CANCER SURVIVORS

Senescent cells accumulate in mouse tissues following DNA damage (66) and chemotherapy (17) and their removal by genetic or pharmacological deletion ameliorates the side effects of such therapies in mice, including hypo salivation (67), fibrosis (68), and ulceration (69). Further, the tissues of cancer survivors have short telomeres and increased numbers of senescent cells (70–73) and oral mucositis, a major side effect of cancer therapy, has been linked to keratinocyte senescence (74).

ORAL SUB MUCOUS FIBROSIS (OSMF)

OSMF is a debilitating and pre-cancerous condition caused by the chewing of "paan" which contains areca nut and sometimes tobacco (10, 16). In early OSMF, there is epithelial atrophy, juxta-epithelial inflammation and the sub epithelial connective tissues become avascular and thickened which leads to collagen accumulation. In advanced disease, the fibrosis extends into the deeper tissues and is associated with an increased inflammatory infiltrate (17). In OSMF, biologically active alkaloids and flavonoids in areca nut stimulate fibroblasts to increase collagen synthesis (18, 19); concurrently, there is reduced collagen degradation due to increased stability of the collagen structure (20, 21) and reduced collagenase activity (22). These events are mediated by changes in the regulation of several SASP factors such as TGF-β1, plasminogen activator-1 and matrix metalloproteinases (MMPs) and their inhibitors. The result is progressive hyalinization and fibrosis of the oral submucosa.

The role of senescence in OSMF has been comprehensively reviewed recently (75). It has been attributed to areca nut alkaloids (76, 77) and occurs in keratinocytes, fibroblasts and endothelial cells; in fibroblasts, the mechanism is associated with ROS, irreparable DNA damage and an increase in mitochondrial mass and membrane potential (76). Depleting the fibroblast population of senescent cells in OSMF drastically reduces the levels of MMP-1 and MMP-2 in conditioned medium suggesting that, as with CAFs, there are both non-fibrogenic and pro-fibrogenic fibroblasts (76). Senescence in keratinocytes, however, leads to epithelial atrophy and down-regulation of basal stemness, whilst senescence in endothelial cells accounts for a decrease in vascularity which leads to the development of a hypoxic state. The consequence of cellular senescence is the generation of a SASP, ROS generation and the induction of DNA double strand breaks in keratinocytes (78). Only when the keratinocytes escape from senescence does malignant transformation ensue but the myofibroblasts persist because they evade immune clearance.

THE ROLE OF SENESCENCE IN OTHER ORAL DISEASES

The oral cavity is the site of trauma, is susceptible to toxin and drug exposure, responds to the impact of systemic disease and aging and is exposed to microbial infection. Whilst senescence has been implicated in the pathobiology of odontogenesis (79–81), changes in the supporting structures of the teeth (82, 83), the pathology of salivary glands (84) and disruption of homeostasis in the oral mucosa, it is thought to play a fundamental role in the most prevalent human disorder, namely periodontal disease (82). It has been proposed that Gram-negative bacterial infection leads to a DNA damage response in both gingival keratinocytes and fibroblasts, chronic inflammation results in ROS-mediated oxidative DNA damage and keratinocyte senescence leads to breakdown of the cervical epithelial barrier (62). Further, bacterial toxins induce local immunosuppression resulting in a failure to remove the senescent cells and the perpetuation of the disorder.

TARGETING SENESCENT CELLS IN DISEASE

Inhibiting SASP Production/Function (Senostatics)

From an early stage, proteins of the SASP have been considered as therapeutic targets in the treatment of cancer (**Figure 2**). Recent data, however, has shown that the SASP is cell-type specific, thus making this approach less practical. A recent review has highlighted some potential drug targets, particularly those associated with inhibition of the inflammasome and plasminogen activator inhibitor-1 (85). Such targets include p38 mitogen-activated kinase (MAPK14; (86), p38 MAPKAPK2/3 (87), Janus-activated kinase 1 (JAK1; (88) and steroids (89); these drugs have been shown to ameliorate the effects of frailty (88) and type 2 diabetes in mice (90). The MAPK14 inhibitors and steroids have been reported to inhibit the secretion of a diverse array of SASP proteins (86, 89), but JAK1 inhibitors have only been reported to target a subset of the inflammasome (88). Regulators of alternative splicing such as PTPB1 have been shown to inhibit the SASP and may also be attractive targets for therapy (91). In addition, rapamycin, a promoter of autophagy has been shown to ameliorate the effects of oral mucositis linked to senescence (74) in mice, but not as yet in humans. A major problem with senostatics, however, is that they are likely to require continuous administration, thereby increasing the cost per patient (92).

Senolytics

The above approaches are likely to be specific for certain senescence-derived molecules and whilst this will reduce the likelihood of side effects, an alternative approach is to use drugs which selectively target senescent cells. Senolytics (**Figure 2**) include BCL-XL inhibitors, finestin, ouabain, quecertin and dasatinib (92). In addition, FOXO4 can bind with the p53 protein

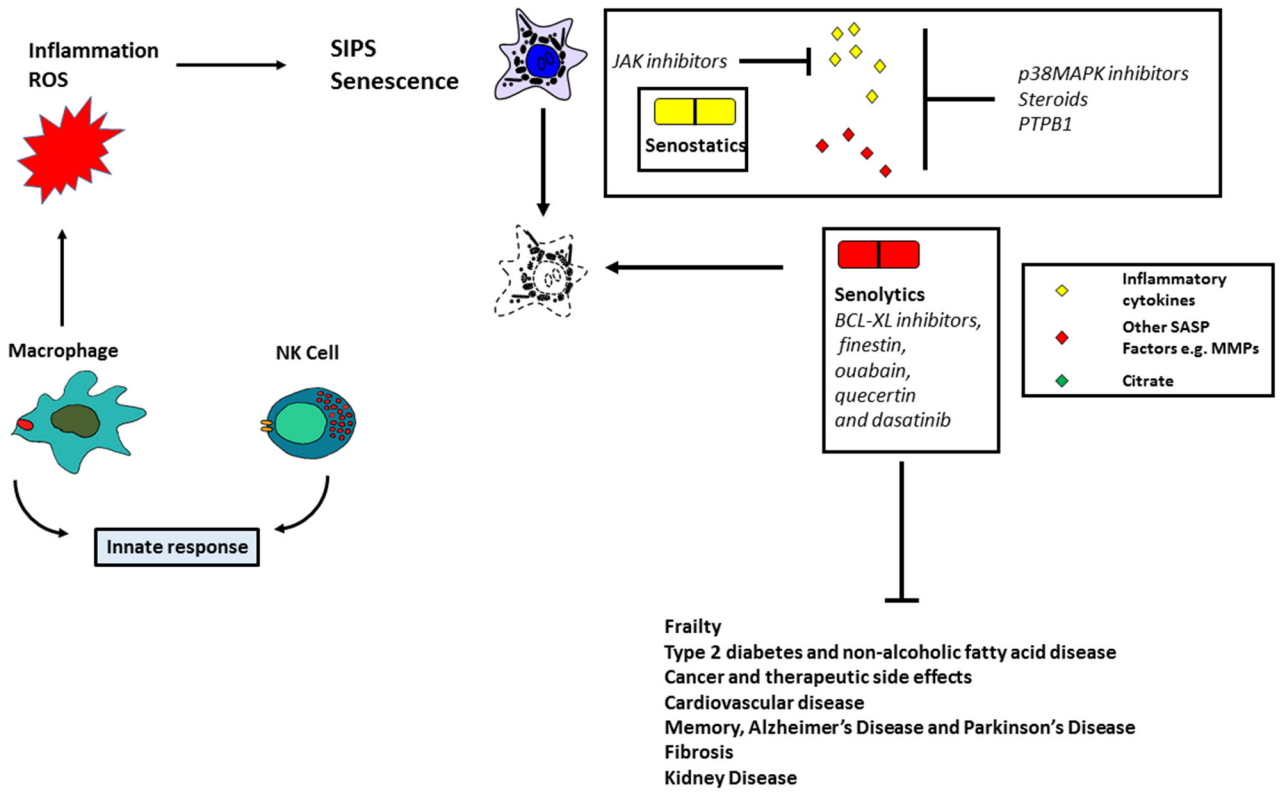

FIGURE 2 | Targeting senescent cells therapeutically. The figure summarizes the instrumental role of senescent cells in human pathologies and the potential role of antioxidant, senostatic, and senolytic therapies.

to induce cellular senescence and a peptide competing with FOXO4 can act as a senolytic by excluding p53 from the nucleus (93). These drugs ameliorate the effects of many age-related diseases in mice and clinical trials have begun to test their effects in human disease. Whilst senolytics can only be administered for a short time due to known side effects (e.g., thrombocytopenia), there is some evidence that they can ameliorate idiopathic pulmonary fibrosis (94) and can transiently clear senescent cells in the adipose and skin tissue of diabetic kidney disease patients (95). Another approach is to use chimeric antigen receptor (CAR) T cells to target senescent cells (96). Senescent cells become hyper-inflammatory, in response to pathogens and repress anti-viral gene expression in non-senescent cells by a paracrine mechanism (97); senolytics improve the survival of old mice infected with pathogens such as SARS-CoV-2. Apart from the known side effects of senolytics (98), it is cautionary to note that they do not always work (92) suggesting some level of cell-type/mechanism specificity. New drugs, therefore, will need to be identified. Further, SIPS is mediated in part by $p16^{INK4A}$ (see above) which is silenced in subsets of mammary cells in middle-aged females and is thought to be a precursor of breast cancer (85). Therefore, although removal of $p16^{INK4A}$-positive cells in mice might improve age-related pathologies without a concomitant cancer risk, this might not be the case in humans.

PREVENTING THE ACCUMULATION OF SENESCENT CELLS

The two approaches described above are currently being heavily pursued but suffer from the fact that both require long term administration if employed alone and approaches that reduce the production of senescent cells will need to be considered in parallel. Such approaches might include stimulating the immune system, antioxidants such as mitochondrial catalase inhibitors (99–101), activators of DNA repair (102) and inducers of telomerase activity (103, 104) all of which would delay senescence in different ways (**Figure 2**).

TARGETING SENESCENT CELLS IN ORAL DISEASES

As yet, no clinical trials have been published that target senescent cells in oral disease. However, there are many mouse models which show that senolytics might be effective in ameliorating OSMF; steroids block many components of the SASP (89) and are one of the treatments known to be beneficial in OSMF (105). Inhibitors of senescence have not been used in the treatment of OSMF, but it has been proposed that they may be of value for the alleviation of both radiation-induced fibrosis (106) and ulcers (83), and for the treatment of pre-cancerous lesions (107).

DETECTING SENESCENT CELLS

Several clinical trials have now assessed the potential of senolytics in human disease (108) but one of the problems is providing evidence that they are actually working. Even in severely ill patients, senescent cells are infrequent and SASP factors are either undetectable (94) or require the screening of several factors to verify senescence (95, 109); even when a few SASP factors are detectable, they are not consistent from patient to patient (95).

Several metabolites do accumulate in the extracellular milieu of senescent fibroblasts *in vitro* (19) and, indeed, a metabolite (15-deoxy-delta-12,14-prostaglandin J2) indicative of senolytic activity has been identified (110). However, no metabolite has been tested for its utility in senolytic- or senescence-related therapies in humans.

DISCUSSION

Whilst it is clear that senescent cells are instrumental in a wide variety of age-related pathologies in mouse models, translating these findings to humans remains a significant challenge. Senescent cells occur at very low frequencies in diseased tissues and most of the SASP factors overlap considerably with other processes, most notably oxidative damage and inflammation. Deleting senescent cells in humans may also have an associated cancer risk. The levels of SASP factors in the plasma/sera in published studies to date are very low and inconsistent, thereby making proof-of principle of many approaches very difficult. Senolytic therapies hold some promise, but safer and cell type-specific versions are likely to be required. It is clear that much further research will be required to identify and eliminate senescent cells in human disease including those of the oral cavity.

AUTHOR CONTRIBUTIONS

EP produced the first draft of the manuscript and figures. EP and SP performed the literature searches and modified and edited the manuscript. Both authors contributed to the article and approved the submitted version.

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