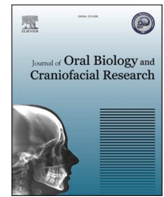
Journal of Oral Biology and Craniofacial Research 14 (2024) 231–237

Contents lists available at ScienceDirect

Journal of Oral Biology and Craniofacial Research

journal homepage: www.elsevier.com/locate/jobcr



Senescence and fibrosis in salivary gland aging and disease

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A R T I C L E I N F O

Keywords:
Senescence
Fibrosis
Salivary glands
Irradiation
Sjogren's Disease

A B S T R A C T

Salivary gland hypofunction is highly prevalent in aged and diseased individuals leading to significant discomfort and morbidity. One factor that contributes to salivary gland hypofunction is cellular aging, or senescence. Senescent cells can impair gland function by secreting paracrine-acting growth factors and cytokines, known as senescence-associated secretory phenotype (SASP) factors. These SASP factors stimulate inflammation, propagate the senescent phenotype through the bystander effect, and stimulate fibrosis. As senotherapeutics that target senescent cells have shown effectiveness in limiting disease manifestations in other conditions, there is interest in the use of these drugs to treat salivary gland hypofunction. In this review, we highlight the contribution of senescence and fibrosis to salivary gland pathologies. We also discuss therapeutic approaches to eliminate or modulate the senescent SASP phenotype for treating age-related salivary gland diseases and extending health span.

1. Introduction

Progressive loss of tissue and organ function often accompanies the aging process. In the salivary gland, loss of saliva production, or salivary hypofunction, is associated with significant morbidity, and reduced quality of life with only a few options available for management of the condition.[1] Many age-related changes in saliva have been documented, including decreased flow, increased ionic concentration, increased viscosity, and increased mucin degradation, reviewed in Refs. 2,3. These age-related changes in saliva lead to the sensation of dry mouth, or xerostomia, taste aberration, a suboptimal salivary pellicle and a subsequent increase in dental caries.[4] While salivary hypofunction occurs with aging, it also occurs with salivary gland disease, such as the autoimmune disease, Sjögren's Disease (SjD) and in response to irradiation (IR) for treatment of head and neck cancers.[1] Like older adults, patients with SjD and patients that have been treated for head and neck cancers also suffer from reduced saliva volume and saliva quality that can be debilitating.[5–7] A better understanding of the causes of salivary hypofunction can lead to development of new, more effective therapeutics.

Reduced organ function in the elderly can in large part be attributed to cellular aging, or senescence. Senescence, which increases with aging, is a cellular response to limit proliferation of aged and/or damaged cells. Senescent cells can propagate signals to other cells leading to organ decline. However, declining organ function can also be attributed to fibrosis, or the excess accumulation of extracellular matrix (ECM) and changes in the ECM organization.[8] Fibrosis, which can occur in response to tissue injury, senescence, or chronic inflammation, disrupts normal tissue architecture and contributes to organ dysfunction.[9] Senescence, fibrosis, and chronic inflammation have been implicated in SjD[10,11] and senescence is a known side-effect of IR treatment.[12] Although few studies have focused on the contribution of senescence to salivary gland disfunction; recent studies have begun to evaluate differences in young and aged salivary glands and to evaluate the potential for remediating senescence as a therapeutic strategy for hyposalivation. Here we will review current knowledge regarding senescence, inflammation, and fibrosis in salivary gland aging and disease. We will also explore the potential for use of senotherapeutics, which target senescent cells, for applications in treating or preventing salivary hypofunction.

2. Senescent cells

Cellular senescence is a permanent cell cycle arrest in response to

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https://doi.org/10.1016/j.jobcr.2024.02.009
Received 28 June 2023; Received in revised form 7 January 2024; Accepted 23 February 2024
Available online 13 March 2024
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DNA damage, mitochondrial dysfunction, and other causes.[13] In limiting unregulated cell proliferation after DNA damage, senescence-associated (SA) cell-cycle arrest functions as a tumor-preventative strategy in adult cells. As senescent cells increase with aging, they promote progressive organ dysfunction and pathology.[14] A principal cause of senescence is DNA damage, which activates p53 and the downstream cell cycle inhibitors $p21^{CIP1}$ and $p16^{INK4A}$ to inhibit the formation of the cyclin D-CDK4/6 complexes to cause cell cycle arrest (reviewed in Ref. 15). Metabolic changes that occur with senescence lead to altered mitochondrial function[16] and production of secreted factors (Fig. 1A). Senescent cells also accumulate DNA damage and undergo genome reorganization,[17] which induces changes in gene expression, including secretion of paracrine-acting factors, which is known as the senescence-associated secretory phenotype (SASP).[18]

Although the detection of senescent cells in vivo is challenging due to their heterogeneity, there are several molecules that are used as markers. The phosphorylation of histone H2AX on Ser139 occurs in response to DNA damage and, in its phosphorylated form, γH2AX is often used as marker for DNA damage and senescence.[19] The metabolic changes that occur in senescent cells lead to the increased expression of the lysosomal enzyme, senescence-associated beta-D-galactosidase (SA-β-gal) that is detectable at pH 6, and is the most commonly used senescence marker[20] (Fig. 1A). Four molecules ($p16^{INK4A}$, $p21^{CIP1}$, SA-β-gal, and γH2AX) are widely used as senescent cell markers, and all have been detected in senescent salivary gland cells[21,22] (Fig. 1B).

However, as these classical senescence markers are not necessarily exclusive to senescent cells and are differentially expressed in different states of senescence, the use of SA-β-gal combined with a cell cycle marker and a core SASP marker has been proposed to validate senescence; additional SASP and senescence subtype markers were proposed in a second phase to identify senescent cell subtypes.[23] Methods in which multiple markers per cell can be detected, such as single-cell RNA sequencing are thus currently being applied to detect senescent cells.[23,24] To address the need for additional senescent cell markers, efforts are underway to better characterize senescent cells, such as the SASP Atlas,[25] and characterization of senescence gene expression in salivary glands is needed.

Transgenic mouse models and novel probes have been applied to the challenge of detecting senescent cells in vivo.[26–30] Tracking p16-expressing cells in live mice has been accomplished with the p16-3MR (tri-modality) reporter mouse,[26,31] which expresses luciferase, red fluorescent protein, and the herpes simplex virus 1 thymidine kinase (HSV-tk). As HSV-tk can induce the prodrug ganciclovir to become toxic and induce apoptosis, this mouse can also be used to ablate senescent cells. Other mice that have been used to label senescent cells in vivo include a p16-tdTomato mouse[32] and a p16-CreERT2; ROSA26tdTomato double transgenic reporter mouse,[33] among other alternatives reviewed in Ref. 34.

Specific probes have been developed to detect senescent cells in vivo. One example is the BOD-L-βGal probe, which is a small molecule that binds to SA-βgal that can be detected with ratiometric imaging. Once delivered in vivo and activated by light, BOD-L-βGal emits fluorescence at 730 nm in the presence of SA-βgal and at 580 nm in its absence. BOD-L-βGal was delivered with nanoparticles to detect senescent cells in atherosclerotic arteries in mice.[35] Molecular tools for labeling senescent cells could have applicability to the study of senescent cells in salivary glands.

3. The senescence-associated secretory phenotype (SASP)

The SASP is characterized by the secretion of highly variable molecules that promote inflammation, including pro-inflammatory cytokines and chemokines by chronically senescent cells.[25,36–38] The core SASP effectors are considered to include TGFβ, IL6, IL1, CXCLs, CCLs, MCP1,

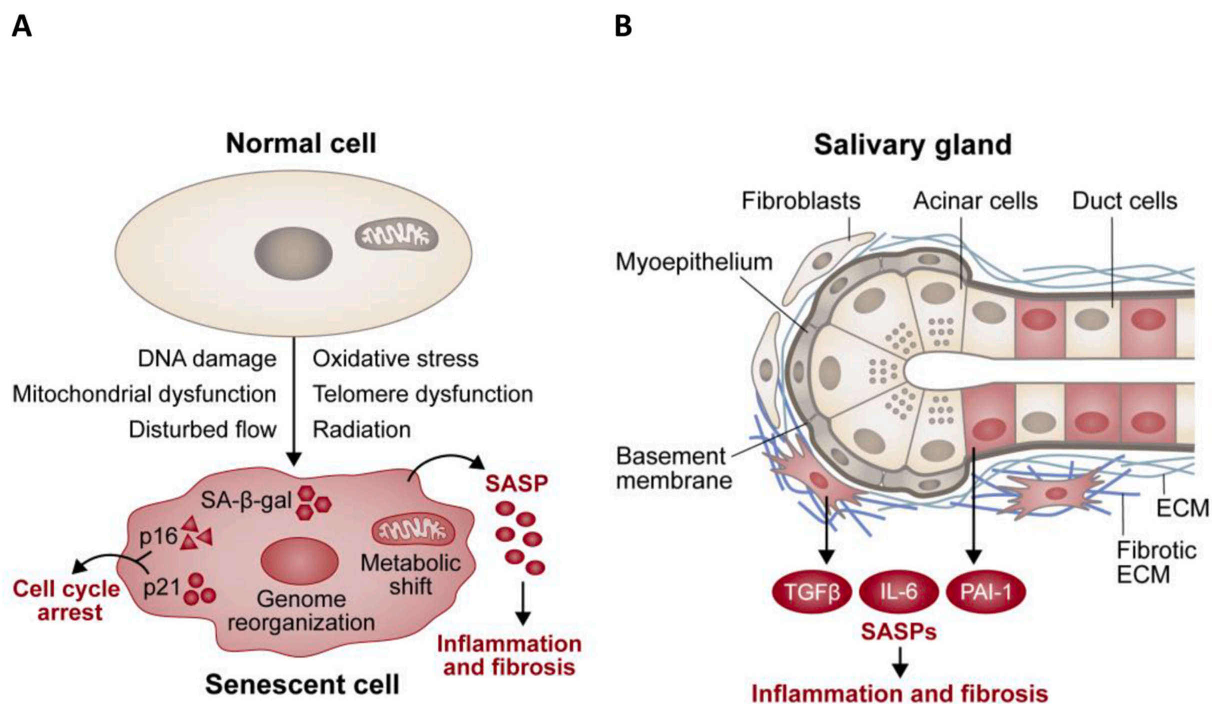

Fig. 1. Senescent cells in the salivary gland. (A) Senescent cell characteristics. In response to multiple insults, including DNA damage, oxidative stress, mitochondrial dysfunction, telomere dysfunction, disturbed flow and radiation, normal cells undergo global changes to become senescent. Senescent cells (red) express cell cycle inhibitors, including p16 and p21 to undergo cell cycle arrest and global changes in metabolism and genome reorganization. Senescent cells also generally produce SA-β-gal and SASP factors that propagate inflammation and fibrosis. (B) Senescent cells in the salivary gland. The primary senescent cell population that has been detected in salivary glands following radiation treatment for head and neck cancers is the ductal cells. However, there are also other senescent cell types. The primary SASP factors that have been linked with senescent salivary glands are TGFβ, IL-6, and PAI1, which additionally promote fibrosis and inflammation. In response to fibrosis, fibrogenic cell populations overproduce ECM.

PAI1, TNFα, and MMPs.[36,39,40] However, a recent study that used a proteomic workflow to catalog the SASP factors produced by human lung fibroblasts and renal cortical epithelial cells found that the SASP profile of the fibroblasts and epithelial cells was unexpectedly largely distinct and much more extensive than was previously appreciated.[25] Another study focused on endothelial cells and used machine learning to compare SASP factors produced by cells in different conditions from eight previously published datasets. This study identified both core senescence genes and also 13 SASP genes that are senescent endothelial cell-specific.[41] Although SASP factors in the salivary gland have been incompletely characterized, TGFβ1 and IL-6 were significantly increased in aged male (82 weeks) vs young (6 weeks) old mice, correlating with increased $p16^{INK4a}$ and $p21^{CIP1}$ expression and also decreased amylase and aquaporin-5 expression, suggestive of reduced secretory function in the glands.[22] Characterization of SASP factors in senescent salivary gland cells will facilitate therapeutic efforts.

4. Role of SASP in the bystander effect and inflammaging

As senescent cells can persist in tissues for a long period of time, SASP factors can have many effects on other cells within affected tissues. One effect of senescent cells is the triggering of secondary senescence in other cells (Fig. 2). The SASP perpetuates senescence throughout affected tissues and perhaps throughout the organism in a phenomenon known as the bystander effect.[42,43] In this way, cells that are adjacent to senescent cells can take on a senescence phenotype without exposure to the primary stimulus. In aging organisms, the effects of senescent cells and other aging cells on intracellular communication that stimulate increased communication with the immune system and propagate inflammation is referred to as "inflammaging".[44] Inflammaging, which is through to result from remodeling of the innate and adaptive immune system, is associated with a low level inflammation driven by chronic inflammatory cytokine production to promote functional decline associated with aging.[45] The long term effects of the SASP can contribute not only to aging but also to age-related diseases, including cancer.[46]

5. Role of SASP in fibrosis

During normal wound healing, senescent cells promote transient deposition of ECM proteins to provide a provisional matrix that promotes healing[26] and, as the ECM is degraded during resolution, the senescent cells are cleared by natural killer cells and macrophages.[47,48] However, as senescent cells accumulate in tissues in response to aging or damage, they contribute to fibrosis by promoting persistent fibroblast activation, excessive ECM deposition,[49] and progressive loss of tissue function.[50] The accumulation of fibrillar collagens involves both increased collagen deposition as well as aberrant collagen and ECM remodeling, which when excessive and prolonged, creates pathological fibrosis with a profound disease burden.[8] SASP-mediated increases in tissue inflammation also synergize with tissue damage to further promote fibrosis.[51–55]

Thus, SASP contributes to a non-resolving fibrotic environment upon tissue injury causing progressive organ dysfunction. While senescent cells have been reported to promote fibrosis progression,[16] a recent study has also linked senescent salivary gland cells to fibrosis. Researchers found an increased expression of senescence markers and pro-inflammatory cytokines, including IL-4, in SMGs of human patients with the chronic fibroinflammatory disease of IgG4-induced sialadenitis.[56] Both acinar and ductal epithelial cells were shown to undergo senescence. The connection between IL-4, senescence, and fibrosis was demonstrated in this same study by the induction of fibrosis in mice injected with IL-4 and also by the increased expression of $p16^{INK4a}$ and ECM proteins in a salivary gland cell line treated with IL-4. The cell culture studies revealed an increase in reactive oxygen species (ROS) and p38 map kinase (p38MAPK) in response to IL-4. Others have shown that the persistent tissue damage and inflammation associated with fibrosis can lead to further DNA damage and cellular stress, triggering senescence in various cell types.[57] Recent work has addressed the molecular contribution of senescent cells to modulation of the ECM. In the tendon, senescent cells were shown to have specific effects upon the ECM that differed from aging.[58] In this study, senescence and aging independently led to alterations in ECM gene expression and alterations in the matrix degrading enzymes, matrix metalloproteinases, (MMPs) in cultured tendon explants. Thus, characterizing the relationship between senescence, fibrosis, and inflammation may provide therapeutic opportunities for targeted treatment of inflammatory and fibrotic diseases of the salivary gland.

6. Salivary gland pathologies, senescence, and SASP

The primary drivers of loss of salivary gland function include aging, injury, autoimmune conditions such as SjD, radiation treatment for head and neck cancers, and side effects of some common medications.[59] Several recent studies have begun to investigate differences in old and young salivary glands. A recent study that compared young and old female human minor salivary glands reported an increase in atrophy of

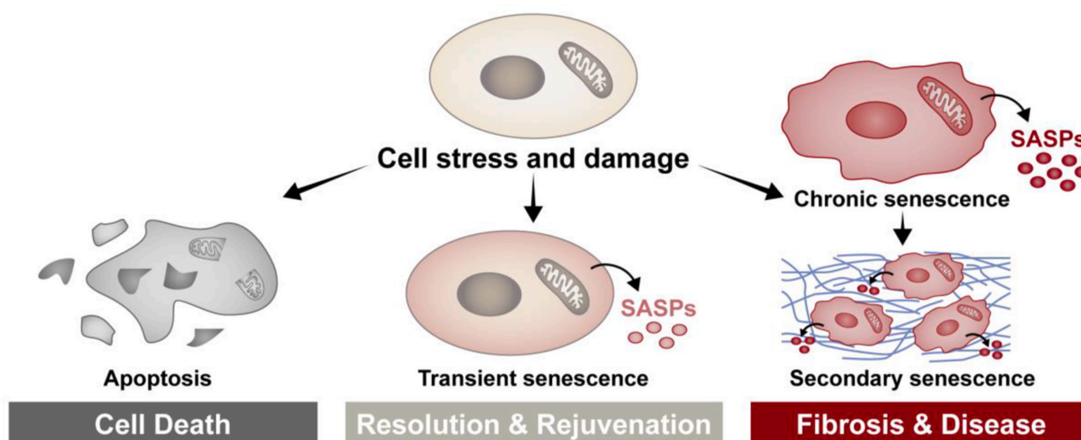

Fig. 2. Senescence, SASP, and fibrotic progression. In response to various cell stresses, cells can undergo apoptosis, or cell death. In response to other stresses, cells can undergo transient senescence and production of SASP factors resolved as the wound heals. With chronic stress, cells that become chronically senescent can contribute to fibrotic disease as they overproduce SASP factors and ECM and propagate senescence to other cells through the bystander effect, which is also called secondary senescence.

acini, increased inflammatory cells, altered immune responses, and the accumulation of both lysosomes and autophagosomes in the old glands relative to the young glands,[60] all of which could relate to decreased gland function. Another study found an increase in Adiponectin (ADIPOQ) and lipogenesis-associated senescence in aged relative to young adult human submandibular salivary glands (SMG) and parotid glands.[61] Silencing of ADIPOQ in human SG epithelial cells in this study reduced SA-β-Gal and inflammation while increasing expression of acinar genes, amylase and aquaporin 5, suggesting a link between lipogenesis-associated senescence and salivary hypofunction.

Senescent cells seem to be an active player in SjD. Senescent cells, as detected by $p16^{INK4a}$ expression, accumulate in the salivary gland ductal cells of primary Sjögren's disease (pSjD) patients.[11] pSjD do not have any other complicating disease that could be attributed to this phenotype. Further, human salivary gland stem cells isolated from parotid gland biopsies of primary SjD patients, showed reduced organoid forming capacity, shortened telomeres, and increased p16 expression, suggesting a premature aging phenotype as a causative agent in pSjD.[11] Consistent with these findings, salivary glands in rodent mouse models and patients with SjD exhibit salivary gland senescence, including an increased expression of $p16^{INK4A}$ in acinar and duct cells and putative epithelial progenitor cells that is concomitant with the accumulation of immune cell infiltrates and hyposalivation.[62] Fibrosis is also increased in salivary glands of SjD mouse models and human patients independently

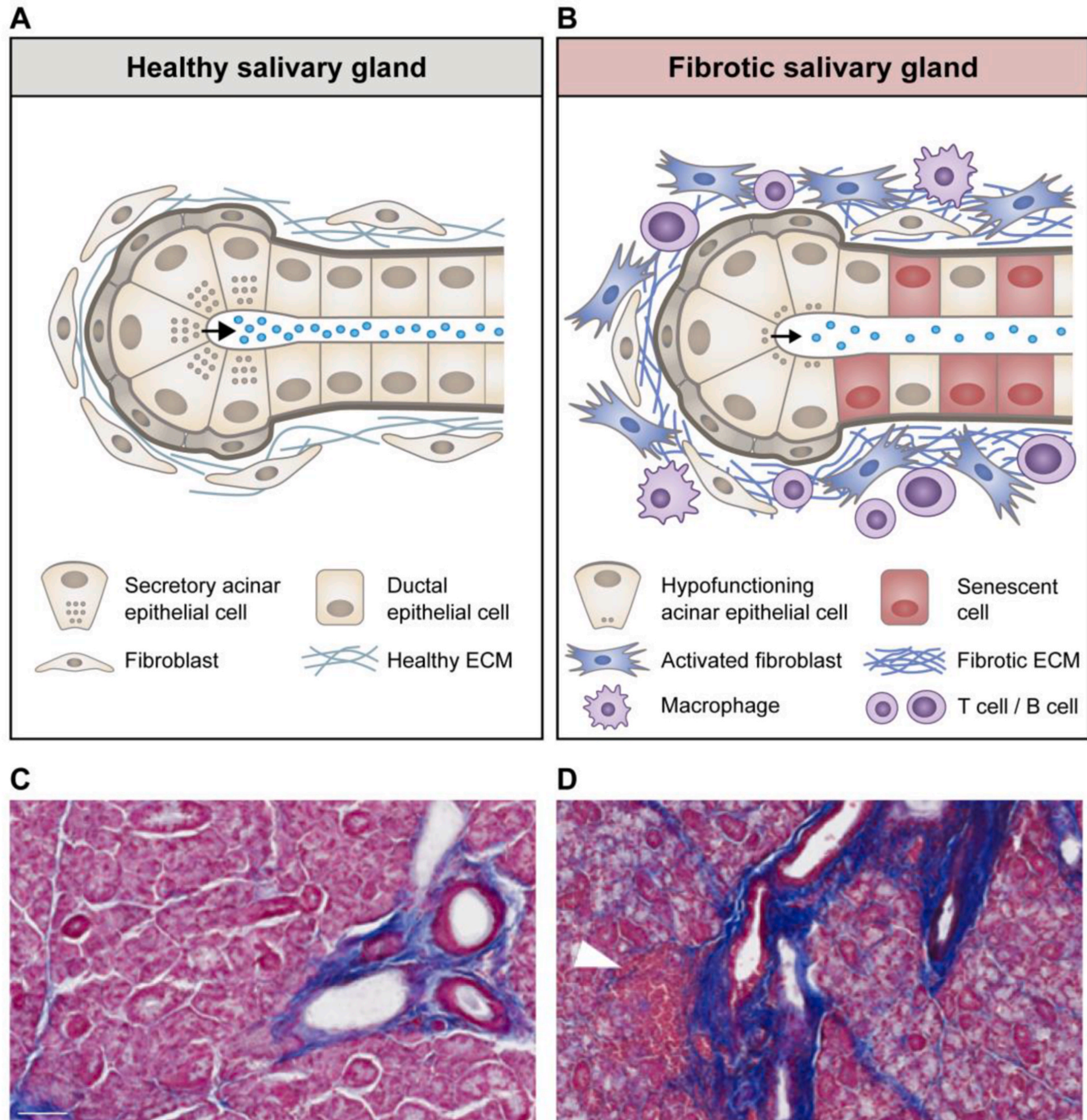

Fig. 3. Healthy vs Fibrotic Salivary Glands. (A) In healthy salivary glands with normal tissue architecture, saliva is produced by acinar cells and modified by ductal cells enroute to the oral cavity. (B) In fibrotic salivary glands, hypofunctioning acinar cells produce less saliva, and fibrogenic cells produce excess ECM (blue). Senescent cells (red) may promote both the fibrotic response and inflammatory response. Particularly in the case of autoimmune conditions such as SjD, B and T lymphocytes are recruited into the gland, accumulating primarily near the ducts. (C) Masson's trichrome-stained 20-week old normal female mouse CD-1 submandibular salivary gland, reveals ECM (blue) primarily around ducts and vessesls with very little interstitial ECM detectable, Scale, 50 μM (D) trichrome-stained 20-week old female NOD/ShiLtJ (SjD model) mouse submandibular salivary gland reveals a marked increase in ECM, including interstial ECM, (blue) and infiltrating lympocytes (red) near ducts and vasculature (arrowhead).

of aging (Fig. 3).[63,64] Studies in other organs have demonstrated that epithelial cells can undergo a transforming growth factor beta (TGFβ)-stimulated epithelial to mesenchymal (EMT) transition to directly participate in fibrosis through ECM production and also in the perpetuation of inflammation by increased cytokine production.[63] The cytokines IL-17 and IL-22 have been shown to drive EMT-dependent salivary gland fibrosis.[65] Further research to delineate the contribution of senescent cells to SjD pathology will provide insights into future therapeutics.

Senescence also contributes to salivary hypofunction induced by irradiation. Exposure to ionizing IR induces salivary gland degeneration, senescence, fibrosis, and hyposalivation in both rodent models and human patients.[21,66,67] Increased expression of γH2AX in mouse submandibular gland duct cells at 7 days[66] and of SA-β-gal 8 weeks after irradiation are suggestive of senescence. The presence of SASP factors was shown to decrease the tissue's regenerative potential following IR.[67] In IL-6 deficient mice, the senescence phenotype was reduced upon exposure to radiation. Interestingly, IL-6 deficient mice and mice treated with IL-6 before irradiation showed a marked increase in saliva flow rates 8 weeks after irradiation relative to control mice. This somewhat unexpected result is thought to be due to stimulation of a DNA-damage response. Consistent with this finding, another study in mouse SMG showed that transient expression of sonic hedgehog *(Shh)* three days post-IR treatment reduced senescence and increased activation of DNA repair genes.[66] Thus, these studies demonstrate that modulation of senescent cells and SASP factors holds promise as a therapeutic strategy to remediate senescence-driven salivary gland hypofunction.

Rodent models are being used to understand the drivers of fibrosis. Ductal ligation of the salivary gland, which models the clinical condition of sialothiosis, or salivary stones, induces loss of acinar cell differentiation, increased senescent cell gene expression in ducts,[68] and fibrosis.[69,70] Excessive deposition of ECM with aberrant remodeling resulting in tissue fibrosis occurs in response to ligation, and TGF-β pathway dysregulation is a primary contributor to this fibrotic phenotype[69] and also in SjD.[67] Stromal fibroblast cells expressing Platelet derived growth factor alpha (PDGFRα) and PDGFRβ are considered to be primary cell types mediating fibrosis, and *Pdgfra*$^+$, *Pdgfrb*$^+$ cell types were recently implicated as the fibrogenic cell population in the murine submandibular and sublingual salivary gland ductal ligation model for fibrosis.[70] Interestingly, *Pdgfra*$^+$ dental pulp mesenchymal stromal cells have been shown to undergo senescence due to chronological aging or by replicative senescence with slightly different outcomes but with reduced self-renewal capacity being common to both mechanisms.[71] Elucidating the interplay of the immune and stromal fibroblast populations and molecular mechanisms by which these cells orchestrate the tissue damage that leads to loss of gland function is an important avenue of research. Single-cell sequencing technologies are being employed to identify these mechanisms and therapeutic targets.[9]

7. Senotherapeutics

The accumulation of senescent cells has been hypothesized to contribute to various age-related phenotypes and diseases, including tissue degeneration, chronic inflammation, hyperplasia, and cancer.[72] Senotherapeutics, or drugs that target senescent cells, are garnering significant interest to the medical community as they offer the possibility of improving quality of life and extending the health span. Recent data support the idea that senolytic drugs, which selectively eliminate senescent cells from target tissues or organs, can mitigate inflammatory/fibrotic pathologies.[73] Indeed, senolytics have demonstrated efficacy in reducing pathological burden in diverse tissues and organisms to delay tissue degeneration and improve tissue function.[55,61,74,75] For example, treatment with the senolytic drugs, dasatinib and quercetin, improved lung function in idiopathic pulmonary fibrosis (IPF) lung models in mice[55] and has had promising results in treating human disease.[76] Murine models that harnessed the activity of p16^{INK4A} to abate senescence were shown to limit age-related declines in cognitive, adipose, cardiac, and renal function,[77] demonstrating the benefit of senescence abatement to age-related loss of organ function. Elimination of senescent cells with a SA-β-galactosidase-targeted prodrug led to reduced inflammation and generally improved physical health in multiple assays spanning strength, balance, and cognition.[78] Senolytic treatments have thus shown significant benefits in multiple age- and disease-related contexts.

Senolytic treatments have also shown efficacy in remediating salivary hypofunction. For example, depletion of p16-expressing cells in salivary gland organoids derived from p16-3MR mice with gangciclovir *in vitro* increased the efficiency of organoid formation, as an index for salivary gland progenitor cell activity.[79] In addition, treatment of irradiated mice with the senolytic drug, ABT263, improved salivary gland morphology and function by increasing stem cell self-renewal capacity and preventing tissue degeneration.[79] This work establishes proof of concept for senotherapeutic strategies to improve salivary gland function.

While senolytics have demonstrated efficacy in diverse organ pathologies, including IPF, the unilateral destruction of senescent cells is not always beneficial. For example, elimination of senescent cells in blood vessels is detrimental as they are needed for wound healing[80] and senescent fibroblasts have been shown to act as sensors of inflammatory signals and promote epithelial regenerative responses.[81] Current efforts are focused on development of senomorphic drugs that would not eliminate senescent cells but rather change their gene expression. As these drugs modulate senescent cell gene expression to minimize the SASP without senescence cell ablation, they are likely to have fewer side effects.[82] Continued development of senomorphic strategies are important areas for novel therapeutic development.

8. Conclusions and future directions

Characterizing the tissue-specific molecular mechanisms by which senescence, SASP, inflammation, and fibrosis drive organ pathology and dysfunction is critical to alleviating disease burden in our aging population. Since little is known about the contribution of senescent cells to salivary gland dysfunction, continued research into the basic biology of senescent cells in specific contexts is needed. Comprehensive characterization of molecular mediators of senescence and SASP and the effects of these cells in diverse contexts including aging, regeneration, and disease needs to be performed in the salivary glands to facilitate therapeutic development for salivary hypofunction. Mouse models for SjD as well as experimental models of gland injury by ligation (ductal ligation), and IR-treatment show promise as models to characterize and probe senescent cell functions. Organoids have also shown efficacy in assessing senescent cell functions. Organoids have the added benefit of allowing assessment of rodent and human cells alike for translational benefit.

Senolytic therapies that eliminate senescent cells have shown promise and should continue to be examined in salivary gland disease. However, as side effects of these drugs can be debilitating, particularly in a chronic setting, less toxic drugs should be explored. Senomorphic strategies for modulating the senescence phenotype also hold promise. As non-coding RNAs regulate SASP,[83] strategies targeting non-coding RNAs are one approach of interest for development of novel senomorphic drugs, but these will require an understanding of the relationship between miRNAs and senescent salivary gland cells.[84,85] Restoring permissive ECM composition and homeostatic remodeling to rebuild normal organ architecture and function are also attractive targets for the development of more refined therapeutics.[86] The field of senotherapeutic development is still in its infancy and there is still much to learn about how these drugs can be applied to age-related diseases of salivary hypofunction to extend health span.

Acknowledgements

This work was supported by the National Institutes of Health grants R01DE027953 and R01DE030626 to M.L., R15CA274603 to J.A.M., R56DE033253 to M.L. and J.A.M., and the University at Albany, SUNY's Aligning UAlbany Strengths with National Funding Priorities Seed Funding Award. The authors thank Jennifer Morrissey and Steven Higgins for the images of mouse submandibular salivary glands and Dr. Pauline Carrico for helpful suggestions.

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