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Skeletal Aging and Osteoporosis: Cellular Senescence and Beyond

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Abstract: Bone is a dynamic organ maintained by tightly regulated mechanisms. With old age, bone homeostasis which is maintained by an intricate balance between bone formation and bone resorption, undergoes deregulation. Oxidative stress-induced DNA damage, cellular apoptosis and cellular senescence are all responsible for this tissue dysfunction and the imbalance in the bone homeostasis. These cellular mechanisms have become a target for therapeutics to treat age-related osteoporosis. Pharmacological and genetic mouse models have shown the importance of senescent cell clearance in alleviating age-related osteoporosis. Senescent cells have an altered secretome, which may have autocrine, paracrine, or endocrine function. The current review discusses the current and potential pathways which lead to a senescence profile in an aged skeleton. The review was written following an extensive literature survey of published studies, mostly excluding articles published on pre-print servers. The review discusses potential therapeutics targeting cellular senescence and the senescent secretome as an underlying pathogenesis of an aging bone.

Keywords: Osteoporosis, Senescence, SASP, Aging, Radiation, Senotherapeutic

Introduction

Bone as a tissue has its own complexities with one of the largest pools of diverse cell types. These complexities are stressed to its maximum limits during old age leading to osteoporosis. Advent of loss of bone with aging are also early signs of increased fracture risk, morbidity, and mortality. Osteoporotic fractures exceed incidences of cardiovascular disease or cancer by ~3-4 fold[1] and is a substantial strain on the economy. Only 31-36% people above the age of 70 have normal bones, with the remainder suffering from some form of osteopenia or osteoporosis. Moreover, it is well understood that loss of estrogen is a key driver of bone loss in women and to some extent in men, which is only exacerbated by aging [2, 3]. However estrogen is not the only cause of bone loss during aging[4], and it has been recognized and discussed in detail that aging stands as a separate entity with distinct mechanisms[5]. This review will expand on known and potential mechanisms underlying the pathogenesis of skeletal aging, mainly DNA damage and cellular senescence, with special emphasis on certain proteins such as poly(ADP-ribose) polymerase 1 (PARP1) due to their role in DNA repair, telomere maintenance, senescence and in the production of pro-inflammatory cytokines (Fig.1).

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Biology of skeletal aging

Bone is a dynamic organ incorporating several cell types which generally work synchronously and maintain the bone homeostasis resulting in the deposition of a mineralized bone matrix. The two processes which maintain this homeostasis are bone formation and bone resorption which are under an equilibrium in a normal physiological condition. Cells of the mesenchymal origin regulate the bone formation process including bone marrow stem cells (BMSCs) which are the progenitors which give rise to osteoblasts, cells responsible to deposit mineral and form the collagen enriched bone matrix supported by the multifaceted osteocytes, the most abundant cell type with an extensive canalicular network.

Osteoclasts, a large multinucleate cell, having a hematopoietic origin, is responsible to resorb the bone matrix and is regulated by extracellular signals secreted by the osteoblasts and osteocytes while being supported by progenitors such as bone marrow monocytes or macrophage precursors [6-8]. Differentiation of osteoclasts require binding of receptor activator of nuclear factor kappa-B (RANK) ligand (RANKL) to the RANK receptor on the osteoclast surface [9, 10] together with the secretion of macrophage colony-stimulating factor (M-CSF) by osteoblasts and bone marrow stromal cells [11] which then activates the proliferation of the osteoclast precursors by binding to the colony stimulating factor-1 receptor (CSF-1R) also known as c-FMS. Parathyroid hormone (PTH), 1,25-vitamin D3, IL-1, IL-6, IL-11 and tumor necrosis factor (TNF) are some of the factors that directly or indirectly influence osteoclast differentiation[12]. Osteoprotegerin (OPG), a humoral tumor necrosis factor (TNF) receptor family protein, secreted by several cell types including osteoblasts, acts as a decoy receptor to RANK blocking the binding of RANKL to RANK (Fig.2). Reduction of osteoprogenitors and osteoblasts with age reduces the OPG levels which in turn allows the activation of osteoclast-based resorption, thus tilting the balance of bone homeostasis and causing osteopenia and osteoporosis. Resorption of bone matrix that include type I collagen, the predominant component of the matrix [13] allows the release of matrix associated proteins such as transforming growth factor β1 (TGF-β1) and Insulin-like growth factor type I (IGF-1), which then promote mesenchymal cell differentiation to form mature osteoblasts [14-17]. Many such factors, some of which are secreted by the osteoclasts as well, are known as coupling factors. Osteocytes, the terminally differentiated osteoblasts, were a major source of RANKL thus promoting osteoclastogenesis and an osteocyte specific deletion of RANKL resulted in osteopetrosis [18].

Osteocytes are one of the most abundant cell types in the bone tissue and contribute to sensing mechanical load [19], through their extensive network of lacuna-canalicular area amounting to 215m^2 [20]. Osteocytes may positively or negatively regulate bone remodeling [21], a dynamic which tilts to a negative regulation with aging during which the lacunar density declines[22].

Age related functional decline in osteoblasts due to increased apoptosis[23], decreased proliferation, impaired osteoblast differentiation[24], increased osteoblast senescence[25] and dysfunctional osteoprogenitors[26], leading to more marrow adipogenesis as the favored pathway[27]. The decline in bone mass with old age is inversely proportional to the bone marrow adipose tissue (BMAT) accumulation. BMAT is also reported in post-menopausal women, due to immobilization as seen in spinal cord injury and with steroid treatments, with age being a synergistic confounder.

Senescence

For many years it was believed that cells *in vitro* could grow uncontrollably. It was first shown by Hayflick and Moorehead that cells in culture do undergo replicative senescence[28]. Replicative senescence became the surrogate term for cellular aging for many years suggesting that cells that are required for tissue homeostasis, regeneration and during development, may be depleted over a period. The term senescence was loosely used for many years to describe organ aging among various pathologies[29-33]. Apart from replicative senescence, which is linked to telomere shortening during cell cycle, cytotoxic stress-induced premature senescence (SIPS) can be triggered by oncogene activation[34],

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accumulation of free radicals, reactive oxygen species, DNA damage in general and of the telomeres, proteostasis, mitochondrial dysfunction, activation of pro-survival pathways, etc.[35, 36]. These changes eventually lead to a cellular morphology which looks enlarged and flattened, with ruffled cellular surfaces[37], increased cellular debris, and often accompanied with chromatin modification, also known as senescence-associated heterochromatin foci (SAHF)[38]. While the triggers for senescence may occur during normal physiological aging, they are aggravated during pathological conditions such as disease or their treatments, such as corticosteroids, chemotherapy, and radiotherapy, etc. Senescence has now been well established as one of the major mechanisms of aging, but the cellular pathways that play a role in the regulation of cellular senescence are only partially discovered. Increased life and health span has been achieved by clearance of senescent cells using pharmacological and genetic mouse models[39, 40]. In this review we have discussed the role of DNA damage repair (DDR) pathways in cellular senescence and skeletal aging.

Two signaling pathways, ataxia telangiectasia mutated (ATM)/p53/$p21^{Cip1}$ and $p16^{INK4a}$/RB, regulate the senescence spectrum. DDR initiates the stabilization of tumor suppressor, p53 which in turn induces cyclin dependent kinase (CDK) inhibitor (CDKi), $p21^{Cip1}$, which initiates cell cycle arrest [41, 42]. $p16^{INK4a}$ was shown to directly bind and inhibit the catalytic activity of CDK4[43] and CDK6[44]. The ultimate result of activation of $p21^{Cip1}$ or $p16^{Ink4a}$ and the blockade of D type cyclin and CDK4/6, thereby activating the tumor suppressor retinoblastoma protein (RB). Accumulation of $p16^{Ink4a}$ has been shown to promote tumor progression and age dependent co-morbidities, and clearance of p16-positive cells starting from mid-life suppressed tumor progression with aging, and other age-related tissue dysfunction[45].

Cellular senescence has been shown to play a role in development, tissue reprogramming and wound healing. While these are some beneficial roles of senescent cells, it is better understood now that senescent cells may belong to a reversible and an irreversible phase. Moreover, during normal physiology, senescent cells are cleared by the immune system, and thus a short presence of these senescent cells in a tissue environment may promote programming and regeneration. However, with normal physiological aging the senescent cells accumulate, and the immune system is often incapable to clear all these senescent cells. Often the senescent cells are in tissues where clearance by the immune system may be a physical challenge. One such example is the bone cell osteocyte, which is embedded in the matrix and a senescent osteocyte is hard to clear by the immune system. Immune cells have also been shown to undergo senescence implicating a much larger systemic profile of senescence[46, 47].

Inducers of Cellular Senescence and osteoporosis - Since the early descriptions of cellular senescence, several inducers of senescence have been defined over the decades including telomere dysfunction, DNA damage, chromatin aberrations, reactive oxygen species (ROS), and oncogenes, among others.

DNA Damage and Genomic instability- Accumulation of mutations and DNA damage throughout life is a major factor for aging. DNA is continuously exposed to exogenous as well as endogenous threats leading to genetic lesions. Chemical exposure, physical damage and biological agents are exogenous agents causing DNA damage. Endogenous threats include error prone DNA replication, generation of reactive oxygen species (ROS) and hydrolytic reactions [48]. Genetic lesions caused by these agents lead to compromised genetic integrity and gradually aging. DNA damage triggers a DDR response which is regulated by several pathways. A damaged DNA may undergo: (i) simple reversal in an error-free manner capable of fixing simple alkylated bases, (ii) a base excision repair (BER) attends to oxidative, deamination, alkylation and abasic single base damage, (iii) nucleotide excision repair (NER) addressing bulky base repairs, (iv) mismatch repair (MMR) which maintains the replicative fidelity, (v) inter cross-link repair (ICL) fixes covalent linkages of adjacent DNA strands, and (vi) the DNA break repair which include single strand break (SSB) repair and double strand break (DSB) repair.

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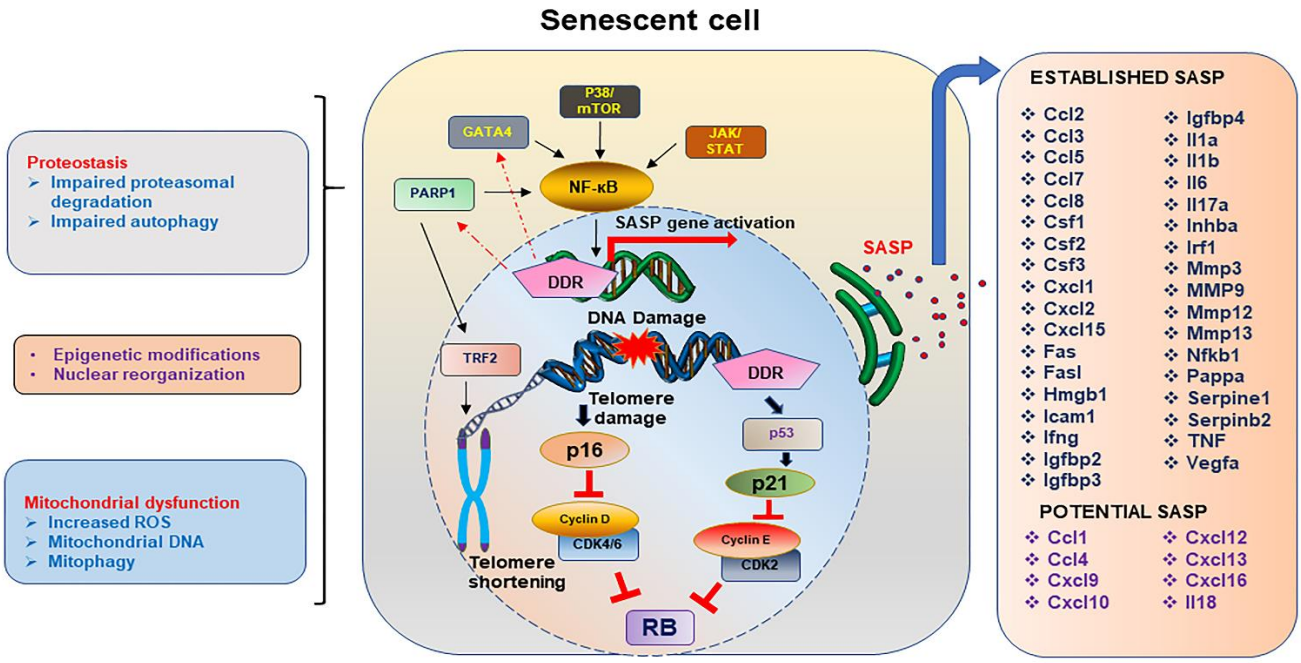

Figure 1. Spectrum of changes in a senescent cell.

DNA damage response (DDR) is one of the key inducers of cellular senescence, and if the DNA damage is in the telomere sites, this drives the cell towards a senescent state which has several characteristics, also acting as sustainers or inducers of the senescent state of the cell. Telomere shortening or damage driven DDR initiates the $p16^{Ink4a}$ or p21 driven pathways which block the cyclin D, CDK2/4/6, and cyclin E to thereby stabilizing the Rb protein, allowing the cell to enter the arrest phase. Activation of NF-κB through indirect activation of PARP1, GATA4, p38/MTOR or JAK/STAT pathways activate the transcription of SASP genes. Proteostasis, either by impairment in the ubiquitin proteasome system or the autophagy pathway, allows aggregation of unwanted proteins, contributing to senescent profile of the cell. Mitochondrial dysfunction, including changes in the mitochondrial DNA, increased ROS and altered autophagy of the mitochondrial compartments, contributing to the overall stressed environment leading to senescence. Chemokines, interleukins, and matrix modifying enzymes form the bulk of the proinflammatory SASP genes which may work in an autocrine, paracrine, or endocrine manner. The list of SASP proteins is incomplete and several potential proteins actively expressed in senescent cells may be characterized as SASP proteins based on their pro-inflammatory profile.

Key proteins of these pathways are also used to often define DDR pathways such as, ATM kinase, ataxia telangiectasia and Rad3 related (ATR) kinase, PARP1] and three DSB repair pathways [classical nonhomologous end joining (c-NHEJ), alternative (alt)-NHEJ, and homology-directed repair (HDR)].

One of the earliest reports showing accelerated aging caused due to a mutation in Xeroderma pigmentosum (XP)-type D (XPD), a gene encoding a DNA helicase that functions in both repair and transcription. Mutation in this gene resulted in a human disorder trichothiodystrophy (TTD). TTD mice were found to exhibit many symptoms of premature aging, including osteoporosis[49]. Reduced bone mass was observed in ATM kinase deficient mice with defects on osteoblast differentiation and increase in osteoclastogenesis [50]. ATM-/- mice also reported reduced osterix protein levels in the calvarial osteoblasts. A similar reduction in bone mass was observed in an inducible deletion of ATR kinase, together with other premature aging phenotypes[51]. Excision repair cross complementary group 1–xeroderma pigmentosum group F (ERCC1-XPF) is an endonuclease that plays a role in several DNA repair pathways. Genetic mutations in the ERCC1-XPF gene in humans have been shown to have progeria like state with osteoporosis as one of the phenotypic pathologies. *Ercc1*-null and hypomorphic mice both displayed severe osteoporosis, with bone resorption outpacing

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bone formation[52]. These mice also displayed increased cellular senescence and SASP, which was reduced by downregulating the nuclear factor kappa B (NF-κB)[52].

Exogenous DNA damage caused by ionizing radiation (IR) has also been shown to be partly responsible in reduction of bone forming cells in mice. Anabolic agents such as PTH 1-34 and neutralizing antibody against sclerostin (Scl-Ab) and anti-resorptive drug zoledronate have been shown to counter DNA damage seen in radiated bones or BMSCs[53-55]. Stabilization of DNA repair proteins Ku70 and DNA-PKC were also shown to protect osteoporosis in radiated bones[56].

Telomere dysfunction- Telomeres are repeated DNA sequences of TTAGGG and may comprise up to a thousand repeats located at the end of the chromosome forming a cap of proteins. Telomeres serve as a molecular clock and maintain the replicative potential of a cell. Telomere length is generally maintained by an enzyme complex called *telomerase*, comprising of an RNA subunit and a catalytic protein subunit called telomerase reverse transcriptase (TERT). Exhaustion of telomeres is a major factor of normal aging and with each cell division there is shortening of the telomere length [57, 58]. Apart from replicative senescence, telomere damage due to oxidative stress can also lead to cellular senescence. Telomere dysfunction accelerates aging as was evident from experimental evidence in mice [59]. The damaged telomere is identified as a double strand break (DSB) and initiates a DDR [60]. Recruitment of DDR pathway proteins follows the initial triggers and colocalization of DDR proteins to the telomere have been successfully used to identify dysfunctional telomeres, defined by different acronyms such as Telomere dysfunction-induced foci (TIF)[25], [61] or Telomere associated foci (TAF). These events trigger the activation of $p53/p21^{Cip1}$ and $p16^{Ink4a}$ senescent pathways which ceases the growth of the cell[62].

Telomere sequence is generally protected by the shelterin complex, which in humans consists of six distinct proteins, TRF1, TRF2, Rap1, TIN2, TPP1 and POT1[63]. In absence of shelterin, the telomere is identified as a DNA damage site and has been shown to be targeted by six different DDR pathways, including ATM and ATR kinases, PARP1, c-NHEJ, (alt)-NHEJ and HDR [63, 64]. Different shelterin subunits have been shown to suppress different DDR pathways.

Two human genetic diseases namely Werner's syndrome (WS) and dyskeratosis congenita with premature aging symptoms such as osteoporosis, which was confirmed in an accelerated model of aging in mice where WS helicase and telomerase were genetically removed[65]. It was later reported that single mutation in the telomerase gene (*Terc*) and double mutants of WS helicase and telomerase (*Wern-/-Terc-/-*) showed accelerated age-associated osteoporosis[66].

Epigenetic alterations- DNA methylation, histone modification and transcriptional changes are associated with aging. The Sirtuin family of proteins have been extensively studied as potential anti-aging targets. Overexpression of Sirtuin members increased the longevity, sirt6 expression in mice increased the lifespan [67]. Altered heterochromatin, also known as senescence-associated heterochromatin foci (SAHF) has been linked to senescent cells [68, 69].

Epigenetic alterations are shown to be associated with age-related osteoporosis, with several such markers are also used as predictors of bone loss with aging. Osteoporosis and osteoarthritis were correlated with methylation levels at CpG loci in aged women [70]. Trimethylation of the histone H3 at lysine 27 (H3K27) by histone methyltransferase EZH2 has been shown to regulate osteogenesis [71-74]. A genome-wide methylation analysis among osteoporotic and osteoarthritic populations identified unique methylation sites, suggesting a role of epigenetic regulation in the two bone pathologies [75]. However, detecting DNA methylation in blood was not found to be a good sample type as a predictor for osteoporosis in aged patients [76].

Loss of proteostasis- Aggregated or misfolded proteins are known to induce age related disorders like, Parkinson's and Alzheimer's diseases. Accumulation of proteins occurs due to a

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dysfunction of the cellular machinery which breaks down proteins, shared between autophagy and the 26S-proteasome system. Timely removal of unfolded/misfolded- and short-lived-proteins is performed by the proteasome, while most long-lived proteins, protein aggregates or degenerated micro-organelles, are degraded by the autophagy-lysosomal pathway. Replicative or hyperoxia-induced senescent cells were shown to have reduced protein turnover correlating well with reduced 26S proteasome activity, resulting in accumulation of oxidized or cross-linked proteins [77-79]. A reduction in autophagy causes loss of proteostasis leading to cellular senescence[80]. A genetic deletion of the autophagy related 7 (ATG7), a key component of the autophagy machinery, showed deterioration in bone mass in mass[81]. In another study autophagy inhibitor 3-methyladenine made BMSCs senescent reducing their osteogenic ability, while autophagy induced rapamycin could restore bone mass in aged mice [82].

In some other cell types, proteasome inhibition induces senescence[83-85]. Proteasome inhibitors are successful therapeutics for treatment of multiple myeloma and negatively affect cancer cell growth. While proteasome function is important during aging and any reduction in function leads to senescence, this story is not without caveats. Based on our work and others, proteasome inhibition improves osteoblast function and improves bone formation, while suppressing osteoclast-based resorption and suppressing proteasome function at least by certain inhibitors, has anabolic effects on bone formation[56, 86, 87]. The question then becomes, why some cells undergo senescence on proteasome inhibition, while bone cells seem to be unaffected. The only explanation which can rationalize this is that there is a different threshold for different cell type which is also dependent on how metabolically active a cell is. Bone cells are mostly quiescent, and hence the proteasome is not very active, while proliferating cells with a high protein turnover and a hyperactive proteasome. It was shown that higher the metabolic activity of a cell, the more susceptible the cell is to undergo senescence due to proteasome inhibition, while quiescent cells are shown to be resistant to proteasome inhibitor induced toxicity[88]. Endogenous proteasome suppression during aging does result in senescence, and in bone loss. This can be attributed to the cumulative cellular events such as impaired autophagy, mitochondrial dysfunction, and impaired endoplasmic reticulum.

Mitochondria and ROS- Being the cellular powerhouse, mitochondria utilize the maximum intracellular oxygen, while producing energy and generating ROS in the process. ROS produced by mitochondria in turn causes DSB in the DNA and activates the DDR. Oxidative stress has been a known inducer of senescence shown in cells grown in high oxygen concentration[89]. It was recently reported that in the absence of mitochondria, senescent cells had reduced ROS, reduced cytoplasmic chromatin fragmentation and a reduced pro-inflammatory SASP profile[90]. Low levels of ROS can maintain bone homeostasis and a balance between osteoblasts and osteoclasts [91]. Abnormal levels of ROS have been shown to cause cell death in osteoblasts and osteocytes and reduction in bone architecture [92]. Increase in ROS and a reciprocal decrease in antioxidant levels accounts for an elevated osteoclast activity and reduced osteogenic potential of osteoblasts causes bone deterioration as seen in human studies [91, 93, 94]. Osteoclasts are multinucleated cells, and thus have a high energy requirement provided by the mitochondria, which helps in the acid production during bone resorption.

Mitochondrial DNA is another focus of research in aging and its associated comorbidities. mtDNA polymerase gamma (Polg), a lone DNA polymerase found in mitochondria, when mutated, showed accelerated age-related osteoporosis with reduced osteogenic potential and increased osteoclasts activity[95].

Cellular Senescence and skeletal aging

The earliest studies that defined the role of cellular senescence in bone deterioration came in the senescence accelerated mice (SAM-R/3 and SAM-P/6)[96]. Phenotypically these mice showed all the characteristics of aging and over the years there were several strains emerged that incorporated more of age-associated co-morbidities[97]. By 2001 cellular senescence was not considered as a mechanism for osteoporosis[98].

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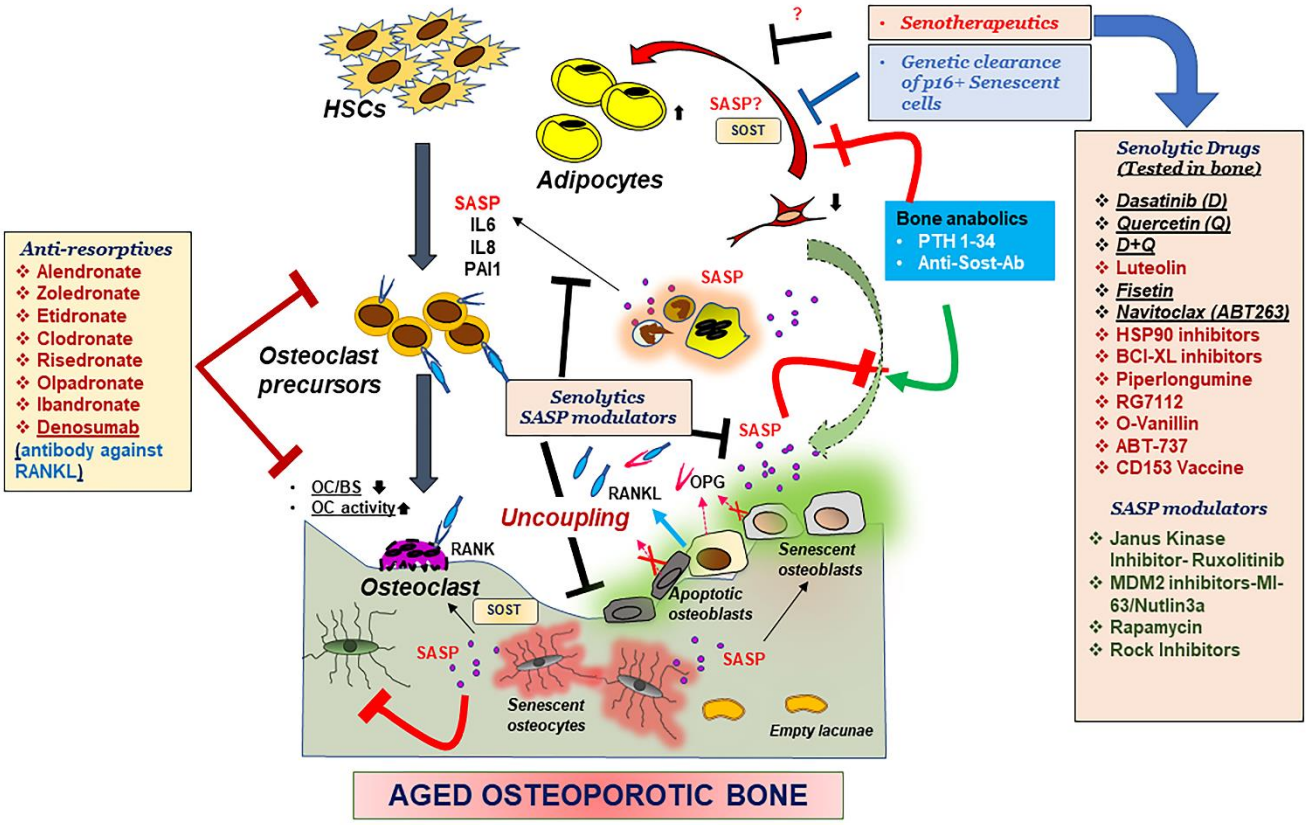

Figure 2. Mechanisms underlying an aging skeleton and potential therapeutic options.

Bone formation which entails recruitment of BMSCs to the bone surface, differentiation into osteoblasts and mineralization by the osteoblasts is followed by further differentiation of osteoblasts into osteocytes, which embed in the matrix, thereby communicating with other osteocytes, or cells in the bone environment through canalicular networks. HSCs and precursors to the osteoclasts are activated by the binding of the RANKL to the RANK receptor, promoting osteoclastogenesis and bone resorption. OPG a decoy receptor to RANKL, secreted by the osteoblasts, blocks the binding of RANKL to RANK and blocks osteoclastogenesis. With aging, osteoblasts and osteocytes undergo apoptosis or cellular senescence, and in the process, this internal regulation by OPG is disturbed, leading to more resorption. Production of pro-inflammatory SASP exacerbates the suppression of osteoblast function while triggering an activation of osteoclast precursors towards osteoclastogenesis. Moreover, reduction in BMSCs due to an altered fate to adipogenesis, also contributes to the suppression of osteoblast function. Reduction in osteoclast numbers, but increased activity, also disturbs the recruitment of more BMSCs to the bone surface, thus causing uncoupling of the bone homeostasis. Bone anabolics such as PTH 1-34 and neutralizing antibody against sclerostin (Sost), and anti resorptives as shown in the figure have been effective treatments for post-menopausal osteoporosis, but their efficacy in an aging population is not determined. Genetic removal of senescent cells was shown to restore bone homeostasis in aged mice hence pharmacological targeting senescent cells became a lucrative therapeutic option. Drugs that can remove the senescent cell (Senolytic drugs) or suppress the production of SASP (SASP modulators), collectively called senotherapeutics, may remove the triggers for uncoupling and restore bone homeostasis. Several of these senotherapeutics are listed in the figure and the ones which have been tested in some form of skeletal aging are underlined.

In fact, it was not until recently that senescent osteoblasts, osteocytes and myeloid populations were identified during physiological aging [25] and in a pathological model of accelerated aging using focal radiation[61]. Markers of senescence *p21*, $p16^{Ink4a}$ and *p53* were identified not only in mice but in aged bones from human biopsies [25]. Targeted removal of senescent cells, either pharmacologically, using senolytic drugs or genetic clearance of p16-positive cells in *INK-ATTAC* mice, or by the targeted inhibition of Janus kinase pathway, which in turn blocked SASP production, alleviated age-related osteoporosis in mice [99]. p16-3MR mice, is another genetic model for the clearance of p16-positive cells. Clearance of p16-expressing cells failed to show any recovery in the age-related bone loss [100]. However, the

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p16-3MR mice was not a good model for clearance of senescent osteocytes [100] as seen in the *INK-ATTAC* mice [99]. Indeed, the p16-3MR mice showed that the clearance of senescent osteoclast progenitors did not have any effect on the bone architecture of aged mice. These data suggest a direct role of senescent osteocytes in the pathophysiology of age-related osteoporosis. A genetically targeted clearance of senescent osteocytes may answer this question in future. In a model of high oxidative stress induced senescence, it was shown that countering senescent cells with senolytic drugs could alleviate radiation-induced skeletal aging like phenotypes[61].

Senescence Associated Secretory Phenotype (SASP)

Senescent cells have a unique secretome, known as senescence-associated secretory phenotype (SASP), pro-inflammatory in nature becoming one of the hallmarks of senescent cells. SASP proteins may have diverse functions, but the primary function is to recruit immune cells for the clearance of senescent cells[101]. When the senescent cells overwhelm the body as seen during aging, the impaired immune function fails to remove the senescent cells, resulting in a sustained SASP production causing systemic morbidity.

As discussed earlier, SASP production is dependent on ROS production and can distinguish quiescent cells from senescent cells. SASP proteins are produced in response to a DDR[102] and may comprise of proteins such as cytokines, chemokines, and interleukins. ROS induces a DSB, which triggers a DDR finally resulting in activation of NF-κB stimulating the SASP secretion. It was shown that activation of DDR induces the transcriptional upregulation of GATA4, which then activates NF-κB and elevated SASP gene activation[103]. The idea that a senescent cell is always associated with a SASP was questioned by the findings when studies showing senescent cells with $p16^{Ink4a}$ expression, were reported without a significant SASP[104]. Hence production of SASP in a senescent cell relied on the presence of a DDR. Mechanistic target of rapamycin (mTOR) pathway has also been shown to play an important role in cellular senescence and aging[105, 106], and the activation of p38/mTOR pathway is required for a sustained SASP production[107]. Glucocorticoids, such as corticosterone and cortisol were shown to suppress the SASP production without the reversal of the senescent state of the cell[108].

The list of proteins that can be called SASP factors is ever growing (Fig. 1)[109]. Production of SASP during physiological skeletal aging shares some common features with pathological skeletal aging[25] such as that seen with radiation[61]. Suppression of SASP using Janus kinase inhibitors (JAKi) alleviated age-related bone loss[99]. A better understanding of heterogeneity of SASP production was seen in an enriched population among different bone cells in mice, with varied expression levels, with larger fold changes seen in myeloid cells of aged bones, as compared to aged osteocytes (Fig.1)[25]. These results were largely replicated in human bone biopsies[25] and radiated mouse bones[61]. In another instance increasing doses of radiation induced proportional levels of senescence and gene expression for SASP markers in rat BMSCs[110]. Lipopolysaccharides have also been shown to induce senescence in alveolar bone together with the SASP factors such as Icam1, Il6, Mmp13 and TNF-alpha[111]. However, with a better understanding of senescence as a driver of age-related osteoporosis, but not post-menopausal osteoporosis [4], the correlation between senescence and bone loss in general is not a linear relationship. The SASP profile in the bones of mice which have undergone either orchidectomy or ovariectomy in young mice did not have resemblance with aged bones and remained mostly non-significant. Similar results were obtained with *INK-ATTAC* mice, in which ovariectomy induced bone loss was not recovered post-clearance of *p16*-positive senescent cells, and clearance of senescent cells did not have any effect on senescence markers. However, a short-term estrogen treatment could suppress age-related senescence and SASP markers[4], suggesting that estrogen may regulate senescence-pathways during old age. Since DDR is a key factor in SASP production, there may be several kinds of pathological osteoporosis where SASP is different from age-related osteoporosis. It was recently shown that ATM, other DDR proteins and NF-κB pathways were greatly elevated in Ercc1 deficient mice, in which the NER pathway of DDR was affected. These mice had a higher senescence and SASP profile which was reduced following the suppression of ATM kinase[112]. These studies suggested that targeting ATM pathway could slow the progression of aging, however there are contradictory studies as well where ATM activation alleviates senescence[113]. Moreover, histone variant macroH2A1, an epigenetic modified form of

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the canonical H2A histone and a marker for SAHF, is one of the recent additions to the proteins that in response to oncogene activation, may regulate SASP production and a persistent DDR, controlled by both positive and negative feedback loops[114]. Variants of macroH2A1, macroH2A1.1 and macroH2A1.2 increase with old age[115]. While a lot has not been reported on the role of macroH2A in bone homeostasis, macroH2A1.2 has been shown to negatively regulate breast cancer-induced osteoclastogenesis, by cooperating with Ezh2 [116]. Interactions between macroH2A1.1 and PARP1 regulate mitochondrial activity and a stress response, which can then regulate the SASP production, an area open for further exploration.

PARP1: Role in senescence and skeletal aging

PARP1 belongs to a family of transferases which is localized in the nucleus and is an important DNA damage response (DDR) protein. Association of PARP1 with DNA repair process [117] and telomere maintenance [118, 119] push the researchers to find the evidence of its role in longevity. PARP1 is known to be a general caretaker of the genome as it participates in major repair pathways and can be called as a first responder DDR protein. Several *in vivo* studies have supported the role of PARP1 in longevity. Telomeric DNA was approximately reduced by 30% in PARP knockout mice [120] as also observed with PARP knockdown or inhibition in cell culture [119]. This regulation of telomere length by PARP1 at molecular level is due to interaction with telomeric repeat binding factor 2 (TRF2) and thus affecting its binding to telomeres [118, 121]. PARP1 modifies target proteins by covalently linking PAR (poly(adenosinediphosphate-ribose)) moieties, a post-translational modification process known as PolyADP-ribosylation or PARylation. PARylation status among 13 mammalian species strongly correlated with their maximum life span, wherein, PARylation was found to be 5 times higher in PBMCs of humans as compared to rodents [122]. Furthermore, PARylation levels in PBMCs were reported to decline with age [123]. Intriguingly, centenarian humans showed higher PARP activity than the young subjects [124, 125]. Dynamics of PARP activity also changes with restriction of cell proliferation which leads to accumulation of age-related macromolecular changes including DNA [126]. Human-PARP1 overexpressed mice had prolonged disease-free survival, reduced tumor burden, but were more susceptible to aging related metabolic disorders. This has raised a question whether PARP1 is the probable candidate for longevity.

Besides, PARP1 is reported to play a role in inflammation [127] and caspase independent cell death [128, 129], hence could act as an aging promoting factor. PARP1 is known to be a transcriptional coactivator of NFκB [130], which is an important mediator of inflammatory signaling and aging [131, 132]. Severe DNA damage and NFκB directed inflammation could hyperactivate PARP1 that leads to necrosis due to depletion of NAD and ATP pool of a cell [133]. PARP1 dependent pathologies to some extent accumulate and lead to neurodegenerative disorders and aging. Therefore, PARP1 acts as a double-edged sword, where it acts as a longevity factor as well as an age promoting factor. PARP1 is an interesting player which exhibits contrasting roles in cell.

PARP1 has an inverse relationship with SIRT1, a longevity associated enzyme belonging to the sirtuin family (NAD dependent deacetylases). PARP activity limits the bioavailability of NAD for SIRT1 activity and henceforth reduces the deacetylation of certain transcriptional factors including PGC1α which would affect mitochondrial biogenesis and ultimately aging [134]. Recent work by Zha & colleagues, 2018 propose the use of PARP inhibitors to maintain mitochondrial function and function of aging induced endothelial progenitor cells (EPCs) by SIRT1 activation [135]. These findings suggest PARP1 as a longevity regulator where it can be a positive or negative regulator in a context dependent manner. There is a need to recognize the scenarios where PARP activity balances genomic integrity and metabolism to regulate aging.

PARP1 in senescence- Persistent DNA damage stimulates senescence in cells and PARP1 being a DNA repair enzyme do play a role in cellular senescence. A major non histone chromatin

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component, DEK protein has a role to play in metabolism and DNA repair. Increased DEK levels are known to favor immortalization by impeding senescence and apoptosis, while DEK deficient cells during genotoxic stress induces senescence [136]. Moreover, DEK is PARylated by PARP1 and hence regulates its activity in response to genotoxic stress [137]. Interestingly PARP1 inhibition increased the cellular senescence, while p21 deletion enhances PARP1 activity and DNA repair by NHEJ, thereby reduces DNA damage and subsequently cellular senescence [138]. PARP1 is a new target for treating various tumors and some studies have elucidated the role of PARP inhibitors in senescence. In ovarian cancer cells, low dose administration of olaparib has induced cellular senescence rather than apoptosis. The study suggested that olaparib induces senescence via p16-Rb or p53-Rb signaling axis and thereby inhibited the cell proliferation [139]. Intriguingly, PARP1 and its family members play key roles in regulating the SASP factors, cytokines and metalloproteases. PARP1 is reported to be associated with the promoters of cytokines, TNFα and IL1β[140]. Histone variant macroH2A1 plays a crucial role in regulating certain SASP genes at transcriptional level. Further, macroH2A1.1 is reported to regulate PARP1 activity either by recruiting it to chromatin [141] and hence could mediate SASP gene expression through PARP1[114]. As already discussed above, PARP1 regulates mitochondrial function and metabolism, hence, macroH2A1 and PARP1 axis could play a key role in senescence and aging which needs to be investigated. Thus, there is a high probability of PARP1 contributing to senescence and its associated phenotypes.

PARP1 role in metabolism and effects on cellular aging- Metabolism is considered to slow down with age, whereby metabolic abnormalities are key hallmarks of aging. Dietary restriction (DR) is testified to extend the lifespan of an organism and thus could affect the longevity and good health in humans, but further research is required to prove the DR effects keeping in criteria the early or late onset of DR [142]. Various research has linked PARP1 with the aging associated metabolic diseases [143-145] as well as brain diseases [146]. PARP1 is known to affect metabolism either directly or indirectly, wherein, PARP activation limits the metabolic fitness of a cell. PAR signaling could affect the activity of enzymes like hexokinase and hence glycolysis [147, 148]. Moreover, PARP utilizes NAD a critical metabolic cofactor, thus hinders cellular energy production [149]. PARP activation and NAD consumption in response to DNA damage sometimes shift the metabolism from oxphos to glycolysis resulting in damaged cell survival [150]. Recent preclinical results have highlighted the role of NAD metabolism in aging and hence restoration of NAD levels in old animals could extend lifespan and promote good health [151]. Researchers are exploring ways to boost NAD levels in cell to attain healthy aging and longevity. NAD supplementation, activation of NAD biosynthetic enzymes and inhibition of NAD degrading enzymes are three main approaches to increase NAD levels. Sirtuins and PARPs are two major NAD consuming enzymes and hence targeting them would be a beneficial strategy in aging. In this context, inhibition of the PARP1 enzyme would prevent degradation of NAD and would thus maintain NAD levels in cells and further delay in aging.

Besides genomic instability, mitochondrial dysfunction is another key player in cellular aging. Mitochondrial DNA (mtDNA) mutations originate either due to oxidative stress or as replication errors by the mitochondrial DNA polymerase. Such mtDNA mutations thereby contribute to age associated diseases and aging phenotypes [152]. Besides, nuclear DNA damage initiates nucleus to mitochondrial signaling which may regulate mitochondria function and aging. This signaling network involves nuclear sirtuins and PARPs that regulate genomic stability as well as mitochondrial integrity [153]. Elucidation of PARP signaling and mitochondrial function relationship would provide a new direction to research on aging. PARP when hyperactivated was shown to affect metabolism and mediate cell death and senescence [154]. *in vitro* and *in vivo* inhibition of PARP1 boosted NAD levels, enhanced SIRT1 activity, mitochondrial content and augmented oxidative metabolism [143]. It will be enlightening to study how PARP connects to mitochondrial function and mitophagy in the aging process.

PARP1 role in skeletal aging- ADP ribosylation (PARylation) is proposed to regulate the differentiation of bone cells and hence has an impact on bone health. PARP1 has been shown to regulate osteoclastogenesis[155] and osteogenic differentiation[156]. Accumulation of PARP1 leads to biomineralization of bone and vasculature triggered by a DDR, leading to excessive extracellular matrix calcification [157], also associated with senescence[158].

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Vascular calcification and bone loss are major disorders associated with aging. Bone mineral density and vascular calcification has an inverse relationship seen specifically in women, but not men[159]. PARP1 expression[160] and activity [161] has been found to increase in calcified aortic valves and vascular smooth muscle cells, respectively. PAR moieties have high affinity for calcium and thus assist in bone mineralization[157]. Although there is no direct evidence of PARP1 in skeletal/ bone aging, but its role cannot be neglected keeping in view its involvement in bone development and homeostasis. Further work is required to identify the connection between PARP1 and bone aging.

Therapeutics for aging bone

Parathyroid Hormone (PTH)

Parathyroid hormone (1-84amino acid; PTH) is an important regulator of calcium homeostasis, where the blood calcium level is controlled by the release of calcium from the existing bone, by a calculated action of osteoblasts over the osteoclasts. PTH is one of the first hormones whose efficacy was considered for the treatment of senile osteoporosis. PTH 1-34 (Teriparatide; Forteo®) is a biosynthetic drug composed of the first 34 amino acids of human parathyroid hormone. It was one of the first anabolic drugs approved for osteoporosis in the European Union and in the US by the FDA[162-164]. Intermittent teriparatide treatment is prescribed for patients who are at high fracture risk. It is currently approved as an injectable and is very effective in improving the overall BMD. The anabolic effect of teriparatide is not fully understood and while it has been shown to improve osteoblast function, increase osteoblast formation and decrease in osteoblast apoptosis, the exact mechanism of the conversion of the progenitors in osteoblasts, role of blood vessels and the movement of cells during bone formation is still under investigation. The use of teriparatide as a treatment of osteoporosis is limited for two years, a limitation assigned based on the high rate of occurrence of osteosarcoma in animal studies[165]. However, long-term follow up in humans, have not reported a single case of osteosarcoma in patients who have received teriparatide treatment[166].

Several studies including ours have investigated the efficacy of teriparatide to counter triggers of senescence by promoting DNA repair mainly through the activation of the Wnt pathway[54, 167]. PTH administration was shown to downregulate senescence by inhibiting $p16^{Ink4a}$ and alleviated the age-related progression of osteoarthritis[168].

Anti-Sclerostin Antibody

Sclerostin, a glycoprotein encoded by the gene SOST, is secreted by osteocytes, has inhibitory effects on the osteoblast function by negatively regulating the Wnt and bone morphogenetic protein (BMP) signaling. A humanized antibody against Sclerostin (Romosozumab) is an emerging therapeutic, which has reached Phase III in clinical trials. The limitation of the Sclerostin action within the skeleton makes it a good candidate for osteoporosis, with fewer concerns of systemic effects. Sclerostin's anabolic function and as a possible therapeutic for osteoporosis, is based on the high bone mass phenotype in patients of sclerosteosis with a genetic deficiency of sclerostin[169, 170]. Similarly, a genetic deletion of sclerostin in mice also resulted in a high bone mass[171]. A pre-clinical study in rats confirmed the efficacy of anti-sclerostin antibody in a model of postmenopausal osteoporosis[172]. In large scale clinical trials, it was reported that romosozumab was associated with an increase in BMD[173], with a better anabolic effect than teriparatide[174]. Unlike teriparatide, romosozumab had no carcinogenicity concerns in animals or humans[175]. Effects of romosozumab were reversible when discontinued and required a subsequent treatment of denosumab, an antiresorptive monoclonal antibody against RANKL[176] and by a single dose of zoledronate, which preserved the anabolic bone accrual initiated by romosozumab, for an additional 2 years[177].

Sclerostin has been shown to negatively regulate several cellular processes in the bone. Sclerostin levels are elevated in elderly people[178, 179]. Similar level of elevation in sclerostin

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levels is observed in osteoclasts from aged mice[180]. Elevated levels of sclerostin were also reported in radiated bones, another model of skeletal aging[55]. It was shown that sclerostin may be responsible in generating radiation-induced DNA damage, since use of a neutralizing antibody against sclerostin promoted DNA repair, suppressed radiation-induced adverse changes in bone marrow including adiposity and alleviated loss in bone architecture due to radiation damage [55]. These studies suggest that sclerostin may be an inducer of senescence.

Anti-resorptives

The class of drugs that suppress the osteoclast-based bone resorption are termed as "anti-resorptives". Bisphosphonates are a class of anti-resorptives which have a pyrophosphate-like chemical structure which allows them to bind strongly to calcium and may work as a beacon to the bone tissue. The nitrogen-containing bisphosphonates which are not limited to etidronate, clodronate, risedronate, alendronate, olpadronate, ibandronate and zoledronate[181, 182]. These nitrogen-based bisphosphonates target farnesyl diphosphate synthase (FPP synthase), an enzyme in the mevalonate pathway, thereby suppressing the osteoclast function. Zoledronate has become one of the most widely accepted anti-resorptive and as a treatment for osteoporosis. It was recently reported that zoledronate can improve DNA repair in MSCs[53], suggesting that it may work as regulator of cell senescence and hence be used as a therapeutic for skeletal aging, which is markedly different from post-menopausal osteoporosis. Denosumab, a monoclonal antibody against RANKL, blocks binding of RANKL to its receptor RANK on the osteoclast progenitors, suppresses osteoclast function and thus protects bone loss as an anti-resorptive. While denosumab may work to alleviate senile osteoporosis, but there is no evidence to suggest that it may have a role in regulating senescence as a mechanism.

Senolytics and SASP modulators

Identification of senotherapeutic drugs were based on the compounds that would selectively kill senescent cells without affecting proliferating cell termed senolytic drugs, or drugs that suppressed cell senescence or SASP termed senomorphic, while some other compounds that are toxic to cells, have no effect on senescent cells, increase senescent cells or increase in proliferation, were excluded as senotherapeutics [183].

Since the identification of senescent osteoblasts and osteocytes in bone tissue, it was speculated that the senescent nature of these cells together with the SASP regulated bone remodeling. Clearance of senescent cells and suppression of SASP hence became lucrative methodologies to treat physiological and pathological skeletal aging. Genetic clearance of $p16^{Ink4a}$ has been shown to improve age related health and life span[39], and age-related osteoporosis[99]. Pharmacological clearance of senescent cells using a senolytic drug cocktail of Dasatinib and Quercetin was also effective in restoring bone architecture as seen in physiological aging [99]and in a pathological model of skeletal aging as seen with radiation-related osteoporosis[61]. However, some other senolytic drugs which were shown to be effective in curing some aspects of age-related comorbidities, were ineffective in radiation-associated bone loss[61], suggesting varied mechanisms of actions among the senolytic drugs. Clinical trials are currently underway to assess the efficacy of senolytic drugs to treat age-related comorbidities, including osteoporosis.

Hence, while anabolic agents can promote bone formation, and anti-resorptives can suppress osteoclast function, senolytic drugs can eliminate the senescent cells responsible for instigating osteoclast activity and suppression of bone formation, making senolytic drugs as a promising treatment strategy for age-related osteoporosis.

Several novel drugs have been explored as senolytic, including Dasatinib (D), Quercetin (Q), D+Q[184], Luteolin[185], Fisetin[186, 187], Navitoclax (ABT263)[188], BCL-XL inhibitors[187], HSP90 inhibitors[183] Piperlongumine[189], RG7112[190], O-Vanillin[190], ABT-737[191] and CD153 Vaccine[192] and aspirin[193, 194], reviewed by Robbins et. al. recently[195] (Fig.2).

Drugs that do not kill the senescent cells but counter the pro-inflammatory protein production are termed as SASP modulators or senomorphics. JAKi, ruxolitinib have been shown to

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be effective in alleviating age-related osteoporosis, by possible suppression of specific factors such as IL6, IL8 and PAI1, which were shown to activate osteoclast formation[99]. Inhibitors for the Mdm2 can block the interaction between Mdm2 and p53 and block p53 degradation, hence can lead to high p53 and p21 expression. However, the same Mdm2 inhibitors, Nutlin3a and MI-63, have been shown to suppress SASP factors[196]. Rapamycin and Rapalogs (analogs of Rapamycin) are also reported to suppress the SASP[197, 198].

Future Directions

With the increase in identification of several target molecules that positively or negatively regulate the bone, advent of new and more effective therapeutics is inevitable. These newer therapeutics should have minimal side effects and their efficacy during other disease conditions should also be explored. So, while the patient is treated for osteoporosis, the drug should not interfere with the function of the drug for a secondary disease. This could be achieved by using single, alternate, or combinatorial treatment and determining the efficacy or toxicity of both or either of the drugs. Alternatively bone anabolic agents could be fused with a "homing" molecule which would guide the drugs only to the bone surface, minimizing the systemic effects on other organs.

An osteoblast specific loss of RICTOR, an mTOR complex2 protein resulted in age-related bone loss[199]. Sirt-3, an important protein in mitochondrial metabolism, activates the mTOR pathway to regulate osteoclastogenesis, increased adipogenesis and bone loss[200].The role rapamycin and similar mTOR inhibitors as senolytic or senomorphic has not been studied in the context of an aging skeleton, but rapamycin was able to alleviate periodontal diseases in aged mice[201].

PARP1 is another interesting DDR protein, which not only plays a role in DDR, but also plays a role in inflammation by regulation of the NF-κB pathway. As seen with ATM kinase levels, PARP1 levels beyond a certain threshold may be detrimental for certain age-related co-morbidities. One such example where PARP1 inhibition initiated prevention of neurodegeneration seen during Parkinson's disease, by restoring degradation of alpha-synuclein[202]. There are other examples where PARP1 inhibition alleviated age-related cellular[135] and tissue dysfunction[203] and could be a potential therapeutic option for osteoporosis.

Some of the novel senotherapeutic drugs which have shown efficacy *in vitro*, may be tested as a treatment of osteoporosis. However, judgement should always side with caution, since senolytic drugs may work for recovering some aged related tissue dysfunction[188], but not for osteoporosis, as in the case with Navitoclax/ABT-263[204]. Another example is Fisetin which also worked as a senolytic and alleviated several age-related phenotypes[186, 187], but not skeletal aging seen with radiation exposure[61]. Fisetin may work for resorption-based osteoporotic diseases as it counters the osteoclast function[205, 206], but its role in age-related osteoporosis is yet to be determined.

Future treatments of osteoporosis and other bone ailments may include senotherapeutics which may be explored to be given in combination with the more established bone anabolic drugs.

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AC conceptualized the review, performed literature survey, wrote the manuscript, and made the figures. JR performed literature survey, wrote the manuscript, and co-edited the figures. Both the authors have approved the final version of the manuscript.

Conflicts of interest:

The authors declare no conflicts of interest.

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