The Fountain of Youth by Targeting Senescent Cells?

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Opinion

The Fountain of Youth by Targeting Senescent Cells?

Peter L.J. de Keizer[1,*]

The potential to reverse aging has long been a tantalizing thought, but has equally been considered mere utopia. Recently, the spotlights have turned to senescent cells as being a culprit for aging. Can these cells be therapeutically eliminated? When so? And is this even safe? Recent developments in the tool box to study senescence have made it possible to begin addressing these questions. It will be especially relevant to identify how senescence impairs tissue rejuvenation and to prospectively design compounds that can both target senescence and stimulate rejuvenation in a safe manner. This review argues that to fulfill this niche, cell-penetrating peptides may provide promising therapeutics. As a candidate approach, the author also highlights the potential of targeting individual FOXO signaling pathways to combat senescence and stimulate tissue rejuventaion.

Trends

Semigenetic clearance of senescent cells delays features of aging in fast and naturally aged mice establishing senescence as their underlying cause.

Viability screens with existing compounds lead to discovery of the first generation of antisenescence compounds as quercetin/dasatinib and pan-BCL inhibitors. Further optimization is required due to suboptimal selectivity or toxicity.

Cell-penetrating peptides can steer very specific protein–protein interactions and have been successful in various clinical trials. They are a potent option for forward design of antisenescence therapies.

Senescent cells can impair their environment through juxtacrine and paracrine signaling of SASP factors. This may be caused by keeping neighboring cells permanently locked in a state of dedifferentiation (Figure 3), leading to reduced tissue rejuvenation potential.

FOXOs regulate $p21^{Cip1}$, a prominent factor in senescence growth arrest, and they inhibit the stemness regulator β-catenin. As such, they could be ideal therapeutic targets to counter senescence, while promoting tissue rejuvenation.

Targeting Senescence: Why?

As we age, the ability of our tissues to fulfill their function gradually declines and eventually, most of us come to suffer from **age-related diseases** (see Glossary) in one way or another [1]. The average population life expectancy is projected to increase [2]. To ensure that these extra years are lived in good health, it is essential to invest in methods that delay **aging** and counter age-related diseases. **Senescent cells** have been found in a large number of these diseases [3], but for a long time, it has been simply impossible to address whether they were the cause or the consequence of a given pathology. **Senescence** (see Supplemental Information online) is largely absent in yeast, flies, and nematodes. Recent advances in the development of constructs that allow senescence to be longitudinally visualized (and semi-genetically removed) in mice [4–9] have finally allowed this question to be addressed (Figure 1). Using models carrying such constructs, it was found that the clearance of senescent cells could markedly extend the health and **life span** of naturally aging mice [10]. This sparked a 'gold-rush' to identify therapeutic compounds that can target senescence, and hopefully at some point, also human diseases. Several fundamental milestones have been recently surpassed, thereby forming the basis for an antisenescence therapy to become reality. In the following sections, the author discusses which criteria remain important to address, including how senescent cells hinder their own environment, whether specific subsets of senescent cells can be selectively targeted, and how tissue stimulation can be induced after senescence clearance.

Senescence in Health and Disease: The Good, the Bad (and the Ugly)

Senescence can be described as a persistent damage response that develops in healthy cells experiencing irreparable stress for a sustained period. Through independent activities of **$p16^{Ink4a}$** and **$p21^{Cip1}$**, irreversible molecular changes are induced, leading to permanent cell cycle arrest [11,12]. In the majority of cases, senescent cells develop a defined, but heterogeneous, secretory profile termed **senescence-associated secretory phenotype (SASP)** [13–15]. This comprises a range of different proteins, including several proteins known to play a role

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in aging and age-related diseases, including matrix metalloproteases such as MMP3 [16], growth factors, chemokines such as CCL2 and CLL11 [17], and prominent interleukins (ILs) such as **IL1, IL6,** and IL8 [18].

Senescence is already detectable during embryonic development where it aids in tissue patterning [19,20]. From an evolutionary point of view, it is thought that senescence is favorable over apoptosis early in life as apoptosis would lead to decreased tissue function and body size. This is for instance illustrated in progeroid mice that deteriorate extremely fast, such as DNA repair-deficient *Ercc1*$^{\Delta/-}$ [21,22], *Xpg*$^{-/-}$ [23], or *Csb*$^{-/-}$/*Xpa*$^{-/-}$ [24], all of which show high levels of apoptosis, severe tissue malfunction, and are indeed very small. Senescent cells, by contrast, remain present while being at least partially functional. Indeed, fast aging models that have a balance leaning more towards senescence (see Box 1 and Figure 1), such as *Xpd*$^{TTD/TTD}$ [25–27], BubR1$^{H/H}$ [5], and Polg^{D257A} [28] are mouse models, which are less frail and present higher body weight than the previously mentioned apoptotic models. In addition, after development, senescence can be beneficial. For instance, in skin punch mouse models, senescence aids in wound healing [7] and it can limit fibrosis following acute damage [29]. Thus, senescence can be beneficial early in life, or under transient conditions of injury.

Box 1. Animal Models to Study Senescence in Aging

Senescence predominantly appears to be a process of higher eukaryotes. For studying the effects of senescence on human age-related diseases, mouse models may therefore be ideal. In two directions, useful models have been developed:

(i) For Tracking and Eliminating Senescence

While senescent cell populations are detectable by combinations of markers, many individual senescence markers are either not solely limited to senescence (e.g., SASP factors, **alarmins**, or DNA-damage markers) or heterogeneously expressed within the population. A uniform phenotype among all senescent cells is that of persistent growth arrest, which can independently be induced and maintained by cell cycle inhibitors p16^{Ink4a} and p21^{Cip1} [11,12]. While p21^{cip1} can also be activated after transient damage [11,12,50] or by nonsenescence-related cues [51], p16^{Ink4a} appears to be unique to senescence. As such, the recently developed mouse models to track and eliminate senescence all make use of (various sizes of) the *p16*^{Ink4a} promoter. For visualization, bioluminescence and fluorescence have been placed under control of this promoter [4,9]. In addition, two elegant constructs have been designed, INK-ATTAC and **p16::3MR**, to allow for drug-inducible suicide genes to selectively eliminate p16^{Ink4a}-positive senescence at any time [5,7]. p16^{Ink4a} has been used as a biomarker for aging [30] and in these models, it was confirmed that (p16-driven) senescence increases with age [6,9].

Unfortunately, it remains difficult to track individual senescent cells for prolonged periods and the field would do best in investing in such reporters. For instance, this can be done by using p16^{Ink4a}-driven near-infrared imaging probes for intravital imaging, an addition likely to be exploited in the future [93,94].

(ii) For Studying Senescence in (Accelerated) Aging

To investigate the effects of senescence on aging and age-related diseases, it would be best to track and eliminate these cells in naturally aged mice. However, such experiments are time consuming and costly. Moreover, like humans, animals age at a different pace and therefore, the biological 'noise' is relatively high in these. Fast-aging mouse models could provide a solution to these limitations as experiments could be performed faster, at a lower cost, and deterioration occurs at a more homogeneous pace. It is important to use mice where deterioration is predominantly caused by the senescence phenotype as opposed to other mechanisms (e.g., apoptosis). In this regard, models that age at an extreme rate, for example, Ercc1$^{\Delta/-}$, *Csb*$^{-/-}$/*Xpa*$^{-/-}$, and Xpg$^{-/-}$, may be less ideal due to phenotypes related to high levels of apoptosis [21–24]. Milder fast-aging mouse models may be more suited, including BubR1$^{H/H}$ [35] and Polg^{D257A} [28], which have shown high levels of senescence and semigenetic clearance of senescence proved to be beneficial [5].

Other secondary processes in these may influence disease development and should be taken into account, such as aneuploidy, as in the case of *bubR1*$^{H/H}$ mice [35] and mitochondrial dysfunction, leading to the distinct secretion profile in *polg*^{D257A} animals [28]. Models with a relatively normal life span, but a disease development more dedicated to senescence, such as *Xpd*$^{TTD/TTD}$ [25,26], may be of great use in the future.

Glossary

ABT-263/ABT-737: pan-inhibitors of the BCL family of antiapoptotic guardians: BCL-2, BCL-XL, and BCL-W.
Age-related diseases: broad summary of diseases that mostly manifest at older age.
Aging: progressive loss of tissue function over time. Often attributed to accumulated cellular DNA damage.
Alarmin: secreted factor from cells triggering an immune response in the environment.
Antagonistic pleiotropy of senescence (theory): one cause having opposing consequences. In case of senescence, these are beneficial effects early in life and during wound healing (transient), while deleterious late in life (chronic).
Autophagy: a turn-over mechanism of cells to recycle unnecessary or dysfunctional cellular components, resulting in energy and building blocks.
Cell-penetrating peptide (CPP): a class of therapeutics making use of peptide sequences to pass a membrane and deliver a cargo. Common membrane-passing sequences involve the TAT sequence of HIV.
Dasatinib: inhibitor of the Src family of kinases.
Forkhead box 'Other' (FOXO): family of transcription factors with prominent roles in aging/longevity, growth/stress signaling, and differentiation/stemness. Can regulate expression of p21^{Cip1} together with p53 and stemness signaling through interaction with β-catenin.
Inflammaging: theory suggesting that low, but chronic, levels of inflammation can drive an age-related decline in function.
INK-linked apoptosis through targeted activation of caspase (INK-ATTAC): a construct driven by part of the *p16ink4a* promoter containing enchanced green fluorescent protein and a version of caspase-8, which is activatable by the drug AP20187. Using this construct, p16ink4a-positive senescent cells can be visualized and eliminated by this drug. As elimination only occurs in cells carrying this construct, it is semigenetic.
Interleukin-1 (IL1): an interleukin that is responsible for activation of much of the SASP and can be considered a master regulator.

Box 2. The Clinician's Corner

- Senescent cells are associated with a spectrum of diseases:
  - Main categories are: metabolic (obesity, diabetes), neurological (AD, PD), muscle, bone, and cartilage related (sarcopenia, osteoarthritis, kyphosis, herniated discs) or tissue dysfunction related (lung emphysema, renal disease, and atherosclerosis).
- Cellular senescence can be triggered by medical treatments such as chemotherapy or ischemia-reperfusion injury during organ transplantation.
- Proof-of-concept in mouse models shows senescence as being causal for a number of these pathologies. Anti-senescence therapies are thus being developed in an attempt to counter these conditions.
- Low off-target toxicity is critical for clinical translation of antisenescence therapies. This has not yet been achieved.
- Cell-penetrating peptides can specifically redirect protein–protein interactions. Where tested, they have been safe and effective in humans. They are potent candidates for prospective design of antisenescence compounds as they can selectively impair signaling that is crucial to the viability of senescent cell subsets.

It is later in life, when the levels of senescence have exponentially increased [30], that actual health problems arise (Box 2). With progressing age, there is an organism-wide increase in the expression of major SASP factors, for example, IL6 [31,32]. After crossing an arbitrary threshold, such factors can significantly impair tissue function [18,33]. Comparable to substance addiction, chronic SASP secretion by senescent cells may impair the functioning of neighboring cells. As such, senescent cells are thought to be major contributors of **inflammaging**; a theory stating that low, but chronic, levels of inflammation are drivers of age-related decline in function [34]. In agreement, senescence and SASP are elevated in a number of fast-aging mouse models [28,35,36] and, where tested, senescence clearance delays their decline in health [5]. Together, this paradoxical role of senescence is defined as the **antagonistic pleiotropy of senescence,** where its beneficial effects occur early in life and during transient wound healing, yet it is deleterious late in life, when chronically present. It is therefore at that stage that targeted therapies might be beneficial.

Three Milestones for Development of Therapies Against Senescence: Proof-of-Concept, Safe Therapeutics, and Reversal of Pathology

For antisenescence treatment to be considered a realistic option against aging, at least three fundamental milestones have to be surpassed: (i) Identifying whether senescence is a cause of aging and whether its elimination stalls this process. (ii) Determining whether senescence can be selectively targeted by compounds safe enough to not affect healthy cells. (iii) Identifying whether clearance of senescence can also be applied retrospectively to counteract features of aging that have already manifested.

The first milestone was reached by experiments performed in fast-aging (*Bubr1*$^{H/H}$) mice crossed into a systemic model for semigenetic clearance of p16-positive cells [**INK-linked apoptosis through targeted activation of caspase (INK-ATTAC)**, Figure 1]. These mice rapidly develop a range of pathologies and genetic loss of p16^{Ink4a} blunted the disease phenotype [35]. In pursuit of this result, semigenetic clearance of (p16^{Ink4a} driven) senescent cells also delayed the pathology development [5], arguing that senescence was indeed a major underlying cause. Remarkably, semigenetic elimination of senescence in naturally aged mice prolonged healthspan and caused an extension in life span of more than 18% [10]. Thus, while removal of functioning cells from frail animals may have seemed dangerous at first, it thus proved to be beneficial. These results unequivocally establish cellular senescence as one of the drivers of aging.

The second milestone, namely, obtaining safe therapeutic approaches for targeting senescence, has been partially reached in initial screens of existing compounds. Two approaches were reported to clear senescent cells: (i) individual or combinational treatment of cells with **quercetin/dasatinib** [37], and (ii) pan-B-cell lymphoma (pan-BCL) inhibitors **ABT-263/ABT-737** [38,39]. Quercetin had already been found to be beneficial against aging [40]. An

Interleukin-6 (IL6): prominent SASP factor (also activated by other processes) that can have strong implications on tissue function when highly expressed.
Ischemia-reperfusion injury: stress response in cells and tissues that are initially deprived from oxygen (ischemia), followed by sudden reflux (reperfusion). A common process during organ transplantation.
Life span: the length for which an organism lives.
Nanog: a stem cell marker indicating pluripotency.
Organoids: 3D cultures mimicking a mini organ for *in vitro* culture.
p16::3MR (trimodality reporter): a construct driven by part of the *p16ink4a* promoter containing *Renilla* luciferase (for bioluminescence), red fluorescent protein (for fluorescence), and thymidine kinase (TK) from the herpes simplex virus. The latter can phosphorylate a substrate ganciclovir (GCV). When GCV is added and taken up by cells it is incorporated into the DNA without apparent negative effect. Phosphorylation by TK however results in double-strand breaks and cell death. GCV addition would thus eliminate p16^{Ink4a}-positive senescent cells carrying this construct.
p16^{Ink4a}: protein expressed from (part of) the *CDKN2A* locus. It is an inhibitor of the cyclin-dependent kinases (CDKs) 4/6, and its expression results in dephosphorylation and activation of the retinoblastoma tumor suppressor pRb, which inhibits E2F-mediated S-phase transition in the cell cycle and a persistent cell cycle arrest. Detected in the majority of senescent cells, though not all. Often used marker for senescence.
p21^{Cip1}: protein expressed from (part of) the CDKN1A locus. Transcriptional target of the p53 tumor suppressor in response to DNA damage. Often activated in senescent cells, but also after acute DNA damage. Can induce cell cycle arrest and has prosurvival functions.
Pluripotency: having the ability as a cell to differentiate into other types of cells during a senescence-stem lock.
Quercetin: a flavonoid found in various fruits and vegetables.
Semigenetic clearance of senescence: drug-induced removal of senescent cells through the use of a construct expressed in these cells. Two examples are INK-ATTAC and p16::3MR.

antisenescence effect would be exciting, as this compound is frequently found in fruits and vegetables and is thus easy to come by. Dasatinib is an Src kinase family inhibitor and has been used against certain types of cancer, as in the case of acute myeloid and lymphoid leukemia [41]. Unfortunately, both compounds were later deemed to be nonspecific toward senescent cells, or their effects were at least cell-type dependent [38]. Consequently, their potency against senescence-driven diseases may be limited, even if promising for other indications.

The pan-BCL inhibitors, however, have been more consistently found to be selective against senescence [38,39]. They can inhibit the entire BCL family of proteins: BCl-2, BCL-XL, and BCL-W, but specifically seem to target senescent cells through inhibition of the latter two [39]. Applied with success against cancer, for example, small cell lung cancer [42], these have already been tested in humans. An important downside of these drugs is that BCL proteins, especially BCL-2, are major general inhibitors of apoptosis [43]. As they are also widely expressed [44,45], pan-BCL inhibition could thus pose a danger to other cell types. As indeed evidenced, they were found to induce off-target toxicity at least in the gastrointestinal tract, as well as in the immune and hematopoietic systems [46–48]. Another disadvantage of pan-BCL inhibitors is that they have also led to decreased organ function, as evidenced for instance, by increased aspartate aminotransferase levels in blood, indicative of reduced hepatic filtering capacity [42]. Age-related diseases can be a severe burden; however, the majority are typically not lethal, at least not in the short run [3]. While acceptable for immediate life-threatening diseases such as late-stage cancer, off-target toxicity would not be acceptable for such indications. Therefore, together with their dose-limiting effects, the use of these inhibitors may be restricted in humans to the treatment of severe indications, as in the case of terminal types of cancer [42,49], and less applicable to age-related diseases. Nevertheless, given their current potential, it will be interesting to determine whether further optimization or more selective targeting can overcome the limitations of these inhibitors.

The third milestone consists of assessing whether aging can also be countered by targeting senescence. The stalling in the deterioration and the countering of the effects of acute damage are of a different complexity than reversing damage that has already developed over a lifetime. The aforementioned compounds were found to counter the damaging effects of irradiation, showing a marginal phenotypical health benefit in progeroid mice [37–39]. However, first, it remains unclear whether these improvements are caused through clearance of senescence (and for which **semigenetic clearance of senescence** would be a critical control). Second, it remains to be determined whether it is possible to counter loss of tissue homeostasis and age-related diseases after natural aging. Future may determine whether this is the case. In doing so, it is important to realize that the mice, and eventually hopefully the patients, that are to be treated are in a very poor condition and drug safety would be of the highest importance.

Senescence: A 'Tower of Babel' Phenotype. What to Target and When?

As many targets for therapy are expressed in healthy cells as well, the question is whether it is at all possible to achieve a level of safety in which senescent cells are targeted, but their healthy counterparts are left untouched. To design the most optimal antisenescence therapy, it is therefore important to understand how senescence and SASP deregulate their neighbors and whether specific senescent cells might be more suitable targets than others. For this, better insights into the molecular mechanisms driving senescence and SASP are needed. The delicate problem here is that, as much as senescence is widely accepted to exist, its definition remains relatively loose. The complexity lies in the fact that very few senescence markers are omnipresent in all types of senescence, and neither are they often to senescence alone. For instance, though all senescent cells are permanently arrested, not all express $p16^{Ink4a}$ or $p21^{Cip1}$; and, moreover, the latter especially can be activated more transiently [11,12,50] or independently from senescence-initiating cues [51]. Likewise, SASP expression is variable between different types of

Senescence: chronic stress response after irreparable damage, associated with a state of persistent growth arrest and often an SASP. The growth arrest can be maintained by chronic expression of $p16^{ink4a}$ (predominantly) or $p21^{Cip1}$ and potentially others.

Senescence-associated secretory phenotype (SASP): the persistent secretion phenotype, which causes senescent cells to influence their environment. Most, but not all, senescent cells develop an SASP. The composition is heterogeneous per cell and type of senescence.

Senescence cells: Cells that reside in a senescent state.

Senescence stem-lock model: proposed model on how senescent cells may impair the function and rejuvenation of their environment by inducing a persistent state of pluripotency in neighboring cells. Individual SASP factors such as IL6 have been shown to induce reprogramming to pluripotency. As SASP secretion by senescent cells is chronic, this state would be permanent, causing these cells to be 'locked' in their stemlike state.

Unfolded protein response: stress response activated by misfolded proteins, for instance after oxidation, leading to activation of chaperones and heat-shock factors to induce proper (re)folding.

	Tools for detection and elimination of senescence Benefits/Considerations	Refs.
p16::FLUC	+ FLUC cloned into exonic region of p16 gene. Allows detection of endogenous p16 detection + p16-driven FLUC expression is very well vivible throughout the body – Disruption of endogenous gene affects endogenous p16 expression and life span – No option for fluorescence-based detection or sorting – Only allows detection of p16. No option for clearing p16-positive cells	[6,9]
p21::FLUC	+ FLUC cloned into exonic region of p21 gene. Allows detection of endogenous p21 + The p21-driven FLUC expression is very well vivible throughout the body + Can be combined with p16-based constructs – p21 is not solely associated with senescence and a less ideal marker than p16 – Disruption of endogenous gene affects endogenous p21 expression – Only allows detection of p21. No option for clearing p21-positive cells	[4,8]
p16::ATTAC (eGFP–CASP8) AP +	+ eGFP is functional. Allows for fluorescence-associated cell sorting + Multiple integrations of the construct, allowing robust detection of p16 expression – AP20187-inducible cell death through CASP8 is expensive – Mice have shortened life span (due to competition with endogenous locus?) – Short fragment (2.6 kb) of p16 promoter might miss endogenous regulators	[5,10]
p16::3MR (RLUC–RFP–TK) GCV +	+ Ganciclovir-inducible cell death through thymidine kinase (TK) is cheap + Single integration of construct. Near-endogenous detection of p16 expression + Relatively normal life span – RFP is nonfunctional or poorly expressed – Detection by RLUC is less optimal than FLUC for distant sites/extremities – The length of the promoter fragment (50kb) sensitizes expression to many cues	[7,38]

	Models for accelerated senescence and aging Benefits/Considerations	Refs.
BubR1$^{H/H}$	+ Genetic evidence shows many of its health problems are caused by senescence + Fast development of pathologies – Dies from causes unrelated to senescence	[5,35]
Polg^{D257A}	+ Perturbed mitochondrial signaling, leading to a unique secretion profile - MiDAS – Does not develop a classical SASP	[28]
Ercc1$^{\Delta/-}$	+ Very short life span. Sucessfully used for life span studies + Develops a wide variety of health issues – Senescence is detectable, but at mild levels – Role of senescence in pathology uncertain due to high levels of apoptosis	[21,22]
Xpd$^{TTD/TTD}$	+ Decreased healthspan, but long life span allows longitudinal analysis of therapy + Develops a range of health issues + Senescence easily detectable in various organs – Not well suited for life span studies	[25–27] Unpublished

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Figure 1. Detecting and Eliminating Senescent Cells in Mouse Models for Aging. The figure provides a comparison of the selection of tools available to detect and eliminate senescent cells and address their importance in aging. Top: Overview of constructs used to detect and eliminate senescence in mouse models, designed around promoter regions of the prominent senescence genes encoding p16^{Ink4a} and p21^{Cip1}. Bottom: A selection of models where senescence and aging occur at an accelerated pace (along with other complications), and where targeting senescence appears to be beneficial to health [4–10,21,22,25–28,35,38]. Abbreviations: eGFP, enhanced green fluorescent protein; FLUC, firefly luciferase; RFP, red fluorescent protein; RLUC, *Renilla* luciferase; SASP, senescence-associated secretory phenotype.

senescence [28,13,52], may even be entirely absent [53], and many individual factors are also produced under conditions that have little to do with senescence, such as acute inflammation [54,55]. Similar facts are true for other markers of senescence. For instance, the condensation of DNA often accompanying the growth arrest [56] only occurs in a subset of senescent cells expressing $p16^{ink4a}$ [57]. Moreover, the release of noncanonical SASP-secreted factors such as high mobility group B1 is dependent on activation of the p53 arm of senescence that also regulates $p21^{Cip1}$ expression, but which may not always be active [58].

Given the importance of SASP on tissue function, and potentially on therapeutic responses, it will be important to improve our understanding on how senescent cells regulate their secretion profile. This may be random, but more likely, it is an orchestrated event. Circadian rhythms, or the cell cycle state at the time of senescence induction, might dictate SASP development regardless of later events. Once established, however, SASP may also be under active regulation of protein quality-control mechanisms. Indeed, increased protein stress, through interference with the **unfolded protein response,** can enhance SASP expression [59]. Similar effects have been shown upon inhibition of protein turnover through **autophagy** inhibition [60,61], whereas autophagy kick-starters such as rapamycin have been recently found to blunt SASP expression [62,63]. To complicate matters further, all of these processes may be under regulation of external cues.

In summary, although semigenetic clearance of senescence has proved to be effective in delaying aging, designing antisenescence therapies adds a different layer of complexity. For such a therapy to be effective and safe, we need to better understand what mechanisms influence the secretion profile of senescent cells and how they influence therapy responses (Figure 2 and Outstanding Questions). It will therefore become important for the field to better define the various subtypes of senescence. A one-size-fits-all therapy might work, but it would be even more attractive to develop methods that only target those subsets of senescent cells that are truly deleterious.

What about Tissue Rejuvenation after Senescence Clearance?

Once a successful antisenescence therapy has been developed, it is equally important to consider what happens once senescence has been successfully removed. To address this, it needs to be understood how senescence, and especially SASP, impairs the local environment. One explanation is that they may interfere with tissue rejuvenation by blocking the renewal of lost or damaged cells. Plausibility for this scenario comes from several observations showing that SASP molecules can influence stem cell signaling and differentiation. For instance, chronic IL1 exposure can induce aged appearance of the hematopoietic system by hampering stem cell differentiation [64]. IL1α and IL1β are master regulators of SASP and induce the expression of a range of downstream factors, such as IL6 [65]. IL6 has been shown to be required for induction and maintenance of **pluripotency** [66] and in general, SASP factors such as IL6 favor the emergence of more stemlike cells by regulating the expression of pluripotency factors such as **Nanog** [67,68]. Indeed, senescence was recently shown to trigger tissue reprogramming *in vivo*, leading to Nanog-positive cells in the vicinity of areas of senescence [95]. Senescent cells might thus trigger reprogramming of neighboring cells into more pluripotent cells. However, since the release of SASP factors (such as IL6) is continuous, they would effectively make this change permanent and keep their neighboring recipient cells locked in this stemlike state. This gives credit to a 'senescence-stem lock' model to explain how senescence may reduce rejuvenation and promote aging (Figure 3). On the one hand, young tissues with few senescent cells would be able to replenish damaged and lost cells by a transient SASP response, causing temporary cell reprogramming and subsequent proliferation/differentiation responses. Aged tissues, on the other hand, contain high levels of senescent cells, which due to chronic SASP secretion, would inflict permanent reprogramming on their neighboring cells. As such, they prevent tissue rejuvenation by 'locking' these cells in a state of de-differentiation and

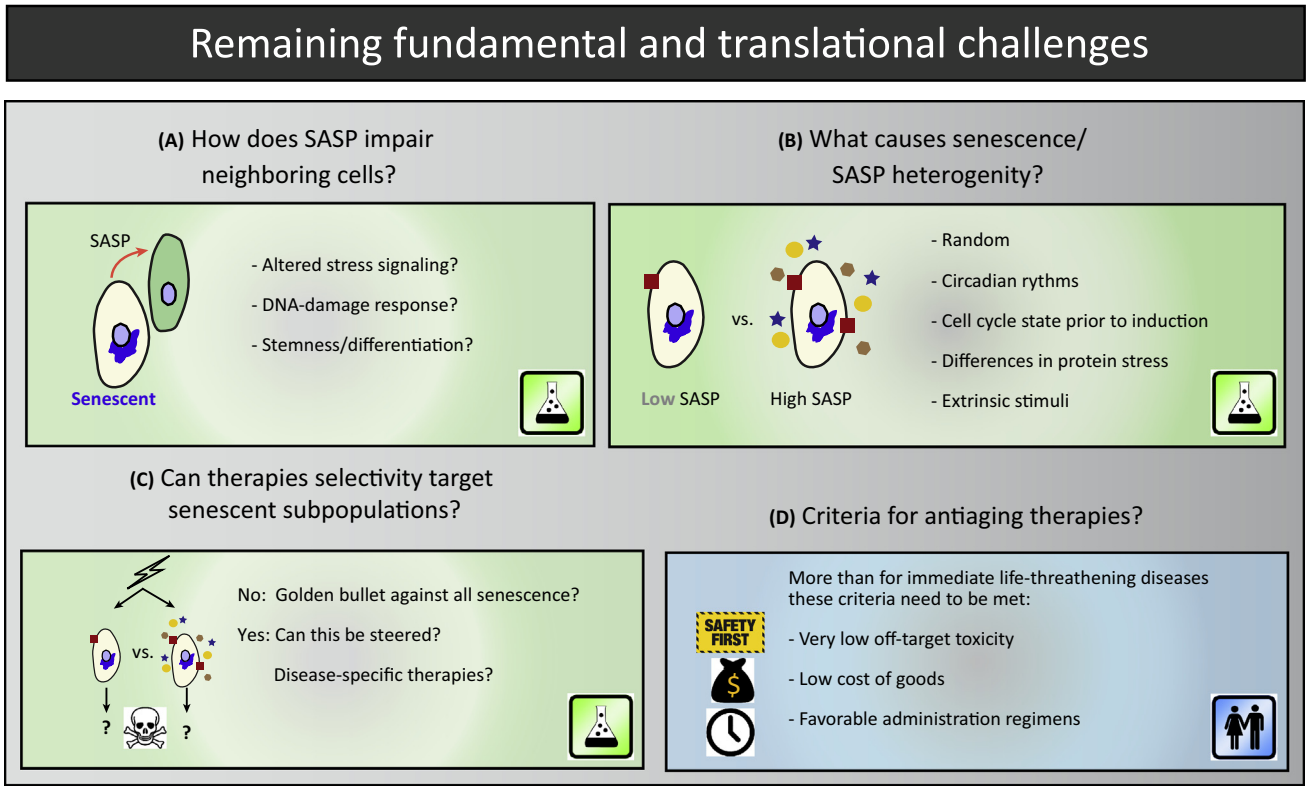

Figure 2. Fundamental Questions and Considerations for Clinical Translation of Antisenescence Therapies. (A) It is evident that senescent cells impair their surrounding milieu and produce a SASP, the individual components have been shown to alter signaling in recipient cells. It remains largely unclear, however, how SASP causes dysfunction in neighboring cells. Specific SASP factors may induce changes in recipient cells that differ from other SASP factors. Identifying their consequences may aid in the development of more tailored therapies. (B) While most senescent cells develop SASP, there is considerable heterogeneity in the expression of individual SASP factors. Several possibilities account for these differences as listed. (C) Using these differences in senescence/SASP expression, is it possible to design therapies to selectively target subsets of senescent cells? (D) Even when A–C have been answered and a successful preclinical therapy is designed, there are considerations for clinical translation. These especially concern the safety of the treatment, costs-of-goods, and dosage frequency. Abbreviation: SASP, senescence-associated secretory phenotype.

thereby, impair the replacement of lost cells. To challenge this model, how senescence – and especially clearance – influences stem cell function and tissue renewal *in vivo* needs to be investigated. From ongoing research, it will soon become clear whether senescent cells are a source of reprogramming through Nanog or other factors. The implications of such results are of great importance for determining the role of senescence in the aging process and for optimizing therapeutic discovery. As an analogy, when taking a defective car for repair, it is not sufficient to remove rust and defective parts if these are not replaced by new ones. A similar truth is applicable to antisenescence treatment. Targeting senescence may be effective in clearing out the malfunctioning parts of an organ, but stem cell differentiation would likely need to be stimulated as part of an antisenescence therapy.

Targeting Senescence and Stimulating Rejuvenation: Veni, Vidi, FOXO?

The most ideal compounds to counteract loss of tissue function would be those that target senescence and stimulate rejuvenation. Of particular interest in this regard, the members of the forkhead box O family might serve this function (Figure 4). **FOXOs** are important cell cycle inhibitors [69]. They are well-known players in longevity in nematodes, flies, and mammals, serve as targets of the insulin/insulin-like growth factor 1 pathway [70], and are mediators of antioxidant responses [71,72] and DNA damage repair [73]. Together with p53, FOXOs are prominent

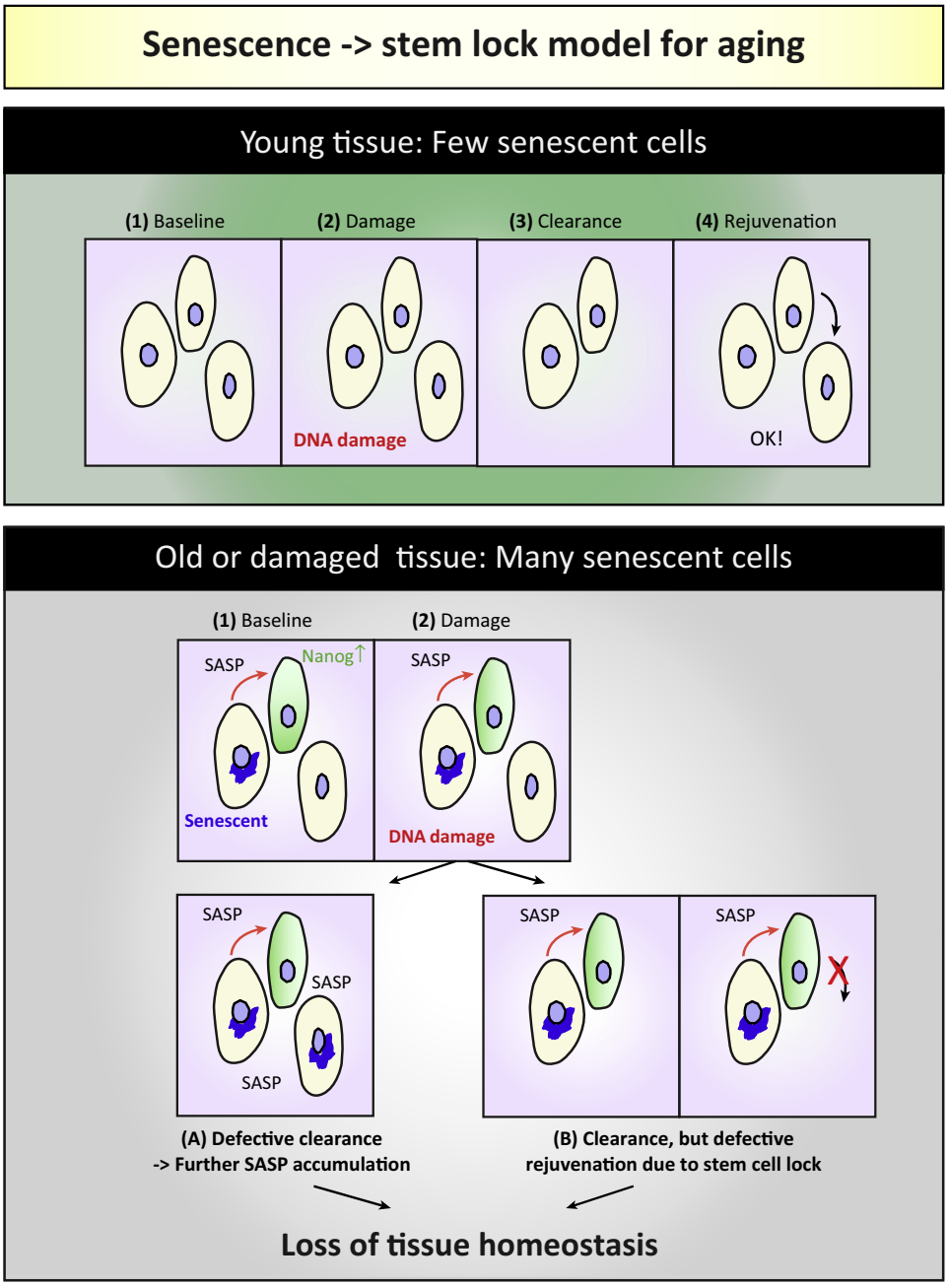

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Figure 3. 'Senescence Stem-Lock' Model for Impaired Tissue Rejuvenation During Aging. Top: When cells become damaged to the extent where they need to be replaced, stem cell differentiation can facilitate tissue rejuvenation. In young tissues, very few senescent cells are present to interfere. Bottom: Senescence is elevated in aged tissues or tissues that have experienced high levels of stress, for example, after medical procedures such as chemotherapy or **ischemia-reperfusion injury.** These senescent cells secrete SASP factors, which can induce neighboring cells to reprogram into a pluripotent state, for example, characterized by Nanog expression. Given that SASP secretion is persistent, these cells would remain 'locked' in this state and would not easily differentiate to replenish lost cells in case of tissue repair. When surrounding cells in aged tissues become damaged, two scenarios would occur: A, Damaged cells fail to turn over as fast as would occur in young tissues. B, When tissue rejuvenation is needed in case there is turn over, the cells surrounding the senescent cells are less able to replace lost cells as they are 'locked' in their stemlike state. Both the prolonged survival of damaged cells and the loss of capacity to replenish lost cells would lead to a decrease in tissue function. The ideal antiaging therapy would target senescence, while promoting tissue rejuvenation. Abbreviation: SASP, senescence-associated secretory phenotype.

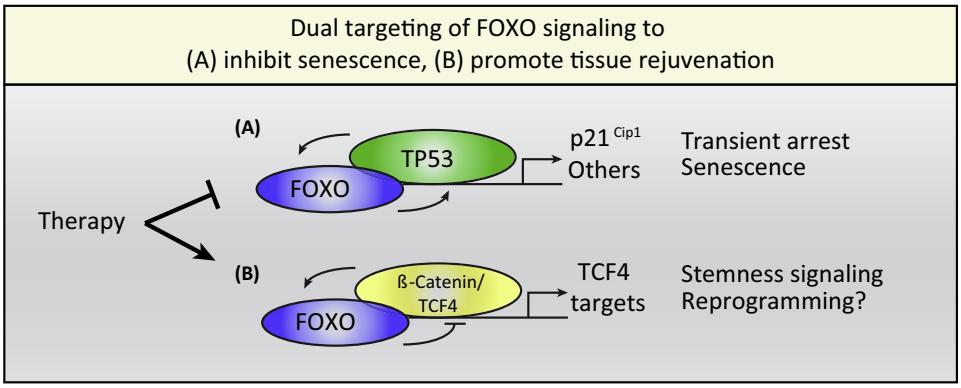

Figure 4. FOXOs: An Option for Dual Targeting of Senescence and Tissue Rejuvenation. FOXOs play diverse roles in cell cycle arrest, survival, and stem cell signaling. (A) In situations of cellular damage, FOXOs can interact with p53 to regulate the expression of $p21^{Cip1}$ to induce cell cycle arrest and senescence. This response would also lead to very strong prosurvival cues. (B) Independently, FOXOs can physically associate with β-catenin to repress stemness signaling. A therapy to interfere with FOXO/p53 and stimulate FOXO/β-catenin could both target senescence, while stimulating rejuvenation.

activators of the cell cycle arrest gene $p21^{Cip1}$, which is often elevated during senescence [74]. $p21^{Clp1}$ is reported to have prosurvival functions [75], and conversely, the p53-mediated $p21^{Cip1}$–senescence response can counteract apoptosis very potently [76]. p53–$p21^{Cip1}$ can actively block reprogramming [77] and expression of stemness markers such as Nestin [78]. Similarly, FOXOs can block differentiation [79] and physically impair β-catenin function leading to a loss in expression in stemness signaling [80,81]. Joint signaling by complex formation of FOXO/β-catenin has recently been linked to $p21^{Cip1}$ expression, further suggesting a connection between these pathways [82]. As FOXOs and p53 physically interact [83], this association may represent an exceptionally interesting point of therapeutic intervention. For doing so, it will be important to unravel how on the one hand, the interplay between FOXOs and p53 influences senescence, and on the other hand, how FOXO/β-catenin might influence pluripotency and differentiation within senescence-deprived tissue (Figure 4). Development of a method for dual targeting of both would be ideal, but also a major challenge. A class of therapeutics that may be well suited for this includes **cell-penetrating peptides (CPPs),** which can specifically interfere with defined protein–protein interactions.

Cell-Penetrating Peptides: Therapeutics against Senescence?

It is currently unclear how senescent cells choose a state of permanent arrest over cell death. Once it is known how they maintain their viability, it should be possible to develop compounds that more selectively avoid clearing nonsenescent cells. One way to limit off-target toxicity of antisenescence therapies would be to design compounds that not only show high selectivity for senescent over normal cells, but also specifically target the subset of senescent cells that are truly deleterious. These therefore need to be very specific and CPPs may fulfill this niche [84]. CPPs are typically designed to mimic surface-exposed protein–protein binding motifs and are generally fused to a moiety to allow cellular uptake at low energy cost [85]. Common uptake mediators in this regard are highly positively charged sequences such as the TAT sequence of HIV (e.g., reviewed in [86]). CPPs have several advantages over broad-range inhibitors, making them interesting candidates for antisenescence therapeutics. First, one of the biggest advantages of CPPs is that they are not restricted to enzymatic pockets, such as the ATP-binding cassettes on which so many kinase inhibitors rely. This way, they can be designed to predominantly target defined protein–protein interactions by competing for one of the endogenous interaction partners. This can indeed be effectively applied to perturb cellular signaling, as was

recently shown for the enzymatic regulation of a FOXO-related Forkhead protein, FOXM1 [87]. In doing so, CPPs do not necessarily inhibit the entire function of their target, but may steer parts of a signaling cascade into a desired direction, while leaving the remainder unaffected. This would be ideal to lower off-target toxicity. Second, given that CPPs are not limited to predefined binding pockets, the choice of targetable domains is vast. This poses an advantage over broad-range inhibitors that are for instance so often used to tune down kinase activity, rendering them ideal for the specific manipulation of a critical pathway that would otherwise be dangerous to interfere with. Finally, due to their positive carrier charge, CPPs are usually predominantly hydrophilic. This makes them ideal for intravenous systemic therapies. A potential risk might be that for systemic therapies, high concentrations of peptides might be needed. This in turn might become toxic. However, their general rapid cellular uptake *in vitro* and *in vivo* argues against this concern. One of the first successful peptides to be used *in vivo* was D-JNKi, a c-Jun N-terminal kinase (JNK) targeting CPP, which proved to be neuroprotective after systemic application in rodent models of stroke [88]. Various successful clinical trials using CPPs have been undertaken, further underscoring their potency. For instance, D-JKNi was well tolerated in clinical Phase I trials administered systemically or locally in individuals with intraocular inflammation [89,90], and proved to be effective in a randomized double-blinded Phase II trial for hearing loss [91]. Moreover, systemic treatment with a p53-targeting CPP (named p28) was well tolerated and proved to be successful in a Phase I trial against solid tumors [92]. Thus, systemic treatment with CPPs can indeed be safe and therapeutically effective. As such, CPPs might be effective against key pathways selectively important for senescent cell viability (Box 2).

Concluding Remarks

Can Targeted Therapies Against Senescence Reverse Aging, or does it Remain Fiction?

Given the recent high-profile reports on this topic, the idea of fighting the effects of aging by targeting senescence is at least plausible. However, it is surprising that in decades of modern research, and the roughly half a century in which senescence has been known, nobody has discovered compounds that are beneficial to health by influencing senescence. It is therefore important to separate fact from speculation and temper unrealistic expectations. Targeting senescence may simply not lead to the fountain of youth. That being said, with anti-senescence therapies we are the furthest we have ever been on the path to healthspan extension and restoration of the loss of health experienced during aging.

From ongoing research, it will become clear to what extent senescent cells can indeed inflict a permanent lock in the stemlike state of their surrounding cells and whether targeting senescence may influence tissue repair and rejuvenation. By extension, the possible role of FOXOs as therapeutic targets may become clear. Targeting senescence and stimulating rejuvenation might at least potentially counter individual age-related diseases and in doing so, we might be getting closer to achieving the goal of developing a 'therapy' against aging (see Outstanding Questions). Coming years will undoubtedly see exciting developments to come.

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Supplemental Information

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Outstanding Questions

How do senescent cells impair the function of their neighbors?

Senescent cells secrete a range of proinflammatory chemokines and cytokines. It is still unclear how all of these come together to deregulate their environment. Effects on reprogramming and tissue rejuvenation are seen (see also the postulated **senescence stem-lock model**). How these are regulated remains elusive and identification will aid in therapy development.

What causes the heterogeneity in senescence and the SASP?

Many senescence markers are only detectable under certain conditions and even vary in seemingly homogenous senescent populations. This suggests that either there are different subsets that need to be better defined or the expression of these markers is volatile. Identifying 'good' from 'bad' senescent cells will aid in optimization of antisenescence therapy.

How does heterogeneity influence antisenescence treatment response?

The mechanisms that cause senescence heterogeneity may also influence therapy responses. Steering these may therefore lead to compounds to clear all senescent cells in a 'golden bullet' approach, or to only hit specific subsets.

Can the complexity of senescence be recapitulated in 3D *ex vivo*?

The interplay of senescent cells with the environment is a crucial component of why senescence is deleterious to healthspan. This is difficult to properly address in 2D. Mouse models are a powerful solution, but these are timely, expensive, and ethically burdened. Development of 3D **organoid** cultures or tissue slice cultures may provide *ex vivo* solutions.

What is needed for clinical translation of antisenescence therapies?

In contrast to immediate life-threatening diseases such as late-stage cancer, many age-related diseases are more of a (serious) nuisance. Off-target toxicity, high costs of goods, and

impractical administration schedules may be justifiable for the former, but are unacceptable for the latter. Answering the outstanding questions might improve the odds of clinical translation.

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