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Concise Review

Mesenchymal Stem/Stromal Cell Senescence: Hallmarks, Mechanisms, and Combating Strategies

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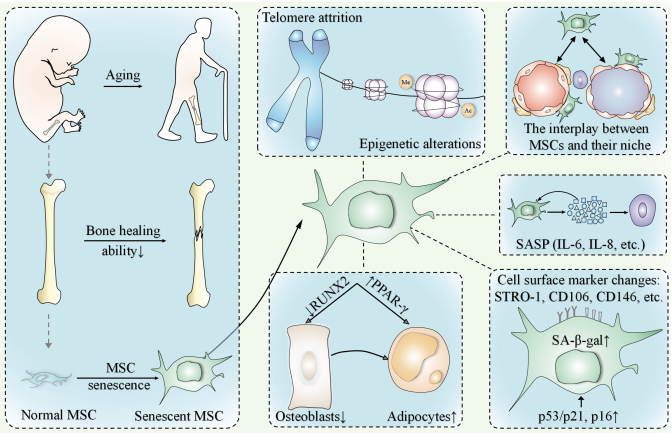
Abstract

Aging is a multifaceted and complicated process, manifested by a decline of normal physiological functions across tissues and organs, leading to overt frailty, mortality, and chronic diseases, such as skeletal, cardiovascular, and cognitive disorders, necessitating the development of practical therapeutic approaches. Stem cell aging is one of the leading theories of organismal aging. For decades, mesenchymal stem/stromal cells (MSCs) have been regarded as a viable and ideal source for stem cell-based therapy in anti-aging treatment due to their outstanding clinical characteristics, including easy accessibility, simplicity of isolation, self-renewal and proliferation ability, multilineage differentiation potentials, and immunomodulatory effects. Nonetheless, as evidenced in numerous studies, MSCs undergo functional deterioration and gradually lose stemness with systematic age in vivo or extended culture in vitro, limiting their therapeutic applications. Even though our understanding of the processes behind MSC senescence remains unclear, significant progress has been achieved in elucidating the aspects of the age-related MSC phenotypic changes and possible mechanisms driving MSC senescence. In this review, we aim to summarize the current knowledge of the morphological, biological, and stem-cell marker alterations of aging MSCs, the cellular and molecular mechanisms that underlie MSC senescence, the recent progress made regarding the innovative techniques to rejuvenate senescent MSCs and combat aging, with a particular focus on the interplay between aging MSCs and their niche as well as clinical translational relevance. Also, we provide some promising and novel directions for future research concerning MSC senescence.

Key words: mesenchymal stem/stromal cells; senescence; rejuvenation; stem cell niche; mitochondrial dysfunction; reactive oxygen species (ROS); cell signaling.

Graphical Abstract



Mesenchymal stem/stromal cell (MSC) senescence is manifested by distinctive phenotypic changes, including flattened and enlarged cell morphology, SASP, biomarker changes, telomere attrition, epigenetic alterations, impaired differentiation potential, and declines in proliferation ability. Abbreviations: MSC, mesenchymal stem/stromal cell; RUNX2, runt-related transcription factor 2; PPAR-γ, peroxisome proliferator-activated receptor-γ; SASP, senescence-associated secretory phenotype; IL-6/8, interleukin-6/8; SA-β-gal, senescence-associated beta-galactosidase; pRB, phosphorylated retinoblastoma.

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Introduction

Mesenchymal stem/stromal cells (MSCs) are nonhematopoietic cells derived from the mesoderm with potent multipotency and regenerative properties, attributing to adult somatic stem cells.[1] In 1968, MSCs were first isolated from bone marrow[2] and subsequently obtained from various tissue sources, such as umbilical cord blood, trabecular bone, periosteum, synovium, placenta, pancreas, adipose tissue, skin, lung, and thymus.[3] In 2006, the International Society for Cellular Therapy (ISCT) proposed the standard for defining MSCs, which emphasized their trilineage (osteogenic, adipogenic, and chondrogenic) differentiation ability in vitro, thus making MSCs hold great promises for stem cell-based therapy.[4,5] Moreover, MSCs are classically known for having peculiar immunomodulatory functions, which enables them to regulate the surrounding immune microenvironment. For instance, MSCs have been shown to inhibit T-cell proliferation and immunological defense responses, suggesting their immunosuppressive property.[6] Besides, MSCs possess low immunogenicity and thus could be transplanted autologously or allogeneically, potentially having many applications in stem cell-based therapy for a variety of diseases,[7,8] although additional rigorous studies concerning MSCs engraftment, differentiation, or tumorigenicity in vivo are needed. Furthermore, MSCs are considered a much safer source for stem-cell therapy due to their lower risk of tumorigenesis than embryonic stem cells or induced pluripotent stem cells.[9] Based on the favorable characteristics described above, MSCs are currently being tested in the context of regenerative medicine for their potential use in cell and gene therapy for numerous human diseases and disorders.[10]

Senescence is a cellular response characterized by a stable cell cycle arrest that limits the proliferative potential of cells. To date, 4 types of senescence have been distinguished: replicative senescence (RS), oncogene-induced senescence (OIS), stress-induced premature senescence (SIPS), and developmental senescence .[11-14] Mounting evidence has shown that MSCs in culture exhibit a finite proliferation capacity before entering a state of replicative arrest, which was initially described by Hayflick in 1965 (ie, Hayflick limit) when culturing fibroblasts.[15] In general, the life span of MSCs ranges from 30 to 40 population doublings (PDs) in vitro.[16] After extended culture, the aging process toward replicative arrest could seriously impair the function of MSCs both in vitro and in vivo. Mesenchymal stem/stromal cells from older donors, for example, have hindered proliferation and differentiation capacity comparing with MSCs from younger donors.[7] Besides, MSCs have been shown to progressively lose their stemness during the expansion in vitro,[17] indicating an inverse relationship between MSCs' function and their divisional history. Therefore, understanding the detailed molecular processes behind MSC senescence is critical for optimizing the therapeutic effect of MSCs and developing plausible strategies to prevent or even reverse the malfunction of senescent MSCs, and hopefully, rejuvenating individuals' overall well-being and mitigating age-related diseases. Here, we summarized the morphological, biological, and phenotypic changes of senescent MSCs, cellular and molecular mechanisms that underlie MSC senescence, and current knowledge and strategies to prevent MSC senescence in this review, with a special focus on the reciprocal interactions between aging MSCs and their niche as well as clinical translational relevance.

Characteristics of MSC Senescence

In general, MSC senescence is associated with a series of distinctive phenotypic changes, including morphological and biological changes, such as enlarged and flattened shape, loss of proliferative and differentiation ability; moreover, alterations in biomarkers, as well as qualitative and quantitative changes in the secretome.[18]

Morphological and Biological Changes

Morphologically, the aging process is described and characterized as flattened and enlarged cells with constrained nuclei, granular cytoplasm, as well as more podia and actin stress fibers.[19-21] An imaging system has been adopted to analyze the enlargement process of MSCs during long-term culture, which showed MSCs from passages 1-3 exhibited a standard size, whereas they began to enlarge at passage 5, resulting in a 4.8-fold larger size at passages 6-9 compared with passage 1.[22] The mechanisms underlying the senescence-associated morphological changes have been reported to rely on the status of the scaffolding protein caveolin-1 (CAV-1), which could regulate actin stress fiber formation and focal adhesion kinase activity in the senescent cells.[23] Hence, CAV-1 has been proposed as a master regulator in the scenario of cellular senescence.[24]

Interestingly, the aberrant morphologies are directly linked to the functional state of cells. Plenty of studies have proved the tight relationship between morphologic features and the biological function of senescent MSCs, although the mechanisms involved are incompletely understood. For example, the adenosine triphosphate (ATP) level and the senescence-associated galactosidase (SA-β-gal) activity in large-sized MSCs are equivalent to those in the aged MSC population, while in small-sized MSCs the ATP and SA-β-gal levels are comparable to the young MSCs and exhibited a youthful phenotype, making cellular size a potential criterion to distinguish senescent MSCs.[25] Besides, morphologic features have been found to be highly predictive of the osteogenic ability of MSCs; through cellular and nuclear morphology, the mineralized capacity was accurately forecasted within the initial 3 days upon osteogenic induction.[26] In addition, abnormal biosynthesis or growth rate can be inferred from the observation of cell size non-uniformity.[26,27] In recent years, an autofluorescence-based method has been proposed as a quantifiable and non-invasive senescence assay, which is also conducted and determined through the increased cell size and cytoplasmic granularity during senescence.[28]

The decline in proliferative capacity in senescent MSCs has been widely reported both in vitro and in vivo. In fact, cellular senescence was initially identified as a loss of proliferative capacity after extended culture, which is known as RS.[29] The colony-forming unit-fibroblast (CFU-F) assay is one of the most frequently used qualitative methods to estimate and evaluate MSCs' proliferation potential in vitro. Senescent MSCs exhibit a decreased level of CFU-F, and, more importantly, they display smaller colony sizes.[30] The phenomenon of RS can also be recapitulated by the repeated passage of cells in culture.[31] For instance, MSCs from aged donors exhibit decreased passaging ability (24 ± 11 vs 41 ± 10 PDs) and proliferation rate (0.05 ± 0.02 vs 0.09 ± 0.02 PDs/day) compared with those from young donors.[21]

In addition to changes in proliferation ability, the multiple differentiation capacity of MSCs has been found to undergo

substantial changes during senescence. Studies have revealed that MSCs progressively lose their differentiation potential during extended culture, eg, diminished adipogenic differentiation and complete loss of osteogenic differentiation potential.[32] Consistently, transcriptome analysis revealed that long-term cultivation of MSCs in vitro results in the downregulation of genes involved in cell differentiation. To illustrate, runt-related transcription factor 2, also known as core-binding factor α1, is an essential osteoblastic transcriptional mediator whose expression level is observed with a decline during MSC senescence.[33] In contrast, a highly elevated expression level of receptor activator of nuclear factor-κB ligand is observed, which is essential for osteoclast differentiation. Besides, the expression of alkaline phosphatase (ALP) and the osteocalcin are both downregulated in senescent MSCs, which is in line with the decrease of bone-forming efficiency during long-term culture.[34] However, changes in differentiation remain controversial, especially the phenomenon of skewed lineage differentiation. In common, it is accepted that MSCs are preferentially differentiated toward adipose during senescence based on available evidence.[33,35] Plenty of molecules and signaling pathways have been found to anticipate the regulation of the skewed lineage differentiation of senescent MSCs. Peroxisome proliferator-activated receptor-γ (PPAR-γ) is regarded as an adipogenic-specific transcription factor; the increase of PPAR-γ during senescence shifts the fate of MSCs toward adipogenesis. On the contrary, Wnt/β-catenin (WNT) signaling could restrain adipogenesis and favor the MSC differentiation toward osteoblasts,[36] making WNT signaling a significant mechanism of balancing adipogenesis and osteogenesis and regulating cellular senescence. A general decrease in WNT signaling with age has been found,[37] while intriguingly, excessive activation of WNT signaling could also trigger MSC aging.[38] As for changes in chondrogenesis, relevant studies are limited, but the tendency is similar to osteogenesis, suggesting a negative correlation between advancing age and the chondrogenic potency of MSCs.[39,40] Therefore, aging negatively influences MSCs proliferation and can shift MSC differentiation bias. In other words, differentiation abilities of MSCs decline with aging, especially osteogenetic ability, as a result, aging facilitating adipogenesis instead of osteogenesis.

Phenotypic Changes

As is well known, one of the critical criteria to define MSC, proposed by ISCT, is specific surface antigen expression. In particular, the MSC population must express CD73, CD90, and CD105, as measured by flow cytometry, and must lack expression of CD45, CD34, CD14 or CD11b, CD79α, or CD19 and human leukocyte antigen-DR isotype.[4] These MSC surface markers remain expressed stably and show little association with culture passage or aging status. In contrast, some other markers, including STRO-1, CD106, and CD146, demonstrate downregulated expression levels in vitro and/or in vivo during senescence, making it possible to identify senescent MSCs through surface marker expression profiles.[41-43] Apart from senescence-related downregulation, CD106 expression decreases in differentiated MSCs dramatically, suggesting its potential role as a marker to identify undifferentiated MSCs.[43] Meanwhile, it is reported that CD146+ MSCs exhibited increased migratory potential toward degenerated tissues[44]; thus, it is tempting to speculate that the decrease of CD146 expression may be associated with the impaired migratory capacity in senescent MSCs. Besides, unlike the downregulated MSC markers mentioned above, CD295 (also known as leptin receptor or LEPR) and CD264 have been found to increase during MSC senescence. CD264+ MSCs exhibit increased SA-β-gal activity and decreased proliferation rate and differentiation ability, while CD295+ MSCs display reduced proliferation capacities, and CD295/LEPR distinctly marks the subpopulation of dying cells.[45,46] Overall, to the best of our knowledge, despite the above-mentioned indicative cell surface markers for distinguishing senescent MSC populations, a consensus is yet to be reached. Solid experimental results are in urgent demand to determine some markers as the gold standard for senescent MSCs.

Speaking of the gold standard for evaluating cellular senescence, SA-β-gal is the most widely accepted marker for senescence assessment since first published by Dimri in 1995,[47] which can be detected via cytochemistry or histochemistry and fluorescence-based methods.[48] The SA-β-gal assay is based on the fact that β-galactosidase (β-gal) activity in senescent cells is significantly enhanced due to the increased lysosomal activity and altered cytosolic pH.[49] However, it can still yield false-positive or -negative results in some exceptional circumstances.[50] For example, SA-β-gal activity is expressed from *GLB1*, the gene encoding lysosomal β-gal when cells have *GLB1* deficiency or defective lysosomal β-gal, SA-β-gal activity is undetectable at late passages even though the cells indeed underwent RS.[51] Recently, another lysosomal enzyme, senescence-associated α-L-fucosidase (SA-α-Fuc), has been identified as a novel biomarker in all types of cellular senescence. Senescence-associated α-L-fucosidase is thought to be a more sensitive and robust biomarker than the classical marker SA-β-gal.[52-54] Based on the above finding, an α-Fuc-responsive aggregation-induced emission probe was developed, which was designed to complement conventional marker, namely SA-β-gal, and has been proved to identify senescent cells lacking β-gal expression.[55]

The senescent state is primarily characterized by durable cell cycle arrest. Activation of cyclin-dependent kinase (CDK) inhibitors $p16^{INK4A}$ and $p21^{CIP1/WAF1}$ is essential for senescence-associated growth arrest, which antagonizes CDK to block cell cycle progression. During cellular senescence, the expression level of $p16^{INK4A}$, p53, and $p21^{CIP1/WAF1}$ was shown to be upregulated both in vitro and in vivo, as evidenced by the fact that $p16^{INK4A}$+ cells were concomitantly positive for SA-β-gal and overexpression of p53 and $p21^{CIP1/WAF1}$ elevated SA-β-gal expression.[56] Besides, inhibiting $p16^{INK4A}$, $p21^{CIP1/WAF1}$, or p53 may also reduce the number of senescent MSCs or restore their proliferation ability.[57] However, it is worth noting that unlike $p16^{INK4A}$ as a reliable biomarker, increased $p53/p21^{CIP1/WAF1}$ level is also found during the process of apoptosis and transient cell cycle arrest, thus making $p53/p21^{CIP1/WAF1}$ merely be convincing in detecting senescent MSCs precisely when in combination with other indicators, such as SA-β-gal.[7,58,59]

Senescent cells produce and secrete many factors, including interleukin-1(IL-1), IL-3, IL-4, IL-6, IL-8, IL-17, epidermal growth factor (EGF), fibroblast growth factors-2 (FGF-2), FGF-4 and FGF-8, hepatocyte growth factor, insulin growth factor-1 (IGF-1), platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and vascular endothelial growth factor, which are collectively referred to as the senescence-associated secretory phenotype (SASP) factors. At late passages during culture, the expression of SASP factors is increased, which drives responses that reinforce senescence

through both cell-autonomous and non-cell-autonomous manners, thus creating a vicious cycle.[18,60] Indeed, blocking the SASP-associated feedback loop, for example, depletion of IL-6R, IL-8R, or IGF-binding protein 7, was proven to delay the onset of senescence or even prevent the entire process.[61-63] In addition to self-regulation, SASP factors also tend to influence neighboring cells via paracrine mechanisms to accelerate senescence.[64] For instance, SASP factors secreted by aged MSCs could induce premature senescence by limiting the proliferation rate of neighboring young hematopoietic stem cells (HSCs).[65] The interaction between SASP and the immune system is complicated. On the one hand, the SASP recruits immune cells, including macrophages, natural killer cells, neutrophils, and T lymphocytes, which eliminate SASP factors. On the other hand, apart from promoting clearance of senescent cells by the immune system,[66] SASP from aged MSCs can also exhibit immune-suppressive function[67,68] and also interact with immune cells to avoid elimination.[69] For instance, Toll-like receptor-4 (TLR-4) can stimulate MSCs to acquire an immune-suppressive phenotype by secreting high levels of soluble factors in an inflammatory environment.[70] Interestingly, as is well known, MSCs have potent anti-inflammatory functions, whereas senescent MSCs play a pro-inflammatory role due to SASP, which has been considered a major cause of aged MSCs' detrimental effects.[71] In recent years, an elegant study has confirmed that, during aging, MSC-derived secretomes (SASP) contribute to activating an inflammatory transcriptome in HSCs that may ultimately impair their functionality.[65] The specific components of SASP vary from cell types and senescence inducers, among which IL-6 and IL-8 remain the 2 universal markers for SASP and can be detected using the commercially available enzyme-linked immunosorbent assay kits.[62] However, SASP remains unspecific in distinguishing senescent cells due to its extensive involvement in multiple physiological and pathological processes.

Mechanisms of MSC Senescence

Stem cell aging is well recognized as a complicated process regulated by multiple mechanisms. The most studied mechanisms implicated in the process of MSC senescence will be discussed in the following subsections, from the perspectives of DNA damage, reactive oxygen species (ROS), mitochondrial dysfunction, telomere attrition, stem cell niche, and epigenetic alterations. Hopefully, characterizing the mechanisms of senescent MSCs in different scenarios contributes to developing specific and optimal anti-senescence therapeutic strategies.

DNA Damage

Mesenchymal stem/stromal cells accumulate DNA damage throughout time as a consequence of growth-promoting stimuli, such as DNA replication mistakes and telomere attrition. Furthermore, endogenous and exogenous mutagens such as oncogene activation, irradiation, chronic inflammation, and particularly oxidative stress, induced by the buildup of ROS, are also proven to be sources of DNA damage. DNA damage response (DDR) network is perceived as a dominant regulator of cell cycle arrest, which is one of the hallmarks of MSC senescence and is primarily regulated by the $p21^{CIP1/WAF1}$ and $p16^{INK4A}$ signaling pathways[72,73] (Fig. 1).

Encoded by *CDKN1A*, $p21^{CIP1/WAF1}$ is a CDK inhibitor, which is induced as a result of DNA damage and transcriptionally activated by p53. The activation of $p21^{CIP1/WAF1}$ decreases the phosphorylation level of retinoblastoma (RB), namely decreases phosphorylated RB (pRB), by inhibiting CDK2, and thereby enabling RB to retain function and keep suppressing the E2 transcription factor (E2F), a key regulator of genes needed for cell growth control and proliferation,[74,75] eventually leading to cell cycle arrest and prevention of re-entry.[76,77] As aforementioned, CDK-mediated phosphorylation tightly regulates pRB activity. Similarly, $p16^{INK4A}$, encoded by *CDKN2A*, can induce cellular growth arrest by blocking CDK4 and CDK9 and keeping pRB in a hypophosphorylated activation state.[78,79] The $p14^{ARF}$ protein ($p19^{ARF}$ in mouse) protein links the p53/$p21^{CIP1/WAF1}$ pathway and $p16^{INK4A}$ pathway, which is also encoded by the *INK4* locus and inhibits p53 degradation by blocking human monocyte-derived macrophages-2 (hMDM-2) ubiquitin ligase, thus favoring senescence.[80]

It is worth mentioning that the DDR and $p21^{CIP1/WAF1}$/$p16^{INK4A}$-mediated cell cycle arrest are not a one-to-one match. For one thing, $p21^{CIP1/WAF1}$ and $p16^{INK4A}$ can also be activated in a DDR-independent manner; for another, stimuli that transiently cause DDR may result in DNA repair activities rather than senescence via the p53 pathway.[7,81] In addition, MSCs are more resilient to genotoxic damage than cycling cells, and such resistance has been attributed to the induction of p53, which is modulated by ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad3-related), 2 central kinases in sensing and responding to DNA damage in DDR signaling network.[82-85]

Reactive Oxygen Species

Reactive oxygen species, a metabolic byproduct, is critical for MSC proliferation and differentiation when accumulated in a physiological amount.[86] Under pathological circumstances, however, excessive ROS buildup causes MSCs' progression to senescence.[87] Not only is excessive accumulation of ROS a key stimulus triggering DDR but it also leads to a wide variety of other detrimental responses, including mitochondrial malfunction, autophagy suppression, telomere attrition, and protein degradation, all of which contribute to cellular senescence.[17]

Reactive oxygen species promotes MSC senescence by causing oxidation and a cascade of secondary metabolic reactive species (Fig. 1). As a member of mitogen-activated protein kinases (MAPKs), p38 MAPK is known to be an indispensable molecule in mediating ROS-induced senescence,[88] and the inhibition of the p38 MAPK pathway results in the prevention of senescence to some extent.[89-91] By directly or indirectly affecting MAPK/ERK kinase kinase 1/2/3/4 (MEKK1/2/3/4) or mixed lineage kinase 3, ROS stimulates MAPK kinase 3 and MKK6 and phosphorylates p38, activating the p38 MAPK pathway.[92] Furthermore, activated p38 MAPK pathway promotes p53 phosphorylation, making ROS lead to senescence through the p53/$p21^{CIP1/WAF1}$ pathway as well.[93]

Besides, phosphatidylinositol-3-kinase (PI3K)-protein kinase B (AKT) signaling pathway is also responsible for the ROS-activated MSC senescence. Through direct stimulation of PI3K, ROS catalyzes the synthesis of phosphatidylinositol 3,4,5-triphosphate and amplifies its downstream signaling, such as AKT, which successively promotes the transcription of its target genes, including mechanistic target of rapamycin 1, forkhead box protein O3 (FOXO3), and p53, eventually

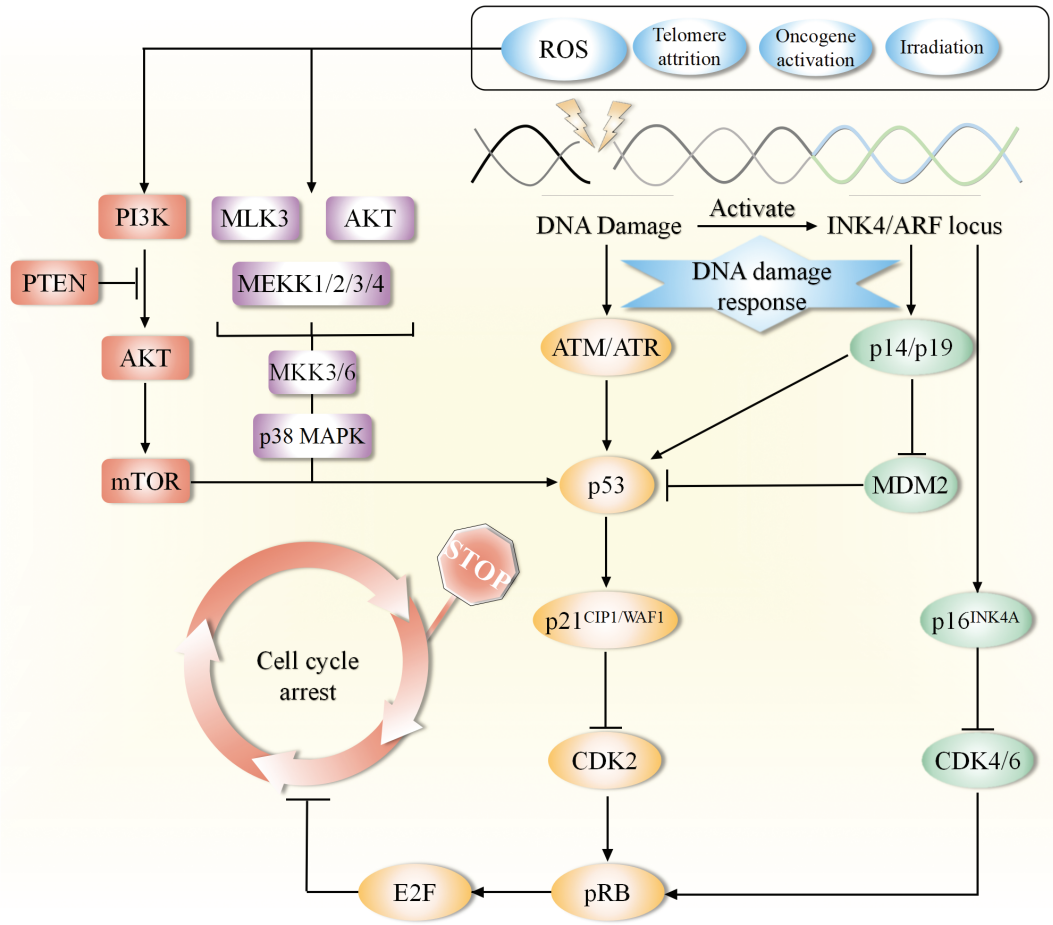

Figure 1. DNA damage response network in MSC cell cycle arrest. DNA damage response is triggered by endogenous and exogenous stresses in senescent MSCs, such as ROS, telomere attrition, oncogene activation, and irradiation, resulting in activation of the 2 main senescence-related signaling pathways, namely p53/p21$^{CIP1/WAF1}$ and p16^{INK4A}, which leads to cell cycle arrest and MSC senescence. Abbreviations: DDR, DNA damage response; ROS, reactive oxygen species; MSC, mesenchymal stem/stromal cell; PI3K, phosphatidylinositol-3-kinase; PTEN, phosphatase and tensin homolog; AKT, protein kinase B; mTOR, mechanistic target of rapamycin; MLK3, mixed lineage kinase 3; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MEKK, MAPK kinase kinase; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia mutated and RAD3 related; MDM2, monocyte-derived macrophages-2; CDK, cyclin-dependent kinase.

leading to the MSC senescence.[94-96] Of note, early studies of phosphatase and tensin homolog (PTEN), a protein that has been confirmed to regulate AKT negatively, showed that its activity was able to promote cell cycle arrest and apoptosis and inhibit cell motility.[97] Collectively, it has been well documented that inhibiting the PI3K/AKT/mTOR/FOXO3 pathway delays senescence and facilitates the anti-apoptosis response.[98-100]

In general, regulation of ROS and oxidative repair become less efficient during aging, ROS accumulation causes molecular damage and MSC senescence, and senescent MSCs, in turn, produce more ROS, forming a positive feedback loop.[101] Thus, the production of ROS and ROS-derived damage may be a major player throughout the MSC aging process, especially the casual link between mitochondrial dysfunction and ROS.[102]

Mitochondrial Dysfunction

Mitochondria and mitochondrial dysfunction hold a prominent place in cellular senescence (Fig. 2). As aforementioned, mitochondria are essential components in the positive feedback loop of ROS-induced senescence. It is well known that increased ROS levels lead to increased mitochondrial DNA (mtDNA) damage, and due to the absence of efficient repair mechanisms, mtDNA is deemed to be more susceptible to mutation than nuclear DNA.[103] Meanwhile, based on the free radical theory of aging proposed by Denham Harman, age-related mtDNA abnormalities also lead to increased ROS expression, thereby forming a vicious cycle that disturbs the balance between ROS and antioxidants.[104,105] Another theory has been postulated that ROS-induced DNA damage promotes mitochondrial biogenesis through the mTOR/proliferator-activated receptor-gamma coactivator-1α/β axis, which in turn results in an elevated number of mitochondria and an augmented mitochondrial ROS production, establishing another vicious cycle.[106]

Mitochondria, as energy centers of cells, are essential for cellular respiration; therefore, their malfunction correlates with aberrant respiratory chain, such as abnormal NAD+/NADH and ATP/ADP ratios, both of which associate with MSC senescence.[107] The imbalanced ATP/ADP ratio could trigger the activation of 5′-AMP-activated protein kinase (AMPK), leading to senescence through the p53/p21$^{CIP1/WAF1}$ pathway.[108] As for the abnormal NAD+/NADH ratio, usually associated with decreased cytosolic malate dehydrogenase and nicotinamide phosphoribosyltransferase,[109,110] it has been considered as a unique type of senescence termed as

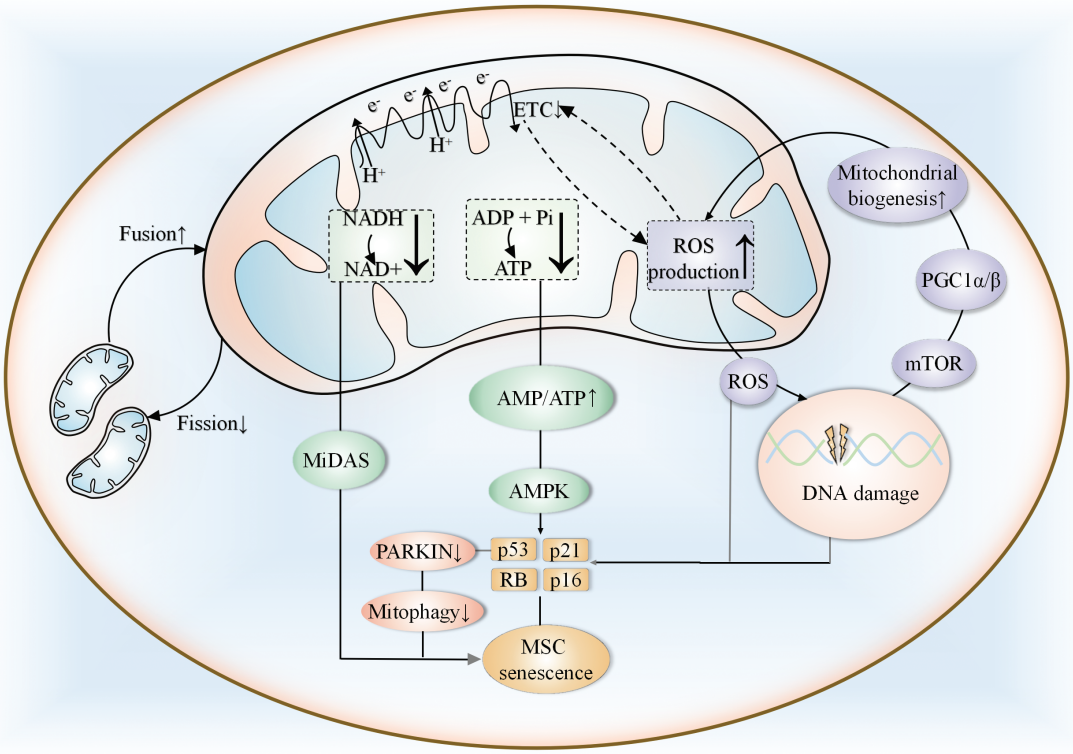

Figure 2. Mitochondrial dysfunction in MSC senescence. Senescence-associated mitochondrial dysfunction is one of the hallmarks of MSC aging, including dysregulated mitochondrial biogenesis, decreased mitophagy, and hyper-fused mitochondrial networks. During aging, AMP/ATP and NAD+/NADH ratios are metabolically disturbed, initiating downstream signaling cascades. Of note, excessive ROS generated by dysfunctional mitochondria may cause DNA damage and ROS accumulation, which in turn exacerbates mitochondrial function, forming a positive feedback loop. Abbreviations: ETC, electron transport chain; NADH, reduced nicotinamide adenine dinucleotide; NAD+, oxidized nicotinamide adenine dinucleotide; ADP, adenosine diphosphate; ATP, adenosine triphosphate; AMPK, 5′-AMP-activated protein kinase; PGC-1α/β, proliferator-activated receptor-gamma coactivator-1α/β; MiDAS, mitochondrial dysfunction-associated senescence.

mitochondrial dysfunction-associated senescence (MiDAS), which drives senescence with an IL-1-deficient SASP via the AMPK-induced p53 pathway.[111] However, the precise biological function of MiDAS is unclear.[112]

Mitochondrial quality control is regulated by mitophagy, which refers to the autophagy of damaged or malfunctioned mitochondria, a critical physiological process for maintaining cellular homeostasis. It is evidenced that insufficient mitophagy is involved in senescence-related cellular injuries.[113,114] Thus, the inhibition of mitophagy induces senescence through the accumulation of damaged mitochondria and metabolic dysfunction.[115,116] Recently, it has been uncovered that reduced PARKIN translocation to damaged mitochondria, mediated by the p53 pathway, results in defective mitophagy and thereby failed clearance of damaged mitochondria.[117] Furthermore, inhibition of p53 has been proved to ameliorate the functional decline of mitophagy in vivo.[118,119] Besides, ROS accumulation and sirtuin (SIRT) deficiency are also proved to inhibit PARKIN-mediated mitophagy, which leads to cell senescence.[120-122]

Another notable change of senescence-associated mitochondrial dysfunction is the imbalance between fusion and fission events.[123] Under normal circumstances, mitochondrial fission generates tiny spherical mitochondria, facilitating the elimination of defective mitochondria that have been exposed to stimuli or stress. While under the senescent process, mitochondria turn to a pro-fusion state with an extended and highly fused mitochondrial network, disabling effective mitophagy to take place.[123,124] Moreover, the mediators of the fission process, such as phosphoglycerate mutase 5, PTEN-induced putative kinase 1, dynamin-related protein 1, and mitochondrial fission protein 1, have been found to contribute to fewer fission events, making them potential targets in developing therapeutic interventions to combat aging.[125-128]

Telomere Attrition

The telomere is a nucleoprotein cap found at the end of linear chromosomes, which serves to maintain chromosomal integrity and regulate cell division. Telomeres shrink with each division as MSCs expand (approximately 30-40 PDs),[16] and senescence occurs when they reach a length where chromosomal stability is no longer guaranteed.[129] However, due to considerable variability in telomere length from donor to donor, predicting or monitoring MSC senescence based on telomere length is not reliable.[22,130]

Telomere length is mainly maintained by telomerase through the constant restoration of deleted 5′-TTAGGG repeats, which have been proposed to interact with the p53 and TGF-β1 signaling pathways.[131-135] In recent years, researchers have confirmed the intimate connection between SIRT1 and telomerase. Inhibiting SIRT1 in MSCs leads to decreased telomerase activity, thus inducing cellular senescence,[136] whereas overexpression of SIRT1 in aged MSCs could reverse the senescence phenotype.[137] Besides, SIRT1 is a downstream target of dysfunctional telomeres, and boosting SIRT1 activity could stabilize telomeres.[138] Additionally, human telomerase reverse transcriptase (hTERT) has been found to play a critical role in regulating cellular senescence, whose overexpression leads to

telomerase activation, telomere elongation, and subsequently, MSCs acquiring increased resilience to oxidative stress.[139]

Stem Cell Niche

Multiple types of cells, vascular networks, secreted metabolic and physical factors, and extracellular matrix (ECM) constitute a unique microenvironment surrounding stem cells, known as the stem cell niche, which was first conceptualized and proposed by Schofield.[140] The stem cell niche has been shown to support and regulate stem cell function, such as survival, stemness, activation, and migration,[141-143] as well as maintain stem cells in a quiescent state, preventing genetic damage.[144] The natural aging process has been proved to cause substantial changes in the stem cell niche, which will be reviewed and discussed in the following sections, along with its direct impact on MSCs (Fig. 3).

Numerous studies have revealed that aging is associated with increased pro-inflammatory cytokine production by senescent cells, such as IL-1, IL-6, IL-8, interferon-γ, and collectively referred to as SASP. Moreover, aging is characterized by a persistent, chronic, low grade inflammatory state, which has been termed inflammaging.[145] Senescence-associated secretory phenotype plays a pivotal role in inflammaging via autocrine and paracrine manner. Among all cellular components, adipocytes, one type of niche supporting cells, have been postulated as the primary source of pro-inflammatory cytokines, resulting in a pro-inflammation milieu for MSCs.[146,147] Furthermore, adipocytes have been found to accumulate in the bone marrow niche along with aging, limiting skeletal fracture repair due to impaired stem cell-based hematopoietic and bone regeneration.[148,149] In addition, during bone regeneration, macrophages have also been found to play a critical role in the recruitment and regulation of MSC differentiation, which is compromised with aging.[150] Up to date, oncostatin M, prostaglandin E2, and bone morphogenetic protein-2 have arisen as the best recognized paracrine mediators in the crosstalk between macrophages and MSCs; however, further studies are still warranted.[150] Throughout the aging process, HSCs, osteoblasts, fibroblasts, and endothelial cells in the stem cell niche are in close proximity with MSCs, altogether forming a delicate cellular regulatory network.[151]

Mesenchymal stem/stromal cell (MSCs') functionality has also been found to deteriorate with age due to age-related ECM alteration, which is another reason to cause impairment of MSCs' functionality. Physiologically, the ECM serves as a "hotbed" and provides a structural scaffold for resident cells, preserving the self-renewal ability and differential potential of MSCs.[152,153] The ECM produced from aged MSCs exhibits a compromised stemness maintenance function, which is thought to be related to MSC-ECM interactions as well as MSCs' stiffness-dependent regulatory mechanism.[154,155]

The stem cell niche of MSCs is well established for its perivascular localization, including the arteriolar niche and the sinusoidal niche, more MSCs associated with sinusoids than with arterioles given the overall abundance of the former.[156,157]

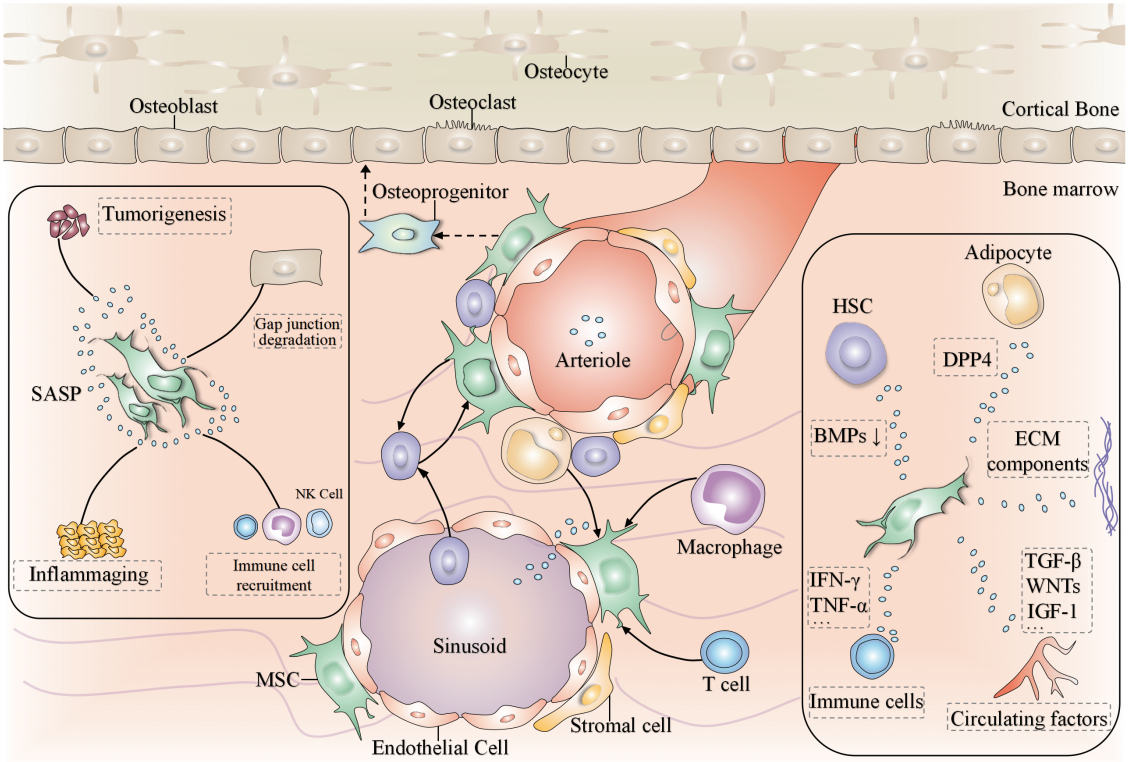

Figure 3. The interplay between senescent MSCs and their niche. MSCs are resident in a supportive microenvironment termed stem cell niche. The stem cell niche contains multiple types of cells, vascular networks, secreted metabolic and physical factors, and ECM. On the one hand, resident MSCs are profoundly affected by their niche. On the other hand, secretome changes of senescent MSCs, such as SASP-related factors, result in stem cell niche remodeling and creating a pro-inflammatory milieu. Abbreviations: ECM, extracellular matrix; SASP, senescence-associated secretory phenotype; NK cell, natural killer cell; HSC, hematopoietic stem cell; WNT, Wnt/β-catenin; TGF-β, transforming growth factor-β; IGF-1, insulin growth factor-1; DPP-4, dipeptidyl peptidase-4; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; BMP, bone morphogenetic protein.

Therefore, it is reasonable to assume that circulating factors have a substantial impact on MSC aging. Indeed, the serum from old rats has been found to promote MSC senescence and hamper the proliferation and survival of cells in vitro.[38] Moreover, taking advantage of heterochronic parabiosis, the impact of circulating factors is convincingly elucidated in vivo. In brief, 2 mice of different ages are surgically unioned, resulting in a shared blood circulation, which demonstrates that exposure to youthful circulation improves osteoblastic differentiation capacity of aged MSCs in vivo and reverses the diminished fracture repair phenotype of old mice.[158] This rejuvenation effect suggests the existence of some detrimental substances in aged circulation, limiting MSCs' functionality and resulting in cellular senescence. So far, increased β-catenin levels have been identified as one of the "harmful" circulating factors, and blocking the canonical WNT pathway could ameliorate the effects caused by aging serum.[38,158] It is worth noting that canonical and noncanonical WNTs work in different ways in regulating MSC biological behaviors. For example, WNT3a and WNT5a, 2 well-known prototypical canonical and noncanonical WNT ligands, the former suppresses osteogenesis upon osteogenic induction, whereas the latter increases ALP expression and favors osteogenesis,[159-161] creating a possible regulatory model implicated in age-related stem cell niche change. Besides WNT signaling, there are some other highly conserved regulators mediating the MSC niche, such as IGF-1 and its associated insulin/IGF-1 signaling and TGF-β signaling.[162,163] Studies found that insulin receptor (IR) blockage, deletion of insulin receptor substrate 1 (IRS1), and/or IRS2 contribute to inhibiting MSC senescence or functional restoration of aging MSCs.[164,165] As for the TGF-β signaling, members of the TGF-β family ligands are key components of the stem cell niche and orchestrate diverse responses in different types of stem cells, including MSCs.[166] Transforming growth factor-β has been shown to accelerate aging by activating $p16^{INK4A}$ and $p21^{CIP1/WAF1}$.[167] Coincided with TGF-β, SMAD3 is upregulated in a dose-dependent manner during senescence, and inhibiting the TGF-β receptor has been validated to enhance the expansion of undifferentiated MSCs.[168] However, more basic research is needed to decipher the precise functions of the above-mentioned signaling molecules concerning age-related stem cell niche change.

Epigenetic Alterations

Although the key mechanisms of MSC senescence remain enigmatic, it is widely accepted that alterations in the epigenetic landscape, including histone modification, chromatin remodeling, and DNA methylation, are common events implicated in the MSC aging process and may drive MSC senescence-related manifestations.

Histone modification significantly influences the transcriptional activities of surrounding genomic regions. Histone deacetylases (HDACs) are a group of enzymes that modulate histone acetylation levels and thereby determining the chromatin as relaxed euchromatin forms, in which it has open configuration and is associated with active transcription, or condensed heterochromatin forms, which represents as an inactive conformation.[169] In aging MSCs, HDACs deficiency upregulates jumonji domain-containing protein 3 (also known as lysine-specific demethylase 6B or KDM6B) and downregulates polycomb group genes through RB/E2F pathway, working as a transcriptional activator of $p16^{INK4A}$ by demethylating H3K27me3 and leading to senescence.[170] Besides, HDAC inhibitors have been proved to accelerate aging by activating the transcription of $p21^{CIP1/WAF1}$ through H3 and H4 acetylation.[171] Moreover, HDAC inhibitors could regulate cellular senescence gene *CDKN2A*, encoding $p16^{INK4A}$, through multiple microRNAs, such as miR-23a, miR-26a, and miR-30a.[172]

Chromatin remodeling, such as global heterochromatin loss and redistribution, is a process that alters chromatin landscape, yielding either transcriptional activation or repression, which is catalyzed by chromatin remodeling complexes, including switch/sucrose nonfermentable (SWI/SNF) and INO80 complexes.[173] As the ATPase subunit of SWI/SNF, Brahma-related gene 1 (BRG1) is considered the critical factor in global chromatin modulation, and dysregulation of BRG1 was associated with MSC senescence via NANOG methylation and γ-isoforms of heterochromatin formation.[174,175] In addition to chromatin remodeling complexes, factors directly related to the chromatin structure, such as KRAB-associated protein 1 (KAP1) and Condensins, also play a critical role in chromatin remodeling.[176] Condensins and KAP1 abnormality may promote MSC aging via excessive ribosome biogenesis and destabilization of nucleolar heterochromatin.[177,178]

DNA methylation profile also connects to MSC senescence. In general, DNA methylation status is regulated by DNA methyltransferases (DNMTs), comprising 3 major members: DNMT1, DNMT3A, and DNMT3B. Specifically, DNMT1 maintains the methylation pattern during DNA replication, whereas DNMT3A and DNMT3B perform de novo methylation.[179] It has been reported that the expression levels of DNMT1 and DNMT3B are significantly decreased during senescence.[180] Besides, inhibition of DNMT could directly demethylate CDK inhibitor genes *CDKN1A* (encoding $p21^{CIP1/WAF1}$) and *CDKN2A* (encoding $p16^{INK4A}$) and thereby induce cellular senescence in MSCs.[180,181]

Until now, few studies investigated mRNA modification during the process of MSCs aging. Given that N^6-methyladenosine (m^6A) modification is highly conserved with considerable impacts on the biological function of MSCs, it deserves more investigation and would be a promising direction for future research.

Hyperglycemia

Hyperglycemia, one of the most prominent features of the diabetic milieu, has been shown to alter MSC's characteristics and functions and result in MSC senescence.[182,183] In a hyperglycemic environment, adipose-derived MSCs (AD-MSCs) have been shown to display a decreased proliferative profile and a greater rate of senescence which highly reduced their therapeutic efficiency.[184] Besides, it has been reported that AD-MSCs under hyperglycemic settings demonstrate diminished immunomodulatory capabilities, angiogenesis, and migratory capacity, as well as increased SASP and insulin resistance.[185] Furthermore, when compared with umbilical cord-derived MSCs (UC-MSCs) collected from healthy pregnant women, UC-MSCs acquired from patients with gestational diabetes mellitus showed decreased cell growth and earlier cellular senescence.[186]

The impact of hyperglycemia on the differentiation potential and cell marker expression of MSCs undergoing senescence is yet to be clarified. Currently, most studies agree that hyperglycemia impairs osteogenic and chondrogenic differentiation, while adipogenic differentiation is less affected.[186-188] As for cell marker alterations, it has been reported that

the expression levels of stem cell markers in UC-MSCs or AD-MSCs from diabetic donors is similar to their counterparts from non-diabetic donors[186,189]; however, it has also been reported that hyperglycemia leads to a reduction of stemness-related markers of UC-MSCs.[187] These perplexing findings might be attributed to different donors or different tissue origins of MSCs. Overall, the effects of hyperglycemia on the senescence of MSCs should be elucidated for a better understanding of MSC and organismal aging and developing strategies to suppress MSC senescence in a hyperglycemic environment.

Strategies to Combat MSC Senescence

Mesenchymal stem/stromal cell senescence is a complex process with comprehensive mechanisms. Strategies allowing the generation of a large number of MSCs with retained stemness and lineage plasticity are in urgent demand for therapeutic purposes. Therefore, many alternative approaches have been attempted to bypass, prevent, or reverse senescence and improve the clinical application of MSCs. Here, we summarized the strategies for combating MSC aging based on the previously discussed mechanisms of MSC senescence.

The accumulation of ROS is the fundamental source of oxidative stress that contributes to senescence. Antioxidants and other senescence suppressors that functional against oxidative stress have shown the potential of alleviating aging. Ascorbic acid is a novel antioxidant, inhibiting ROS production in MSCs through AKT/mTOR signaling.[190] Other antioxidants, such as Cirsium setidens, lactoferrin, and *N*-acetyl-l-cysteine, have also been reported to inhibit ROS production or scavenge ROS directly.[191,192] Interestingly, hypoxic culture works as an efficient method for inhibiting MSCs senescence with retention of stem cell properties and enhancement of growth kinetics compared to normoxia, and more importantly, without increasing tumorigenicity.[193-195] Besides, inhibitors of p38/MAPK signaling could also ameliorate the cellular injury caused by ROS. Overall, ROS controllment seems to be an ideal strategy to extend the survival of MSCs with functional integrity. However, the specificity of antioxidants and the dosage remain unclear, especially when facing different types of senescence, and overdosage of antioxidants applied to proliferating MSCs may even cause DNA damage and play an opposite role.

Sirtuins are a NAD-dependent HDAC family critically involved in glucose metabolism, insulin secretion, protein homeostasis, and circadian rhythm, thus playing a pivotal role in forestall cellular senescence and age-associated diseases.[196] Sirtuins family is evolutionarily conserved across eukaryotic species, from yeast to humans, of which there are 7 isoforms (SIRT1-7) characterized in humans.[197,198] To date, at least 4 members of SIRTs, ie, SIRT1, SIRT3, SIRT6, and SIRT7, have been proved to function as potential anti-aging factors. During MSC senescence, SIRT1 exhibited decreased expression levels, while its overexpression inhibited or delayed senescence.[199] Moreover, reduction of SIRT1 could impair the differentiation ability, while overexpression of SIRT1 could enhance MSCs osteogenic capacity by promoting deacetylation and nuclear translocation of B lymphoma Mo-MLV insertion region 1 (also known as polycomb group ring finger 4 or PCGF4).[200,201] Consistently, SIRT1 expression was spontaneously upregulated upon osteogenic differentiation.[202] The mechanisms involved in the anti-aging effects of SIRT1 remain unraveled, but may attribute to the maintenance of genomic stability and metabolic efficiency. Similarly, SIRT3 also exhibited decreased expression levels as MSCs were expanded ex vivo, depletion of SIRT3 accelerated aging and inhibited MSC differentiation into osteoblasts and adipocytes, and that overexpression of SIRT3 in late-passage MSCs could restore their differentiation capacity, reduce oxidative stress and senescence.[121,203] SIRT6 specifically deacetylates histone lysine residues H3K9, H3K18, and H3K56 to modulate the recruitment of transcription factors and facilitate a repressive heterochromatin structure, which is essential for maintaining genomic integrity.[204-206] SIRT6 deficiency in human MSCs displayed accelerated functional decay, predominately characterized by increased sensitivity to oxidative stress and dysregulated redox metabolism. Mechanistically SIRT6 deacetylates H3K56 and decreases heme oxygenase-1 (HMOX-1, or HO-1) expression via nuclear factor erythroid 2-related factor 2, eventually leading to accelerated senescence.[207] Recently, SIRT7 has emerged as an important regulator that delays MSC senescence, which safeguards chromatin architecture to control innate immune regulation via cGAS (cyclic GMP-AMP synthase)-STING (stimulator of interferon gene) pathway and ensures geroprotection during human MSC aging.[208,209]

Genetic approaches, such as genetic engineering, have been proposed to slow down MSC senescence effectively. It has been reported that knockdown of migration inhibitory factor (MIF) could induce senescence in young MSCs, while MIF overexpression leads to the rejuvenation of aged MSCs.[210] Alternatively, hTERT overexpression confers increased replicative life span of MSCs, accompanied by preserved normal karyotype, promoted telomere elongation, and abolished cellular senescence.[211] Moreover, miR-195 knockdown could activate telomere re-lengthening by upregulating the expression levels of hTERT, contributing to rejuvenation of MSC senescence.[212] Recently, overexpression of Erb-B2 receptor tyrosine kinase 4 in aged MSCs also has been demonstrated to reversed their senescent phenotype and enhanced their resistance to oxidative stress.[213] A similar rejuvenation effect can be observed with overexpression of Yes-associated protein and forehead box D1 in aged human MSCs.[214] It is noteworthy that genetic modulation, such as knockdown or depletion of specific genes, can remarkably restore the proliferation and differentiation potential of MSCs; however, we always have to bear in mind the risks of malignancy, which may emerge due to dysregulation of genes that promote tumorigenesis.

In recent years, multiple high-throughput techniques have been developed and used for the purpose of anti-aging drug screening. Pharmacological and nutritional supplements, as well as cytokines, hormones, and other signaling molecules, have been widely investigated to assess their ability to prevent or slow down the senescence of MSCs. Among them, FGF-2, PDGF, and EGF supplementation could increase MSC proliferation ability and delay the aging process of MSC.[215] In addition, the exogenous application of rapamycin, MIF, and lysophosphatidic acid has also been reported to confer extended life span of MSCs and/or rejuvenation of aged MSCs in culture.[216-218] As discussed in the previous subsection, telomerase maintains telomere length to avoid telomere erosion and combat senescence; therefore, molecular compounds that can activate the endogenous telomerase, such as aspirin,[219] vitamin C[220] and FGF-2[215] have been used to rescue the proliferative potentials of senescent MSCs and recover their

osteogenic capacity as well. Furthermore, mitochondrial malfunction is another typical phenotype during MSC senescence and restoration of which contributes to retarding aging. To this end, melatonin has been found to restore mitochondrial function and mitophagy through upregulation of heat shock 70 kDa protein 1 like protein (HSPA1L), thus alleviating MSC senescence.[221] Anti-aging benefits could also be acquired from drug repurposing. Metformin is a lead candidate and a good example, well known as a first-line medication for treating type 2 diabetes, which has shown potential benefits on aging and healthspan via its effects on cellular metabolism and reduction of oxidative stress.[222]

Concluding Remarks

Mesenchymal stem/stromal cell is one of the most studied types of adult somatic stem cells. For decades, MSCs have undergone extensive investigation in experimental and clinical scenarios due to their easy accessibility, immunoregulatory functions, and therapeutic potential in regenerative medicine.[10,223,224] However, some significant issues concerning safety and efficacy have to be taken into consideration and overcome before clinical implementation. One of the critical hurdles of MSCs' clinical application is their senescence when cultured and expanded in vitro to acquire sufficient quantity. In particular, the aging process significantly impacts and dampens the proliferative and functional activity of MSCs, making it challenging to apply MSCs effectively on stem-cell therapy or tissue engineering.

Besides, from a broader perspective, stem cell aging is one of the leading theories of organismal aging.[225] According to it, multicellular organisms undergo physiological and functional declines at tissue, organ, and individual levels as they age, primarily owing to changes in different stem cell populations of various tissues, such as HSCs and MSCs. Currently, MSC senescence is well recognized as a complex and comprehensive process where multiple signaling pathways and molecules are involved and intertwined. Moreover, the pathways regulating MSC senescence are usually shared in multiple important biological processes under physiological conditions. Hence, a crucial scientific question in the field of stem cell research is to decipher the underlying mechanisms that keep stem cells youthful and vital without causing detrimental side effects.

As for current anti-aging strategies, there are still certain limitations regarding each technique. For instance, elimination of ROS to prevent premature senescence sounds attractive but may lead to adverse effects since ROS act as secondary messengers in various cellular pathways, including those which protect against or repair damage.[226,227] Genetic approaches to reverse MSC senescence remain unreliable due to off-target effects and risks of malignancy. Pharmacological and nutritional supplementations are usually dose-dependent and vary from individual to individual, which may generate uncontrollable results. In contrast, SIRTs might be the breakthrough of rejuvenating senescent MSCs and anti-aging treatment with relatively safe outcomes and fewer side effects. Last but not least, current relevant studies are mainly based on sequencing bulk samples for genetic analyses, which neglects the key feature of MSCs. Therefore, with the advancements of experimental techniques, a deeper understanding of MSC senescence may be provided through single-cell RNA sequencing (scRNA-seq) and/or spatial transcriptomics in the near future.

Overall, we have discussed the typical characteristics of senescent MSCs, the cellular and molecular mechanisms underpinning MSC senescence, the possible strategies to rejuvenate aging MSCs and combat aging, and raised some promising future directions based on currently available evidence.

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Conflict of Interest

The authors declared no potential conflicts of interest.

Author Contributions

B.L., Z.W., and Y.W. wrote the manuscript. B.L., Z.Z., and L.L. critically reviewed and edited the manuscript. Z.W., T.O., H.L., X.Q., and C.W. contributed to the material collections and analysis. B.L., Y.W., and T.O. contributed to the design of the figures. All authors approved the final version of the manuscript.

Data Availability

No new data were generated or analyzed in support of this research.

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