Senescent cells as a target for anti-aging interventions: From senolytics to immune therapies

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ABSTRACT

Aging and age-related diseases are major drivers of multimorbidity and mortality worldwide. Cellular senescence is a hallmark of aging. The accumulation of senescent cells is causally associated with pathogenesis of various age-associated disorders. Due to their promise for alleviating age-related disorders and extending healthspan, therapeutic strategies targeting senescent cells (senotherapies) as a means to combat aging have received much attention over the past decade. Among the conventionally used approaches, one is the usage of small-molecule compounds to specifically exhibit cytotoxicity toward senescent cells or inhibit deleterious effects of the senescence-associated secretory phenotype (SASP). Alternatively, there are immunotherapies directed at surface antigens specifically upregulated in senescent cells (seno-antigens), including chimeric antigen receptor (CAR) therapies and senolytic vaccines. This review gives an update of the current status in the discovery and development of senolytic therapies, and their translational progress from preclinical to clinical trials. We highlight the current challenges faced by senotherapeutic development in the context of senescence heterogeneity, with the aim of offering novel perspectives for future anti-aging interventions aimed at enhancing healthy longevity.

Key words: cellular senescence, small molecules, seno-antigens, immune therapy, age-related diseases

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OVERVIEW OF CELLULAR SENESCENCE

SEnescence phenotypes and their pathophysiological functions

Cellular senescence is a biological process in which the normal cell cycle halts irrevocably in response to various intra- and extra-cellular stressors. The senescent phenotype typically encompasses a flattened and enlarged morphology, apoptosis resistance, altered gene expression and the senescence-associated secretory phenotype (SASP).[1] Depending on the stimulus, cellular senescence is categorized into four distinct types: replicative senescence (RS), stress-induced senescence (SIS), oncogene-induced senescence (OIS), and therapy-induced senescence (TIS). RS is mostly observed in *in vitro* cultures of epithelial cells and fibroblasts, arising when cells exhaust their proliferative capacity as their telomeres shorten below a critical length.[2] In the presence of deoxyribonucleic acid (DNA)-damaging factors such as oxidative stress, heat shock, irradiation, and genotoxic drugs potentially leading to oncogene activation, cells stop in their proliferation programs to prevent the passing down of genetic mutations as a tumor suppressive mechanism.[3] Additionally, senescence can be induced *via* paracrine and endocrine signaling through the SASP, which comprises pro-inflammatory cytokines, chemokines, metalloproteinases, growth factors, lipids and micro ribonucleic acids (miRNAs).[4]

The effect of cellular senescence varies across different developmental stages and tissue microenvironments. Physiologically, senescence cells contribute to wound healing, fibrosis resolution, recruitment of immune

cell, suppression of tumorigenesis, and development of forelimbs and neural tube during embryogenesis.[3,5,6] Pathologically, senescent cell accumulation leads to impaired tissue regeneration, chronic inflammation and fibrosis. Cytokines and chemokines in the SASP establish a immunosuppressive tissue environment prompting malignant cancer cell phenotypes such as stemness, immune evasion, and metastasis in the surrounding tissue.[3,7,8] The presence of TIS senescent cells in the tumor microenvironment confers resistance to chemotherapy and immunoblockade therapy.

Moreover, senescence cell accumulation is causally implicated in the pathogenesis of various age-related conditions, such as frailty, sarcopenia, Alzheimer's and cardiovascular diseases.[4,9] Previous studies demonstrated that small amounts of senescent cell transplantation to young mice can lead to physical dysfunction and shortened lifespan. In contrast, multiple transgenic models of senescent cell depletion and treatment with senolytics have been demonstrated to not only alleviate, but also delay the onset of age-associated disorders.

Established transgenic and genetic models for studying senescence

Various genetic models of senescence cell depletion and premature senescence have been established to facilitate understanding of molecular mechanisms of cellular senescence. The earliest models were those of constitutive multiple tumor suppressor 1 (p16) and cyclin-dependent kinase inhibitor 1A (p21) knockout and overexpression, which were used to study the pathophysiological effects of singular senescence regulators *in vivo*.[10,11] These models are imperfect in that they do not differentiate adult phenotypic changes resulting from senescent cell depletion to those arising from a differential embryogenesis program due to a lack of p16 or p21. This was resolved by subsequent models, where transgenic systems were introduced into model organisms to enable inducible systemic removal of senescent cells upon treated with specific chemicals. For example, the INK-ATTAC and p21-ATTAC transgenic mice for selective depletion of $p16^{Ink4a+}$ and $p21^{CIP1+}$ cells, respectively, upon intraperitoneal injection of AP20187(synonyms: B/B Homodimerizer). Another mice model, p16-3MR, makes use of a trimodal reporter to monitor p16 high senescent cells *in vivo*, and their systemic clearance upon administration on ganciclovir.[12] Other transgenic models have used other reporter genes such as enhanced green fluorescent protein (EGFP), tdTomato or luc to visualize senescent cell burden *in vivo*, as characterized by high expression levels of p16 or p21.[13,14] However, due to the lack of cell-type specificity, these models typically do not differentiate between the phenotypic changes resulting from local to systemic senolysis, or the other effects associated with loss of p16 and p21-expressing cells that are independent of the senescent program. More recently, inducible models of senescent cell monitoring, modulation and elimination were generated using Cre-LoxP recombination, such as the p16-LOX-ATTAC and p21-Cre mouse that monitor and manipulate $p16^{Ink4a+}$ and p21 high cells in mice,[15,16] facilitating deeper insight into the link between senescent cell burden and organ-specific pathologies. These are currently the state-of-the-art technologies in the field to quantify and study senescence *in vitro* and thus their usage is recommended.

Compared to models of physiological aging, mice models of progeria are useful and time-efficient genetic backgrounds to study senotherapeutic interventions. These mice typically show a five- to six-fold acceleration in aging and copy many phenotypes of naturally aging mice and humans, including senescent cell accumulation, loss of tissue homeostasis and shortened healthspan and lifespan. Common underlying causes of these progeria syndromes are genomic instability, compromised nuclear envelope, defective mitosis and oxidative stress as a result of accumulated reactive oxygen species (ROS). Accordingly, genetic manipulations interfering with DNA repair (*Ercc1*$^{-/\lrcorner}$, *Xpd*$^{TTD/TTD)}$, nuclear envelope maintenance (*Lmna*$^{G609G/G609G}$, *Zmpste24*$^{-/-}$), mitotic spindle assembly (*BubR1*$^{H/H}$) and radical scavenging mechanisms (*Sod1*$^{-/-}$) have been used to generated models of premature aging and senescence,[17] which are now widely employed when investigating the efficacy of senotherapeutics in healthspan and lifespan extension.

Although extensively tested and used, inherent limitations exist in these genetic and transgenic models of senescence. Firstly, given the heterogeneous nature of cellular senescence, characterizing senescent cells based on single molecular marker may not reflect the exact condition of senescent cell accumulation *in vivo*. Additionally, in spite of their phenotypic resemblance, the underlying aging drivers between genetically manipulated progeria and naturally aged animals may be different. As the former are typically nonexistent outside of the laboratory context, animal models of physiological aging are still irreplaceable as the most accurate models to understand the effect of senotherapeutics on healthy longevity in humans. Therefore, the research community would greatly benefit from the design of animal models that bear not only phenotypic, but also genotypic and mechanistic resemblance to the human aging process. Moreover, develop transgenic models that identify senescent cells based on multiple molecular biomarkers, rather than just one, can be greatly helpful to better characterizing the senescent landscape *in vivo* and develop targeted therapies.

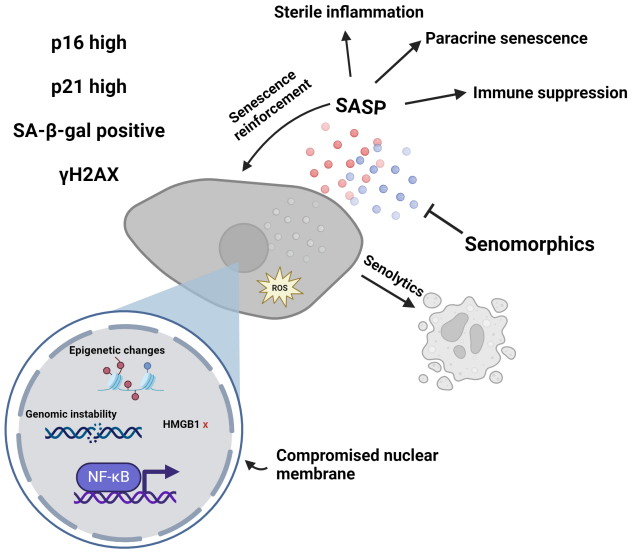

Figure 1: Metabolic changes during cellular senescence and mechanisms of senotherapeutic interventions. Cellular senescence are primarily characterized by elevated p16, p21, SA-β-gal, DNA damage, and pro-inflammatory SASP. The two types of chemical senotherapeutics are senolytics and senomorphics, which promote senolysis and inhibit the SASP, respectively. Created using BioRender.com. p16: multiple tumor suppressor 1; p21: cyclin-dependent kinase inhibitor 1A; SA-β-gal Senescence-associated beta gal; SASP: senescence-associated secretory phenotype; γH2AX: γ H2A histone family member X; HMGB1: high mobility group box 1; NF-κB: nuclear factor kappa-B; ROS: reactive oxygen species.

Senescent cell biomarkers in the context of senescence heterogeneity

Cyclin inhibitors p16 and p21 are arguably among the most important biomarkers of *in vitro* senescence, as they capture the fundamental mechanism of cell cycle arrest which induces the senescent cell fate. Senescence-associated beta gal (SA-β-gal) is another popular laboratory standard for quantifying senescent cells, reflecting the heightened activity and abundance of lysosomal enzymes that enable the cleavage of terminal β-d-galactose from β-D-galactosides at pH = 6 (Figure 1). Additional senescence features, such as genomic instability, chromatin remodeling, compromised nuclear membrane integrity, extracellular translocation of alarmins, SASP upregulation, and morphological changes are often tested as auxiliary markers to complement the abovementioned biomarkers. Experimental methods for evaluating these markers including immunohistochemistry staining for DNA damage markers histone variant γ H2A histone family member X (γH2AX), P53-binding protein 1 (53BP1), and promyelocytic leukemia (PML), the loss of nuclear protein lamin B1, intracellular absence of high mobility group box 1 (HMGB1) and bulk RNA-seq profiling for SASP factors.[18,19] While a rigorous characterization of senescent cells *in vitro* can generally be obtained by multiplexing three or more of these consensual markers, quantification of senescence *in vivo* is often complicated by the fact that tissue homogenates are composed of varied cell types and expression levels, among which senescent cells account for a relatively small percentage. There is also the need to unambiguously differentiate between senescent and quiescent cells, which exhibit phenotypic resemblance but are fundamentally different in that cell cycle arrests in quiescence are reversible and transient. Apart from cell type differences, expression of senescence biomarkers in individual cells are also subject to changes depending on temporal dynamics. For example, a single-cell omics study revealed that cells of the same origin can take on different trajectories during senescence initiation and ultimately transition into distinct clusters. In this particular case, diploid WI-38 human cells, upon senescence induction, resulted in two clusters with opposite regulation in oxidative phosphorylation and guanosine triphosphate hydrolases (GTPase) activity.[20] Furthermore, false positives can occur when senescence biomarkers are expressed by non-senescent cells. It has been well documented that mature macrophages are upregulated in $p16^{Ink4a}$ and, like senescent cells, can be removed by the senolytic combination dasatinib and quercetin (D + Q) with high efficiency,[21] warranting caution in future studies and clinical trials. Although single binary markers of senescence commitment have long been used in preclinical research, their utility as clinical parameters have so far been limited due to the wide-ranging heterogeneity of senescence *in vivo*, which often renders estimates of senescent cell quantification imprecise. As highlighted in a recent review, tissue senescent cell abundance obtained under similar conditions may vary up to ten-fold across different studies and experimental techniques.[22] This inconsistency is in part due to the fact that senescence markers of proliferation and cell cycle arrest typically do

not reflect the wider process of cellular aging, including the accumulation cellular changes in pre-senescent cells or cells already senesced for prolonged periods of time. Expression profiles of cells during these stages can differ greatly from those observed of senescent cells in culture and are relevant for the characterizing of senescence landscape *in vivo*, which has so far remained unexplored.[23] Single-cell and single-nucleus sequencing that provide highly resolved insight into senescence heterogeneity in terms of senescence stimuli, localization and accumulation kinetics.[16,24] For example, a machine learning model trained on published transcriptomic data from 602 samples and 30 cell types, named senescent cell identification (SenCID), classified senescent cells to six distinct senescence identities (SIDs) of varying senescence baselines, functions and susceptibilities to senolytic treatment. When applied to single-cell RNA sequencing (scRNA-seq) data of of tissue during aging and age-related disorders, the model revealed heterogeneous senescence trajectories, and enabled genome-wide mapping of hierarchical modulators of senescence.[25] The use of integrated multi-omics techniques RNA-seq, assay for transposase-accessible chromatin with high throughput sequencing (ATAC-seq) and chromatin Immunoprecipitation sequencing (ChIP-seq) enables researchers to further identify epigenetic elements, key interacting genes N-acetyltransferase 1 (NAT1), Pre-B-cell leukemia homeobox transcription factor 1 (PBX1), RNA recognition motif (RRM). Using a multi-omics analysis and genetic manipulations, another study identified early 2 factor transcription factor 4 (E2F4), transcriptional enhanced associate domain transcription factor 1 (TEAD1) and activating protein-1 (AP-1) to be key factors regulating the RS transcriptome and epigenome.[26,27] Whereas single-cells technologies are typically devoid of original tissue context, a recent work engaging multi-organ spatial transcriptome sequencing (stRNA-seq, spatial transcriptomics RNA sequencing) provided a panoramic multi-organ characterization of tissue senescence in aged mice and identify the aggregation of immunoglobulin G (IgG) as a marker and inducer of tissue senescence, unveiling a novel molecular target for senescence-targeted interventions.[28]

SMALL MOLECULE SENOTHERAPEUTICS

Senolytics and senomorphics

Small molecule senotherapeutics represent a class of chemical compounds that specifically target and eliminate senescent cells or attenuate their pro-inflammatory SASP, without compromising the viability or functions of their proliferating counterparts (Figure 1). Mechanistically, the majority of senolytics induce senolysis by targeting and inhibiting of senescence cell antiapoptotic pathways (SCAPs), among which are ephrin-B 1/3 (EFNB1/3), p53, the heat shock protein 90 (HSP90), and anti-apoptotic members of B-cell lymphoma-2 (BCL-2) family proteins, such as BCL-extra large (BCL-xL) and BCL-w.[29-31] Senomorphics, on the other hand, primarily inhibit various inflammatory factors in the SASP, such as interleukin-6 (IL-6), IL-10 and tumor necrosis factor α (TNFα) *via* disrupting upstream signaling pathways, among which are nuclear factor kappa-B (NF-κB), protein 38 mitogen-activated protein kinase (p38MAPK), silent information regulator transcript 1 (SIRT1), Janus kinase/signal transducer and activator of transcription (JAK/STAT) and nuclear factor erythroid 2-related factor 2 (NRF2).[32] While the abundance of available senolytics and senomorphics precludes an exhaustive summary, we aim to offer a panoramic view on important milestones, and focus on candidates with the most clinical relevancy, that is, have demonstrated promising senotherapeutic effects on *in vivo* models of age-related disorders.

Clinically approved pharmaceutics

Clinically approved drugs are a promising source of senotherapeutic agents due to their established safety profile in patients with relatively well-understood toxicities (Figure 2). Some of the earliest identified senolytic compounds, including tyrosine kinase inhibitor dasatinib (D) and Bcl-2 inhibitor navitoclax (N), are repurposed from chemotherapeutics for their unique ability to selectively induce apoptosis in senescence cells. Fenofibrate, a peroxisome-proliferator-activated receptor α agonist (PPARα agonist) originally prescribed for dyslipidemia, can efficiently reduce senescence burden in aged chondrocytes, improving osteoarthritis conditions in human patients, alleviating cartilage degradation, and mitigating inflammatory responses linked to osteoarthritis and reduced the Western Ontario and McMaster Universities Arthritis Index (WOMAC) osteoarthritis index.[33] In a proof-of-concept study, zoledronic acid, a bisphosphonate commonly used in treatment of osteosarcoma and cancer-induced osteoporosis, was found to be senolytic and senomorphic under *in vivo* conditions.[34] Notably, zoledronic acid has been previously associated with lifespan extension in progeroid mice.[35] And when used in naturally aged mice, zoledronic acid reduced circulating SASP factors and led to improved physical function. Metformin, the most widely prescribed treatment for type 2 diabetes mellitus (T2DM) and well-known geroproector, has been reported to delay or alleviate senescence phenotypes across multiple cell types and senescence causes.[36,37] It has also been shown to exhibit senomorphic effect, attenuating SASP factors including IL-6, IL-1βand C-X-C motif chemokine ligand 1 gene (CXCL-1), predominantly *via* inhibition of NF-κB pathway.[38,39] Geroprotective effect of Metformin has been reported in multiple animal models including lifespan

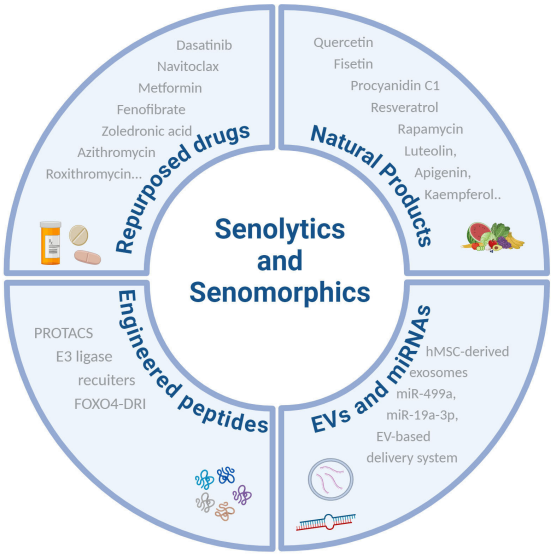

Figure 2: Sources of identified small molecule senolytics and senomorphics. Existing clinical treatments and natural products make up most of these senotherapeutic agents. Synthesis of senolytic peptides and EVs and miRNA-based senolytics are also in development. Created using BioRender.com. miRNA: micro ribonucleic acid; EVs: extracellular vesicles.

extension in *Caenorhabditis elegans*, *Drosophila melanogaster* and mice, and deceleration of aging clocks in male nonhuman primates.[41] Despite its therapeutic benefits, the optimization of treatment dosage remains an important consideration limiting the clinical translation of Metformin. Metformin concentrations used to elicit therapeutic or geroprotective *in vivo* settings typically range from 100 mg/kg to 200 mg/kg concentrations, much higher than what is practically achievable in human clinical trials.[41,42] This concern is especially relevant considering Metformin's dosage-dependent toxicity and associated adverse events such as lactate acidosis, vitamin B12 deficiency and its associated anemia and neuropathy.[43] Another important consideration for clinical translation is the gender difference in geroprotective benefit of Metformin. In clinical practice female patients are often prescribed lower dosages of Metformin and report more gastrointestinal side effects. This is consist a lifespan-extending dosage in male mice can lead to the opposite effect in female mice.[44]

Natural products

Screening natural products library revealed diverse phytochemicals with senotherapeutic potential (Figure 2). These compounds are derived from plants, fruits, and vegetables and can be incorporated into dietary supplementation with ease, enabling a speedier route to the market than repurposed drugs (Table 1). Flavones are a family of antioxidant and anti-inflammatory compounds with prophylactic benefits for various age associated diseases such as neurodegeneration and type II diabetes.[45] Many members of the flavonoid family, including quercetin, fisetin, rutin, apigenin, luteolin and kaempferol have all been found to have some degree of senolytic efficacy.[46-48] As with repurposed drugs, these compounds often pleiotropically target multiple key senescence- and SASP-associated signaling pathways.[32] The earliest flavonoid to be used in senolytic studies, quercetin, when used with dasatinib, led to synergistic effect for lifespan and healthspan extension in mice of multiple morbidities.[30] One of the most potent flavonoids in senotherapy, fisetin, when administered late in life in wild type mice, reduced senescence burden across multiple organs *in vivo*, improved tissue homeostasis, alleviated age-related pathologies and led to increased median and maximum lifespan.[46] A novel senotherapeutic flavonoid, procyanidin C1 (PCC1), primarily found in grape seed extract, is a senomorphic at low concentrations and exerts senolytic effect at higher concentrations. Remarkably, intermittent administration of PCC1 in both old and senescent cell-transplanted mice markedly improved late-life survival without compromising healthspan or increased morbidity.[49] Additionally, other plant-derived molecules ouabain, piperlongumin and synthetic analogs such as EF24 (curcumin) and GL-V9 (wogonin) have all demonstrated senolytic activity *in vitro*, through elevation of ROS levels, BCL-2 protein degradation or Na^+/K^+ ATPase inhibition.[32] Although preclinical *in vivo* results are encouraging, the bioavailability of most natural products are limited by their relatively short half-life and relatively low abundance. Therefore, exploration into optimized delivery methods and mass production strategies are imperative toward their successful translation.

Engineered peptides and synthetic drugs

In addition to traditional senolytic development approaches,

Table 1: Overview of senolytic therapies: small molecule and immune-based approaches

Classification		Advantages	Potential limitations	Development Stage	Examples
Small Molecules	Repurposed clinical drugs	Clinical availability	Dose-dependent toxicity and side effects e.g., thrombocytopenia, neutropenia	Phase I and/or Phase II	D + Q, navitoclax, HSP90 inhibitor, zoledronic acid
	Natural products	Low toxicity, ease of translation as dietary supplements	Low bioavailability	Phase I and/ or Phase II (Quercetin, Fisetin), preclinical	Quercetin, Fisetin, PCC1, rutin, luteolin, kaempferol
	Engineered peptides and synthetic drugs	High specificity and localized effect	Applicability in heterogeneous *in vivo* context	Preclinical	FOXO-DRI, PROTACs, ARV825, KSL0608-Se
	Biologically derived molecules	Low toxicity, transient effect		Preclinical	MSC-derived EV, miR-499a, miR-19a-3p mimetic
Immune-based senotherapies	Senolytic CAR-T	Prophylactic effect, long-term efficacy	Cytokine release syndrome, high cost, complex procedure	Preclinical	uPAR directed CAR-T, NKG2DL directed CAR-T
	Senolytic vaccine		Non-specific of normative physiology	Preclinical	GPNMB vaccine

D + Q: dasatinib and quercetin; HSP90: heat shock protein 90; FOXO-DRI: fork head box O transcription factor-D-Retro Inverso; PROTACs: Proteolysis targeting chimeras; MSC: mesenchymal stem cells; EV: extracellular vesicle; uPAR: urokinase plasminogen receptor; CAR-T: chimeric antigen receptor T cells; GPNMB: glycoprotein nonmetastatic melanoma protein B.

synthetic approaches of medicinal chemistry are also being engaged in senolytic discovery (Figure 2). Fork head box O transcription factor 4-D-Retro Inverso (FOXO4-DRI), a synthetic isoform of the transcription factor FOXO4, triggers apoptosis of senescent cells by disrupting the interaction between FOXO4 and p53 and releasing p53 into the cytosol. In $Xpd^{TTD/TTD}$ mice of accelerated aging and in aged mice, FOXO4-DRI rescued aging phenotypes such as renal failure, frailty and liver dysfunction.[50] Proteolysis targeting chimeras (PROTACs), are bifunctional constructs that bind simultaneously to E3 ubiquitin ligases and their target proteins, leading to their ubiquitination followed by degradation in the proteasome. Bromodomain and extra-terminal family protein degrader (BETd), was found to induce autophagy-dependent apoptosis in senescent cells by activating autophagy genes *via* degradation of bromodomain-containing protein 4 (BDR4). In models of obese mice, BETd reduced senescent hepatic stellate cell burden and suppressed carcinogenesis.[51] Recently, KSL0608-Se, a photoactivable senolytic drug capable of single cell-resolution senolysis was designed using beta-galactosidase (β-gal) targeted prodrug and target-site anchoring strategies. The drug specifically targets and binds senescence-associated (SA)-β-gal positive senescent cells through SA-βgal-dependent activation and biorthogonal anchoring. Upon light irradiation, the drug induced apoptosis of targeted cells through the *in-situ* production of cytotoxic singlet oxygen. Moreover, KSL0608-Se was effective in suppressed senescence markers and delaying age-related dysfunction in naturally aging mice.[52] The highly specific structural design of synthetic senolytics restricts the scope of their effect to protein interactions and cellular processes, relieving cells from side effects arising from complete inactivation of the targeted transcriptional regulators yet retaining their ability to induce apoptosis in senescent cells (Table 1).

Extracellular vesicle (EV) and mirna-based senolytics

EVs and their component miRNAs have long been identified as important regulators in metabolism, intracellular communication and changes in tissue environment. Mesenchymal stem cell-derived extracellular vesicles, particularly exosomes, have therapeutic benefits for a variety of age-related diseases including osteoarthritis, diabetes, cardiovascular diseases and renal failure. Recently, studies revealed conditioned media and EVs isolated from human mesenchymal stem cells (MSCs) are potent in reducing senescent cell burden and may serve as novel senolytic strategy (Figure 2). In fibroblasts of high-glucose induced senescence, small extracellular vesicles (sEVs) isolated from human decidua-derived mesenchymal stem cells suppressed p21 and SA-βgal, whose mechanism is associated with receptor for advanced glycation endproducts (RAGE) inhibition, reduced ROS production, and Smad activation.[53] More importantly, EVs derived from human embryonic stem cells was also found to reduce senescence burden and extend the healthspan of *Ercc1-/⊿* progeroid mice.[54] miRNA regulate various key pathways in cellular senescence, and the enrichment of certain miRNAs have been proposed as novel markers of senescence. Apart from senolytic EVs, EV-based delivery

vehicles have also been used to enhance efficacy of existing senolytic treatments. Using DNA recombinant technology, researchers generated EVs expressing Streptavidin-fused surface protein prostaglandin F2 receptor inhibitor (PTGFRN), incubated with biotinylated antibodies against senescent cell surface antigen glycoprotein nonmetastatic melanoma protein B (GPNMB).[55] The EVs containing D + Q were then able to eliminate senescent cells with increased efficiency, compared to the traditional method of oral administration.[55] Recent studies have found that the inhibition of certain miRNA types is able to effectively eliminate senescent cells *in vitro*, suggesting that miRNAs may indeed have a causal role in senescence. Adipose-derived stem cells (ADSCs) cocultured with senescent human umbilical vein endothelial cells (HUVECs), when transfected with miR-499a, displayed a broad-spectrum downregulation of senescence associated genes.[56] A structural mimetic of miR-19a-3p, upon delivery in oxidative stress-induced senescent osteoblasts reduced senescent cell number as quantified by SA-β-gal.[57] These findings offer a new perspective into developing agents that harness the benefit of RNA-based therapeutics, such as improved safety due to the transient nature of their effect. However, the precise mechanism of senolytic messenger RNAs (mRNAs) and its *in vivo* applicability is still poorly understood. And it remains a challenge how different miRNA can be designed to target senescence program across cell types and divergent transcriptional profiles.

Translational prospects

Mounting evidence from preclinical studies have underscored the potential of senotherapeutics in mitigating age-related pathologies and extending healthspan and lifespan in naturally aging organisms. Here we summarize *in vivo* evidence suggesting the therapeutic effect of senolytic in age-related diseases and outline the current progress in their clinical translation.

Cardiovascular diseases

Senescent cell burden in the cardiovascular system are linked to impaired muscle contraction, hindered blood flow, increased susceptibility of plaque formation, and heightened risk of cardiovascular diseases.[58] In multiple mice models of cardiovascular disorders, senolytics demonstrated therapeutic potential for cardiac fibrosis, atherosclerosis, heart failure, and hypertension.[58] Digoxin, HSP90 inhibitor 17-DMAG (alvespimycin), and navitoclax, among other senolytic agents, have been shown to significantly decrease atherosclerotic lesion formation and plaque burden in *ApoE*$^{-/-}$ mice of atherosclerosis.[59-61] In mice of doxorubicin- and angiotensin II (Ang II)-induced heart failure, navitoclax reduced inflammation, cardiac fibrosis and dysfunction.[62,63] Age is associated with poor prognosis after myocardial infarction (MI), and the presence of senescent cluster of differentiation 57^{+} (CD57^{+})CD8^{+} T cells have been correlated with incidence of post-myocardial infarction (post-MI) cardiovascular mortality. Treatment with navitoclax and D + Q in post-acute myocardial infarction mice both effectively eliminated senescent cardiomyocytes, improved myocardial remodeling and overall survival rate.[64]

Neurodegenerative diseases

Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive memory decline in which senescent cell accumulation plays a pivotal role. Primary etiologies of AD, such as amyloid β (Aβ) aggregation and tauopathy, have been associated with accumulation of p16^{Ink4a} positive oligodendrocyte progenitor cells and astrocytes.[65,66] In microtubule associated protein Tau (MAPT) P301S PS19 mouse model of tau-dependent neurodegeneration with INK-ATTAC transgene, elimination of p16Ink4a-expressing glial cells, alleviated gliosis, prevented tau hyperphosphorylation and aggregation, hippocampal neuron degeneration and improved cognitive function.[66] Additionally, D + Q treatment of AD mice reduced senescent oligodendrocyte progenitor cells in Aβ plaque microenvironment, reduced Aβ burden and inflammation, and improved cognitive function.[65] Parkinson's disease (PD) is another neurodegenerative condition of common occurrence in aged adults, which is characterized by the loss of dopaminergic neurons and impaired motor function.[67] Senescence-associated oxidative stress and neuroinflammation is known to exacerbate PD pathology.[68,69] Astragaloside IV (AS-IV), a neuroprotective and antioxidant agent isolated from Chinese medicine, was found to eliminate astrocytes undergoing replicative senescence and lipopolysaccharide/1-methyl-4-phenylpyridinium (LPS/MPP^{+})-induced premature senescence. Additionally, AS-IV restored dopaminergic neurons and alleviated PD-associated motor function decline in mice with chemically induced PD, revealing a potential PD therapeutic candidate with senolytic benefits.[70]

Musculoskeletal diseases

Senescent cell density is positively correlated with the progression of osteoarthritis (OA), a degenerative joint disease primarily characterized by cartilage breakdown and chronic inflammation. Senolytics have been found to alleviate multiple key OA pathologies, and a few have progressed into clinical trials. p53 inhibitor UBX0101-induced was found to be a potent senolytic *in vivo*, reduced oxidative stress, promoted partial cartilage repair and suppressed inflammation.[71] However, a phase 2 clinical trial using UBX0101 for OA treatment (NCT04349956) failed to meet its primary endpoint, as no significant differences were observed between the treated and

placebo groups. Intra-articular navitoclax injection on mice with post-traumatic OA effectively mitigated the proinflammatory microenvironment, restored subchondral bone damage, and enhanced cartilage maintenance.[72] Intervertebral disc deterioration (IVDD) is a prevalent condition among the elderly and a common cause of back pain and stiffness. Chronic accumulation of senescent cells with age is associated with IVDD pathogenesis in human and multiple model animals.[73] Long-term administration of D + Q in C57BL6 mice led to compartment-specific effects on delaying disc degeneration, alleviating senescence markers, and reducing SASP factors.[74] A study using poly (lactic-co-glycolic acid) nanoparticles for localized delivery of navitoclax effectively inhibited intervertebral disc degeneration and promoted structural restoration.[75]

Current and completed clinical trials

There are currently more than 20 ongoing clinical trials testing the efficacy of senolytics in the background of age-related diseases, with an increasing number of trials being initiated each year.[4] The earliest clinical trial involving senolytics was a two-center open-label pilot study (NCT02874989) involved a cohort of 14 patients diagnosed with idiopathic pulmonary fibrosis (IPF). Intermittent administration of D + Q treatment over a three-week period led to in clinically meaningful improvements in physical function.[76] A follow up study on IPF patients observed increased urinary levels ofα-Klotho post D + Q administration.[77] In diabetic kidney disease, a Phase 1 open-label trial following a similar intermittent D + Q treatment procedure (NCT02848131) reduced senescent cell count in skin epidermal and adipose tissues and lowered circulating SASP factors.[78] In another Phase 1 feasibility trial, the safety profile of intermittent D + Q treatment was confirmed in patients of AD (NCT04063124), and there was an overall trend of improvement in SASP and markers of AD pathology. Notably, D and Q exhibited varying central nervous system (CNS) penetrability. While D was detectable in cerebrospinal fluid in 4 of the 5 participants, Q was not.[79]

Coronavirus disease 2019 (COVID-19) is another excellent background to study senolytic therapy, due to the remarkable correlation between age and symptom severity and mortality from COVID-19. In a randomized phase 2 trial (NCT04861298), two-week oral Q treatment accelerated recovery from mild to moderate COVID-19 and improved symptoms of acute COVID such as increased serum lactate dehydrogenase (LDH) levels.[80] COVID-19 of fisetin (COVID-FIS), a multicenter, placebo-controlled trial is being carried out where fisetin is used for treating elderly COVID-19 positive patients in skilled nursing facilities (NCT04537299). Incidence of COVID progression is used as a primary marker to evaluate treatment efficacy. Secondary markers, such as senescent cell count, tolerability and other functional tests are also measured.[81] The trials described above, alongside other ongoing trials targeting COVID-19 (NCT0447695, NCT04771611), are expected to provide helpful knowledge for future study designs aimed at testing senolytics in viral infections and vaccination response.

Among the candidate senotherapeutic agents, Metformin is the most extensively tested in clinical trials for a variety of age-related pathologies. The vast majority of trials indicate positive clinical outcomes associated with Metformin-treated T2DM patients for frailty, cancer, metabolic disorders, cardiovascular diseases and overall mortality.[82-84] However, clinical trials investigating Metformin in the context of cognitive decline has shown some conflicting results. While a number of longitudinal studies indicate inverse correlation between T2DM and neurodegeneration, a cohort study of 4651 T2DM patients with 12-year follow up, Metformin treatment was associated with increased risk of Parkinson's disease, Alzheimer's disease and all-cause dementia.[85] This contradiction can be explained by the prevalence of comorbidity in T2DM patients and concomitant usage of other prescription drugs, which may have undesirable interactions with Metformin. Nevertheless, it highlights the importance of future controlled trials involving larger cohorts to further understand Metformin in the context of neurodegeneration in non-diabetic aging individuals. The first-of-its-kind metformin clinical trial targeting aging with metformin (TAME), currently in its initiation stage and is expected to recruit 3000 elderly adults, who will be chronically administered clinically relevant dosages Metformin over six years and assessed for effects on parameters and indications of aging.[86] Results from this trial can be expected to greatly strengthen the understanding of geroprotective benefits of Metformin in humans, including gender differences and side effects from chronic application at clinically relevant dosages.[87]

A significant limitation in clinical trials involving senolytics is the lack of a clinically relevant endpoint to accurately measure the efficacy of senolytic treatments. Current clinical benchmarks for monitoring *in vivo* senescent cell populations are limited to tissue biopsies and circulating SASP factors in bodily fluids such as blood, urine, and saliva.[4] However, in order to establish the therapeutic benefit of senolytics on aging, readily obtainable, easily measurable and reliable indicators of biological age and senescent state that correlate with clinically observed aging phenotypes are greatly needed. Other considerations for senolytic trials include optimal dosage and delivery scheme for maximum healthspan benefits. Longitudinal studies would also be beneficial, as they're critical to evaluate long-term effects of senolytic treatment on the overall health

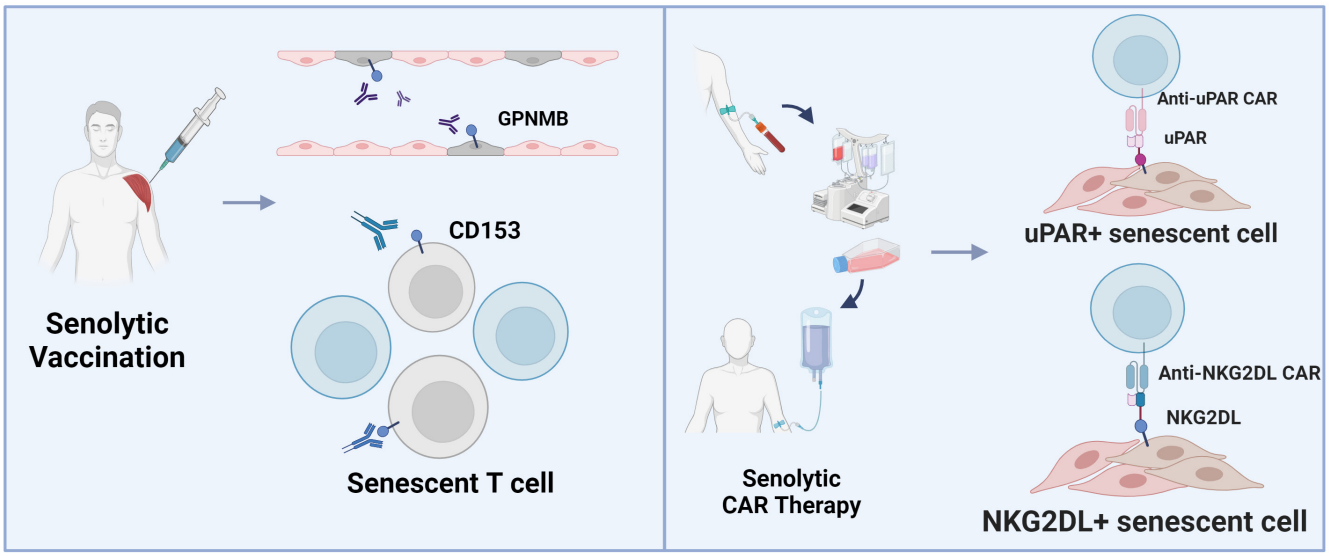

Figure 3: Seno-antigen targeted immune cell therapies and vaccines. Specifically upregulated surface proteins in senescent cells are referred to as seno-antigens which targeted to induce antibody production and elimination by genetically manipulated or endogenous immune cells. Created using BioRender.com. GPNMB: glycoprotein nonmetastatic melanoma protein B; CAR: chimeric antigen receptor; uPAR: urokinase plasminogen receptor; NKG2DL: natural killer group 2 member D ligand.

status of the patient.

IMMUNOTHERAPY FOR SENESCENT CELL CLEARANCE

The endogenous senescent cell population is regulated by both innate and adaptive immunity, with clearance mechanisms of specific cell types varying depending on their surface antigen expression.[88] Key mechanisms involved in this process include natural killer (NK) cell-mediated granule exocytosis, cytotoxicity mediated by $CD8^+/CD4^+$ T cells, macrophage-driven efferocytosis and indirect clearance through dendritic cell regulation of T cells. However, the immunosuppressive effects of the SASP impedes the immune clearance of senescent cells, leading to their accumulation with age and the pathogenesis of age-related diseases.[5] Strategies are being established to harnessing adaptive immunity for the purpose of senescent cell clearance such as vaccines and immune cell therapy (Figure 3). which rely on the identification of seno-antigens, which are highly specific and conserved surface antigen that unambiguously characterize senescent cells. Here we review the known progress in the development of these immune therapies and identify key questions for future investigations.

Seno-antigen directed vaccines

Vaccines against multiple age-related morbidities, including AD, T2DM, hypertension, atherosclerosis, and cancer have been conceived and developed over the years, a few of which are currently tested in clinical trials.[89] Due to the peculiar role of senescence in aging and chronic diseases, there has been growing interest in developing vaccines against senescent cells to achieve therapeutic or even preventative effects for these outcomes. For instance, based on the observation that senescent T cells ($CD4^+$ $PD\text{-}1^+$ $CD153^+$) highly express CD15, Yoshida *et al.* generated an anti-CD153 vaccine with a CpG adjuvant ($CD153^-$CpG, Figure 3). When mice of high-fat diet induced obesity were immunized, the vaccine led to effects including the production of IgG2 antibodies and the depletion of $CD153^+$ senescent T cells in visceral adipose tissue, along with improved obesity phenotypes such as enhanced insulin sensitivity and glucose tolerance.[90] Another study revealed GPNMB to be a potent seno-antigen. During atherosclerosis, GPNMB is broadly upregulated in upregulated in vascular endothelial cells and leukocytes. Anti-GPNMB vaccine was found to be an effective senolytic in HUVECs, eliminating senescent cells *via* NK-cell-mediated antibody-dependent cellular cytotoxicity (ADCC, Figure 3). Phenotypic changes also include improved metabolic function in high fat dies mice, reduced atherosclerotic lesion in *ApoE*$^{-/-}$ mice, enhanced physical function in middle-aged mice, and improved survival in *Zmpste24*$^{-/-}$ progeroid mice.[91]

While these studies have opened up important avenues for further investigation, much work remains to be done before these seno-antigen vaccines can be translated for practical application. First of all, it is imperative to understand the physiological roles of the target antigens and to ensure their senolytic benefits do not come at the cost of overall

health. Importantly, the research group which developed GPNMB vaccines also reported that GPNMB is an important regulator of lysosomal activity, and that genetic depletion of GPNMB can lead to shortened replicative lifespan in vascular endothelial cells, vascular dysfunction and increased formation of atherosclerotic plaques, with its effect on other tissues and organs remaining unclear. Moreover, as seno-antigen vaccines target both high- and low-expressing cells, it's important to differentiate whether the therapeutic benefits are attributed to the elimination of seno-antigen enriched senescent cells, or other cells in which seno-antigens are expressed but are independent of the senescence program. Finally, the extent of immune response induced by seno-antigen vaccines should be well controlled, eliminating senescent cells with high efficacy without inducing overt immune responses such as acute inflammation or cytokine release syndrome (CRS). The optimal dosage, vaccination frequency and antibody retention post vaccination should also be characterized should these vaccines progess into clinical application.

Immune cell therapies

In the past decade, chimeric antigen receptor (CAR)-T cells have emerged as a promising strategy for personalized immunotherapy in the realm of immune-oncology. Mechanistically, CAR-T cells circumvent cancer immune evasion mechanisms and eliminate cancerous cells in an major histocompatibility complex (MHC)-independent manner. CAR-T therapy for hematological malignancies and various solid tumors have been tested in multiple clinical trials and exhibited favorable safety and efficacy profiles.[92] There are currently five CAR-T therapies approved by the Food and Drug Administration (FDA) for clinical usage. Beyond the context of oncology, CAR-T is also being employed to target seno-antigens to achieve *in vivo* senescent cell clearance. The first seno-antigen used for this therapeutic approach is urokinase plasminogen receptor (uPAR), a cell surface receptor and activator of intracellular signaling related to inflammation, cell proliferation, migration and adhesion (Figure 3). uPAR is upregulated in cells RS, Kras-induced OIS and TIS as well as human tissues of multiple age-related disorders such as IPF, diabetes and atherosclerosis. *In vivo* experiments in mice with lung adenocarcinoma and mitogen-activated protein (MEK) and cyclin-dependent kinase 4/6 (CDK4/6) inhibition-induced senescence, the urokinase-type plasminogen activator receptor (uPAR)-directed CAR-T cells effectively depleted senescent tumor cells and prolonged survival. When used to treat liver fibrosis induced by carbon tetrachloride (CCl_4) exposure and non-alcoholic steatohepatitis (NASH), there was an improvement in multiple pathological in fibrotic tissue.[93] In a more recent study, uPAR was found to improve physical aging phenotypes and alleviate metabolic syndrome. Additionally, when used in young mice, CAR-T cells were able to persist at considerable levels for up to 12 months post initial treatment and limit the onset of metabolic and physical disorders later in life.[94] Another surface antigen explored for CAR-T therapy is the natural killer group 2 member D ligand (NKG2DL), widely upregulated in tumor and senescent cells to mediate immunosuppression and evasion from NK cell clearance but not in normal cells (Figure 3). CAR-T cells against NKG2D ligand demonstrated potent senolytic ability across a variety of senescence stimuli such as irradiation, oncogenic mutation, and genotoxic etoposide. *In vivo* mice models of induced senescence and physiological aging, murine NKG2D ligand-directed CAR-T therapy alleviated various age-related disorders. In naturally aged nonhuman primates, CAR-T cells engineered with human NKG2D ligand suppressed senescence markers and SASP factors.[95] CAR-T could be advantageous over certain small molecule therapeutics which require daily or intermittent administration, due to their persistence of senolytic capacity for prolonged periods after treatment.

However, the abovementioned studies hold a few limitations. First of all, the assessment of treatment efficacy in these studies are limited to the a reduction of senescent cell count as indicated by single markers such as p16 and SA-β-gal which, as mentioned earlier in this review, may not be an accurate representation of the exact populations targeted by the senolytic CAR-T. Characterization at single-cell level would be beneficial to understand the specific cell types involved, and to elucidate the extent to which the anti-aging benefits of senolytic CAR-T could be ascribed to their elimination of deleterious senescent cell populations, as opposed to an inhibition of other physiological processes. Moreover, considering the seno-antigens typically have important roles in intracellular signaling and regulation of immune responses, it remains to be investigated whether the inhibition of uPAR expressing cells could result in side effects such as impaired immune response, and their long-term effect on health status and lifespan remains poorly understood. This is particularly relevant considering the persistence of CAR-T cells *in vivo*, which could pose difficulty for timely treatment termination. Translation of CAR-T therapy also has inherent limitations such as high cost, complex individualized production procedure and eliciting CRS, a well-documented side effect of this therapeutic strategy. By contrast, CAR-NK cell could be a potentially favorable alternative vehicle immune senolytic therapy. These cells favor "off-the-shelf" production due less stringent requirements for autologous immune cells and less risk of graft-versus-host-disease in the allogenic setting. The likelihood of CRS is also lowered due to the difference in secretome profile between activated NK cells and T cells.

The former predominantly releases interferon-γ (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF), which are less relevant to CRS pathogenesis, compared to interleukins (IL-6, IL-8, IL-10, IL-1a), tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1), among others, which are released by CAR-T cells. The cost issue surrounding current CAR-T therapies can also be mitigated by CAR-NK, which can be derived from a variety of sources such as the NK92 cell line, induced pluripotent stem cells (iPSCs) and peripheral blood mononuclear cells (PBMCS).

New horizons: novel regulators of cellular senescence in aging

Research has revealed many insights into regulatory mechanisms of cellular senescence and organismal aging, nevertheless, novel signaling pathways and regulators of senescence are continuously being identified, unveiling new ways to alleviate senescence associated detriments and targeting senescent cells. Here we highlight select research findings that could offer valuable insight into discovery of future candidate translational senotherapeutics.

Novel roles of senescence associated signal transducers

IL-11 is a pro-inflammatory cytokine of the IL-6 family with important roles in hematopoiesis, platelet formation, fibrosis, inflammation, and tumorigenesis.[96,97] Previously, IL-11-mediated activation of MEK/extracellular signal-regulated kinases (ERK) was known to play a role in senescence-related pulmonary fibrosis in mice models of stress-induced accelerated senescence.[98] However, IL-11 had not been associated with other aspects of senescence and aging. In a recent seminal study, IL-11 was found to causally partake in multiple age-related chronic conditions. Age-associated chronic inflammation was found to be mediated by IL-11 through activating the ERK-adenosine monophosphate-activated protein kinase (AMPK)-mechanistic target of rapamycin complex 1 (mTORC1) axis, and Janus kinase (JAK)-signal transducer and activator of transcription (JAK-STAT) signaling pathway. Genetic depletion of IL-11 in mice led to marked lifespan extension of over 20% in both male and female mice. The use of IL-11 inhibitor extended both lifespan and healthspan in mice, while alleviating multiple age-related aliments such as sarcopenia, frailty and metabolic dysregulation.[99] This evidence suggest that IL-11 could be an important upstream regulator of biological aging and pleiotropic target to combat various hallmarks of aging, including cellular senescence. Due to their shared signal transducer and close resemblance in biological functions, the discovery of IL-11 as a causal player in aging could lead to insights into previously undefined role of IL-6 family protein in aging and related conditions.

Novel strategies towards reversal of cellular senescence

Phenotypic and transcriptomic changes during cellular senescence have all been liked to changes at the epigenetic level.[100] It has been demonstrated that the activation of various reprogramming transcription factors, such as the Yamanaka factors Oct4, Sox, Klf4 and cMyc (OSKM), or chemical cocktails, can lead to the dedifferentiation of cells into pluripotent state, essentially reversing cell age. Reprogramming of senescent and aged cells has garnered the attention of the research community, as they may offer a novel paradigm for alleviating senescence-related aliments without eliminating them. However, as continuous *in vivo* expression of OSKM almost inevitably results in cell identity loss and teratoma formation, partial reprogramming techniques have been used as an alternative to reverse adverse effects of cell aging while preserving cell identity.[101] Ocampo *et al.* were the first to demonstrate that transient reprogramming through short-term cyclic expression of OSKM with an inducible cassette removed aging phenotypes in cells and prolonged lifespan in mice with Hutchinson-Gilford progeria syndrome (HGPS).[102] Further studies revealed transient upregulation of OSKM, Lineage 28 (LIN28) and nanog homeobox gene (NANOG) through mRNA transfection improved phenotypic and transcriptomic in fibroblasts and endothelial cells isolated from elderly individuals.[103] Moreover, ectopic expression of OSK factors in inducible changes in the epigenome (ICE) mice developed by Yang *et al.* improved senescent phenotypes such as nuclear envelope integrity, reversed transcriptomic markers of aging and restored youthful DNA methylation landscape.[100] Nevertheless, should be noted that the reverse of cellular senescence has inherent limitations, restoring of proliferative potential lead to propagation of deleterious mutations and genetic aberrations, which are abundant in senescent cells. Hence, any translational efforts aimed at reversing senescent cell age should be proceeded with caution.

DISCUSSION

The selective elimination of senescent cells with molecule chemicals represents an innovative approach to target the hallmarks of aging. Since the discovery of D + Q as the first senolytic agent, numerous clinical drugs, synthetic products and natural compounds have been identified as candidate senolytics and senomorphics. The therapeutic benefit of senolytics is supported by evidence from preclinical studies in diseased and physiological aging models, which has fueled their progression into multiple clinical trials. Mechanistically, most senolytics inhibit survival pathways to induce apoptosis in senescent cells with relatively little harm to normal, proliferating cells. On the other hand, senomorphics inhibit SASP expression and

reduce inflammation in the surrounding tissue without inducing cell death, offering an often equally effective but safer alternative to senolytic drugs. Despite the efforts of ongoing clinical trials, the safety profile of chronically administering these small molecule senotherapeutics remains to be validated. A primary consideration is the tradeoff broad-spectrum senolytic effect and the negative effects on normal proliferating cells, due to the lack of clearly defined boundaries between senescent and cells undergoing milder, but non-senescence-inducing stresses, or even non-senescent cells that express markers of senescence. For example, some well-known side effects of continuous navitoclax treatment include thrombocytopenia, internal bleeding, and neutropenia, which might arise from the inhibition of anti-apoptotic Bcl-2 family proteins in platelets and neutrophils (Table 1).[29,104] The same is true for other chemotherapeutic-derived senolytics, whose inherent genotoxicity may pose unwanted risks for normal cells. In contrast to small molecules, seno-antigen directed immune therapies offer a more targeted approach to senescent cells with uniquely upregulated surface markers. Preliminary studies have demonstrated efficacy in reducing senescent cell burden and improving physical parameters. However, there remains the question whether established seno-antigens are sufficiently representative of the heterogeneous senescent cell population, and to what extent they are able to ameliorate senescent cell burden *in vivo*. To date the number of candidate seno-antigens remains relatively few, yet emerging technologies such as single cell proteomic and multi-omic analyses may dramatically enhancing the efficiency of seno-antigen discovery.

During the development of senescent cell targeted therapies, it is important to note that the elimination of senescent cells may not always be beneficial. Cellular senescence is known to play beneficial roles during embryogenesis, wound healing, tumor suppression and maintenance of tissue integrity. Transient initiation of senescence as a response to liver damage or cutaneous injury is known to promote tissue regeneration and prevent excessive fibrosis. Studies have shown that genetic depletion of p16 high cells may lead to disrupted physical barrier and fibrogenesis in the liver, as p16-enriched sinusoids are eliminated without eliciting replacement by new cells.[21] Where the elimination of senescent cells is unfeasible or may lead to adverse effects, reversing the senescent cell age through epigenetic reprogramming could be an alternative solution. Proof-of-concept studies with partial reprogramming have been successfully carried out through transient activation of Yamanaka factors or administration of chemicals. Although the full mechanism behind this epigenetic-mediated rejuvenation effect remains to be elucidated, it is nevertheless an intriguing research avenue awaiting future exploration.

To date, a number of senotherapeutics have progressed into clinical phase and tested in those with age-related disorders. Studies with longer durations in larger patient cohorts utilizing composite markers for a comprehensive evaluation of senescence are needed to thoroughly assess the long-term systemic effect of senotherapeutics in combating diseases and aging, hence their overall translational potential.

Since the characterization of the first senolytics, the field of senotherapeutics has expanded rapidly to encompass nearly all aspects of translational medicine. By integrating principles of pharmacological treatment and immunotherapy to eliminate or rejuvenate senescent cells, it is possible to achieve therapeutic effects superior to symptomatically intervening on aging-related diseases. Although unresolved challenges exist, we maintain a positive outlook that the safety and applicability of senotherapeutics will be improved. Continued efforts in this area of study, particularly in rigorous studies in discovery science and collaboration to validate the effectiveness and safety of senotherapeutics in clinical trials, hold great importance in combating aging and improving human longevity.

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No additional data is available.

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