

## Senescent mesenchymal stem/stromal cells and restoring their cellular functions

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### Abstract

Mesenchymal stem/stromal cells (MSCs) have various properties that make them promising candidates for stem cell-based therapies in clinical settings. These include self-renewal, multilineage differentiation, and immunoregulation. However, recent studies have confirmed that aging is a vital factor that limits their function and therapeutic properties as standardized clinical products. Understanding the features of senescence and exploration of cell rejuvenation methods are necessary to develop effective strategies that can overcome the shortage and instability of MSCs. This review will summarize the current knowledge on characteristics and functional changes of aged MSCs. Additionally, it will highlight cell rejuvenation strategies such as molecular regulation, non-coding RNA modifications, and microenvironment controls that may enhance the therapeutic potential of MSCs in clinical settings.

**Key Words:** Mesenchymal stem cells; Senescence; Features; Function; Rejuvenation strategy

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**Core Tip:** Mesenchymal stem cell (MSC) administration is a promising therapeutic strategy for various human diseases. However, cell aging limits MSC function and therapeutic properties *via* reducing their activities. We review the morphological changes, molecular expression alterations, and functional degeneration of aged MSCs, and the effects of aged MSCs on immune cells and other target cells. Additionally, we summarize the strategies to rejuvenate aged MSCs to enhance their clinical potential.

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## INTRODUCTION

Mesenchymal stem/stromal cells (MSCs) are multipotent progenitor cells that can retain postnatal capacity for both self-renewal and multilineage differentiation. The minimal criteria for MSCs as defined by the International Society for Cellular Therapy in 2006 are adherence to plastic under culture conditions; positivity for cell surface markers CD44, CD90, CD105, and CD73; negativity for hematopoietic markers CD45, CD34, CD14, CD11b, CD79 $\alpha$ , CD19, and human leukocyte antigen-DR; and multi-differentiation potential of osteogenesis, chondrogenesis, and adipogenesis<sup>[1]</sup>. They are a heterogeneous population of cells isolated from a variety of mesodermal tissues. These cells are involved in a wide range of physiological and pathological processes, such as bone development, adipogenesis, fibrosis, and inflammatory regulation<sup>[2]</sup>. Over the past few decades, the amount of MSC-focused research has grown exponentially. These studies include both preclinical and clinical trials of either autologous or allogeneic MSCs. Infusion of MSCs has been performed to evaluate their safety and therapeutic efficacy in diseases of the immune<sup>[3]</sup>, hematological<sup>[4]</sup>, cardiovascular<sup>[5,6]</sup>, nervous<sup>[7,8]</sup>, respiratory<sup>[9]</sup>, digestive<sup>[10]</sup>, skeletal<sup>[11]</sup>, endocrine<sup>[12]</sup>, and reproductive<sup>[13]</sup> systems<sup>[14]</sup>. To date, more than 1000 MSC-based clinical trials have been registered in the United States National Institute of Health database<sup>[15,16]</sup>. It is well recognized that MSC administration is a safe and effective strategy in the treatment of a variety of diseases.

Emerging evidence has demonstrated that multiple factors, including cell species, tissue source, isolation method, culture conditions, and cellular status, may explain the inconsistency in the features and characteristics of MSCs in some preclinical and clinical trials. A recent study showed that aging is an important factor affecting MSC properties and functions<sup>[17]</sup>. Age-dependent decline in MSC number and function was found in old individuals<sup>[18]</sup>. Additionally, MSCs from young donors may also become senescent because of excessive cell passage, oxidative stress, and other injuries<sup>[19,20]</sup>. The senescent cells manifest a sequence of progressive changes in cellular morphology, biological function, and molecular expression<sup>[21,22]</sup>, as well as weakened efficacy in cell-based therapies<sup>[23]</sup>. Therefore, appropriate quality controls or cellular rejuvenation processes are required to obtain clinical-grade MSCs. In this review, we will focus on investigations that have assessed the molecular features and functional changes of aged MSCs and highlight rejuvenation strategies that will enable more effective clinical translation.

## CHARACTERISTICS AND FUNCTIONAL CHANGES OF AGED MSCS

Aging MSCs exhibit morphological changes and undergo a progressive decline in homeostasis, which contributes to the age-dependent deterioration of MSC function<sup>[24]</sup>. These changes in senescent MSCs include a general decrease in their regenerative capacity, a switch in differentiation potency, and weakened regulatory functions (such as immunosuppressive effects)<sup>[25]</sup>. A full understanding of these characteristics is fundamental for the development of strategies to delay or even prevent MSC

senescence. In view of this, the phenotypes and functional characteristics of senescent MSCs will be summarized in this section.

### **Morphological changes in aged MSCs**

The most noticeable changes in aged MSCs are morphological. *In vitro* imaging analysis demonstrated that MSCs from early passages (P1-P3) were remarkably uniform in size<sup>[24]</sup>. At P5, they exhibited a flattened and enlarged morphology compared with those at P1. Additionally, gradual telomere shortening is a typical characteristic of aging in MSCs<sup>[26]</sup>. Moreover, these changes in morphology represented the heterogeneous response to the cellular microenvironment *in vitro* and *in vivo*.

### **Alterations of activity**

In aged MSCs, the balance of homeostasis is disrupted, the proliferative ability declines, and mitochondrial dysfunction increases. In addition, both the DNA repair and retrogression of anti-oxidative capacity are reduced<sup>[18,27]</sup>. Senescent MSCs display delayed self-cloning as well as restricted differentiation potency<sup>[28]</sup>. Additionally, they exhibit a shift in differentiation potency from osteoblasts to adipocytes<sup>[29-33]</sup>. Genetic stability, another biological index affecting MSCs activity, is involved in biosafety issues and therapeutic efficacy of these cells<sup>[34]</sup>. Mounting evidence suggested that long-term cultured MSCs acquired genetic alterations, with the promotion of cell senescence and potential increased risks of transformation. However, the relevance of increased genomic instability with culture passages is still being debated<sup>[35-38]</sup>. Roselli *et al.*<sup>[39]</sup> reported that MSCs were genetically stable in long-term cultures at least up to passage 10, and abnormal MSC clones showed neither growth advantage nor senescence resistance. Some authors suggested that senescent cells are unlikely to undergo malignant transformation, even if the presence of few tumorigenic cells can not be excluded<sup>[40]</sup>. The inconsistencies may be caused by tissue sources, culture conditions, culture times, and culture passages<sup>[41]</sup>. Nevertheless, it is of critical importance to evaluate MSC genetic stability before clinical application.

### **Biomarkers for aged MSCs**

Several methods may be used to identify MSC senescence. The most widely used measures include increased senescence-associated (SA) beta-galactosidase activity (SA-beta-gal), cell cycle arrest, and persistent DNA damage response signaling<sup>[22]</sup>. Furthermore, specific markers for senescent MSCs have been discovered. Analysis of the MSC compartment revealed that MSC subpopulations differ between developmental and aged stages. CD271(-)CD146(+) cells only appeared in fetal bone marrow (BM)-containing colony-forming-unit-fibroblasts. The dominant MSC subset in pediatric and fetal samples was the CD271(bright)CD146(+) population, whereas the main cell type in adult samples was CD271(bright)CD146(-)<sup>[42]</sup>. The proportion of CD11b+, CD3+, Gr-1+, or F4/80+ cells is upregulated in BM from aged mice, while the percentage of B220+ cells was significantly decreased compared with those from young mice BM<sup>[43]</sup>.

Recently, novel specific biomarkers were found to demonstrate the senescent state of MSCs<sup>[21]</sup>. MSC-derived microvesicles (MVs) are one such biomarker<sup>[44]</sup>, and senescent late passage MSCs displayed a smaller MSC-MV size compared with early passage MSCs. Additionally, when comparing late and early passage MSC-MVs, there was a lower ratio of CD105+ cells and decreased osteogenesis in late passage MSC-MVs<sup>[45]</sup>. When the percentage of CD264+ cells was greater than 35%, CD264 expression was inversely correlated with the regenerative potential of MSCs. Above the 75% threshold, MSCs were enlarged and showed a decreased proliferation and differentiation potency.

CyBC9, a senescence-specific fluorescent probe, is a promising tool used to rapidly identify both early and late senescent phenotypes in clinically relevant MSCs<sup>[46]</sup>, and it can be applied to live cells as a nontoxic probe. The mitochondrial *Cox1* gene containing the differentially methylated CpG island 4 was upregulated in MSCs from human fetal heart tissues. This demonstrated that CpG hypo-methylation in mitochondria might serve as a biomarker for senescence of human fetal heart MSCs induced by chronic oxidative stress<sup>[20]</sup>.

### **Portraits of expression profiles**

Recent studies have demonstrated significant changes in the expression profiles (including transcriptomic, proteomic, epigenetic, and non-coding RNAs) of senescent MSCs. Transcriptome drift even preceded replicative exhaustion and other aging metrics<sup>[47]</sup>. Utilizing a microarray assay, transcriptome analyses were performed using

various types of aged MSCs (Table 1).

**Transcriptomics:** Assays for gene expression profiles have been performed on multiple types of aging MSCs. These MSCs were obtained from various species and tissues, and subjected to different treatments. Benisch *et al.*<sup>[48]</sup> investigated the transcriptional profiles of human BM-MSCs from five elderly patients (79-94 years old) who had osteoporosis (hMSC-OP), the age-matched control group (hMSC-old; donor age 79-89 years), middle-aged donors (hMSC-C; donor age 42-67 years), and healthy middle-aged donors (42-64 years old) until they entered senescence (hMSC-senescent). By using hMSC-C as control cells, they found a small overlap of gene expression in the hMSC-old, hMSC-senescent, and hMSC-OP groups. By comparing the gene expression profiles of hMSC-OP, hMSC-old, and hMSC-senescent with hMSC-C, special transcriptomic features in each group were obtained. The differentially expressed genes in the three groups are mainly involved in proliferation, differentiation, osteoclastogenesis, and DNA repair<sup>[48]</sup>. Wu *et al.*<sup>[49]</sup> identified six hub genes and eleven transcription factors related to adherens junctions, DNA damage induced by oxidative stress, attribution of telomeres, differentiation, and epigenetic modulation by comparing the gene alterations between hMSC-C and hMSC-old. Yoo *et al.*<sup>[50]</sup> revealed that 19 genes were downregulated and 43 upregulated in senescent human BM-MSCs relative to young MSCs. And these genes were mainly involved in metabolic functions and cell adhesion. Additionally, 394, 1073, and 2077 genes were significantly up-regulated in BM-MSCs from pesticide exposed, P14 MSCs, and MSCs from aged donor, compared with control MSCs (P3), respectively<sup>[51]</sup>. And 218, 1077, and 1571 genes were down-regulated in BM-MSCs from pesticide exposed, P14 MSCs, and MSCs from aged donor, compared with P3 MSCs, respectively. Insulin-like growth factor-1 (*Igf-1*), *prolactin*, *leptin*, and *Cox-2* were identified as key genes of the predicted protein-protein interactions<sup>[51]</sup>. In murine BM-MSCs that were freshly sorted by fluorescence-activated cell sorting, 927 differentially expressed genes were obtained in aged BM-MSCs. These genes contained cytokine receptors, chemokines, markers of cell senescence, and other groups, which were seen in the gene expression omnibus<sup>[52]</sup>.

Human umbilical cord (hUC)-derived MSCs, cultured in chemically defined xeno- and serum-free medium, displayed comparable growth trajectories up to passage (P) 9 and variably approached senescence after P10. However, significant changes in the transcription profiles occurred earlier. Microarray analysis of 14500 human genes in aged hUC-MSCs revealed that a nonlinear evolution of aging MSCs appeared after P5 and accumulated rapidly after P9<sup>[47]</sup>. As for hUC vein-MSCs, young (P9) and senescent (P18) cells were used for transcriptome analysis assays. This study identified 73 differentially expressed genes in senescent cells, compared with young MSCs<sup>[49]</sup>. Among these, 18 upregulated genes were screened out as characteristic molecular signatures of senescence when comparing senescent and young hMSCs derived from donors with normal or constitutional chromosome inversion karyotype. Among them, 11 novel candidate markers for senescence were identified.

In response to IL-2 priming, human adipose-derived MSCs (ADSCs) showed increased expression of genes encoding potent growth factors, cytokines, angiogenic, and anti-apoptotic promoting factors, and they were defined as novel transcriptional signatures closely associated with senescence<sup>[53]</sup>. In CD45-CD31-CD34+ ADSCs from murine inguinal fat pads, aging has been shown to affect cellular signaling and function as well<sup>[54]</sup>. Single-cell transcriptional profiles of ADSCs isolated from both young and aged mice were analyzed by utilizing a microfluidic-based single-cell gene expression platform. About 70 gene targets related to stemness, vasculogenesis, and tissue remodeling were evaluated and used to define ADSC clusters in each group. Ingenuity Pathway Analysis of a subset of this heterogeneous cell collection was performed. This analysis suggested that deficiency of a putatively vasculogenic subpopulation of ADSCs was a potential risk for age-related impairments in ADSCs function (particularly with regard to wound healing)<sup>[54]</sup>.

**Proteomics:** Proteomics is an efficient and accessible tool used to determine protein expression profiles. An SA secretome, also known as SA secretory phenotype (SASP), usually contains the expression of growth factors, cytokines, and extracellular proteases that modulate the microenvironmental phenotypes caused by senescent cells<sup>[55]</sup>. The SASP is useful for the development of biological markers and rejuvenation strategies in aged MSCs<sup>[22]</sup>. Secretome analyses for secretory protein profiles in senescent MSCs are summarized in Table 2. For example, elderly MSCs exhibited increased levels of pro-inflammatory factors, including interleukin-6 (IL-6), IL-8 (IL-8/CXCL8), and monocyte chemoattractant protein-1 (MCP-1/CCL2). Neutralization of these factors improved their immunomodulatory function<sup>[56]</sup>. In the conditioned

**Table 1 Summary of various transcriptomics analyses studies of senescent mesenchymal stem cells**

Ref.	Species	Tissue sources	Classification	Cells	Groups	Database	Differentially expressed genes (DEGs)	Identification of targets
Benisch <i>et al</i> <sup>[48]</sup> , 2012	Human	Bone marrow of femoral heads	Affymetrix Gene Chip	Cultured in DMEM/Ham's F-12 (1:1) medium supplemented with 10% fetal calf serum (FCS), 1 U/mL penicillin, 100 µg/mL streptomycin, and 50 µg/mL L-ascorbic acid 2-phosphate. Used after 1 to 2 passages	hMSC-C: Middle-aged donors (42-67 yr old); hMSC-old: The age-matched control group (79-89 yr old); hMSC-OP: Patients (79-94 yr old) who had primary osteoporosis; hMSC-senescent: Healthy donors of middle-age (42-64 yr old) until they entered senescence	GEO accession numbers: GSE35955; GSE35956; GSE35957; GSE35958; GSE35959	One gene was upregulated and seven downregulated in all three groups, compared with the hMSC-C group; 38 genes with enhanced and 36 genes with reduced expression in hMSC-OP and hMSC-old groups, compared with hMSC-C; 2477 genes with higher and 1222 genes with lower expression in hMSC-OP, in comparison with hMSC-old	The reliable or promising candidates for osteoporosis, including susceptibility genes: <i>Lrp5</i> , <i>Spp1</i> (Osteopontin), <i>Col1a1</i> , <i>Sost</i> , and <i>Mab211l</i>
Yoo <i>et al</i> <sup>[50]</sup> , 2013	Human	Bone marrow-derived MSCs	SSH analysis	Purchased from Cambrex Bio Science	Young human MSCs (Y-hMSCs): Approximately 10 population doubling levels (PDL); senescent MSCs (S-hMSCs): Until approximately 30 PDL, at least 80% of the cells were positive for SA-β-Gal staining	NA	Nineteen genes were down-regulated and 43 upregulated in S-hMSCs	Gradually downregulated mRNA in S-hMSCs: <i>Pdia3</i> , <i>Wdr1</i> , <i>Fstl1</i> , <i>Copg1</i> , <i>Lman1</i> , and <i>Pdia6</i> ; significantly upregulated genes: <i>Hsp90b1</i> , <i>Eid1</i> , <i>Atp2b4</i> , <i>Ddah1</i> , <i>Prnp</i> , <i>Rab1a</i> , <i>Psg5</i> , <i>Tm4sf1</i> , and <i>Ssr3</i>
Bustos <i>et al</i> <sup>[52]</sup> , 2014	Mouse	Bone marrow	Affymetrix Gene Chip	Sorted by fluorescence-activated cell sorting (FACS)	BM-MSCs from young (3-mo-old) and aged (24-mo-old) mice; young donor BM-MSCs <i>vs</i> aged ones	GEO accession number: GSE44403	927 genes were differentially expressed	Confirmed by qPCR: Cytokine receptors (15 genes), chemokines ( <i>Ccr7</i> , <i>Cxc3cr1</i> , <i>Cxcr5</i> ), markers of cell senescence ( <i>CDK</i> , <i>p21</i> , <i>p27</i> , and <i>p53</i> ), <i>Marcks</i> , <i>Mmp9</i> , and <i>Timp2</i>
Duscher <i>et al</i> <sup>[54]</sup> , 2014	Mouse	Inguinal fat pads	Microfluidic-based single-cell gene expression platform	CD45-/CD31-/CD34+ cells were sorted	Adipose-derived mesenchymal stem cells (ADSCs) from young (3 mo) and aged (21 mo) mice	NA	Differences in transcriptional profiles of genes related to cell stemness, remodeling, and vasculogenesis: <i>Adam10</i> , <i>Angpt1</i> , <i>Angpt2</i> , <i>Hif1a</i> , <i>Mef2c</i> , and <i>Sod2</i>	Age-related depletion of a subpopulation of MSCs characterized by a pro-vascular transcriptional profile, such as <i>Angpt1</i> , <i>Vegfa</i> , and <i>Sod3</i>
Medeiros <i>et al</i> <sup>[19]</sup> , 2017	Human	Umbilical cord veins	The GeneChip Human Genome U133 Plus 2.0 array	The surface markers including CD105, CD73, CD90, CD14, CD34, and CD45 were analyzed by flow cytometry; differentiation capacity toward three lineages was assessed	hMSCs in the 9 <sup>th</sup> (Y-hMSCs) and 18 <sup>th</sup> passages (S-hMSCs) were used for assays, hMSCs/n from the donor with normal karyotype, and hMSCs/inv from the donor with a constitutional inversion of chromosome 3; the comparisons were as follows: (1) Y-hMSCs/n & S-hMSC/n; (2) Y-hMSCs/inv & S-hMSCs/inv; (3) Y-hMSCs/n & Y-hMSCs/inv; and (4) S-hMSCs/n & S-hMSCs/inv	GEO accession number: GSE56530	73 DEGs in S-hMSCs/n compared with Y-hMSCs/n and 279 DEGs in S-hMSCs/inv compared with Y-hMSCs/inv; 93 DEGs in Y-hMSCs/inv compared with Y-hMSCs/n; 425 DEGs in S-hMSCs/inv compared with S-hMSCs/n. The candidates for senescent markers: <i>Dio2</i> , <i>Foxe1</i> , <i>Galnt5</i> , <i>Has3</i> , <i>Krt19</i> , <i>Krt34</i> , <i>Krtap1-55</i> , <i>Oc730755</i> , <i>Mrv1</i> , <i>Plcb4</i> , and <i>Scube3</i>	Confirmed by qPCR: <i>Ankrd1</i> and <i>Mmp1</i> in S-hMSC/n <i>vs</i> Y-hMSC/n; <i>Sfrp1</i> , <i>Ankrd1</i> , <i>G0s2</i> , and <i>Ndn</i> in S-hMSC/inv <i>vs</i> Y-hMSC/inv; <i>Adora2b</i> , <i>Sfrp1</i> , <i>Kynu</i> , <i>G0s2</i> , <i>Aldh1a1</i> , and <i>Mab211l</i> in Y-hMSC/inv <i>vs</i> Y-hMSC/inv; and <i>Adora2b</i> , <i>Ccl7</i> , <i>Sfrp1</i> , <i>Kynu</i> , <i>Ankrd1</i> , <i>Mmp1</i> , <i>Lamc2</i> , <i>G0s2</i> , <i>Mab211l</i> , and <i>Ndn</i> in S-hMSC/inv <i>vs</i> S-hMSC/n
Wu <i>et al</i> <sup>[49]</sup> , 2019	Human	Bone marrow of femoral heads	Affymetrix Gene Chip	Cultured in DMEM/Ham's F-12 (1:1) medium supplemented with 10% FCS, 1 U/mL penicillin, 100 µg/mL streptomycin, and 50 µg/mL L-ascorbic acid 2-phosphate. used after 1 to 2	Middle-aged group <i>vs</i> elderly group	GEO accession number: GSE35955	156 up-regulated and 343 down-regulated differentially expressed genes (DEGs)	Six hub genes identified by PPI network analysis: <i>Ctnnb1</i> , <i>Ppp2r1a</i> , <i>Fyn</i> , <i>Mapk1</i> , <i>Pik3c2a</i> and <i>Ep300</i> . 11 TFs identified by TFs screening: <i>Creb1</i> , <i>Cux1</i> , <i>Egr1</i> , <i>Ep300</i> , <i>Foxc1</i> , <i>Hsf2</i> , <i>Mef2a</i> , <i>Plau</i> , <i>Sp1</i> , <i>Stat1</i> and

Wiese <i>et al</i> <sup>[47]</sup> , 2019	Human	The perivascular region of Wharton's jelly from umbilical cords	Affymetrix GeneChip U133A 2.0 arrays	passages Provided by Tissue Regeneration Therapeutics, Inc. Positive for the cell surface markers CD73, CD90, CD105, CD10, CD140b, CD146 (40%-60%), CD166, and MHC-I; negative for the cell surface markers CD45, CD31, CD34, and HLA-DR. Exhibit trilineage potential in directed differentiation assays	Human umbilical cord perivascular cells (HUCPVCs) from early passages (P2-P5), mid-passages (P6-P9), and pre-senescent passages (P10-P12)	GEO accession number: GSE119987	The transcriptome of HUCPVCs was stable through P5. A single significantly DE gene was identified at P6 and P7 compared with P2, whereas 5 DE genes were detected at P8 and 27 at P9. The number of significantly DE probe sets increased from 27 (P9) to 301 (P10), then to 1094 (P12)	Usf1 Significant transcriptome drift occurred only after P5
Leveque <i>et al</i> <sup>[51]</sup> , 2019	Human	Bone marrow aspirates from the iliac crest of healthy donors (21 to 26 years old)	RNAseq Analysis	The surface markers including CD34, CD45, CD73, CD90, and CD105 were analyzed by flow cytometry; differentiation capacity toward three lineages was assessed	Four groups: Control MSCs (P3); 21 d pesticide mixture exposed MSCs (P4); long-term cultivated MSCs (P14); and MSCs from aging donor (72 yr old)	The SRA database under accession number PRJNA510912	394, 1073, and 2077 EST were significantly increased from pesticide exposed, P14 MSCs, and MSC from aged donor; 218, 1077, and 1571 ESTs were down-regulated	Confirmed by QPCR: <i>Igf-1</i> , <i>Prolactin</i> , <i>Leptin</i> , and <i>Cox-2</i>

GEO: Gene expression omnibus; DEGs: Differentially expressed genes; hMSC: Human mesenchymal stem cells; MSC: Mesenchymal stem cells; HUCPVCs: Human umbilical cord perivascular cells; FCS: Fetal calf serum; ADSCs: Adipose-derived mesenchymal stem cells; PDL: Population doubling levels; DMEM: Dulbecco's Modified Eagle's medium; FACS: Fluorescence-activated cell sorting; BM: Bone marrow.

medium (CM) from senescent MSCs induced by the HIV protease inhibitor tipranavir, the soluble proteins were evaluated to find dysregulated secreted factors using antibody arrays and liquid chromatography-mass spectrometry (LC-MS)<sup>[57]</sup>. Semi-quantitative antibody arrays and LC-MS analysis identified altered secretion of 86 proteins related to the extracellular matrix, cell adhesion, angiogenesis, and wound healing. Among the identified secreted factors in the proteomic analysis, a series of TGF- $\beta$  targets were significantly upregulated. Further investigation revealed that insulin-like growth factor-binding protein 7 (IGFBP7), one of the targets of TGF- $\beta$ , is independent of any additional factors that induce osteogenesis in hMSCs. IGFBP7 is also essential for the viability of hMSCs during osteogenesis<sup>[57]</sup>.

In the ADSCs from one-year-old male C57BL/6 mice, the anti-senescent protein, telomerase reverse transcriptase (TERT), and the anti-apoptotic transcription factor myocardin were overexpressed to restore their functions. The secretomes in CM and exosome-enriched fractions from the transgenic cells were analyzed using a proteomic approach. This approach involved combining two-dimensional gel electrophoresis with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry<sup>[58]</sup>. The comparative targeted proteomic analysis revealed that both matrix metalloproteinase-2 (MMP-2) and its inhibitor metalloproteinase inhibitor 2 (TIMP2) levels are increased by two-fold in the CM compared with those in mock-transduced cells.

**Epigenetics:** Epigenetic profiles of aged human BM-MSCs have been reported, and briefly reviewed by Cakouros and Gronthos<sup>[59]</sup>. Using the human methylation bead

Table 2 Summary of secretome alteration analysis studies of senescent mesenchymal stem cells

Ref.	Species	Tissue sources	Classification	Cells	Groups	Differentially expressed proteins	Identification of targets
Kizilay <i>et al.</i> <sup>[56]</sup> , 2017	Human	Subcutaneous and pericardial adipose tissue	R&D Systems Human Cytokine Array; multiplex electrochemiluminescence immunoassay V-Plex Pro inflammatory Panel	CD44, CD73, CD105, and CD90 expression was more than 95%; CD45, CD34, CD19, CD14, and HLA-DR expression was less than 5%; differentiation capacity toward three lineages was assessed	E-MSCs: MSCs from elderly ATH patients (> 65 yr old); A-MSCs: MSCs from adult ATH patients (< 65 yr old)	The expression of IL-6, IL-8/CXCL8, MCP-1/CCL2, MIF, IFN- $\gamma$ , IL12p70, IL-13, IL-2, and IL-4 was elevated in E-MSCs relative to A-MSCs	Neutralization of IL-6, IL-8/CXCL8, and MCP-1/CCL2 significantly improved the E-MSCs' immunomodulatory function
Infante <i>et al.</i> <sup>[57]</sup> , 2018	Human	Bone marrow	Semi-quantitative antibody arrays; liquid chromatography-mass spectrometry (LC-MS); Version 4.0.4265.42984, Nonlinear Dynamics	Obtained from Lonza commercially; passages 3-4	Ctrl-hMSCs: Incubated with dimethyl sulfoxide alone; PreA-hMSCs: Treated with the HIV protease inhibitor (tipranavir) every other day until passage 11	A dysregulation in the secretion levels of 42 proteins was detected by antibody arrays; 44 were detected by LS-MS in preA-hMSCs; most of them were overexpressed in preA-hMSCs, in comparison with ctrl-hMSCs	IGFBP7 is essential for hMSCs viability during early osteogenic differentiation
Madonna <i>et al.</i> <sup>[58]</sup> , 2019	Mouse	Peri-epididymal visceral adipose tissue from 1-yr-old male C57BL/6 mice	Two-dimensional gel electrophoresis (2DE); matrix-assisted laser desorption/ionization time-of-flight mass spectrometry	The expression of CD45, CD34, CD133, ASMA, Desmin, CD105, CD73, CD90, CD79, and CD160 was analyzed by flow cytometry	Mock AT-MSCs: Mock-transduced AT-MSCs; rTMAT-MSCs: Rejuvenated by TERT and the anti-apoptotic transcription factor myocardin overexpression	113 protein spots were picked up and identified from the whole CM and exosome-enriched fraction in rTMAT-MSCs	Two novel candidates supporting angiogenesis in the whole CM of rTMAT-MSCs: MMP2 and its inhibitor TIMP2

hMSC: Human mesenchymal stem cells; MSC: Mesenchymal stem cells; TERT: Telomerase reverse transcriptase; MMP2: Matrix metalloproteinase-2; CM: Conditioned medium; LC-MS: Liquid chromatography-mass spectrometry.

ChIP array, researchers identified a series of hypomethylated, hypermethylated, and hydroxymethylated CpG sites in MSCs from aged subjects<sup>[60-62]</sup>. However, differentially methylated CpG sites are a robust age-related DNA methylation signature, illustrating similar DNA changes independently of disease state, sex, tissue, and cell type<sup>[59,63]</sup>. Additionally, aged human BM-MSCs from long-term culture exhibited consistent epigenetic changes *in vitro* when the methylation profile of human BM-MSCs at early and late passages was assessed<sup>[64]</sup>. Following the application of enhanced reduced representation bisulfite sequencing, the global DNA methylation profiling demonstrated a greater breadth than previously reported. The genome-wide analyses using whole-genome bisulfite sequencing provided a better understanding of how the epigenetic modifications alter gene expression and regulate the biological characteristics<sup>[65]</sup>.

**Non-coding RNAs:** It has been previously reported that some non-coding RNAs are associated with cellular senescence in different cell types<sup>[66-70]</sup>. MicroRNAs (miRNAs) are small non-coding RNAs that have highly conserved sequences and regulate target genes in cellular functions of metabolism, proliferation, apoptosis, and senescence<sup>[71,72]</sup>. In senescent MSCs, 43 miRNAs were identified and characterized using a miScript miRNA assay<sup>[73]</sup>. Among them, the expression of 24 miRNAs was closely related to

cellular senescence as referred to previous studies. As for the rest, fourteen miRNAs (miR-10, miR-27b, miR-30b, miR-30d, miR-103a, miR-103a-2, miR-136, miR-140-5p, miR-323-3p, miR-330-5p, miR-361-5p, miR-409-3p, miR-424, and miR-455-3p) were upregulated, and five miRNAs (miR-16-2, miR-29b, miR-199b-5p, miR-454, and miR-618) were downregulated in response to cellular aging. Additionally, miR-29b and miR-199b-5p modulated cellular senescence *via* LAMC networks<sup>[73]</sup>.

In MSCs cultured under hypoxic conditions, miRNA expression profiles of MSCs from young ( $\leq 30$  years old) and aged ( $\geq 60$  years old) donors were analyzed using an Agilent Technologies Bioanalyzer high sensitivity DNA chip<sup>[74]</sup>. Principal component analysis demonstrated differentially expressed miRNAs in normal and hypoxic groups. There was  $> 2$ -fold upregulation of nine miRNAs in young MSCs and two miRNAs in aged MSCs after culturing under hypoxic conditions. Also, the hypoxia induced downregulation of four miRNAs in young MSCs and thirty-one miRNAs in aged MSCs, respectively. MiR-543 and miR-590-3p were identified as regulators of cellular aging in hMSCs through direct binding to the aminoacyl tRNA synthetase-interacting multifunctional protein-3/p18 transcripts and decreasing the protein expression levels<sup>[75]</sup>.

Long non-coding RNAs (lncRNAs) are non-coding transcripts, longer than 200 nucleotides, that play critical roles in the regulation of MSCs senescence. They are not only involved in age-related lineage fate switching but also in the reprogramming of old cells<sup>[76,77]</sup>. LncRNA microarray analysis of young and aged Sca-1+CD29+CD45-CD11b- murine BM-MSCs has demonstrated that 92 lncRNAs showed altered expression<sup>[76]</sup>. Among them, 83 lncRNAs were downregulated, and 9 were upregulated in cells isolated from aged mice. Further investigation demonstrated that the candidate BM stem cell-related lncRNA (BMNCR) was highly expressed in the BM-MSCs of young mice, and significantly decreased during aging. Moreover, the BMNCR levels in human BM-MSCs were negatively correlated with age. The effects of BMNCR were evidenced by Bmncr-KO and Bmncr-Tg mice simultaneously<sup>[76]</sup>.

### **Functional degeneration of aged MSCs**

The senescent MSCs exhibit significant impairments in paracrine functions<sup>[78]</sup>, resistance to oxidative stress, hypoxia, or serum deprivation-induced apoptosis<sup>[79-81]</sup>. The age-dependent decrease in cytokines, chemokines, and growth factors released by MSCs will impact cellular functions such as apoptosis, migration, osteogenesis, angiogenesis, cell adhesion, and immunomodulation<sup>[52,54,82]</sup>. Finally, the aged MSCs delay wound healing and exacerbate tissue injuries<sup>[54]</sup>. In summary, the functional regression of senescent MSCs limits their application in tissue engineering and regenerative medicine.

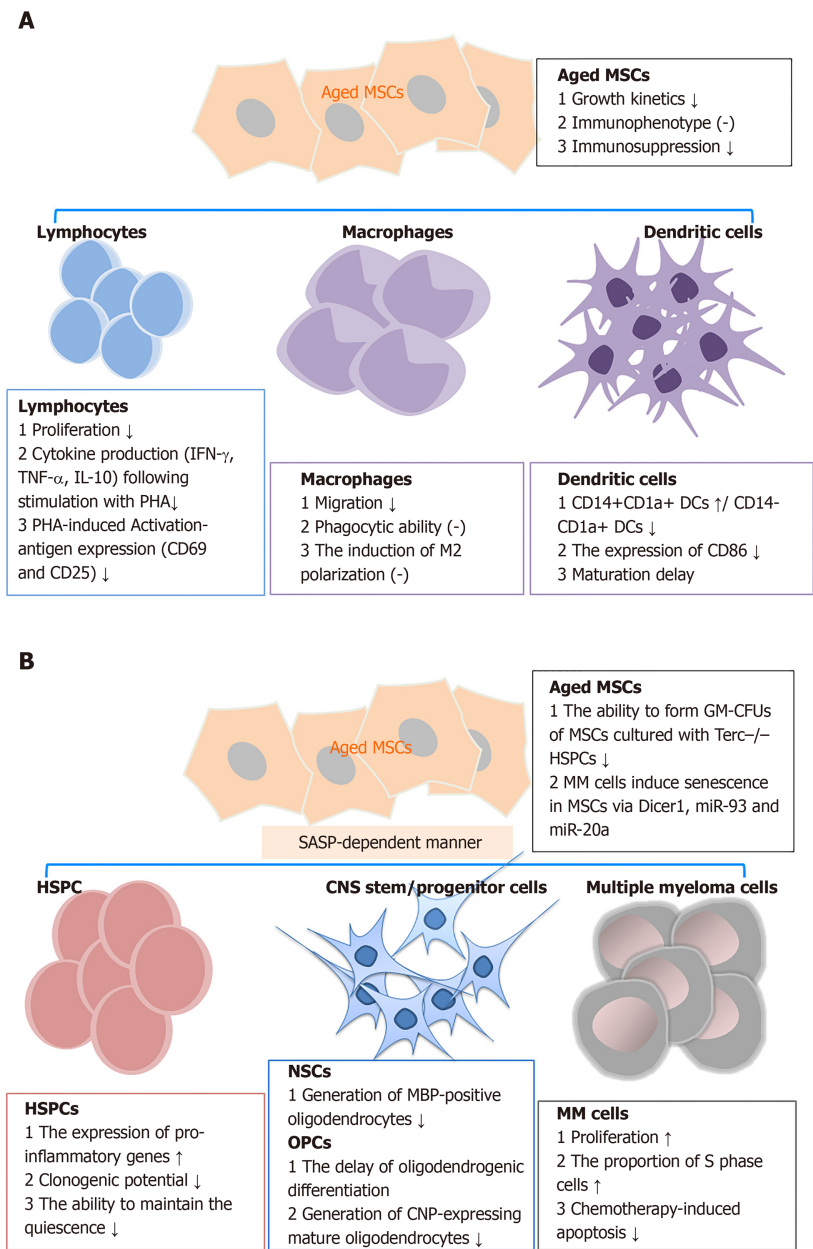
### **Age-related effects of MSCs on target cells**

Bidirectional signaling exists between MSCs and their target cells<sup>[83,84]</sup>. The interaction between MSCs and target cells has been shown to occasionally follow a time-dependent model of regulation and feedback<sup>[85]</sup>. MSC senescence decreases the functions of a large variety of immune cells, hematopoietic stem and progenitor cells (HSPCs), oligodendrocytes, senescent chondrocytes, and other target cells through either direct or indirect cross-talk (Figure 1)<sup>[78,84,86]</sup>.

**Immune cells:** Previous studies have demonstrated that MSC senescence retards immunosuppression in various types of immune cells. Replicative senescence of MSCs derived from BM or adipose tissue showed decreased ability to suppress T-cell, but not natural killer and B-cell proliferation<sup>[87,88]</sup>. Long-term expansion of MSCs reduced the capacity to inhibit CD4+ and CD8+ T cell proliferation. This phenomenon was observed by co-culturing MSCs with alphaCD3CD28-activated peripheral blood mononuclear cells. The inhibitory effect on T-cell proliferation significantly decreased along with increased passage number of hBM-MSCs, and the effect in hUC-MSCs was even more substantial<sup>[88]</sup>. ADSCs derived from elderly subjects also displayed a diminished capacity to suppress the proliferation of activated T cells. Similar results were observed in MSCs isolated in parallel from Lewis and Brown Norway rats of young (less than 4 wk old) and aged (older than 15 mo) groups<sup>[89]</sup>. Aside from proliferation, the senescent MSCs impair the suppressive effects of the activation-antigen expression and cytokine production in phytohemagglutinin-stimulated T cells<sup>[79]</sup>. Soluble factors and direct cell-cell contact partially mediate the decreased suppressive effect of aged MSCs on T cells<sup>[56]</sup>.

In addition to lymphocytes, MSC senescence affects the phenotypes and functions of macrophages and dendritic cells<sup>[29,90]</sup>. When co-cultured with BM-MSCs from young





**Figure 1** Effects of senescent mesenchymal stem cells on target cells. A: Effects of aged mesenchymal stem cells (MSCs) on immune cells; B: Effects of aged MSCs on other target cells, including hematopoietic stem and progenitor cells, neural stem cells, and multiple myeloma cells. DCs: Dendritic cells; HSPCs: Hematopoietic stem and progenitor cells; NSCs: Neural stem cells; OPCs: Oligodendrocyte progenitor cells; MM cells: Multiple myeloma cells; MSCs: Mesenchymal stem cells; PHA: Phytohemagglutinin; GM-CFUs: Granulocyte macrophage-clone formation units; MBP: Myelin basic protein; CNP: 2',3'-cyclic-nucleotide 3'-phosphodiesterase.

mice, a macrophage cell line (RAW264.7 cells) exhibited higher migration rates, although they displayed similar phagocytic ability and induction of macrophage M2 polarization<sup>[29]</sup>. In dendritic cells, cellular maturation was inhibited when cultured with expanded marrow stromal cells relative to the parental MSCs<sup>[91]</sup>.

**Other target cells:** Apart from immune cells, senescent MSCs show impaired activity against multiple target cells. Senescent MSCs enhanced the expression of pro-inflammatory SASP factors in HSPCs and inhibited their clonogenic potentials<sup>[92]</sup>. These cells also have a reduced capacity to maintain CD34+CD38- HSPCs quiescence, as a result of increased IL-6 secretion<sup>[93]</sup>. In a study on telomere dysfunction in MSCs, using Terc-/- mice, Ju *et al*<sup>[94]</sup> found that aged Terc-/- BM-MSCs depressed the functions of HSPCs and early hematopoietic progenitors. Aging MSCs had a reduced ability to induce oligodendrogenic differentiation in neural stem cells. Additionally, the production of 2',3'-cyclic-nucleotide 3'-phosphodiesterase-positive oligodendrocytes in oligodendrocyte progenitor cells was reduced. The impaired differentiation suppressed the generation of myelin-like sheaths during central

nervous system remyelination<sup>[95]</sup>. Also, in the peri-infarct cortex of rats subjected to transient middle cerebral artery occlusion, aged MSC administration resulted in more microglia and reduced pericyte infiltration compared with that for young MSCs. The changes in cellular components probably correlated with reduced expression of brain-derived neurotrophic factor and MCP-1<sup>[96]</sup>. In a myocardial infarction (MI) model, the infusion of old MSCs resulted in a switch of the cellular profile in the infarct region. This was characterized by fewer endothelial cells, vascular smooth muscle cells, and macrophages, and more fibroblasts compared with young MSCs<sup>[97]</sup>. Similarly, senescent MSCs facilitated the growth of multiple myeloma (MM) cells, which aggravated disease progression<sup>[98]</sup>. In turn, MM cells (such as NCI-H929, OPM-2, KMS-12-BM, and primary CD138+ tumors cells) induced senescence in MSCs derived from healthy controls with decreased expression of Dicer1, miR-93, and miR-20a.

## CELLULAR REJUVENATION STRATEGIES

Simple isolation, standardized culture methods, and potential autologous application make MSCs a superior cell source for the treatment of various diseases and injuries<sup>[13]</sup>. Therefore, optimizing the viability and function of MSCs for infusion is of great significance. With an increased understanding of the characteristics of MSC senescence, further investigations are ongoing to resolve challenges linked to the clinical application of cellular therapies. To date, the rejuvenation strategies have demonstrated therapeutic potential including molecular regulation, non-coding RNA modification, and control of the microenvironment.

### **Molecular regulation**

Multiple molecules have been confirmed to restore the proliferative ability and biological function of MSCs *via* gene modification, or the administration of recombinant proteins, agonists, or inhibitors.

**Sirtuins:** The sirtuins (SIRT), which include seven sirtuin homologs, are wellknown for their ability to delay cellular senescence and extend the lifespan of organisms ranging from yeast to humans<sup>[99]</sup>. SIRT1 overexpression in aged MSCs ameliorated the senescence phenotype, recapitulated angiogenesis, and protected cells from oxidative stress. Infusion of SIRT1-modified aged MSCs promoted the expression of pro-angiogenic factors, such as angiopoietin 1, and basic fibroblast growth factor (bFGF). Additionally, SIRT1-modified aged MSCs increased Bcl-2/Bax ratio at the protein level, promoted cellular survival, inhibited fibrosis, upregulated vascular density, and improved heart function in an MI model, compared with vector-aged MSCs<sup>[100]</sup>. SIRT1 pathway activators, including nicotinamide mononucleotide<sup>[101]</sup>, nicotinamide phosphoribosyl transferase<sup>[102]</sup>, cell-deposited decellularized extracellular matrix<sup>[103]</sup>, and SIRT1720<sup>[104]</sup> have been applied in aged human MSCs. They improve cell viability and osteogenesis while inhibiting apoptosis and adipogenesis in aged MSCs. Pretreatment of aged MSCs with SIRT1720 enhanced therapeutic efficacy by promoting angiogenesis and repressing fibrosis following rat MI<sup>[104]</sup>.

Likewise, the overexpression of SIRT3 improves the antioxidant capacity and promotes the survival of old human MSCs through activating forkhead box O3a in the nucleus, manganese-superoxide dismutase, and catalase. In an MI model, the application of old human MSCs overexpressing SIRT3 enhanced cardiac function and decreased infarct size<sup>[105]</sup>. SIRT6 maintains hMSC homeostasis by co-activating the antioxidant nuclear factor erythroid 2-related factor 2 pathway, RNA polymerase II, and heme oxygenase 1<sup>[106]</sup>.

**Growth factors:** Growth factors are a superfamily of proteins that promote cell survival, expansion, migration, and differentiation, as well as prevent disruption of homeostasis *in vitro* and *in vivo*<sup>[107]</sup>. Through stimulation of the FGFR1/2 pathway, LY294002 (a PI3K inhibitor) rescued MSCs from senescence<sup>[108]</sup>. Acadesine activates adenosine 5'-monophosphate-activated protein kinase (AMPK), a downstream signal of FGF21, and abrogates the depletion of FGF21-induced senescence by inhibiting mitochondrial fusion<sup>[109]</sup>. Silencing mitofusin-2 has been found to inhibit MSC senescence induced by the abrogation of FGF21 as well. Knockdown of insulin-like growth factor binding protein 4 restored the osteogenic potency of aged MSCs *via* the activation of Erk and Smad signals<sup>[110]</sup>. Pretreatment of aged MSCs with macrophage migration inhibitory factor (MIF) enhanced the secretion of vascular endothelial growth factor (VEGF), bFGF, hepatocyte growth factor, and insulin-like growth factor,

which promoted their growth, paracrine function, and survival<sup>[80]</sup>. MIF-rejuvenated MSCs release growth factors through interactions with CD74 and subsequent activation of AMPK-FOXO3a signaling, which protects cells from doxorubicin-induced senescence by modulating the PI3K-Akt signaling pathway<sup>[111]</sup>.

**Additional potential regulators:** The AKT pathway plays an important role in the rejuvenation of features and functions of aged MSCs. This pathway can act *via* ERBB4/PI3K/AKT<sup>[112]</sup>, lactoferrin/AKT<sup>[113]</sup>, Vc/AKT/mTOR<sup>[114]</sup>, and fucoidan/FAK-Akt-TWIST axes<sup>[115]</sup>. Administration of rapamycin, an inhibitor of the mTOR signaling pathway, raised the expression level of NANOG, postponed replicative arrest, and enhanced the lifespan increment of BM-MSCs<sup>[116]</sup>. NANOG, an embryonic transcription factor, is a pluripotency marker that facilitates myogenic differentiation and restores the contractile function of senescent MSCs<sup>[117]</sup>. Additionally, a high number of potential regulators involved in senescent MSC rejuvenation have been screened and investigated *in vitro*. For example, both L-carnitine<sup>[118]</sup> and curcumin<sup>[119]</sup> affect the methylation status of the *TERT* promoter, increase the telomerase activity, and consequently alleviate the aging-related features of ADSCs. In human BM-MSCs, the addition of L-carnitine during expansion also elevates cell production<sup>[120]</sup>.

Many *in vivo* experiments using various disease models have been conducted to confirm the efficacy of the rescue strategies to rejuvenate aged MSCs. It is reported that melatonin can protect MSCs from senescence *via* prion protein (PrPc)-dependent enhancement of mitochondrial function<sup>[121]</sup>. Implantation of genetically-modified old human MSCs with tissue inhibitor of matrix metalloproteinase-3 or VEGF promotes angiogenesis, prevents adverse remodeling, and preserves cardiac function to a similar extent compared with young hMSCs<sup>[122]</sup>. Stem cell antigen 1 (Sca-1)<sup>+</sup> MSCs resident in the heart increase angiogenesis, and activate cell proliferation in the infarcted heart, which improves cardiac function after MI<sup>[123,124]</sup>. Overexpression of neuron-derived neurotrophic factor rejuvenates human ADSCs and BM-MSCs from the elderly, reduces the ischemic area, and repairs cardiac function after MI by improving angiogenesis and decreasing apoptosis<sup>[125,126]</sup>. Ethyl pyruvate, a HMGB1 inhibitor, restores the senescent phenotype of BM-MSCs, alleviates clinical signs of lupus nephritis, and prolongs survival of MRL/Mp-lpr/lpr mice *via* TLR4-NF-kappaB signaling<sup>[127]</sup>. These candidates (both *in vitro* and *in vivo*) may be valuable for the identification of suitable targets with utility in the production of clinical-grade MSCs.

### Non-coding RNA modification

Non-coding RNAs are novel genetic regulators involved in regenerative medicine. With respect to aging, transfection of the miR-195 inhibitor restores the expression of anti-aging factors, including TERT and SIRT1, as well as phosphorylation of AKT and FOXO1. The miR-195 inhibitor reduced the expression of SA-beta-gal, which significantly induced telomere relengthening and restored the proliferative abilities of old MSCs. Administration of aged MSCs with miR-195 knockout alleviated infarction size and improved left ventricular function<sup>[128]</sup>. Additionally, miR-10a, miR-29c-3p, and miR-130b have been reported to rejuvenate MSC senescence by targeting different downstream pathways<sup>[128-131]</sup>. The overexpression of miR-10a in aged hBM-MSCs stimulates angiogenesis by inducing the expression of angiogenic factors *via* activated Akt. These cells then enhance implanted stem cell survival and improve cardiac function after MI<sup>[129]</sup>. The miR-29b-3p derived from BM-MSCs regulates aging-associated insulin resistance<sup>[132]</sup>. In multiple myeloma-MSCs, Dicer1 overexpression and upregulation of miR-93/miR-20a could reverse the effects on differentiation and reduce cellular senescence<sup>[98]</sup>. The lncRNA Bmncr regulates the age-related lineage switch between osteogenic and adipogenic differentiation in BM-MSCs. Overexpression of Bmncr (Bmncr-Tg) reduced bone loss and fat accumulation by maintaining extracellular matrix protein fibromodulin and activating the bone morphogenetic protein 2 pathway<sup>[76]</sup>.

### Microenvironment modulation

A conducive microenvironment is essential for maintaining MSC activity<sup>[133]</sup>. When BM-MSCs were treated with BM supernatant from systemic lupus erythematosus (SLE) patients, they demonstrated characteristics of senescence. An inflammatory microenvironment is considered to play a primary role in the senescence of SLE BM-MSCs<sup>[127]</sup>. In unbalanced microenvironments caused by aging, the survival, proliferation, colony formation, migration, and appropriate differentiation of grafted BM-MSCs were significantly suppressed<sup>[43,84]</sup>. In addition, the BM pCO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> levels displayed a close correlation with MSC differentiation and proliferation<sup>[134]</sup>.

Therefore, microenvironment regulation is a promising strategy to reverse the decline of aged MSCs and promote the clinical efficacy<sup>[135,136]</sup>.

Rejuvenating the senescent MSCs is more effective in hypoxic conditions compared with that in normal conditions. The neuroprotective effects of CM from aged human BM-MSCs against cerebral ischemia were enhanced by hypoxia conditioning *in vitro*<sup>[137,138]</sup>. Stem cell-deposited decellularized extracellular matrix can rescue hUC-MSCs from oxidative stress-induced premature senescence and facilitate their clinical application in regenerative medicine<sup>[103]</sup>. The co-culture system is a convenient means of modulating the cellular microenvironment. BM-MSCs co-cultured with young (P3) human umbilical vein endothelial cells demonstrated a higher proliferative ability and decreased pro-inflammatory (cytokines and miRNA) phenotype, compared with the old cells (P13)<sup>[139]</sup>. In conclusion, the enhancement of the microenvironment has a significant effect on the prevention of MSC senescence.

### **Other factors influencing rejuvenation**

Besides restoring the activities of aged autologous MSCs, some researchers have attempted to find an accessible source for the replenishment of autologous MSCs. In the past few decades, the differentiation of pluripotent stem cells into MSC-like cells has been explored to address the problems of viability and scarcity of autologous MSCs derived from old individuals<sup>[140-142]</sup>. Induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs), which acquire a rejuvenation gene signature, are the alternative sources of MSCs<sup>[141,143]</sup>. However, the production protocols used to derive MSCs from iPSCs and ESCs require optimization. The introduction of new technologies, such as 3D culture and gene engineering, might make them more valuable for further clinical application<sup>[142]</sup>.

Biomaterials and various cellular components are potential carriers used for the modification of aged MSCs. MVs carrying mRNAs, miRNAs, non-coding RNAs, proteins, and DNA are a novel and promising tool used to reverse aging in cells by mediating intercellular communication<sup>[44,108]</sup>. For instance, exosomes containing miR-17 and miR-34a from young MSCs rejuvenate aged murine hematopoietic stem cells *via* AKT/autophagy-related mRNAs<sup>[144]</sup>. Extracellular vesicles from human iPSCs can reduce cellular reactive oxygen species levels and alleviate aged phenotypes of senescent MSCs by partially delivering intracellular peroxiredoxin antioxidant enzymes<sup>[145]</sup>. Media supplied with human platelet lysate from younger donors were able to facilitate MSC expansion and osteogenic differentiation<sup>[146]</sup>. Additionally, many bioactive hydrogels<sup>[147]</sup>, biomimetic scaffolds<sup>[148]</sup>, and other biomaterials<sup>[149,150]</sup> have been tested to assess whether they can modify aged MSCs. Removal of senescent cells in a high-throughput manner is another strategy that can be used to address the challenge of senescence<sup>[151]</sup>; this strategy, which has been explored in clinical trials, involves the isolation and enumeration of senescent MSCs from undiluted human whole blood.

Some chemical compounds and foods rejuvenate senescent MSCs. Zinc sulfate significantly reduced the doubling time and increased *TERT* gene expression of rat ADSCs under extremely low-frequency-electromagnetic field<sup>[152]</sup>. It also enhanced telomere length extension in human ADSCs by regulating telomerase and methylation of the *TERT* gene promoter CpG island<sup>[153]</sup>. Besides zinc sulfate, resveratrol mimics the effects of dietary restriction, improves osteogenic function, and promotes mitochondrial activities of senescent MSCs through the regulation of mitofilin<sup>[154]</sup>. NT-020, a dietary supplement containing blueberry, green tea, vitamin D3, and carnosine, rescued the reduced proliferation of MSCs in serum from aged rats<sup>[155]</sup>. Additionally, *Undaria pinnatifida* and its ethanol extracts improve replication ability and ameliorate functional decline in senescent hBM-MSCs (P17)<sup>[156]</sup>.

The rejuvenation methods mentioned above have potentials to optimize the functional status of aged MSCs. However, most of them were *in vitro* or rodent model studies. Further research is needed to evaluate their long-term safety and efficacy before it can be clinically useful.

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## **CURRENT CHALLENGES AND FUTURE PERSPECTIVES**

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Senescence is an inevitable biological process for MSCs obtained from old individuals or long-term cultures. Although recent studies have revealed the characteristics and mechanisms of MSC senescence and attempted to rejuvenate aged MSCs, many issues remain unresolved. First, in studies of age-correlated phenotypic alterations, the expression of CD90 and CD73 in intervertebral disc cells was reduced in older individuals, while CD146 expression was increased<sup>[157]</sup>. However, the expression of

these factors (MSC markers) is rarely compared between young and aged MSCs. The comparison of these two types of cells provides a better understanding of senescent MSCs. Second, the effects of cellular rejuvenation for aged MSCs need to be determined *in vivo*, especially in the context of the multidirectional functions of regulators<sup>[158]</sup>. For example, hypoxia not only promotes the expansion of MSCs<sup>[159,160]</sup>, but also influences the activity of MSCs during osteogenic differentiation<sup>[161]</sup>. Future work *in vivo* can provide more information about clinical efficacy. Additionally, MSCs isolated from specific tissues usually maintain lineage differentiation towards a specific cell type, and this plays a crucial role in regenerative therapy<sup>[161]</sup>. Therefore, the directional differentiation capacities in aged MSCs must be clarified following the increase in available tissue sources. Finally, many newly developing technologies, such as MVs, three-dimensional spheroid culture, and nanobiotechnology, will aid in improving aged MSC function in clinical therapies. Additionally, the functional discrepancies in various rejuvenation factors reported in different studies should be evaluated. For example, although a decline of osteogenesis capacity in aged MSCs was reported, other studies suggested that bone formation capacity was not affected in aged MSCs<sup>[162]</sup>. The function of pigment epithelium-derived factor (PEDF) responding to the senescence is unanticipated to demonstrate the different results in different research teams<sup>[97,163]</sup>. Liang *et al*<sup>[97]</sup> showed that increased PEDF secretion resulted in the impaired therapeutic ability of aged MSCs. However, Cao *et al*<sup>[163]</sup> showed that PEDF delayed cellular senescence and allowed a greater expansion of MSCs by suppressing oxidative stress and preserving differentiation potentials, compared with that in the control group. The different PEDF functions are possibly attributable to MSC heterogeneity, varying research objectives, and the specific experimental models used.

## CONCLUSION

The rejuvenation of aged MSCs holds great promise for the accelerated translation of cell-based approaches (especially autologous cell administration) into clinically relevant therapies.

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