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Targeting Cell Senescence for the Treatment of Age-Related Bone Loss

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Abstract

Purpose of Review We review cell senescence in the context of age-related bone loss by broadly discussing aging mechanisms in bone, currently known inducers and markers of senescence, the senescence-associated secretory phenotype (SASP), and the emerging roles of senescence in bone homeostasis and pathology.

Recent Findings Cellular senescence is a state of irreversible cell cycle arrest induced by insults or stressors including telomere attrition, oxidative stress, DNA damage, oncogene activation, and other intrinsic or extrinsic triggers and there is mounting evidence for the role of senescence in aging bone. Cellular aging also instigates a SASP that exerts detrimental paracrine and likely systemic effects.

Summary With aging, multiple cell types in the bone microenvironment become senescent, with osteocytes and myeloid cells as primary contributors to the SASP. Targeting undesired senescent cells may be a favorable strategy to promote bone anabolic and anti-resorptive functions in aging bone, with the possibility of improving bone quality and function with normal aging and/or disease.

Keywords Cell senescence · Osteoporosis · Aging · Telomere dysfunction · Senescence-associated secretory phenotype · Senolytic drug

Introduction

Features of bone aging include reduction in bone mass and bone mineral content, changes in shape and structural constituents of bone, higher levels of bone marrow fat, and increases in bone turnover [1]. With changes in the matrix, bone architecture is altered by the rearrangement of trabecular struts, subperiosteal expansion, and enlargement of the medullary cavity [2]. There are also changes in the crystalline properties of calcium deposition. When these factors are combined with hormonal changes, decreased physical activity, reduced responsiveness to mechanical loading, and dietary inadequacies in the elderly, disruption of normal bone homeostasis occurs. The overall outcome is age-related loss of bone mass and strength, ultimately leading to osteoporosis, and associated with an increased risk for fractures as well as a reduced capacity for healing after injury [3].

Current interventional strategies for osteoporosis due to aging, bone loss due to co-morbid conditions that occur with aging, and other secondary causes that may be present in older adults include treating underlying causes and using pharmacologic compounds that target unfavorable bone turnover. With respect to the latter, available medications usually employ mechanisms of action that limit excessive osteoclastic resorption or promote osteoblastic function, and to a lesser degree a combination of both. Another approach to age-related bone loss is a paradigm shift based on the idea that targeting primary aging processes will have beneficial effects across multiple aging tissues including bone. This paradigm shift invokes the detrimental effects of senescent cells that accumulate in aged tissues, and, conversely, that clearance of senescent cells (or the inflammatory mediators they produce)

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will restore normal physiological function. In this review, we will explore aspects of bone aging that may be contributed by senescent cells, mechanisms by which senescence may cause bone loss, markers that may be useful in identifying senescent cells in the bone microenvironment, the evidence that senescence may be a factor in non-senile osteoporosis, and potential pharmacological interventions that target senescent cells.

Bone Aging

Peak bone mass is obtained during the third decade of life after which bone mass plateaus and then declines. This loss occurs mainly due to decreased bone formation relative to bone resorption. Age-related decreases in bone volume in men occur primarily as a result of trabecular thinning, whereas in women, it involves loss of whole trabecular components [4]. It can therefore be understood that bone loss with aging in men is less profound but steady, when compared to women who experience bone loss during perimenopause and an increase in bone turnover in the early postmenopausal period. Furthermore, bone remodeling is also directly affected by a reduction in androgen (testosterone) and estrogen levels with aging that implicate uncoupling of bone formation and resorption [5]. Normally, estrogen has an inhibitory effect on osteoclasts and a loss of this inhibition causes higher bone turnover. Estrogen deficiency is a major cause of postmenopausal bone loss and contributes to age-related bone and pathogenesis of osteoporosis in both sexes. Estrogen levels have an effect on several cell types, including T cells, and its deficiency results in increased tumor necrosis factor (TNF) secretion that promotes RANKL-induced osteoclastogenesis. Other indirect effects of estrogen deficiency on bone metabolism include reduction of both intestinal and renal calcium absorption [6]. Given that estrogen is a major regulator of bone metabolism also in men, low estrogen levels lead to increased fracture risks, irrespective of normal testosterone levels.

Additional contributing factors to loss of bone are linked to increases in the levels of PTH which is caused partly due to reduced renal function as well as intestinal calcium resorption that occurs with aging [7]. The latter effect is also triggered by reduced vitamin D levels occurring concomitantly with estrogen reduction [8]. Mechanistically, the key regulatory WNT pathway is affected and results in reduced osteoblast formation, as well as alterations in signaling and activation of osteoclasts [9]. A notable humoral factor contributing to bone aging is insulin-like growth factor (IGF), specifically IGF-1, an important mediator of growth hormone (GH) which plays roles in proliferation, development, and lifespan. With a reduction in IGF-1, there is a notable impairment of bone formation and osteocyte function ultimately contributing to bone loss by causing imbalances in bone remodeling [10, 11]. Together, these critical interconnected activities contribute to bone aging [12]. Bone aging appears to be mediated by complex interactions at multiple levels, including cellular components, and alterations in tissue-intrinsic endogenous growth factors and cytokines, as well as inflammatory factors such as interleukins (e.g., IL-1α, IL-1β, TNFα, IL-6, IL-7) that promote osteoclastogenesis [13].

Bone loss due to aging has also been implicated as a consequence of senescence mechanisms that affect bone cells. Cellular senescence is a process in which cells cease to divide and exhibit phenotypical changes that include changes to the chromatin as well as the secretome due to a variety of triggers (discussed in detail in later sections). In vivo age-related senescence in bone still remains to be fully explored, with a majority of studies focused on in vitro senescence. Farr et al. report cell types within the bone microenvironment that undergo senescence with aging in vivo wherein they report senescent myeloid cells and senescent osteocytes to be the key sources of the senescence-associated secretory phenotype (SASP) in bone and bone marrow, with increased levels of NF-kB, IL-1α, and other major SASP markers including IL-6, IL-8, RANTES, M-CSF, PAI-1&2, matrix metalloproteinases (MMPs), and TNFα [14••]. Additionally, osteocytes from old mice exhibit telomere dysfunction and satellite distension.

It is important to also note that senescence is not restricted to only mitotic (proliferating) cells but also to non-dividing post-mitotic cells, such as osteocytes [14••]. One of the major deleterious effects of senescent cells is their secretion of various factors that cause neighboring cells to become dysfunctional, thereby compromising normal tissue functions. In the context of targeting cellular senescence, given the beneficial effects of clearing senescent cells, recent studies have shown that inhibition of the pro-inflammatory secretome using a Janus kinase (JAK)-inhibitor or senolytic drugs results in lower bone resorption without loss of trabecular bone and with increased cortical bone formation ([15••]). Despite these recent advances, further studies are required to address the challenges in defining the complexity of aging mechanisms as it relates to bone [16].

Features of Cellular Senescence

Cellular senescence is a state of permanent cell cycle arrest and is a process that mammalian somatic cells undergo in response to a variety of stressors or exhaustive proliferation [17]. Characteristic features of senescence include telomere shortening, genome and epigenome instability, oxidative damage, and a viable but non-replicative state. The secretion of pro-inflammatory molecules (i.e., the SASP) has deleterious local and systemic consequences including recruitment of inflammatory cells and pro-fibrotic factors [18, 19], resulting in

the loss of normal cellular and tissue function [20]. Cells undergoing senescence are phenotypically different from quiescent cells in that they have undergone an irreversible cell cycle exit that is characterized by a unique senescence phenotype accompanied by secretion of a plethora of factors that cause dysfunction of nearby cells [21].

Specifically, bone senescence involves a complex interaction of several cellular and structural components of bone tissue with both local and systemic factors contributing to senescence. Local factors that are more specific to the bone microenvironment include the expression of endogenous factors that have direct effects on osteoblasts, osteocytes, osteoclasts, and mesenchymal stem cells (MSCs), along with perturbations to the normal signaling cascades and paracrine mechanisms leading to bone cell aging [22]. Even though the numbers of senescent cells in advanced aged tissues are low, these cells have characteristic negative effects on the bone microenvironment through their SASP and likely distant effects on the entire skeleton and other tissues. In normal physiological aging, senescent myeloid cells and senescent osteocytes appear to be the major contributors to SASP in bone. Senescent cells in the bone microenvironment show an increased expression of $p16^{Ink4a}$ in osteoblast progenitors, osteoblasts, and osteocytes [14••, 15••].

Inducers of Senescence

Cellular senescence is elicited by several intracellular or extracellular factors which include dysfunctional telomeres that act as triggers through the p53 pathway, telomere-independent DNA damage and/or mutations, and oncogene expression, as well as alterations to the heterochromatin (Fig. 1). These multiple stimulatory factors induce pre-senescent cells to attain a senescent phenotype. Senescence can also be induced by increases in p16 expression due to oxidative stress [23], genotoxic stress [24], and mitochondrial injury [25] as well as toxins [26] and chemotherapeutic drugs. These stressors have been linked to the induction of senescence through direct DNA damage or replication stress-induced DNA damage [27, 28].

Telomere Dysfunction Age-associated telomere shortening and uncapping is an important inducer of senescence in bone cells as it reduces proliferation of the cells, and, in the case of MSCs, limits osteoblast differentiation. Studies in mice with loss of telomerase reverse transcriptase (Terc) show lower bone mass with reduced osteoblast differentiation and relatively increased osteoclastogenesis [29]. This has been shown to involve increased p53 expression causing cell cycle arrest, apoptosis, and diminished Runx2 expression [30]. It has also been shown that overexpression of telomerase in MSCs results in maintenance of osteogenic differentiation in vitro as well as increased bone formation in vivo [31, 32, 33•, 34].

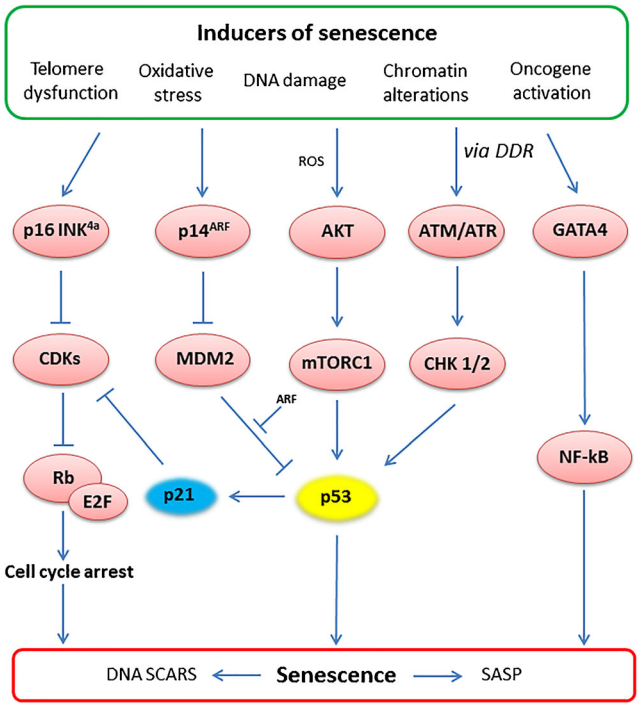

Fig. 1 Cellular senescence induction pathways and mechanisms. Senescence is induced by several factors including telomere dysfunction, oxidative stress, DNA damage, and chromatin alterations, as well as oncogene activation. Key pathways involved in senescence are the $p16^{INK4a}$/Rb and the p53/p21 pathways. Upregulation of the $p16^{INK4a}$ in response to oxidative and other stress leads to inhibition of cyclin D-dependent kinases (CDKs). When Rb is non-phosphorylated, it binds to E2F, thereby causing cell cycle arrest leading to senescence. CDK activity is inhibited by both $p16^{INK4a}$ and p21. Under non-stress conditions, p53 is degraded by MDM2 (mouse double minute 2 homolog, also known as, E3 ubiquitin-protein ligase), and this process is inhibited by ARF. Activation of p53 induces either growth arrest or apoptosis depending on cellular context. Additionally, p53 functions to repress proliferation in part via p21 expression, which is a p53 transcriptional target. Another major pathway involved in senescence is signaling though the phosphoinositide-3-kinase (PI3K)–AKT–mammalian target of rapamycin (mTOR) pathway via mTORC1 which promotes p53-dependent senescence. Additionally, DNA damage response (DDR) results in activation of the checkpoint kinases ATM/ATR and CHK1/2, leading to p53 accumulation. Through p53 activation, persistent DNA damage foci result in DNA segments with chromatin alterations reinforcing senescence (DNA SCARS). The DDR also activates GATA4 that serves as a key regulator in the activation of the senescence-associated secretory phenotype (SASP). Suppression of autophagic degradation of GATA4 leads to upregulation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), which leads to induction of senescence

Using a telomerase-based accelerated aging model, Pignolo et al. have provided evidence that replicative aging of osteoblast precursors is an important mechanism of senile osteoporosis and that age-related osteoporosis is the result of impaired osteoblast differentiation in the context of intact osteoclast differentiation. Consistent with premature senescence, MSCs from these mutant mice possess a reduced in vitro lifespan characterized by impaired osteogenic potential [35••].

DNA Damage Unrepaired DNA damage is another potentiator of senescence. This mode of senescence induction is also known to depend on p53 and p21 pathways (Fig. 1) [36–38]. Since this mechanism also induces p16, there is a secondary arrest of cell replication due to p16 in cells with DNA damage. DNA damage response (DDR) is usually activated by double-strand breaks. Any deficiency in non-homologous end joining, which involves factors such as DNA-PKcs, Ku70, Ku80, and their dimers, accelerates senescence [39]. Another possible association between DNA damage and senescence is BRCA1 and its loss leads to impaired DNA repair. Through p53 activation, persistent DNA damage foci result in DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS) [40, 41]. Mediators of DNA-SCARS include CHK2, p53, and the absence of DNA repair proteins such as Rad51 [42]. Other contributors include genotoxic stressors resulting in inactivation of a signaling cascade involving DNA damage via ATM or ATR repair kinases. Also, the DDR activates GATA4, a key senescence regulator that functions as an important switch in the senescence network to activate the SASP (Fig. 1) [43•, 44]. Mechanistically, upon induction of senescence, ATM and ATR independent of p53 and $p16^{INK4a}$ pathways to induce arrest of cell cycle, also suppress autophagic degradation of GATA4, eventually leading to NF-κB upregulation and the induction of the SASP [43•].

Reactive Oxygen Species Another trigger of senescence is increased oxidative stress which causes damage in the bone microenvironment. With aging, reactive oxygen species (ROS) increase and cause cell death. Apoptotic effects on osteoblasts and osteocytes have been linked to increases in ROS and an associated decrease in glutathione reductase levels. These have been shown to increase the phosphorylation of p53 that in turn regulates senescence. Furthermore, increases in endogenous glucocorticoids and lipid oxidation also contribute to oxidative stress which subsequently downregulates Wnt signaling. Increased ROS promotes osteoclast activity whilst inhibiting osteoblast formation and function [45, 46].

Chromatin Alterations The conformational states of chromatin (active euchromatin or silent heterochromatin) depend mainly on histone modifications. Senescence can be induced by inhibition of the enzyme histone deacetylase (HDAC) which promotes the formation of euchromatin [47, 48]. The senescence response to HDAC inhibition involves p21 and p16 expression as well as the p53 pathway (Fig. 1). Histone trimethylation of Lys9 on histone H3 (H3K9me3) is known to directly influence the formation of global heterochromatin and senescence associated heterochromatin foci [49]. Recent studies show that inhibition of S-adenosylhomocysteine hydrolase induces senescence. Another study highlights EZH2-mediated senescence through two distinct mechanisms: [1] loss of EZH2 in proliferating cells triggers DNA damage before causing an expected reduction in H3K27me3 levels and (2) loss of H3K27me3 resulting in the induction of p16 expression and production of SASP markers IL-1α, IL-1β, IL-6, IL-8, MMP3, CCL2, and CXCL1, both mechanisms underscoring the importance of poly-comb groups (PcG)–mediated epigenetic induction of senescence [50].

Oncogene-Induced Senescence This mechanism of inducing senescence is mediated by RAS/RAF/MAPK signaling owing to oncogenic mutations in RAS or RAF proteins, or mutations in factors that act upstream in the pathway, such as EGFR [51]. Oncogene-induced senescence can also result owing to high transcriptional activity of the elongation factors E2F1, E2F2, or E2F3 which can be caused, for example, by the loss of function of RB1 tumor suppressor (Fig. 1) [52]. $p16^{INK4a}$ plays an important role in senescence regulation as the inhibitor of cyclin-dependent kinases CDK4 and CDK6, which phosphorylate and inhibit RB1 protein [53]. Associated with an increase in $p14^{ARF}$ is the increased expression of TP53 target genes, such as p21, which triggers p53-mediated cell cycle arrest. $p15^{INK4b}$, together with $p16^{INK4a}$, contributes to senescence of mouse fibroblasts [54]. TGF-beta-induced $p15^{INK4b}$ expression is also important for the senescence of hepatocellular carcinoma cell lines [55]. Only one study provides evidence for oncogene-induced bone senescence where $p27^{Kip1}$-induced osteoblast senescence in FASST mice (fibroblasts accelerate stromal-supported tumorigenesis) results in increased osteoclastogenesis, bone metastases, and SASP expression [56].

The Senescence-Associated Secretory Phenotype

Senescent cells secrete several factors, defined as the SASP, that include pro-inflammatory cytokines, growth factors and extracellular matrix remodeling factors, and chemokines, as well as proteases which have potential direct or indirect negative regulation on otherwise non-senescent or healthy cells in proximity. The SASP is a key distinguishing feature of the senescence program and can be characteristically different depending on the tissue, although no descriptive studies comparing the SASPs from distinct cell types currently exist. While a few studies have attempted to report on the SASP resulting from oncogene-induced senescence of fetal lung fibroblasts [57] and identification of the SASP markers at the mRNA level in cells from the bone microenvironment [14••], the SASP remains to be thoroughly documented at the proteome level. The paracrine activity of senescent cells is an important universal feature of the SASP, and it is possible that particular combinations of these paracrine factors are cell or tissue type–dependent. Recent studies show that the SASP may also have some beneficial roles, since transient exposure

to components of the SASP induces cellular plasticity and tissue regeneration [58].

Major proteins known to be associated with the SASP include TNFα, MCP-1, IL-6, IL-1a, PAI, granulocyte-macrophage colony-stimulating factor (GM-CSF), growth-regulated oncogene (GRO)α,β,γ, insulin-like growth factor-binding protein (IGFBP)-7, IL-1α, IL-6, IL-7, IL-8, monocyte chemoattractant protein MCP-1, MCP-2, macrophage inflammatory protein MIP1α, MMP-1, MMP-10, and MMP-3 [59, 60]. The underlying mechanisms for the SASP in cell senescence are yet to be fully explained, but are likely to be associated with DNA damage [61] and oxidative stress [62]. Furthermore, inflammation stimulated by IL-1β treatment has been shown to induce $p16^{INK4a}$ expression, which in turn induces the production of MMPs (MMP-1 and MMP-13), thus linking inflammation to senescence [63]. In cutaneous wound repair, the SASP is characterized by production of platelet-derived growth factor PDGF-AA and CYR61 (also known as CCN1), which have central roles in the induction and maintenance of the senescent state [64, 65].

Deciphering the mechanisms by which the SASP of bone cells alters bone tissue architecture and remodeling remains to be elucidated. It is assumed that the constituents of the SASP are usually specific for each cell/tissue type with some highly conserved proteins pertaining to that particular tissue's function. It can also be supposed that the secretome varies over time and could also depend on the mode of induction (e.g., stress-induced or DNA damage response). Thus, the SASP signature is not a singular set of factors, but is rather cell-dependent and stimulus-dependent. More research is needed to elucidate upstream mechanisms involved in the development of the SASP, to explore differences in the SASP between different bone cell types, and to describe the characteristics of the SASP in senescent cells of the in vivo bone microenvironment rather than in culture-expanded senescent cells.

Very few studies have been conducted on investigating the bone-specific SASP, of which one major work identifies SASP markers at the transcriptional level. This work showed that of the 36 SASP genes that were analyzed, a majority (23 of them) were significantly upregulated in osteocytes of old bones and very few factors were altered in osteoblast progenitors and osteoblasts [14••] with about 12 factors significantly increased in biopsies from older women. Both NF-kB and Il-1α were significantly upregulated in both aging osteocytes and myeloid cells alongside several other major SASP markers including MMPs, TNFα, PAI-1&2, IL-6, IL-8, HMG-1, and RANTES, to name a few [14••]. It is therefore increasingly important to define the SASP at the secreted proteome level which will give tremendous insight into understanding the secretome at high resolution and help identify disease targets and ways in which they can be selectively attenuated and/or removed as part of treatment interventions for chronic age-related diseases including osteoporosis.

Markers of Cell Senescence

As discussed earlier, irrespective of the mechanism of induction, the hallmark of cellular senescence is the loss of proliferative capacity while maintaining cell viability [66]. Cellular senescence likely represents a tumor suppressor program characterized by stable cell cycle arrest. In addition to cell cycle arrest, senescent cells go through morphologic, biochemical, and functional changes. These changes, studied in vitro and in vivo, have identified biomarkers of cellular senescence (Table 1) that are also largely representative of tissue aging including bone, and perhaps even systemic aging.

The loss of proliferative capacity in senescent cells is due not only to cell cycle arrest in late G1 phase but also involves an inappropriate growth-promotion sequence [67]. Senescence can be driven by growth-promoting pathways such as mTOR (Fig. 1), when the cell cycle is arrested [21, 68, 69]. $p16^{INK4a}$ plays an important role in cell cycle regulation by decelerating progression from G1 phase to S phase and $p16^{INK4a}$ increases dramatically as cells and tissues age [70–72]. $p21^{Cip1/Waf1}$ represents a major target of p53 activity and thus is associated with linking DNA damage to cell cycle arrest [73, 74]. p21 has often been considered critical for establishing senescence, whereas $p16^{INK4a}$ may be more involved in the maintenance of the phenotype [74]. However, the roles of $p16^{INK4a}$ and $p21^{Cip1/Waf1}$ in establishing the senescent phenotype appear also to be both cell type specific and dictated by the predominant mechanism of senescence (e.g., replicative exhaustion/telomere dysfunction, oncogene mediated, and macromolecular damage) [20, 75].

Senescent cells show morphologically flattened and enlarged cell shapes [76–78]. Such structural changes can limit functional efficiency and may contribute to growth arrest [79]. Investigation of the cell cytoskeleton shows an increase in

Table 1 Biomarkers of cellular aging

p16*, p21*
Cell enlargement and flattening*
Senescence-associated β-galactosidase*
Senescence-associated α-fucosidase
SASP factors*
MicroRNAs*
Telomere shortening*
Dysfunctional telomeres*
Changes in DNA methylation*
Senescence-associated heterochromatic foci (SAHF)*
Senescence-associated distension of satellites (SADS)*
Ribosomal protein L29

Biomarkers in combination, rather than singly, are more reliable for the identification of senescent cells

*Biomarkers that have been used to study aging in bone tissue

Vimentin in senescent cells [80] while Caveolin-1 plays a role in regulating focal adhesion activity and actin stress fiber formation [81]. Microscopic examination of in vitro and in vivo aged post mitotic cells shows expansion of the lysosomal compartment [82–84]. A lysosomal enzyme, senescence-associated beta-galactosidase (SA-β-Gal) is widely used as a biomarker for senescent cells [85, 86]. It is an abundant lysosomal enzyme with an optimal pH of 4 in young or immortal cells [87]. However, this activity is activated at pH 6 in senescent cells [87–89]. The expression of SA-β-Gal correlates with cellular aging in cultured cells in vitro and tissues in vivo [85, 87]; however, it can falsely stain quiescent cells [90] and there is no evidence linking this enzyme to the generation or maintenance of senescence [87]. Senescence-associated alpha-fucosidase, another lysosomal enzyme, is a more sensitive marker of senescence and is especially useful in cases where SA-β-Gal expression is low [91].

As mentioned above, senescent cells produce pro-inflammatory and matrix-degrading molecules [75] that include inflammatory and immune-modulatory cytokines, growth factors, cell surface molecules, and survival factors. These components of the SASP function in an autocrine manner, reinforcing the senescent phenotype [92, 93], and in a paracrine manner, where they may promote epithelial-to-mesenchymal transition and malignancy in the nearby premalignant or malignant cells [94]. In aging osteoprogenitors, increases in SASP factors such as TNF-α, IL-1β, Sdf1, and MMP-13 among others have been noted [14••, 95, 96].

MicroRNAs (miRNAs) are single-stranded 22- to 24-nucleotide non-protein-coding RNAs that can inhibit protein translation or induce RNA degradation by recognizing and binding to the 3′-untranslated region of specific target mRNAs. A single miRNA can target up to several hundred mRNAs, thus repressing the expression of components within one pathway, or in several related pathways. They can rapidly control cellular processes such as differentiation, proliferation, migration, autophagy, apoptosis, and senescence [97]. An increasing number of studies have identified miRNAs associated with cellular aging and declines in tissue function [98]. For example, miR-195 expression was increased in MSCs from older mice and subsequently targeted telomerase reverse transcriptase (tert) which is important for telomere maintenance. Subsequent removal of miR-195 increased tert, sirt1, and phosphorylation of Akt and FOX01 [99]. In addition, transplanting miR-195 depleted old MSCs into the infarcted mouse heart reversed the impairment of regeneration due to age [99]. Several studies have begun to map the miRNA transcriptome of senescent MSCs and have identified differentially expressed miRNAs that are or may be linked to senescence [100, 101].

Telomere shortening is frequently used as a biomarker for replicative senescence. Telomeres shorten during cell replication and accumulation of DNA damage at telomeres leads to uncapping, dysfunction, and ultimately to cellular senescence [102, 103]. The ends of chromosomes are composed of short TTAGGG repeats with associated proteins that together form a cap [104]. The cap prevents the telomere end from appearing as double-strand breaks (DSBs) and blocks chromosome fusion [38]. Dysfunctional telomeres arising either through natural attrition due to telomerase deficiency or by the removal of telomere-binding proteins are recognized as DSBs. Dysfunctional telomeres detected as telomere dysfunction–induced foci (TIFs) have been used as a senescence marker in bone cells (e.g., MSCs, osteocytes) [14••, 30] as well as in other cell types.

Epigenetic modifications such as DNA methylation show changes in patterning throughout the human lifespan [105]. In general, there is an overall loss of DNA methylation with age which has been termed “epigenetic drift” [106, 107]. However, an increase in methylation of promoter-associated CpG islands is seen in aging individuals, while the more prevalent intergenic CPGs lose methylation, the net effect accounting for decreases seen with increasing age [108–111]. In contrast to the phenomenon of epigenetic drift where methylation decreases with age for an individual but not necessarily in the same way across all individuals, the search for an “epigenetic clock” which could predict age across individuals has identified DNA methylation levels at specific genomic sites that are highly correlated with age in certain tissues [109, 111–113, 114••]. Continued investigation using specific DNA methylation patterns to define the epigenetic clock has the potential to create a powerful database of biomarkers for the study of aging.

Chromatin is reorganized in senescent cells to form characteristic senescence-associated heterochromatic foci (SAHF). SAHF were first visualized in senescent cells as dot-like patterns using 4′, 6-diamidino-2-phenylindole to stain the chromosomes [115, 116]. SAHF regions are enriched in heterochromatic markers and show an increase in high mobility group A proteins [117••]. These regions are thought to be largely transcriptionally inactive with a lack of nascent RNA signals [115, 118, 119] and may contribute to senescence-associated cell cycle arrest by silencing transcription of genes important for proliferation [115, 120]. SAHF are not found in all senescent cells. For example, they are absent from patients with Hutchinson Guilford progeria syndrome [121, 122]. In contrast, senescence-associated distension of satellites (SADS), a process where the peri/centromeric satellite heterochromatin is unraveled, occurs in all models of senescence studied [123]. It occurs early in the progression of senescence prior to SAHF formation and unlike SAHF formation which relies on the p16 pathway [115], SADS are not exclusive to either the p16 or p21 senescence pathways [123, 124].

Changes in nucleolar morphology due to replicative senescence have been noted. Proliferative cells generally show several more compact nucleoli while senescent cells have one

enlarged nucleolus [125, 126, 127••]. Recent studies have shown that the senescent phenotype leads to diminished ribosome biogenesis and the accumulation of rRNA precursors [127••]. In particular, accumulation of ribosomal protein L29 both in vitro and in vivo was an accurate biomarker of senescent cells [127••]. Reduction of ribosome biogenesis factors RSL1D1 (ribosomal L1 domain containing 1), nucleostemin, EBP2 (EBNA1 binding protein 2), or DDX21 (DExD-Box helicase 21) [128–131] can trigger senescence by activating signaling cascades that prevent cell cycle progression [127••].

The molecular pathways involved in triggering and/or maintaining the senescent phenotype are not fully understood. However, single markers currently utilized to detect senescent cells are limited and lack specificity in certain cases [90, 132, 133]. However, combinations of these markers can be used to specifically recognize senescent cells in culture and tissue samples. For example, p16 expression, together with SA-β-Gal and morphological changes, is widely acceptable in combination as indicative of cellular senescence [70].

Bone Pathology and Senescence

The onset of many chronic diseases is linked with aging, and primary age-related processes precede tissue dysfunction that leads to organ system pathology. Although senescent cells accumulate locally in tissues and have deleterious autocrine and paracrine effects, they also cause detrimental systemic effects in remote organs. Osteoporosis has long been considered an aging-related condition, and even when senescence was not well defined it was linked to the pathophysiology of bone loss [134]. In bone, coupling between formation and resorption synchronizes bone remodeling and maintains healthy bone. Both age- and disease-related cellular processes affect this remodeling where the net balance between bone formation and resorption favors the latter [135]. Pathologic bone disorders are observed with specific age-related co-morbidities, such as diabetes, menopause, andropause, and renal disease. Other conditions that result in osteoporosis are spinal cord injury–related unloading, tobacco and alcohol addiction, and metabolic disorders to name but a few. There is growing evidence for the presence of senescent cells and components of the SASP in osteoporotic bone from aged mice and elderly people [14••]. Since senescent cells affect bone homeostasis, it will also be important to understand whether they play a similar role in bone disease secondary to other age-related conditions, unloading, or insults such as injury and toxicities.

As described earlier, cellular senescence usually refers to the cessation of cell proliferation of an otherwise growth competent cell, and this growth arrest is associated with a pro-inflammatory secretome (SASP) [136]. The triggers of cellular senescence, as described earlier, are not limited to changes in chromatin (DNA damage, telomere attrition, and epigenetic changes), changes in mitochondrial function, or changes in protein homeostasis (Proteostasis) [137], and new definitions are being put forth as additional inducers are identified. Mechanisms behind replicative senescence and oxidative stress or oncogenic-induced senescence [138, 139] are now well recognized. Telomere shortening triggers replicative senescence through ataxia-telangiectasia mutated (ATM) kinase, *p53*, and the *Cdkn1a* ($p21^{Cip1/Waf1}$) pathways [37], causing tissue dysfunction [140]. Irrespective of the method of induction, senescent cells are resistant to apoptosis and are metabolically viable, with a very active secretome. The mechanism for senescence in bone cells is characterized by the upregulation of cell cycle inhibitors CDK4/6, *Cdkn2a* ($p16^{Ink4a}$), and *Cdkn1a* ($p21^{Cip1/Waf1}$), in myeloid, osteoprogenitor, osteoblast, and osteocyte cell types [14••].

Bone cell types respond differently to the triggers of senescence and express specific markers of senescence depending on the cell-specific senescence pathways utilized. With age, the number of senescent cells increases in bone tissue, as shown by increased $p16^{Ink4a}$ expression in osteoprogenitors, osteoblasts, osteocytes, and myeloid cells [14••], while $p21^{Cip1/Waf1}$ appears only to be expressed in osteocytes. Expression of $p21^{Cip1/Waf1}$ tends to be induced by conditions that generate oxidative stress [141, 142]. The fate of these senescent bone cells, the changes they undergo, and the factors they secrete (i.e., SASP) affect their microenvironment and ultimately bone architecture (Fig. 2). The SASP factors secreted vary among the types of cells in the bone microenvironment. Furthermore, variation was observed in the SASP markers expressed in human bone as compared to mouse bone tissues. The most consistent SASP markers which were expressed in both human and mouse bones were *CSF-2*, *CSF-3*, *CCL-2*, *CCL-8*, *IGFBP4*, *IRF1*, *FASL*, *TNF*, *PAPPA*, and *ICAM1* [14••].

The most common changes seen in osteoporotic bone are decreased bone formation, increased resorption by osteoclasts, reduced osteoprogenitors, and increased bone marrow adiposity (BMA) [143]. Increased bone resorption has been shown in mouse models of accelerated senescence [144] and in aged mice [15••], correlating with the at least relative increase in osteoclast progenitors and mature osteoclasts [145, 146]. Changes in osteoclast function with age are related to the SASP.

MSCs or osteoprogenitors decrease with age, an observation based on colony-forming unit-fibroblast (CFU-F) assays [147, 148]. Senescence in MSCs is linked to a reduced rate of bone formation and increased BMA, as shown in multiple mouse models of comorbidity-related osteoporosis [143, 149]. In a recent study, deletion of Foxp1 led to premature bone aging, with an inverse relationship to $p16^{Ink4a}$ and increased marrow adiposity [150]. Ionizing radiation (IR) is a known generator of senescence in vitro and has been shown to reduce CFU-F numbers of MSCs, while promoting adiposity, thus depleting the total pool of bone marrow MSCs [149].

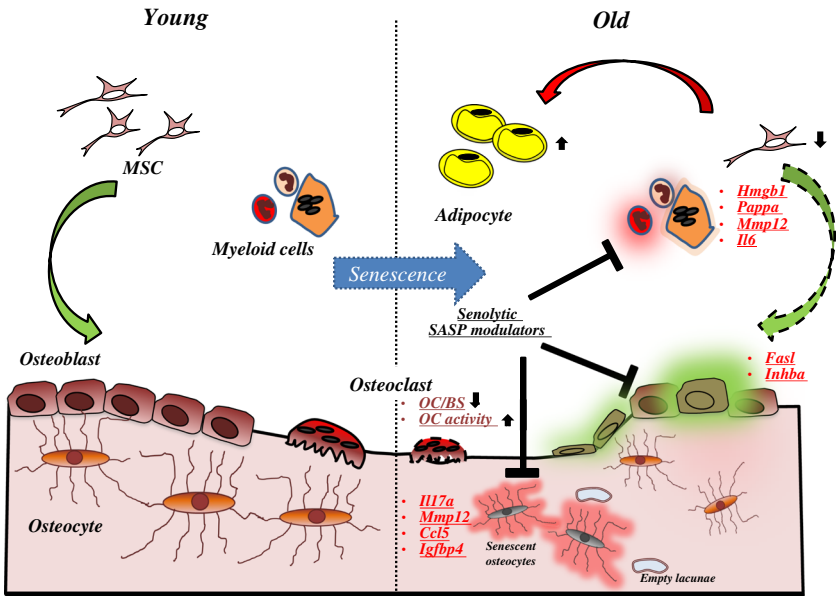

Fig. 2 Cellular changes in the bone microenvironment with aging. Hallmark features of aging bone include empty osteocytic lacunae, shrunken osteocytes, osteoblast apoptosis, reduced osteoclast number/bone surface (OC/BS), increased osteoclast activity (increasing resorption), and increased bone marrow adiposity (reducing the MSC pool), overall leading to reduced bone formation and increased bone turnover. During aging, the bone microenvironment undergoes changes mediated by cellular senescence. The imbalance between osteoblastic bone formation and osteoclastic bone resorption is triggered by pro-inflammatory factors (SASP) from osteocytes, osteoblasts, and myeloid cells. Suppression of pro-inflammatory components of the SASP (by compounds that modulate the SASP) and clearance of senescent cells (by senolytic drugs) promote bone accrual by reinstating (normalizing) bone coupling

Serum from aged animals has been shown to promote adipogenesis, while inhibiting osteogenesis [151], suggesting the presence of factors favoring adipocyte differentiation of MSCs. Hence, MSC cell fate is regulated not only by the senescence status of MSCs itself, but also by its environment.

Senescence in Non-senile Osteoporosis

Diabetes and bone senescence Diabetes mellitus (DM) is predicted to affect more than 50% of the world's population aged 65 years and older. Reduction in insulin levels results in high circulating glucose and the formation of advanced glycation end products (AGEs), leading to senescence in MSCs [152] and inhibition of bone formation. Type-1 (T1) DM causes the most secondary osteoporosis [153] with reduced bone mineral density (BMD) at an early age predisposing to age-, disease-, and menopause-related osteoporosis later in the life. It is unclear whether T1DM-related osteoporosis is also due, at least in part, to senescence. In a streptozotocin-induced mouse model of T1DM, there is bone loss due to reductions in osteoblast and osteoclast function, along with an increase in adiposity and a loss of vascularity [154]. Furthermore, in experiments using endothelial progenitor cells (EPCs) from DM patients or in mouse models of T1DM, senescence has been characterized by increases in p53- and p21-mediated pathways [154, 155]. Oxidative stress is one of the major inducers of senescence and bone marrow mononuclear cells from T1DM mice showed increased mitochondrial reactive oxygen species causing oxidative stress–induced senescence [156].

Secondary bone pathologies linked with T2DM occur in a larger population of DM patients. Even though T2DM is often linked with high BMD, due to altered bone quality, the rate of fractures in this group is high [157]. Importantly, T2DM is more likely to be an age-related comorbidity [158] caused by senescence. Both T1DM and T2DM are associated with AGEs, which in turn have been related to low bone quality and increased fractures [159–162]. T2DM-related insulin resistance leads to high glucose, dyslipidemia, immune dysfunction, and hormonal imbalance, which are all considered predisposing for senescence [163].

Recent studies have identified the role of several SASP factors in the pathophysiology of T2DM. Indirect evidence links increases in IL6, IL8, and MCP-1 in obese adults [164] with the rise in an overall pro-inflammatory environment that results in elevated macrophage function. Several SASP markers such as IL6, IL-1β, HMGB1, and PAI-1 were all identified as predictors of T2DM and also found in aged bone tissues [165].

Androgen decline and bone senescence Decline in androgen in men is associated with increases in fracture rates [166, 167], sexual regression, erectile dysfunction [168], lower urinary tract symptoms, increase in atherosclerosis [169, 170], decline in cognitive abilities [171, 172], sarcopenia [173], and reduction in physical ability. Androgen deprivation results in

immune dysfunction caused by immune senescence affecting both innate and acquired immunity [174]. In studies with aged male macaques, restoration of androgen levels partially rescued immune senescence [174]. Androgen deficiency affects both genders, but predominantly males [175]. In both men and women, androgens are utilized to produce hormones such as testosterone and estrogen. Estrogen is directly related to the maintenance of bone mass [176–178], while testosterone and estradiol deficiency in older men have been linked to osteoporosis [179]. While the role of senescence during androgen deficiency is still not established, studies in ovariectomized mice indicated a premature T cell senescence [180]. The study indicated that ovariectomy led to accumulation of $CD4^{+}CD28^{null}$ T cells, and these effects were reduced by treatment with estrogen [180].

Another study implicated the androgen receptor (AR) in its ability to induce senescence in prostate cancer and normal prostate epithelial cells, via p21-induced senescence, but independent of p53 [181]. AR has also been shown to induce premature senescence in dermal papilla cells, using a DNA damage and p16 mechanism.

Alcoholism and bone senescence Alcohol consumption is considered harmful if taken beyond a certain limit. However, low amounts of alcohol consumption have been linked with positive effects on health. The first report on alcoholism causing changes in bone mass was shown by Saville et al. [182]. Severe cases of alcoholism, as seen with binge drinking are considered to be directly linked with low bone mass, cortical thinning, osteoporosis, and fractures [183, 184]. Cases of mandibular osteomyelitis and facial fractures have been reported with binge drinking [185, 186].

Studies performed in vitro have shown that alcohol causes DNA damage, inhibits proliferation, and promotes senescence in a dose-dependent manner with suppression of osteogenic potential in osteoblasts and MSCs by activating p53. Secondary osteoporosis caused by excessive alcoholism may be due to liver injury, fibrosis, or senescence. Recent studies have shown that components of the SASP or micro RNAs linked with inflammation-induced aging have systemic effects and induce aging in younger animals [187]. Hence, the role of senescence in secondary osteoporotic conditions like alcohol-induced osteoporosis may be further elucidated by understanding the role of circulatory components of the SASP.

Skeletal unloading–induced senescence Skeletal unloading causes rapid bone loss [188–191], and highlights the importance of weight bearing and exercise in the maintenance of bone fidelity. Skeletal unloading may increase with aging, as immobilization due to prolonged bed rest among the elderly is a major cause of lower BMD and fractures. Skeletal unloading, whether due to immobilization, spinal cord injury, or lack of gravity such as in space travel, results in reduced BMD. Age-related osteoporosis is often linked with the lack of responsiveness of the cells to mechanical loading generated by weight bearing. In experiments performed in vitro, senescent MSCs failed to respond to low-magnitude mechanical stimulation as reflected by changes in their secretome [192].

In mice, p53 and $p21_{Cip1/Waf1}$ mRNA expression was found to be increased in bone marrow cells of tail suspension–induced unloaded bones [193]. Interestingly, $p53^{-/-}$ failed to respond to the hind-limb unloading-induced bone loss, indicating that unloading-induced osteoporosis follows a p53/p21 pathway [193, 194]. This also indicates that signaling pathways which regulate cell growth upon loading and mechanical stimulus could be regulating cellular senescence by suppressing the p53/p21 axis.

Glucocorticoid-induced bone senescence Glucocorticoid-induced osteoporosis (GIO) is one of the major causes of fractures [195–197]. Glucocorticoids (GCs) affect bone formation by altering the function of progenitors, osteoblasts, and osteocytes [198]. It has been shown in different cell lines that GCs cause senescence [199–202]. GCs stimulate $p21^{Cip1/Waf1}$ gene expression by directly binding to the steroid-responsive region in rat hepatoma cells [203], but a similar role for p21 induction by GCs was not seen in chondrocyte senescence in vivo [204]. Despite its routine use as a factor in the in vitro differentiation of MSCs, dexamethasone, a type of GC, has been shown to inhibit osteoblast function by promoting cell senescence and activating certain components of the SASP [205, 206]. GCs have been shown to suppress the WNT pathway, and suppression of the WNT pathway has been linked to reduced bone formation, with altered osteoblast activity [207]. GCs also cause telomere dysfunction [208], further supporting that they are inducers of senescence, and suggesting that the pathophysiology of GIO could be related to site-specific or systemic accumulation of senescent cells.

Targeting Senescence to Improve Bone Fidelity

Targeted clearance of senescent cells by agents called "senolytic drugs" result in the improvement of tissue function and extended life span [15••, 209–212]. Detailed reviews have been written about potential senotherapeutics ("senolytic"; inducers of apoptosis in senescent cells and "senomorphic"; suppression of SASP function) [213–216]; hence, we shall review only those senotherapeutics that have been shown or have the potential to maintain or improve bone fidelity with aging.

Genetic studies done with $p16^{Ink4a\text{-}ATTAC}$ mice expressing an inducible caspase 8 suicide transgene showed that senescent cells expressing specifically $p16^{Ink4a}$ were eliminated when treated with Ap20187, an activator of caspase 8–

induced apoptosis [217••]. Genetic clearance of senescent cells in aged *INK-ATTAC* mice increased bone volume and improved bone architecture of both the spine and femur [15••].

Pharmacologic clearance of senescence cells or suppression of the harmful SASP was also tested as a potential therapeutic treatment for age-related bone damage (Fig. 2). A senolytic cocktail (Dasatinib (D), a tyrosine kinase inhibitor plus Quercetin (Q), a plant based flavonol) or Ruxolitinib, an inhibitor of the Janus kinase pathway (JAKi) that can suppress the SASP, improves both the trabecular and cortical architecture of the spine and femur [15••]. While D plus Q significantly improved osteoblast numbers on the bone surface, both D plus Q and JAKi suppressed osteoclast-based resorption by reducing osteoclast numbers. PAI-1, IL-6, and IL-8 were some of the major SASP factors secreted by the senescent osteocyte and myeloid cells [14••]. Bone marrow cells undergoing osteoclast differentiation and pre-treated with medium conditioned by senescent cells in presence of neutralizing antibodies against PAI-1, IL-6, and IL-8 produced fewer mature osteoclasts, suggesting a direct role for these SASP components in increased resorption with age [15••].

Senolytic drugs, such as ABT263 (Navitoclax, an inhibitor of the anti-apoptotic proteins BCL-2 and BCL-xL), were shown to clear senescent bone marrow hematopoietic stem cells and senescent muscle stem cells in mice that were either aged or exposed to whole body irradiation [218]. Inhibitors of BCL-2 and BCL-xL, such as Fisetin, A1331852, and A1155463, cleared senescent cells in vitro [215] and improved health span in mice [219]. Thus, these compounds may also be potential agents for improving bone architecture during aging.

Conclusions and Future Perspectives

The search for new senotherapeutic drugs has increased substantially [220] and the potential for a senolytic or senomodulator to become part of standard of care for age-related osteoporosis seems probable in the near future. Potential concerns about side effects and long-term deleterious consequences of senolytics are partly mitigated by the fact that they can be administered infrequently, owing to the rapid clearance of senescent cells and the time it takes for senescent cells to re-accumulate after clearance. The same argument cannot be made for senomodulators, which do not clear senescent cells, and likely must maintain steady-state levels in order to suppress the SASP. It is also probable that targeting cellular senescence for other than age-related bone loss may be less effective or ineffective. For example, recent evidence suggests that estrogen-deficiency osteoporosis proceeds through mechanism(s) unrelated to cell senescence [221]. A final consideration is whether senotherapeutic agents can potentiate medications currently used for the treatment of osteoporosis.

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Compliance with Ethical Standards

Conflict of Interest Robert Pignolo, Rebekah Samsonraj, Susan Law, Haitao Wang and Abhishek Chandra declare no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- •• Of major importance

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