Online ISSN 2690-1803

Aging Pathobiology and Therapeutics

Age-linked Stem Cell-Mediated dysfunction

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Topic: Age-linked Stem Cell-Mediated dysfunction

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EDITORIAL

Age is linked to pathological conditions and diminished physiological functions. These include neurological and immune decline, hematological disorders such as myeloproliferative disorders, and cardiac dysfunction. It is unclear if the decline occurs by dysfunction in stem cells or if there is a decline in the tissue niche that can no longer support the resident stem cells. This special issue invites papers related to the broad topics of aging and also welcome papers that discuss if aging can be druggable. In this regard, papers describing the complexity of aging would enhance this series. Papers are also welcome on specific diseases.

Topic: Age-linked Stem Cell-Mediated dysfunction

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DOI: 10.31491/APT.2022.12.101

REVIEW

Age effect on mesenchymal stem cell properties: a concise review

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This article belongs to the Special Issue: Age-linked Stem Cell-Mediated dysfunction

Abstract

Mesenchymal stem cells belong to one of the multipotent stem cell types isolated from almost all tissues in the human body. They function to maintain tissue homeostasis with their highly proliferative property, and they also possess immunomodulatory properties. The properties of mesenchymal stem cells can be influenced by multiple factors, among which donor ages have been indicated negatively correlated with the proliferation, migration, and differentiation of mesenchymal stem cells. Telomerase activity, telomere length, and cell senescence have been studied to understand the mechanisms of the age effect on mesenchymal stem cell properties. Rejuvenation treatments are the critical research direction to attenuate the deterioration of mesenchymal stem cell properties by the age effect. This review article summarized the updated research on the impact and mechanisms of aging and age-related factors on different mesenchymal stem cell properties. In addition, the treatments to rejuvenate the aged mesenchymal stem cells will also be discussed. This review article aims to enlighten scientific researchers in better preparing and nursing the autologous mesenchymal stem cells from the elderly for future applications in tissue engineering and regenerative medicine.

Keywords: Mesenchymal stem cells, aging, proliferation, migration, differentiation

Introduction

Stem cells belong to the undifferentiated cells with the ability to self-renewal and differentiation into mature cells. They are essential for tissue growth, development, and homeostasis. Adult stem cells refer to the stem cells found in the developed tissues, and they function to maintain adult tissue specificity by homeostatic cell replacement and tissue regeneration [1]. They are presumed to be inactive within the adult tissues, but they can be stimulated to divide into a stem cell clone and a transiently amplifying cell. The latter will undergo limited divisions before terminally differentiating into mature functional tissue cells. Because of lineage-restricted differentiation for adult stem cells, different types of adult stem cells are equipped with specific functions in different tissues and organs. Apart from the most-studied blood-forming hematopoietic stem cells (HSCs) [2], mesenchymal stem cells (MSCs), also known as marrow stromal cells, belong to another multipotent adult stem cell population with potentials differentiating into the mesodermal lineages, including adipocytes, chondrocytes, and osteocytes [3]. Although MSCs were first identified in bone marrow, they reside within the connective tissues of many organs, including adipose tissue, umbilical cord, and teeth [4]. MSCs isolated from the fetal tissues, including the umbilical cord, umbilical cord

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Received: 02 November 2022 / Revised: 01 December 2022
Accepted: 14 December 2022 / Published: 29 December 2022

blood, Wharton's jelly, placenta, and amniotic membrane, are considered the fetal MSCs, and the alternative is considered adult MSCs. Human MSCs can be sorted with the positive selection by CD29, CD44, CD73, CD90, CD105, CD146, and STRO-1 as well as the negative selection by CD31, CD34, CD45, CD49f, and CD133 [5]. Apart from the expression of specific cell surface markers, MSCs are also defined to be growing in adherence to the plastic surface while maintained in standard culture conditions and are capable to be *in vitro* induced into mesenchymal lineages with the appropriate medium as recommended by the International Society of Cellular Therapy [6].

MSCs have been applied as a therapeutic agent for the treatment of various diseases, such as cardiovascular [7] and neurodegenerative diseases [8, 9]. Autologous MSCs are developed from the patients themselves without immune rejection, whereas allogeneic MSCs are established from the selected donors allowing expansion on a large scale and cryopreservation to provide a readily available source of stem cells. Current allogeneic volunteer MSC donors mostly are of a young age [10]. Yet, there are substantial pieces of evidence demonstrating the aging process adversely affects the properties of MSCs [11]. The impacts of age and age-related factors on the MSC properties are of great importance for autologous or allogeneic MSC transplantation, especially among the elderly. In this review article, we will summarize the donor age effect on MSC properties, together with the underlying molecular mechanisms. In addition, the treatments attenuating or delaying the age effect on MSCs will also be discussed.

Age effect on mesenchymal stem cell yield from tissues

A study on MSCs derived from anterior cruciate ligaments demonstrated that the mean proportion of isolated MSCs was slightly but significantly higher in older donors (67.96 ± 5.22 years) than the younger donors (29.67 ± 10.92 years) [12]. In contrast, a study on MSCs in adipose-derived stromal vascular fraction reported a negative correlation of MSC count with donor age [13]. Yet, human adipose-derived MSCs harvested from the same subjects with a time window of 7 to 12 years apart (initial age of the 3 donors: 17, 21, and 72 years old) show no significant difference was found in cell yield, stromal-vascular fraction subpopulation, proliferation, and tri-lineage differentiation [14]. These studies indicated that the effect of donor ages on the cell yield of MSCs harvested from donor tissues is still controversial.

Age effect on mesenchymal stem cell surface marker expression

A study reported that the subpopulations of bone marrow-derived MSCs harvested from younger donors are composed of more $CD71^+$, $CD146^+$, and $CD274^+$ MSCs than that from older donors, and the fluorescence per cell of CD71, CD90, CD106, CD140b, CD146, CD166, and CD274 is negatively correlated with the donor age [15]. Similarly, human bone marrow-derived MSCs express CD13, CD44, CD90, CD105, and Stro-1 regardless of age, but those from the donors over 40 years old showed significantly lower expression of CD90, CD105, and Stro-1 [16]. Moreover, lower SSEA-4 expression was found in the elderly bone marrow-derived MSCs as compared to the young MSCs [17]. Consistently, our previous study also reported that lower expression of SSEA4 was found in human MSCs derived from periodontal ligaments with a donor age of > 40 years old as compared to those with a donor age of ≤ 20 years old [18]. In contrast, no significant difference in cell surface marker expression was reported in bone marrow-derived MSCs from the pediatric and adult donors [19]. Human adipose-derived MSCs from all age groups also show comparable expression of CD3, CD14, CD19, CD34, CD44, CD45, CD73, CD90, and CD105 [20]. The effect of donor ages on MSC marker expression could be exhibited in donors with older ages.

Age effect on mesenchymal stem cell proliferation

MSC proliferation is related to the availability and abundance of stem cells present to exert a regenerative effect. The number of colony-forming unit-fibroblasts (CFU-F) colonies with alkaline phosphatase (ALP) activity in bone marrow-derived MSCs of younger donors (3–36 years old) is significantly higher than that of the older donors (41–70 years old) [21]. Consistently, there is a significant decline in the CFU-F number in bone marrow-derived MSCs from older donors (21–40 years old) as compared to younger donors (0–20 years old) [16]. Similar results were also observed in adipose-derived stem cells that a 30% decline in CFU numbers and with 38% increase in population doubling time from the donors with age > 50 years old as compared to those with age < 20 years old [22]. Moreover, the cumulative population doubling of bone marrow-derived MSCs from pediatric donors is twice that of young adult donors [19], and the doubling time is 1.7-fold longer in bone marrow-derived MSCs from the older subjects as compared to the younger subjects [23]. In addition, our previous study also found that human MSCs derived from periodontal ligaments with a donor aged ≤ 20 years old show significantly higher proliferation than that of a donor aged 21–40 years old and > 40 years old [18]. On the contrary, no significant differences in CFU numbers of bone marrow-derived MSCs among different donor ages were also reported [24-26]. Interestingly, umbilical cord-derived MSCs from older mothers also show lower proliferative and colony-forming capacity as compared to those from younger mothers [27]. Yet, other studies demonstrated that human fetal membrane-derived MSCs from older mothers show a higher proliferation rate than those from younger mothers [28]. Collectively, there are prominent pieces of evidence that the proliferation of

MSCs would be reduced in donors of older ages.

Age effect on mesenchymal stem cell migration

The movement of stem cells and their capacity to migrate to injury sites are the determining factors of stem cell regenerative potentials. The migration ability of human adipose-derived MSCs is significantly decreased in the elderly donors as compared to the child donors with a significant reduction in *CXCR4* and *CXCR7* expression in the elderly group [29]. Moreover, the migratory activity of human periodontal ligament-derived MSCs with a donor age of 56–75 years old is significantly decreased as compared to those with a donor age of 16–30 years old [30]. Consistently, our previous study also demonstrated that human periodontal ligament-derived MSCs with donor age > 40 years old show significantly lower migration as compared to that with donor age ≤ 20 years old and 20–40 years old, accompanied by lower expression of *PTK2* in the periodontal ligament-derived MSCs with donor age > 40 years old [18]. Notably, bone marrow from the aged mice can induce a slower migration ability of murine MSC cell line C3H10T1/2 as compared to that from the young mice [31]. Collectively, aging, together with the aged tissue microenvironment, could reduce the migration ability of MSCs.

Age effect on mesenchymal stem cell differentiation

MSCs, equipped with multipotent differentiation potential, can give rise to mesenchyme tissue cells, including adipocytes, osteoblasts, chondrocytes, myocytes, and cardiomyocytes. As compared to the younger adipose-derived MSCs, the aged MSCs show decreased chondrogenic and osteogenic potential, but are in favor of shifting towards adipogenic differentiation with increasing age [32]. Yet, another study reported that the osteogenic and chondrogenic potentials of adipose-derived MSCs decline with the donor age, but the adipogenic potential of adipose-derived MSCs is independent of the donor age [20]. Advancing age has been demonstrated to have a significant negative effect on the adipogenic and osteogenic differentiation potentials of human adipose-derived MSCs [29], while no differences in the differentiation efficiency in adipogenesis and osteogenesis between young (≤ 35 years old) and old (≥ 55 years old) adipose-derived MSCs have also been reported [33]. In contrast, the adipogenic and osteogenic potentials of bone marrow-derived MSCs decrease with increasing age while the chondrogenic potential did not change [34]. Besides, the osteogenic differentiation of bone marrow-derived MSCs is more affected by age than the adipose-derived MSCs [35]. No significant differences in the osteogenic differentiation capacity of bone marrow-derived MSCs between young and aged donors have also been reported [15, 26]. Under a moderate level of inflammatory stimuli, osteogenic differentiation of bone marrow-derived MSCs from elderly donors could be greatly diminished, and adipogenic differentiation remains unchanged, while the bone marrow-derived MSCs from young and intermediately aged donors show better osteogenic differentiation but reduced adipogenic differentiation [36]. For human periodontal ligament-derived MSCs, the osteogenic and adipogenic differentiation capacities of human periodontal ligament-derived MSCs are reduced when age increases [30]. Consistently, our previous study demonstrated that the osteogenic, chondrogenic, and adipogenic differentiation abilities of human periodontal ligament-derived MSCs with donor age > 40 years old are all reduced as compared to those with donor age ≤ 20 years old [18]. Collectively, the age effect on the differentiation of different mesodermal lineages of MSCs could be dependent on the originated cell sources and the microenvironments.

Apart from mesodermal lineage differentiation, we have previously demonstrated that human periodontal ligament-derived MSCs and adipose-derived MSCs can be induced into neural and retinal lineages [37-39]. It has been reported that the neuroectodermal differentiation potential of human bone marrow-derived MSCs from old donors (> 45 years old) is completely lost, with no cells showing mature neuroectodermal phenotypes and fewer cells expressing early neuroectodermal marker proteins as compared to that of the young donors (18–35 years old) [40]. Yet, additional studies are needed to validate the age effect on the neural differentiation of MSCs.

Age effect on immunomodulation of mesenchymal stem cells

The allogeneic transplantation of MSCs can be achieved because of the immunomodulatory properties of MSCs. It has been reported that adult adipose-derived MSCs (< 65 years old) inhibit the activated $CD4^+$ T-lymphocytes more effectively than elderly adipose-derived MSCs (≥ 65 years old) with increasing mean $CD4^+$ T-lymphocyte proliferation by 0.5 % for any 1-year increase in age [41]. However, it was also shown that gingival tissue-derived MSCs display effective immunoregulation in a mouse model of lipopolysaccharide-induced acute lung injury irrespective of donor age [42]. Similarly, human dental pulp-derived MSCs have been shown effectively regulate the $CD4^+$ T cells; yet, their effects on Th1 and Th2 cells are not affected by the donor ages [43]. In mouse, the aged MSCs presented with a lower immunomodulatory property to induce T cell apoptosis in the co-culture system as compared to the young MSCs [44]. For our previous study, we demonstrated that human periodontal ligament-derived MSCs with donors ages 20–40 and > 40 years old show higher *IL6* and *CXCL8* expression [18]. Elevated expressions of *IL6* and *CXCL8* are also reported in adult MSCs as compared with pediatric MSCs [45]. These could indicate that the microenvironment around the aged MSCs could be inflammatory, reflected by the accumulation of

inflammatory T and B lymphocytes [44].

Age effect on the neuroprotective effect of mesenchymal stem cells

We have previously demonstrated that human periodontal ligament-derived MSCs can protect retinal ganglion cells from optic nerve injury by secreting the brain-derived neurotrophic factor and interacting with the host cells in the retina [46]. It has been reported that bone marrow-derived MSCs from both young (16–18 years) and old (67–75 years) donors in a co-culture system significantly enhance total neurite length of dorsal root ganglia neurons, and only the MSCs from young donors, but not the old donors, can further be potentiated by the treatment of growth factors [47]. Moreover, under the culture with a conditioned medium of bone marrow-derived MSCs, the rescue ability of MSCs on the reduced survival of rat cortical neurons by trophic factor withdrawal decrease with increasing MSC donor age [48]. In addition, it has been suggested that the composition of the secreted bio-active materials of MSCs derived from human tooth germ is influenced by the passage number of the cells [49]. These indicate that increasing MSC age could weaken its ability to neurotrophic factor secretion and compositions, which leads to the reduced neuroprotective effect of the aged MSCs.

Molecular mechanisms of age effect on mesenchymal stem cells

Telomere length

The length of the telomere is an indicator of the mitotic capacity of a cell. Telomere shortening is considered a hallmark of stem cell aging [50]. It has been reported that the infant adipose-derived MSCs exhibited longer telomere lengths than the elderly MSCs [51]. Consistently, our previous study demonstrated that human periodontal ligament-derived MSCs with a donor age > 40 years old have shorter telomere lengths than those with a donor age ≤ 20 years old [18]. However, the same telomere length, regardless of the donor's age, has also been demonstrated in human adipose-derived MSCs [52]. Similarly, no difference in telomere length was found in bone marrow-derived MSCs from younger (8 months–6 years old) and older (38–58 years old) donors [53]. The telomere lengths in native bone marrow-derived MSC are also not related to the ages of the donors [54]. In placenta-derived MSCs, the telomere lengths could be related to cell division rather than the aging of the mothers [55]. Collectively, the role of telomere length in the age effect on MSC properties is still controversial.

Telomerase activity

Telomerase (telomere terminal transferase) is a reverse transcriptase responsible for maintaining the telomere length via *de novo* telomere synthesis [56]. Telomerase activity is related to the proliferation capability of MSCs [57]. Low levels of telomerase activity were reported in bone marrow-derived MSCs in a study [54], and another study reported no telomerase activity is detected in bone marrow-derived MSCs from different ages of human donors [15]. Yet, the analysis of the microarray datasets GSE97311 and GSE68374 revealed that some of the down-regulated genes in the aged adult bone marrow-derived MSCs are involved in the telomerase activity as compared to the fetal MSCs [58]. In addition, telomerase expression was reported to be lower in bone marrow-derived MSCs from the adult rats as compared to that from the young rats [59]. The role of telomerase activity in the age effect on MSC properties requires further investigations.

Cell senescence

Cellular senescence is a special form of durable cell cycle arrest, leading to the gradual decline in the ability of cell proliferation, differentiation, and physiological function over time. Senescent cells are characterized by durable growth arrest, expressions of anti-proliferative molecules, such as $p16^{INK4a}$, and activation of damage-sensing signaling pathways, including p38 and NF-κB [60]. A significant increase in quiescence of the G2 and S phase was reported in adipose-derived MSCs from the aged donors with increased expression of *CHEK1* and $p16^{INK4a}$ genes with age [22]. The donor age of adipose-derived MSCs is associated with an increase in the expression of senescence-associated β-galactosidase staining with p16 and p21 gene expression higher in adipose-derived MSCs from the aged donors (> 50 years) than the young donor (< 40 years) [20]. The increase in senescence-associated β-galactosidase-positive cells in the elderly human adipose-derived mesenchymal stem cells is accompanied by increased mitochondrial-specific reactive oxygen species production and the p21 expression [29]. Similarly, the percentage of senescence-associated β-galactosidase-positive cells is tremendously increased in bone marrow-derived MSCs from the aged donors (> 60 years old) as compared to the young donors (< 30 years old) [61]. Moreover, the numbers of p21-positive and p53-positive cells were also found to be significantly higher in bone marrow-derived MSCs from the aged donors (> 40 years old) as compared to the young donors (7–18 years old) [16]. Critically, NAP1L2 is a regulator for cell senescence of bone marrow-derived MSCs through the activation of the NF-κB pathway [62], whereas follistatin is a marker for human bone marrow-derived MSC aging [63]. For the gingival tissue-derived MSCs, an increase in p53 and sirtuin-1 expression was shown in MSCs from the elderly donors (59–80 years old) as compared to the young donors (13–31 years old) [42]. Yet, no evidence of cellular senescence was reported in bone marrow-derived MSCs from pediatric and adult donors [19]. Collectively, the pieces of evidence of the involvement of cell senescence in the age effect of MSC properties are substantial (Figure 1).

A hallmark of aging is chronic, low-grade, "sterile" inflammation [64]. Cellular senescence is associated with the production of pro-inflammatory chemokines, cyto-

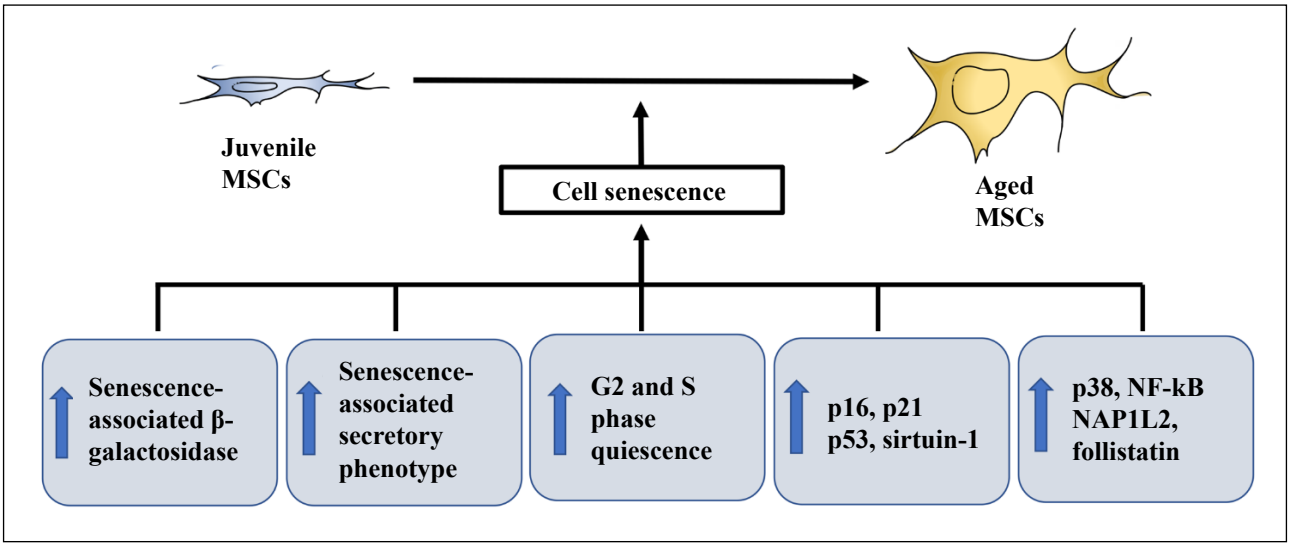

Figure 1. **Cell senescence in aged mesenchymal stem cells**. Increase in senescence-associated β-galactosidase staining, quiescence of G2 and S phases, expressions of p16, p21, p38, p53, and sirtuin-1, activation of nuclear factor-κB (NF-κB), nucleosome assembly protein 1 like 2 (NAP1L2), follistatin, and senescence-associated secretory phenotype (SASP) have been shown contributing to cell senescence in the aged mesenchymal stem cells (MSCs).

kines, and extracellular matrix remodeling proteases, which comprise the senescence-associated secretory phenotype (SASP) [65]. Accumulation of senescent fat progenitor cells has been found in adipose tissue with aging, and the senescent cells acquire SASP and provoke inflammation in adipose tissue with JAK pathway activation in adipose tissue with aging [66]. Exposure to TNF-α could induce the upregulation of SASP components in adipose-derived MSCs, including interleukin (IL)-6, IL-8, and monocyte chemoattractant protein 1 (MCP-1) [67]. It has been reported that transplanting relatively small numbers of senescent cells into young mice is sufficient to spread cellular senescence to host tissues and cause persistent physical dysfunction [68], indicating the endocrine effects of the senescent cells. Consistently, transplanting adipose-derived MSCs from old donors, but not from young donors, induces physical dysfunction in older recipient mice owing to a naturally occurring senescent cell-like population in adipose-derived MSCs primarily from old donors [69]. Therefore, the senescent MSCs could limit the application of exogenous autologous delivery of MSCs from aged donors and impose a potential risk to the shortening of the health- and lifespan of the recipients. Rejuvenation of the senescent MSCs could be helpful to improve autologous MSC transplantation in elderly individuals.

Rejuvenation of the aged mesenchymal stem cells

Rejuvenation refers to the restoration of youthful vigor. Multiple strategies have been studied to rejuvenate the aged mesenchymal stem cells (Figure 2) to improve their properties for treatments.

Sorting of juvenile subpopulations

MSCs are heterogeneous in the population [1]. We have previously isolated the pluripotent neural crest subpopulation from human periodontal ligament-derived MSCs [70], suggesting that there could be "juvenile" cells residing in the aged MSCs as a rare subpopulation. Consistent with our study, 8% of the SSEA-4-positive subpopulation was identified in human bone marrow-derived MSCs from elderly donors and exhibits a "youthful" phenotype that is similar to that of young MSCs with the number of cells increased by 17,000 folds [17]. Moreover, it has been shown that the sorted $CD264^+$ human bone marrow-derived MSCs have elevated β-galactosidase activity, decreased differentiation potential, and are inefficient in colony formation relative to the $CD264^-$ MSCs [71], indicating that $CD264^-$ is a selection method for the "juvenile" MSCs. Yet, CD271 might not be the marker for the isolation of the "juvenile" cells from the aged MSCs [72].

Senotherapeutics

Senotherapeutics refers to a strategy targeting cellular senescence to delay the aging process. Senotherapeutics are composed of analytics (selectively inducing senescent cell death) and xenomorphic (indirectly suppressing senescence by inhibiting SASP to delay the progression of senescence and tissue dysfunction) [73]. Treatment with dasatinib significantly increases the number of apoptotic PE-adipose-derived MSCs from women with preeclampsia as compared to those from normotensive pregnancies by decreasing the gene expression of p16 and SASP components [67]. Cocktail treatment of dasatinib and quercetin can decrease the number of naturally occurring senescent cells and their secretion of frailty-related pro-inflammatory cytokines in explants of human adipose tissue [68], and improve the osteogenic capacity of bone marrow-derived MSCs from the aged mice [74]. Navitoclax (ABT-263) has been demonstrated with a moderate senolytic effect on senescent human bone marrow-derived MSCs by reducing the senescence-associated β-galactosidase expression [75],

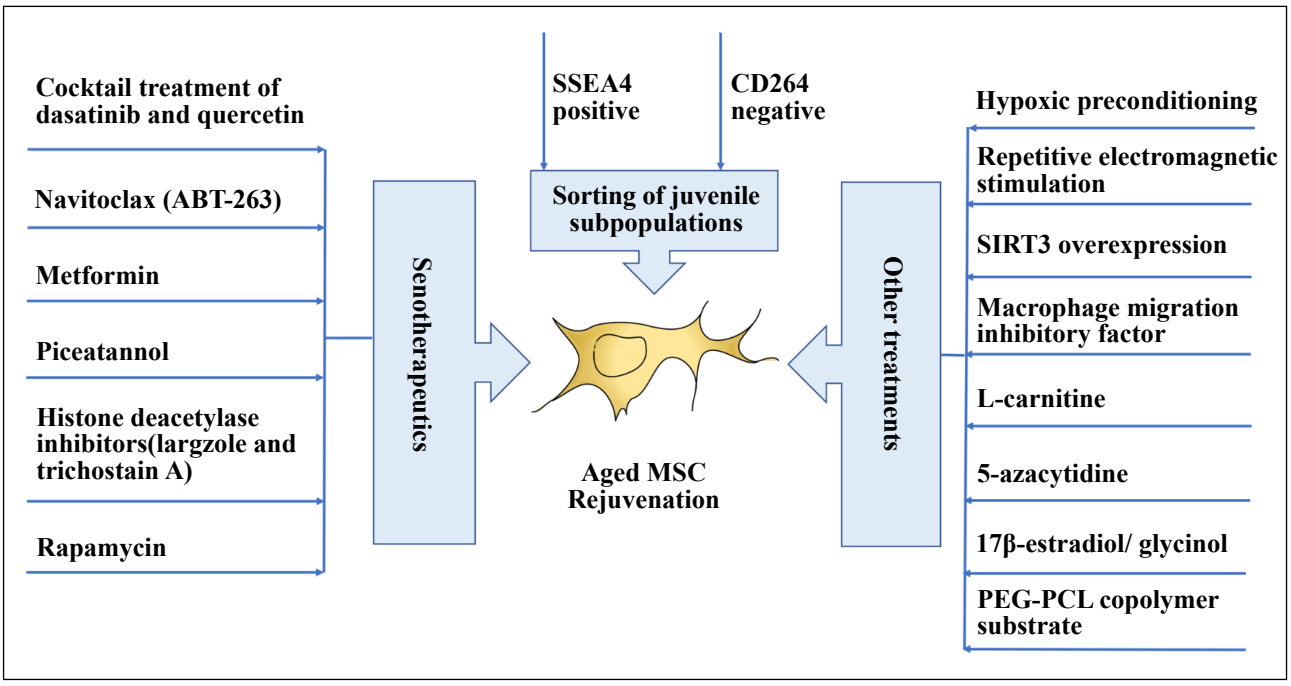

Figure 2. **Rejuvenation of the aged mesenchymal stem cells.** Sorting of juvenile subpopulations among the aged mesenchymal stem cells (MSCs), senotherapeutics, hypoxic preconditioning, repetitive electromagnetic stimulation, sirtuin-3 (SIRT3) and macrophage migration inhibitory factor overexpression, treatment with L-carnitine, 17β-estradiol, glycinol, and 5-azacytidine, and culturing on the poly(ethylene glycol)-poly(ε-caprolactone) (PEG-PCL) copolymer substrate have been studied as the rejuvenation strategies on the aged MSCs.

whereas metformin reduces the replicative senescence and cell death associated with the prolonged cultivation of human adipose-derived MSCs [76]. Moreover, piceatannol has been shown to reduce the number of senescent human bone marrow-derived MSCs after genotoxic stress and in senescent replicative cultures by promoting the recovery of cell proliferation and the stemness of MSCs [77]. Similarly, largazole and trichostatin A, the histone deacetylase inhibitors, can improve human umbilical cord-derived MSCs proliferation and delay its aging [78]. In addition, rapamycin has also been reported to reverse the senescent phenotype and improve the immunoregulation of human bone marrow-derived MSCs from systemic lupus erythematosus patients by inhibiting the mTOR signaling pathway [79]. Collectively, senotherapeutics should be a promising and emerging treatment strategy to remove senescent MSCs from aged donors.

Other treatments

Hypoxic preconditioning induced by 2, 4-dinitrophenol can improve the regeneration potential of aging bone marrow-derived MSCs into pancreatic β-cells [80]. Similarly, hypoxic preconditioning can improve the *in vivo* angiogenic capacities of human adipose-derived MSCs from older donors [81]. Moreover, preconditioning the bone marrow-derived MSCs with repetitive electromagnetic stimulation can enhance CFU-F and cell proliferation in bone marrow-derived MSCs, more effectively from the older donors than the young donors, via transient nitric oxide production and extracellular signal-regulated kinase 1/2 activation [82]. For gene modulation, *SIRT3* overexpression can protect human bone marrow-derived MSCs from older donors against oxidative damage by activating catalase and manganese-dependent SOD through FOXO3a and improved their cell myocardial repair effect [83]. The improvement of myocardial repair by the aged MSCs can also be achieved by modulating the macrophage migration inhibitory factor that overexpressing macrophage migration inhibitory factor in human bone marrow-derived MSCs from older donors can reduce cellular senescence, activate autophagy, induce angiogenesis, prevent cardiomyocyte apoptosis, and improve the heart function and cell survival after myocardial infarction [84]. In addition, treatment with L-carnitine has been demonstrated to increase the gene expression of human telomerase reverse transcriptase and telomere length in human adipose tissue-derived MSCs isolated from healthy aged volunteers [85]. For the osteogenic differentiation, we have previously demonstrated that treatment of 5 μmol/L curcumin can enhance the osteogenic differentiation of human bone marrow-derived MSCs via matrix metalloproteinase-13 expression and activity [86]. Treatments with 17β-estradiol and glycinol have also been demonstrated to rescue the age-related reduction in osteogenic differentiation of bone marrow-derived MSCs isolated from older donors through estrogen receptor signaling [87], whereas treatment of 5-azacytidine induces the proliferation and improves the osteogenic differentiation potential of adipose-derived MSCs from older donors with DNA demethylation and increased *TET2* and *TET3* gene expression [88]. Furthermore, it has been reported that culture of the bone marrow-derived MSCs from aged human donors on a poly(ethylene glycol)-poly(ε-caprolactone) copolymer substrate can decrease levels of detected intracellular ROS levels in the aged MSCs and promoting the osteogenic differentiation [89].

Challenges and prospects

Aging is a life-long process of living toward old age, which is characterized by the progressive loss of physiological functions that could lead to diseases and death. The effect of aging on MSCs is complex and complicated, involving genetic martial deterioration, non-coding RNAs, exosomes, protein imbalance, mitochondrial dysfunction, reactive oxygen species as well as the mTOR, and insulin/IGF-1-like signaling pathways [90]. However, as a life-long process, MSCs are not just influenced by aging. In the real world, other environmental exposures and behaviors can also influence the properties of MSCs [91, 92]. The influences of these personalized factors also need to be considered in the analysis of the donor effect. Refine phenotyping and grouping with larger sample sizes could help to resolve the effects of specific factors on MSC properties. Single-cell and spatial transcriptomics could also help to delineate the specific aging cells among the heterogeneous subpopulations of MSCs [93].

The induced pluripotent (iPS) stem cells [94] is demonstrated as an example of rejuvenation. There is still a lack of consensus on the standard/clinically recognized rejuvenation strategies for aged MSCs although numerous anti-aging strategies have been proposed [95]. Yet, MSCs possess diversified properties for different treatment approaches [9], and different rejuvenation approaches might be needed for different MSC properties. Further studies are needed to optimize the condition and quality of MSCs in the treatment regime for each MSC property. Despite the uncertainties regarding the application of aged MSCs, MSC therapy would still be a promising and important strategy for the treatment of different diseases.

Declarations

Authors' contributions: T.K.N. and H.S.C. conception and design. Y.H. and T.K.N. financial support. C.B.C., X.B., and Y.H. collection and/or assembly of data. C.B.C., X.B., Y.H., and T.K.N. data analysis and interpretation. C.B.C., X.B., Y.H., T.K.N., and H.S.C. manuscript writing.

Financial support and sponsorship: This work was supported in part by the Special Fund of Guangdong Province for Science and Technology (project code: 210714156882753 to Y.H.), and the Joint Regional Basic Science and Applied Basic Science Research Fund of Guangdong Province (project code: 2019A1515110685 to T.K.N.), China.

Conflicts of interest: The authors declare no potential conflicts of interest.

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Cite this article as: Chen CB, Bin X, Huang YQ, Ng TK & Cheung HS. Age effect on mesenchymal stem cell properties: a concise review. *Aging Pathobiol Ther*, 2022, 4(4): 109-118. doi: 10.31491/APT.2022.12.101

Aging Pathobiology and Therapeutics 2023; 5(1): 03-17 ***03***
DOI: 10.31491/APT.2023.03.107

REVIEW

Senescence of mesenchymal stem cells: implications in extracellular vesicles, miRNAs and their functional and therapeutic potentials

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This article belongs to the Special Issue: Age-linked Stem Cell-Mediated dysfunction

Abstract

Senescence is seen as the cellular counterpart of tissue and biological aging, with irreversible stagnation of cell growth, and changes in function and behavior. Mesenchymal stem cells (MSCs) are one of the key therapeutic tools in regenerative medicine, and their regenerative and therapeutic potential declines significantly with the increasing age of cell donors and prolonged continuous culture *in vitro*. MicroRNAs (miRNAs) are regarded as important players in regulating the expression and function of multiple genes and pathways. Emerging evidence suggests that extracellular vesicles (EVs) participate in a complex cell senescence network, at least partially by providing certain miRNAs. Therefore, MSC EVs and miRNAs are implicated in not only contributing to but also influenced by MSC senescence. Here, we will provide an overview of the recent results on roles and mechanisms of miRNAs, particularly EV-miRNAs, involved in MSC senescence, and discuss their implications in functional properties and therapeutic efficacy of MSCs and their EVs.

Keywords: Extracellular vesicles, microRNAs, mesenchymal stem cells, senescence

Introduction

Stem cells offer the foundation of regenerative medicine. Based on the plasticity of stem cells, *in vitro* and *in vivo* induction or gene modification methods can make them transdifferentiate into therapeutic cells to achieve therapeutic purposes. Mesenchymal stem cells (MSCs) are one of the most accepted therapeutic cells in regenerative medicine and tissue engineering [1]. MSCs can be obtained from a rather wide range of adult tissues (*e.g.*, muscles, bone marrow (BM), and adipose tissue) and neonatal tissues (*e.g.*, umbilical cord (UC), placenta, and amnion), and amplified by *in vitro* expansion [2], easily reaching the manufacturing levels. MSCs have the potential for self-renewal and multi-lineage differentiation and exert pro-angiogenesis, pro-proliferation, anti-apoptotic, anti-fibrosis, and anti-inflammatory functions through the interaction between cells and the secretion of many soluble factors [3].

Cellular senescence refers to the irreversible stagnation of cell growth under the action of various stress factors and may be important to prevent the proliferation of damaged cells and acts as a barrier to tumor lesions [4]. However, cells that undergo permanent proliferation arrest may be detrimental to the entire individual, and senescent cells are present in aging tissues and accumulate in an age-dependent manner that accelerates the decline of tissue function and contributes to the development of age-related diseases [5]. The regenerative and therapeutic potential of MSCs decreased significantly with the increasing age of the cell

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Received: 29 November 2022 / Revised: 31 January 2023
Accepted: 16 February 2023 / Published: 29 March 2023

donor. In cell-based therapy and tissue engineering, MSCs require prolonged and large-scale *in vitro* manufacture, in which continuous expansion may lead to replicative senescence [1], likely constraining the manufacturing quantity in return. Senescent MSCs usually show decreased regenerative ability, reduced differentiation ability, and weakened immune-regulatory functions, and thus possibly fail to achieve optimal therapeutic outcomes. In order to manufacture the highest quantity of MSCs with optimal functional properties, there is an urgency to develop technologies to easily assess and delay the replicative senescence of MSCs.

MicroRNAs (miRNAs) are a class of special small RNAs composed of about 22 nucleotides that selectively bind to the 3'-untranslated region (3'-UTR) of the mRNA sequence and regulating the translation and stability of the targeted mRNA, thus altering gene expression without changing the genetic code [6, 7]. MiRNAs are important regulators of senescence-related gene expression. Most miRNAs that regulate stem cell senescence have been shown in MSCs and hematopoietic stem cells (HSCs) by targeting genes associated with metabolism, epigenetics, and DNA damage [8, 9]. Extracellular vesicles (EVs) are heterogeneous vesicles induced by stimuli such as cell differentiation, activation, senescence, and transformation. They are formed by lipid bilayer membranes and contain proteins, nucleic acids, lipids, and their derivatives. EVs are an important participant in cell-to-cell communication and can precisely regulate receptor cell senescence and inflammation under various physiological and pathological conditions [10, 11]. It has been reported that miRNAs released in the extracellular environment by cell-secreted EVs can influence the senescence of surrounding cells. In this review, we will focus on the latest advances in the regulatory role of miRNAs, especially those in EVs, in MSC senescence, and their application potentials.

MSC senescence

Although MSCs originate from the mesoderm, they can differentiate into mesodermal tissues (*e.g.*, adipose, bone, cartilage, and hematopoietic tissues) and non-mesodermal tissues (*e.g.*, neurons and glial cells) [12, 13]. Due to their self-renewal, multipotent differentiation, and immunomodulatory properties, MSCs are considered ideal candidates to replace damaged or lost cells and tissues *in vivo*. Thus far, MSCs are widely used for regenerative medicine and tissue engineering and are currently the focus of over thousands of clinical trials, showing significant therapeutic capacity in a broad range of diseases, such as pulmonary fibrosis [14], myocardial infarction [15], and diabetes mellitus [16].

Senescence is a physiological process of organisms and is associated with a decline in MSC activity, which slows tissue repair and maintenance [17]. *In vitro*, proliferation arrest is the major characteristic of cell senescence. With the accumulation of undegraded macromolecules, senescent cells show morphological enlargement, flattening, and extensive vacuolization [18], accompanied by increased senescence-associated β-galactosidase (SA-β-gal) activity, DNA damage, telomere shortening, and genomic instability.

In tissues or organisms, senescent cells can transmit signals to surrounding tissues through senescence-associated secretory phenotype (SASP), which consists of basic fibroblast growth factor (FGF), cytokines (interleukin-6 (IL-6), IL-1β), chemokines (IL-8, and monocyte chemoattractant protein-1 (MCP-1)), extracellular proteases (matrix metalloproteinases (MMPs)), growth factors (transforming growth factor-beta (TGF-β), hepatocyte growth factor (HGF)), and vascular endothelial growth factor (VEGF) [19]. SASP can in a way help eliminate senescent cells and/or tissue remodeling by promoting phagocytic immune cells and promote the occurrence and development of tumors and age-related diseases by creating a pro-inflammatory microenvironment.

The aging of adult resident MSCs is directly proportional to the old donor, and the functional properties of MSCs deteriorate severely with the increase of donor age. Compared with MSCs from adult tissues, some MSCs from neonatal tissues have a stronger proliferative capacity *in vitro*, especially under hypoxic conditions [20]. The differentiation efficacy of adult MSCs into certain lineage-specific cells is also influenced by the donor age, while their *ex vivo* proliferative potential depends on population doubling (PD) and cell passage [21]. The senescence of MSCs influences their replicative potential and properties (*e.g.*, morphology, function, and biomarker), which may affect their therapeutic efficacy. The functional degradation and potentially harmful effects of senescence have limited the application of MSCs in regenerative medicine and tissue engineering. Therefore, it is important to understand the senescence features of MSCs and identify common methods for assessing the MSC state.

During the long-term culture of MSCs *in vitro*, their proliferative capacity and colony-forming units (CFU) decreased. The proliferation of MSCs slows down at 30-40 PD, stops proliferation, and enters the senescence state when PD reaches a certain level [22]. The number of colonies indicates the clonogenic potential and proliferation ability, and the level of CFU decreased in senescent cells. The CFU of MSCs decreased continuously with the increase of passage and could hardly be detected after the 20th passage [23]. Therefore, detecting PD and CFU indicators of MSCs is a shared method for detecting senescence *in vitro*.

The size and morphology of MSCs changed significantly during senescence. With long-term culture *in vitro*, the early MSCs, similar to spindle-forming fibroblasts form, became larger in size, flattened in shape, and increased in cytoplasmic granules [24]. The *in vitro* imaging system analysis showed that the cell volume of MSCs began to expand at the 5th passage, and the area of the 9th passage cells increased by 4.8 times compared with the 1st passage [25]. The cell size was strongly associated with the increase of SA-β-gal expression and actin stress fibers [26]. Therefore, assessing the morphology and size of MSCs

is also a shared method for detecting senescence *in vitro*. Especially based on the unique morphology of senescent cells, the development of image recognition-related detection technology has excellent application prospects.

MSCs continuously lose their adipogenic and osteogenic differentiation potential during prolonged culture [27]. It has been reported that senescence can transform the osteogenic differentiation potential of MSCs into adipogenic [28]. Rapamycin, an autophagy activator, can restore the biological characteristics of senescent MSCs by increasing proliferation and osteogenic and decreasing adipogenic differentiation [29].

MSCs are involved in regulating the activation and phenotype of innate and adaptive immune cells, including dendritic cells, macrophages, monocytes, natural killer cells, and lymphocytes. When co-cultured with young mice MSCs, mice macrophages retained their original phagocytosis and M2 polarization and showed higher migration rates [30]. With senescence, the protective immunomodulatory functions of MSCs may be altered, such as their reduced ability to inhibit lymphocyte proliferation. With the increase of passages, the ability of MSCs co-cultured with peripheral blood mononuclear cells to inhibit the proliferation of $CD4^+$ and $CD8^+$ T cells were continuously weakened [31]. In addition to proliferation, senescent MSCs attenuated the inhibitory effects of phytohemagglutinin-stimulated T-cell cytokine and activation-antigen production [32].

The secretory properties of MSCs also change with senescence. The expression of growth factors (TGF-β and HGF), inflammatory cytokines (IL-1, IL-6, and IL-8), and extracellular proteases (MMP1, MMP3, and MMP9) increased in SASP secreted by senescent MSCs [33]. SASP-related factors were increased in the conditioned medium of late passages compared with that of early passages. SASP-related factors drive the senescence of their own or neighboring cells in a cell-autonomous manner or paracrine manner, resulting in negative effects on cellular functions (such as cell adhesion, differentiation, proliferation, and migration) [34].

Specific molecules associated with MSCs--CD markers show different expression patterns at early and late stages. The expression of CD264 is up-regulated during the intermediate stage of cell senescence and continues to be up-regulated during cell senescence, which can be used to evaluate therapeutic potential. When the $CD264^+$ proportion is 75%, the regenerative potential of MSCs is severely impaired [35]. On the other hand, the $CD146^+$ proportion decreases with the increase in donor age and generation [36]. The expression of $CD90^+$ and $CD106^+$ is also decreased in senescent MSCs [26]. Leptin receptor (CD295) can be used to mark apoptotic cells and its expression increased with MSCs of advancing biological aging [37].

Telomere shortening and DNA damage are the major mechanisms of senescence. Telomere length is closely related to the replicative potential of cells and tissues. Telomerase prevents telomere shortening and induces elongation by bringing repeated TTAGGG to chromosome ends [38]. However, telomerase almost does not express itself throughout the life cycle of MSCs. Due to the lack of telomerase activity, adult MSCs showed irreversibly shortened telomeres during continuous passages [39]. Oxidative stress is the major cause of DNA damage. Increased oxidative stress-related molecules can induce senescence and growth arrest in MSCs, which are highly sensitive to the accumulation of DNA damage [40]. Elevated intracellular reactive oxygen species (ROS) levels can reduce MSCs proliferation and DNA synthesis [41]. The activity of the antioxidant enzyme (superoxide dismutase (SOD)) decreased in late-generation MSCs, while the levels of nitrogen monoxide (NO), ROS, and gluconate oxidizing enzyme increased [42].

Phosphatidylinositol 3-kinase (PI3K)/v-akt murine thymoma viral oncogene homolog (Akt)/mechanistic target of rapamycin (mTOR) pathways are activated by the high concentration of ROS and are key regulators of the oxidative stress response [43]. Nuclear factor erythrocyte 2-related factor 2 (NRF2) plays an important role as a transcription and regulator factor in oxidative stress response by regulating a variety of antioxidant response element-dependent antioxidant genes [44]. NRF2 activity decreased with the senescence of MSCs. Activation of NRF2 may be an effective method for preventing the deterioration of the MSC growth state under oxidative stress and maintaining stemness [45].

In addition, mitochondrial membrane potential changes in senescent cells, are accompanied by increased cellular oxygen consumption and ROS production [46]. Mitochondrial dysfunction has been shown to contribute to senescence. When mitochondrial function is impaired, oxidative stress increases, leading to apoptosis [47]. Mitochondrial fusion increased and mitochondrial fission decreased in senescence MSCs. The efficiency and function of autophagy gradually decline with age, and enhanced autophagy may prolong the life span of organisms [48]. *In vitro* MSC senescence induced by the high glucose concentration showed increased autophagy levels, while down-regulation of autophagy alleviated the senescence, suggesting autophagy is involved in MSC senescence [49].

MiRNAs in MSC senescence

MiRNAs are important contributors to epigenetic regulation, affecting the translation and stability of targeted mRNAs to regulate post-transcriptional gene expression [50]. Mounting evidence indicates that individual miRNAs participate in the regulation of target mRNAs and mediate numerous cellular processes by influencing different signaling networks [51], including senescence-related multiple signaling molecules and pathways (Figure 1 and Table 1).

Specific miRNA function and expression profiles may reflect unique developmental stage-specific, tissue-specific, or disease-specific patterns. Several miRNAs are expressed differently between young and senescent MSCs (Figure 2). The miScript miRNA assay was used to identify 43

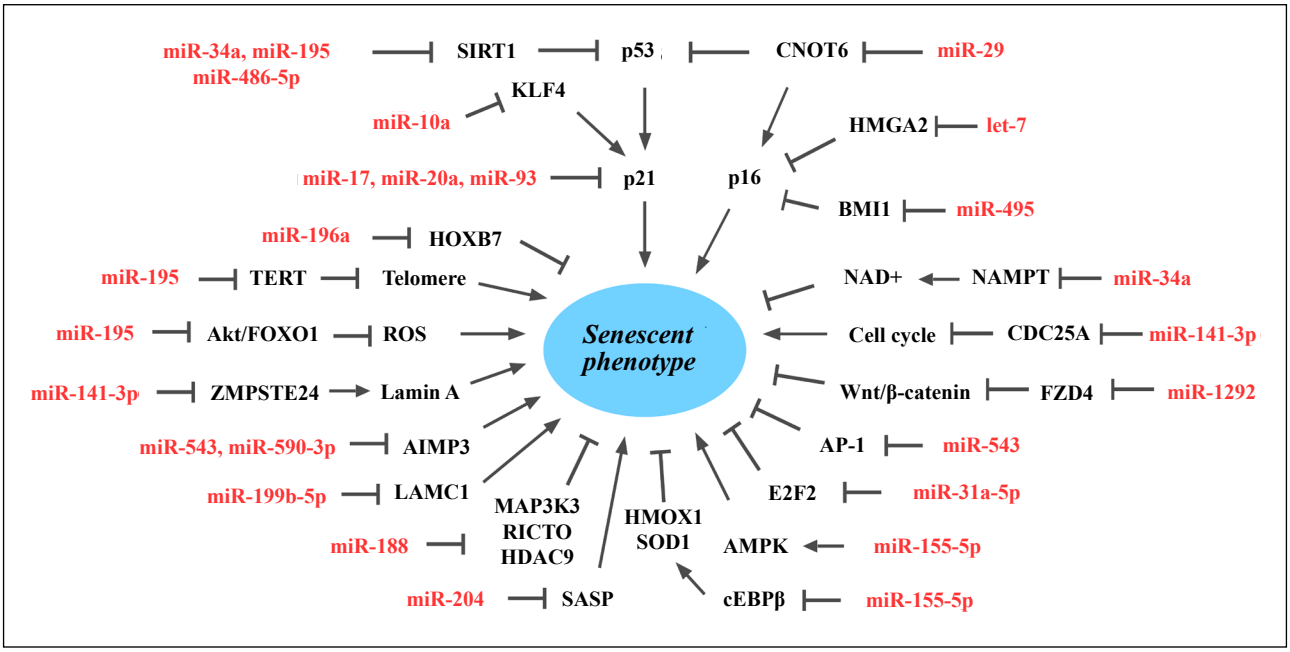

Figure 1. **MiRNAs are involved in MSC senescence.** AIMP3, Aminoacyl-tRNA synthetase-interacting multifunctional protein 3; AMPK, AMP-activated protein kinase; AP-1, activating protein 1; BMI1, B-cell-specific moloney murine leukemia virus insertion site 1; CDC25A, cell division cycle 25A; cEBPβ, CCAAT/enhancer binding protein β; CNOT6, CCR4-NOT transcription complex subunit 6; E2F2, early 2 factor 2; FOXO1, forkhead box O1; FZD4, frizzled-4; HDAC9, histone deacetylase 9; HMGA2, high mobility group A2; HMOX1, heme oxygenase-1; HOXB7, homeobox B7; KLF4, krüpple-like factor 4; LAMC1, laminin gamma 1; MAP3K3, mitogen-activated protein kinase kinase kinase 3; NAMPT, nicotinamide phosphoribosyl-transferase; RICTOR, RPTOR-independent companion of MTOR complex 2; SASP, senescence-associated secretory phenotype; SIRT1, sirtuin 1; SOD1, superoxide dismutase 1; TERT, telomerase reverse transcriptase; ZMPSTE24, zinc metallopeptidase STE24.

Table 1. MiRNAs are involved in MSC senescence.

miRNA	miRNA Target	Mechanism	Reference
let-7	HMGA2	Regulate the p16INK4a/pRB pathway	[55]
miR-10a	KLF4	Reduce p21 expression	[59]
miR-17	SMURF1	Regulate p53 pathway	[61, 62]
miR-20a/93	p21	Regulate p53 pathway	[56- 58]
miR-29	CNOT6	Activate the p16INK4a/pRB and p21/p53 pathways	[63]
miR-31a	E2F2	DNA damage and heterochromatin	[64]
miR-34a	NAMPT	Regulate mitochondrial dysfunction and SIRT1/ FOXO3a activation	[65- 67]
miR-141	BMI1, SDF1, SVCT2, DLX5, ZMPSTE24	Regulate differentiation, migration, proliferation, and cell cycle	[68- 71]
miR-155	Cab39, cEBPβ	Regulate AMPK pathway and ROS production	[72, 73]
miR-188	RICTOR, MAP3K3, HDAC9	Regulates differentiation	[74, 75]
miR-195	SIRT1, TERT,Akt/FOXO1	Shorten telomere length and ROS production	[76]
miR-196a	HOXB7	Repress proliferation	[53]
miR-199b	LAMC1	Regulate LAMC network	[52]
miR-204	SIRT1	SASP expression	[82, 83]
miR-335	AP1	Disrupts immunomodulatory properties and chondrogenic differentiation	[84, 85]
miR-486	SIRT1	Repress cell proliferation and differentiation	[86]
miR-495	BMI1	Increased p16, p21 and p53 expression,SA-β-gal activity, and suppress cell migration	[87, 88]
miR-543/590	AIMP3	Affect differentiation potential	[89, 90]
miR-1292	ALP, RUNX2, FZD4	Regulate Wnt/β-catenin pathway	[91, 92]

miRNAs in senescent MSCs, of which 23 miRNAs were analyzed. 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 up-regulated in response to senescence, and five miRNAs (miR-16-2, miR-29b, miR-199b-5p, miR-454, and miR-618) were down-regulated [52]. MiRNA expressed on MSCs from old donors (39-78 years) and young (3-13 years old) donors were also shown different, and 7 miRNAs (miR-99a, miR-100, miR-196, miR-337-5p, miR-376b, miR-431, and miR-543) were particularly identified, with miR-196 rarely detected in the old-donors [53]. By analyzing the replicative senescence-induced miRNAs expression changes of MSCs derived from young and old donors, twelve miRNAs were shown to be differentially expressed jointly in young and old donor MSCs. Among them, ten miRNAs (miR-150-3p, miR-371a-5p, miR-762, miR-1207-5p, miR-1225-5p,

miR-1915-3p, miR-2861, miR-3665, miR-4281, and miR-4327) were found to be up-regulated and two miRNAs (miR-25-3p and miR-93-5p) were down-regulated [54]. Functionally, overexpression or downregulation of particular miRNAs has been proven to mediate senescence by targeting candidate genes on the $p16^{INK4a}$/pRB and p53/p21 pathways, which primarily control cell senescence.

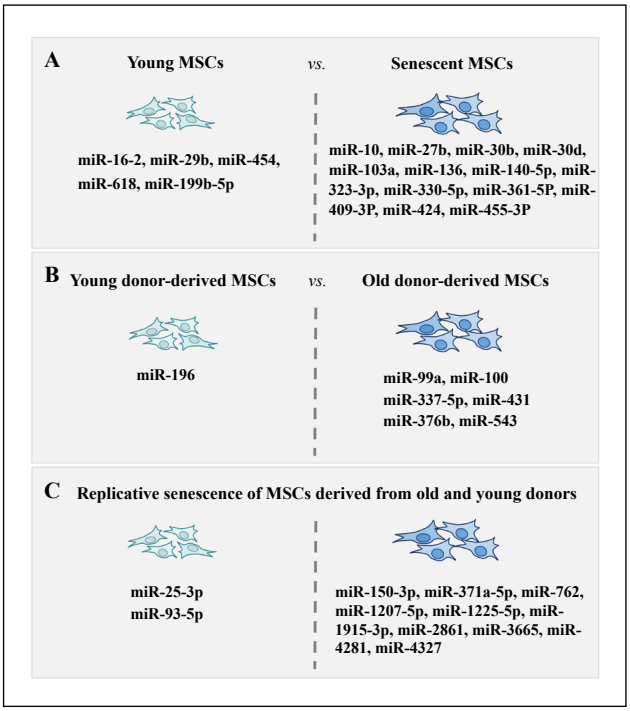

Figure 2. Characteristics of miRNAs profile of young and senescent MSCs.

Histone deacetylase inhibitors induce senescence in MSCs. The up-regulation of miRNAs in the let-7 family can reduce high mobility group A2 (HMGA2) expression during cell senescence [55]. Decreased HMGA2 leads to the activation of the $p16^{INK4a}$ gene, which in turn induces MSCs senescence [56] through the regulation of the $p16^{INK4a}$/pRB pathway by let-7. In senescent MSCs, the expression of miR-20a and miR-93 (members of the miR-17 family) decreased [57]. The decreased expression of miR-20a was critical for the upregulation of p21, and overexpression of miR-20a significantly attenuated senescence [58].

The expression of miR-10a in MSCs also affected by donor age. MiR-10a attenuated cell senescence by inhibiting Krüpple-like factor 4 (KLF4) and increased the differentiation capacity of aged BM-MSCs [59]. KLF4, a zinc finger transcription factor, is involved in the regulation of important processes such as cell cycle, cell growth, and apoptosis. Overexpression of KLF4 can induce cell senescence, which is mainly caused by inducing p21 expression [60].

MiR-17 partially rescues the osteogenic differentiation of senescent MSCs *in vitro* and *in vivo*. Smad ubiquitin regulatory factor 1 (SMURF1), as a direct target gene, is an important contributor to the cascade of p53/miR-17 in osteogenesis [61]. The miR-17 family participates in senescence regulation by directly targeting p21 [62].

The expression of miR-29 showed an increasing trend during the aging of human MSCs. MiR-29c-3p may regulate MSCs senescence depending on the p53 pathway. Overexpression of miR-29c-3p resulted in enhanced SA-β-gal staining and SASP expression, delayed osteogenic differentiation, and reduced proliferation, whereas that of silencing had the opposite results. MiR-29c-3p was shown to target CCR4-NOT transcription complex subunit 6 (CNOT6) and activated the $p16^{INK4a}$ /pRB and p53/p21 pathways in MSCs [63].

The expression of miR-31a-5p was significantly elevated in old rat BMSCs, which exhibited increased adipogenesis and senescence phenotypes. MiR-31a-5p affects osteoblastic and osteoclastic differentiation and mediates the age-related bone marrow microenvironment. MiR-31a-5p induces DNA damage, cell senescence, and senescence-associated heterochromatin foci by targeting E2F2, which is involved in senescence-related changes of heterochromatin [64].

The expression of miR-34a increased in senescent MSCs with continuous passage. MiR-34a promotes apoptosis by regulating mitochondrial dysfunction and activating sirtuin 1(SIRT1)/forkhead box O3a (FOXO3a) and intrinsic apoptosis pathways. In replicative and naturally senescent MSCs, inhibition of miR-34a contributes to the alleviation of senescence-related phenotypic features [65]. MiR-34a is up-regulated by p53 and then down-regulates SIRT1 expression (a p53 inhibitor), thus forming a positive feedback loop [66]. Exception of p53/p21, overexpression of miR-34a reduces cycle-dependent kinases and cyclins. In addition, overexpression of miR-34a in young MSCs induces long-term proliferation, increased SA-β-gal activity, and decreased osteogenic differentiation capacity. MiR-34a significantly reduced SIRT1 activity, nicotinamide adenine dinucleotide $(NAD)^+$ content, and NAD^+/nicotinamide adenine dinucleotide (NADH) ratio by targeting nicotinamide phosphoribosyl-transferase (NAMPT) [67].

In MSCs, miR-141 target genes include B-cell-specific moloney murine leukemia virus insertion site 1 (BMI1), stromal cell-derived factor 1 (SDF1), sodium-dependent from vitamin C-2 (SVCT2), and distal-less homeobox 5 (DLX5), which are involved in the regulation of differentiation, migration, and proliferation. The expression of miR-141-3p depends on histone acetylation at the promoter and increases in senescent MSCs [68, 69]. MiR-141-3p directly inhibited zinc metallopeptidase STE24 (ZMPSTE24) (enzyme for processing pre-lamin A into lamin A) [70]. In the subculture of aged MSCs, the cells have abnormal nuclear morphology due to the increase of pre-Lamin A. MiR-141-3p targeted cell division cycle 25A (CDC25A) leads to inhibiting MSC proliferation by arresting cell cycle at the G1 phase [71].

The expression of miR-155-5p in MSCs from old donors was significantly higher than that from young donors. In young donor-derived MSCs, high expression of miR-155-5p resulted in increased cell senescence. MiR-155-5p increases mitochondrial fusion and inhibits mitochondrial

fission in MSCs through the AMP-activated protein kinase (AMPK) pathway, thereby leading to cell senescence by inhibiting Cab39 expression [72]. In addition, miR-155-5p promotes ROS production. MiR-155-5p suppressed the expression of antioxidant genes (heme oxygenase-1 (HMOX1) and superoxide dismutase 1 (SOD1)) by repressing CCAAT/enhancer binding protein β (cEBPβ, a common transcription factor regulating these genes) [73].

MiR-188 regulates the senescence-associated transition of BMSCs from osteogenesis to adipogenesis and has additional significance in senescence. The expression of miR-188 increased in BMSCs of elderly mice and humans. In lineage-negative myeloid cells, overexpression of miR-188 promotes senescence. MiR-188 targeted genes including RPTOR-independent companion of MTOR complex 2 (RICTOR), mitogen-activated protein kinase kinase kinase 3 (MAP3K3), and histone deacetylase 9 (HDAC9) [74, 75].

The expression of miR-195 increased in senescent and old donor MSCs, and the miRNA directly targeted SIRT1 and telomerase reverse transcriptase (TERT) [76]. SIRT1 is a regulator of p53 deacetylation and exerts an inhibitory role in aging [77]. TERT encodes telomerase, which prevents telomere shortening [78]. MiR-195 affects telomere length changes by targeting TERT. Increased miR-195 expression shortens telomere length in MSCs from old donors. Inhibition of miR-195 significantly reduced SA-β-gal expression in senescent MSCs. MiR-195 also affects the phosphorylation of Akt and FOXO1 [76]. FOXO is a downstream target of the PI3K-Akt signaling pathway, which regulates the ROS pathway during cell senescence [79]. Among them, FOXO1 is a transcription factor involved in the expression of antioxidant enzymes (SOD and catalase) and acts on SIRT1-mediated ROS increase and maintenance during senescence [80, 81].

Expression of miR-196a increased with senescence. Compared with the children group, the expression level of miR-196a increased and Ki-67 decreased in adult MSCs. MiR-196a is negatively correlated with MSC proliferation by directly targeting homeobox B7 (HOXB7). Overexpression of HOXB7 can reduce senescence and improve cell growth, which is related to the increase of basic fibroblast growth factor secretion. HoxB7 acts in cell differentiation, proliferation, and signal transduction, and is a major factor driving the behavioral longevity of progenitor cells to optimize MSC performance [53].

Compared to young (average 21 years) and old (average 65 years) donor MSCs, miR-199b-5p is dysregulated in senescent MSCs. MiR-199b-5p directly represses the expression of laminin gamma 1 (LAMC1) to regulate the LAMC network, thereby indirectly affecting the senescence of MSCs [52]. LAMC1 promotes tumor cell migration and proliferation through the Akt-NF-κB signaling pathway.

The expression of miR-204 is upregulated in senescent human umbilical vein endothelial cells (HUVECs) and stress-induced senescent chondrocytes [82, 83]. In mice, ectopic expression of miR-204 is sufficient to promote osteoarthritis development, while knockdown improved surgically-induced osteoarthritis and repressed SASP expression [83]. SIRT1 is considered to be a key regulator of inflammation and aging. miRNAs post-transcriptionally downregulated SIRT1 during the differentiation of mouse embryonic stem cells, and maintain low levels of SIRT1 expression in differentiated tissues, where MiR-204 was found to be involved in inhibiting SIRT1 protein expression [82].

The expression of miR-335 was increased in BMSCs from old donors and senescent MSCs. Forced expression of miR-335 in MSCs induces a senescent phenotype and disrupts immunomodulatory properties and chondrogenic differentiation ability by repressing activating protein 1 (AP-1), which regulates cell proliferation, differentiation, and migration [84, 85].

MiR-486-5p plays a role in senescence by targeting the SIRT1. In adipose-derived MSCs (AMSCs), miR-486-5p is increased during aging and replicative senescence. Overexpression of miR-486-5p represses cell proliferation and adipogenic and osteogenic differentiation and induces senescence phenotype. MiR-486-5p directly regulates SIRT1 expression and deacetylase activity, and downregulation of SIRT1 can induce senescence [86].

In MSCs, miR-495 increased $p16^{INK4a}$, p21, and p53 expression and SA-β-gal activity by targeting BMI1 [87]. BMI1 is an inhibitor of cell senescence and a regulator of $p16^{INK4a}$ [88]. Conditioned medium collected from MSCs overexpressing miR-495 suppressed the cell migration, which is consistent with the paracrine effect of SASP to trigger cell senescence into healthy adjacent cells [87].

Aminoacyl-tRNA synthetase-interacting multifunctional protein 3 (AIMP3) affects the senescence and differentiation potential of MSCs, and its protein expression level increases with senescence, while miR-543 and miR-590-3p can significantly reduce the expression of AIMP3. Overexpression of miR-543 or miR-590-3p alleviated the late passage MSCs, whereas inhibition of miR-543 or miR-590-3p aggravated senescence by increasing AIMP3 [89, 90].

MiR-1292 acts to accelerate senescence in adipose-derived MSCs and is negatively correlated with osteogenic markers alkaline phosphatase (ALP) and runt-related transcription factor 2 (RUNX2) in bone. MiR-1292 mediates its influence through the Wnt/β-catenin pathway by targeting frizzled-4 (FZD4) [91]. The Wnt/β-catenin signaling pathway is an important contributor to the self-renewal and differentiation of MSCs by promoting the intracellular production of ROS [92].

EV, EV-miRNA in MSC senescence

Based on their differences in size and secretion pathway, EVs are classified into three subtypes: exosomes, microvesicles, and apoptotic bodies [93]. Exosomes (less than 120 nm) originate from the endoplasmic reservoir, producing multivesicular bodies that fuse with the plasma membrane to secrete their contents. Microvesicles (100

to 500 nm) are budding vesicles that may arise from the plasma membrane under various conditions of stress. Apoptotic bodies (500 nm to 5 μm) are released from the plasma membrane of apoptotic cells [94].

EVs are composed of nucleic acids (mRNA, DNA, miRNAs, and long noncoding RNAs), lipids, and proteins [95]. The contents reflect the origin of the cell and convey specific molecules for specific cell types. EV-miRNA exchange between cells may be a key for intercellular communication and the miRNAs encapsulated into EVs are strictly regulated by various microenvironmental conditions and stress stimuli. The miRNA content of EVs may reflect the pathological state of released cells and serve as promising biomarkers for multiple pathologies. EVs are highly enriched for ALG-2 interacting protein X (ALIX), CD63, CD81, and tumor susceptibility gene 101 (TSG-101). Various techniques have been used to characterize EVs, including atomic force microscopy, dynamic light scattering, enzyme-linked immunosorbent assay, electrochemical biosensors, flow cytometry, fluorescence-activated cell sorting, microfluidics, nanoparticle tracking analysis, resistance pulse sensing, scanning electron microscopy, and transmission electron microscopy [96, 97].

Senescence-related EVs can transfer regulatory factors such as miRNAs and proteins to promote the senescence process in autocrine, endocrine, and paracrine ways. Senescent cells secrete high levels of EVs and regulate the microenvironment. P53 regulates the transcription of other endosomal genes associated with vesicle biosynthesis. DNA damage-induced senescence induces an increase in p53-dependent EV biogenesis. Senescent cell-derived EVs are partially dependent on p53 and its downstream target tumor suppressor-activated pathway 6 (TSAP6) [98].

Senescent cell-derived EVs enable neighboring cells to respond particularly rapidly and efficiently to stress by regulating the surrounding environment. On the hand, these EVs may play a role in promoting SASP by transmitting pro-senescence signals, which facilitate the regenerative potential of surrounding cells and the elimination of senescent cells and also enhance local inflammation levels by recruiting immune cells and spreading senescence throughout tissues. A recent study has just shown that senescence-associated exosomes influence the genetic information and immunomodulatory potential of the microenvironment [99].

At present, a variety of inflammation-related miRNAs have been identified in EVs, such as miR-19b, miR-20a, miR-21, miR-126, miR-146a, and miR-155 [100]. The expression pattern of different miRNAs in MSC-EVs changes with senescence [101]. Compared with young rats, the expression levels of miRNA-294 and miRNA-872-3p in MSC-EVs decreased with age [102]. The expression of miRNA-146a was elevated in late passage MSC-EVs compared with the early passage [103]. Mouse senescent MSC-EVs contain miRNA-183-5p, which promotes senescence in young MSCs [104].

Old bone marrow-derived EVs were absorbed by young MSCs and repressed osteogenic differentiation. Overexpression of miR-183-5p reduced Hmox1 protein level and cell proliferation and promoted senescence in MSCs [104]. MiR-34a increases with age in muscle-derived EVs and induces senescence of BMSCs. That is, EVs may induce MSC senescence through miR-34a-5p targeting SIRT1 [105].

MiR-17-3p and miR-199b-5p were decreased in senescent fibroblast-derived EVs. In particular, miR-199b-5p is decreased in senescent MSCs and elderly donor-derived MSCs [52]. MiR-17-3p is also decreased in senescent MSCs and skin fibroblasts as a cellular model. MiR-23a-5p has been proven to regulate the osteogenic differentiation of BMSCs, and its expression was increased in senescent fibroblast-derived EVs [106]. MiR-23a-5p promotes osteogenic differentiation by targeting transmembrane protein 64 (TMEM64), whereas inhibition of miR-23a-5p expression promotes adipogenic differentiation in MSCs [107].

MSC-EVs containing let-7a, miR-21, miR-191, and miR-222 are known to regulate cell proliferation and cycle progression [108]. The expression of miR-21 was decreased in EVs of senescent MSCs and adult MSCs, and this miRNA was also decreased in MSCs from ovariectomized mice and postmenopausal osteoporotic patients [109]. In breast cancer cells, this miR-21 targets E2F2, a downstream effector of p21 and $p16^{INK4a}$ [110].

MiR-31 is a circulating miRNA that is differentially expressed with senescence and increased in the blood of osteoporosis patients. The expression of miR-31 is also elevated in senescent endothelial cell MVs. These MVs repress the osteogenic differentiation of MSCs by targeting FZD3 [111]. MiR-31a-5p was found in senescent MSC-derived exosomes, which trigged osteogenesis of co-cultured bone marrow cells [112]. Compared with young mice, exosomes secreted from older mice-isolated muscle cells are enriched with miR-34a. MiR-34a is related to senescence and inflammation. Myoblast exosomes over-expressing miR-34a can reduce MSCs proliferation and induce senescence by promoting SA-β-gal activity [105].

Induced pluripotent stem cell-derived MSC-EVs (iMSC-EV) enriched with miR-105-5p could rejuvenate senescent nucleus pulposus cells by activating the SIRT6 pathway *in vitro*. miR-105-5p plays a pivotal role in the iMSC-EV-mediated therapeutic effect by decreasing the level of the cAMP-specific hydrolase phosphodiesterase 4D (PDE4D) [113]. It has been reported that suppression of PDE4D expression can promote the migration, invasion, colony formation, and proliferation of colorectal cancer cells [114].

MiR-146a-5p is increased in senescent MSC-derived EVs. This miRNA is known to regulate the NF-κB signaling activation and SASP production of senescent cells [103]. In a mouse model of allergic airway inflammation, MSC-EV suppresses the function of group 2 innate lymphoid cells, reducing inflammatory infiltration and T helper 2 cytokines production by transporting miR-146a-5p [115]. MSC-EV effectively represses the inflammatory response of cardiomyocytes by delivering miR-146a-5p to reduce v-myb myeloblastosis viral oncogene homolog-like 1 (MYBL1) expression [116].

Exosomes enriched with miR-188-3p ameliorate senes-

cence by regulating the mTOR complex. Incubation of old MSCs with this exosome decreased senescence markers and mTOR pathway proteins, and up-regulate the pluripotency markers. Inhibition of miR-188-3p in MSC-EVs significantly increased the expression of RICTOR, decreased the expression and phosphorylation of Akt, and downregulated the proportion of SA-β-gal staining cells [117].

Interestingly, EVs from MSCs of young donors or early passages have been shown to reverse the senescent phenotypes of late passages MSCs or that from pre-mature aged patients. In our study, we found that adding early passage iMSC-EV to the senescent iMSC culture promoted cell growth, downregulated the expression of age-related genes, reduced mitochondrial density, and improved mitochondrial membrane potential (Figure 3). This, even still at the preliminary stage, may suggest that the addition of exogenous exosomes, ideally engineered with elevated expression of specific miRNAs, to the MSC culture, may be feasible for promoting MSC proliferation in culture or scaling-up the manufacture of MSCs to a significant extent.

Prospects of MSC-EV therapy

In initial studies in most animals, MSCs have shown encouraging positive results in various types of cell therapy, but the concerns of human MSC therapy remain unneglectable, including immune rejection and various cancer promotion. Due to the clinical outcomes of MSC-based therapy remaining nonoptimal, so far, a large proportion of the majority of registered clinical trials applying MSC therapy for human diseases have indeed fallen short of expectations.

Emerging evidence suggests that MSC-EV therapy has equal or better efficacy than MSCs in many diseases, and the risk of MSC-based therapy is significantly reduced.

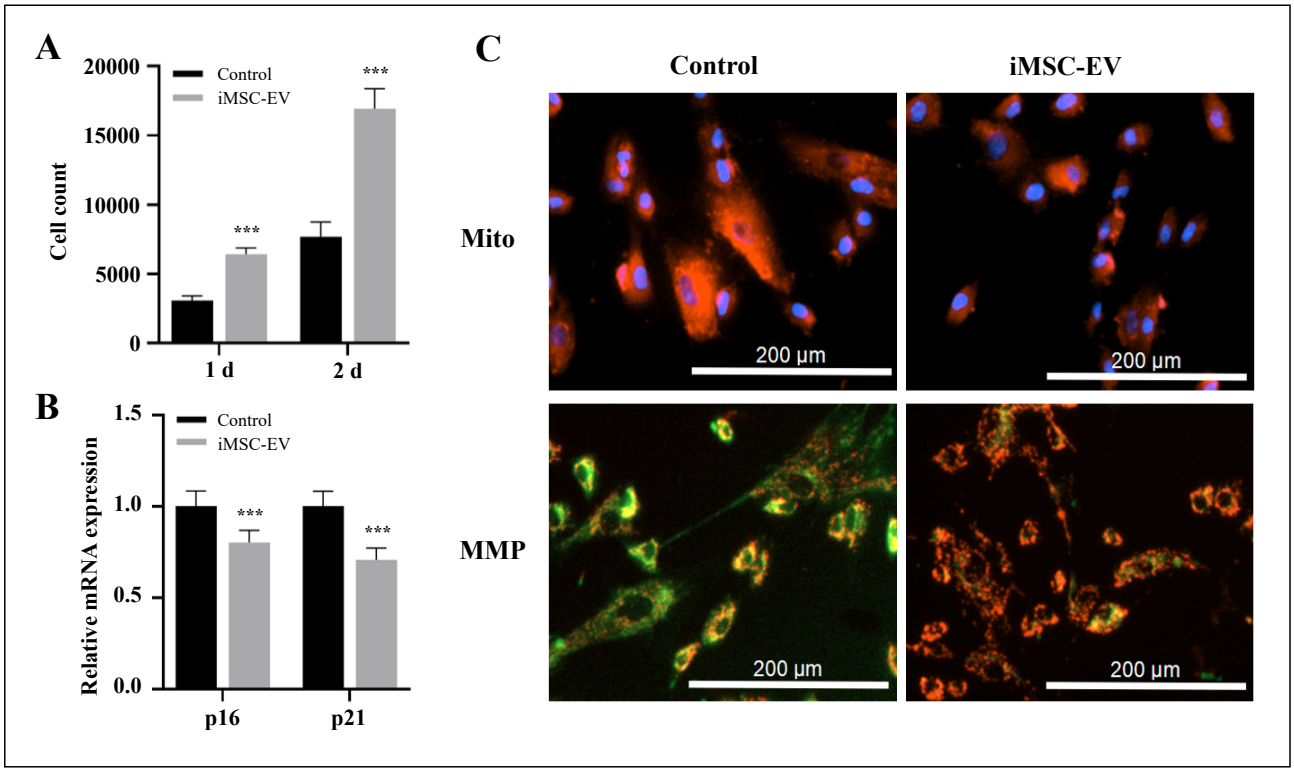

Figure 3. Effect of early passage iMSC-EV on senescence characteristics of late passage iMSCs.A. Changes in cell count of senescent iMSCs after early passage iMSC-EV incubation. B. Expression of cell cycle genes of senescent iMSCs. C. Mitochondrial density and mitochondrial membrane potential (MMP) of senescent iMSCs. $n = 3$, *** $p < 0.001$.

The advantages of cell-free therapies based on MSC-EVs are considerable. The incapability of MSC-EVs to self-replicate greatly reduces the risk of expansion and tumor and increases safety. The small size also provides faster tissue penetration [118]. The potential to stimulate the immune system is limited, reducing the risk during allogeneic transplantation. EVs are easier to handle in transportation and storage, which makes EV therapeutic potential optimal.

The effects of MSC-EVs, including anti-senescence, anti-inflammatory, and wound healing, play a positive role in various diseases. In pilocarpine-induced status epilepticus mice treated with MSC-EVs, EVs reach the hippocampus within 6 h and exert anti-inflammatory and neuroprotective effects, which are coupled with normal hippocampal neurogenesis and cognitive and memory functions [119]. Melatonin-pretreated MSC-EVs affect the ratio of macrophage M2 polarization to M1 polarization by regulating the activation of phosphatase and tensin homolog (PTEN)/Akt signaling pathway, thus suppressing inflammatory

response and promoting diabetic wound healing [120]. In the rat model of skin burn treated with human MSC-EVs, EVs accelerate the re-epithelialization of the wound, promote the nuclear transfer of β-catenin, and enhance skin cell migration and proliferation, thus facilitating wound healing [121].

Although preclinical data have demonstrated the scalability of EV isolation methods and the safety of therapy, the clinical use of MSC-EVs is still limited. Currently, there is a lack of well-defined and standardized optimal culture conditions of parental cells and optimal protocols for EV isolation and storage, optimal therapeutic doses and dosing schedules, as well as reliable potency and safety profiles. Currently, studies have investigated the effectiveness of MSC-EVs in the clinical setting, and most clinical trials are still recruiting and active (Table 2). The delivery routes oral, intranasal administration, intravenous and intraperitoneal injection.

The heterogeneity of MSC-EVs is probably one of the key factors affecting their therapeutic properties. EV variability lies in the contents of RNA and proteins, particularly non-coding RNAs with properties such as inflammation resolution, potency, and tissue regeneration. The development of technology for detecting EV contents is helpful to promote the study of maintaining EV characteristics. Currently, contents are analyzed using chemical, physical, biological, and nanotechnological methods, usually involving the use of multiple antibodies, nucleic acid fitting, or molecular markers as recognition components, coupled with various chemical labels (*e.g.*, redox probes and optical dyes), nanoparticle tags or DNA oligonucleotide [122]. For example, Raman spectroscopy is used to distinguish the overall chemical bond characteristics of EVs based on the spectral patterns generated by vibration and rotation. EV particles are captured on a specially modified plane or spherical interface and fluorescent dye labeling is added

Table 2. List of clinical trials using MSC-EVs.

No.	Condition or disease	Treatment	Trial Phase	Trial ID
1	Cerebrovascular Disorders	Allogenic MSC-EVs enriched by miR-124	Phase I Phase II (Recruiting)	NCT03384433
2	Metastatic Pancreas Cancer With KrasG12D Mutation	MSC-EVs with KRAS G12D siRNA	Phase I (Recruiting)	NCT03608631
3	Chronic Graft Versus Host Diseases	Artificial tears for 14 days of UMSC-EVs 10ug/drop	Phase I Phase II (Recruiting)	NCT04213248
4	Alzheimer Disease	Twice a week for 12 weeks nasal drip of MSC-EVs (5, 10, 20μg)	Phase I Phase II (Recruiting)	NCT04388982
5	Hospital-acquired pneumonia	7 times aerosol inhalation of MPC-EVs (8×10^8 or 16×10^8 particles)	Phase I Phase II (Recruiting)	NCT04544215
6	Acute Respiratory Distress Syndrome	Basic treatment and 7 times aerosol inhalation of MSC-EVs (2×10^8, 8×10^8, or 16×10^8 particles)	Phase I Phase II (Recruiting)	NCT04602104
7	Moderate SARS-CoV2 Infection	Intravenous injection of MSC-EVs	Phase II Phase III (Recruiting)	NCT05216562
8	Degenerative Meniscal Injury	Intra-articular administration of synovial fluid-derived MSC-EVs	Phase II (Recruiting)	NCT05261360
9	Perianal Fistula	Placenta-MSC-EVs	Phase I Phase II (Recruiting)	NCT05402748
10	Retinitis Pigmentosa	Subtenon injection of Wharton jelly-derived MSC-EVs	Phase II Phase III (Recruiting)	NCT05413148
11	Chronic Ulcer	MSC conditioned media	Phase I (Completed)	NCT04134676
12	Novel Coronavirus Pneumonia	5 times aerosol inhalation of MSC-EVs (2×10^8 nanovesicles/3 ml)	Phase I (Completed)	NCT04276987
13	Healthy	Once aerosol inhalation of MSC-EVs (2×10^8, 4×10^8, 8×10^8, 12×10^8, or 16×10^8nanovesicles/3 ml)	Phase I (Completed)	NCT04313647
14	SARS-CoV-2 Associated Pneumonia	Twice a day for 10 days inhalation of MSC-EVs ($0.5\text{-}2\times10^8$ nanovesicles/3 ml)	Phase I Phase II (Completed)	NCT04491240
15	COVID-19 Associated Acute Respiratory Distress Syndrome	Intravenous administration of BM-MSC-EVs	Phase II (Completed)	NCT04493242

Note: * Information obtained from https://clinicaltrials.gov/ on 30 November 2022.

to detect and quantify the membrane proteins and internal miRNAs [122-124]. The analysis and identification of specific contents can be achieved by using surface-sensitive label-free physical analysis methods (*e.g.*, electrical impedance spectroscopy, quartz crystal microbalance, and surface plasmon resonance) or external chemical tags to monitor the binding of EV contents to receptors on the array [125-127]. Although the diversity of EV detection methods has been achieved, the standardization of identification and analysis is still very important. The above detection methods are more or less affected by the difference in the quality of reagents provided by different suppliers. The control of high-quality biologics and the evaluation of binding parameters helps to improve the reproducibility of detection.

Promoting or inhibiting expression levels of specific miRNAs in EVs can improve therapeutic efficiency for specific diseases or specific repair tissues. The culture conditions and external stimuli of stem cells can alter their EV yield and content composition. While the EV components cannot be fully controlled in gene-manipulated cells, currently, breakthroughs have been made in the use of EVs as a carrier for the better delivery of specified molecules, including passive loading (*e.g.*, incubation stimulation) or active loading (*e.g.*, extrusion, electroporation, hypotonic dialysis, sonication, saponin permeabilization, and transfection) [128]. The miRNA enrichment techniques can be achieved by constructing overexpressed cell lines or direct loading miRNAs into EVs by physical or chemical methods. Due to the complex EV loading mechanisms involving the endosomal sorting complex required for transport (ESCRT)/Rab protein family, multivesicular bodies, intracellular tubules, and actin networks, the generated EVs loaded with specific miRNA molecules by transfection of parental stem cells are unreliable and unpredictable. In addition to cell transfection, direct delivery of desired miRNAs into EVs is an efficient and feasible method for enriching miRNAs, which can enhance the interaction of miRNAs with the surface of EVs by using calcium chloride ($CaCl_2$) buffered medium and promote the incubation of selected miRNAs into EVs [129]. The heat shock method can alter the fluidity of EV membranes, and promote miRNA entry into EVs [130]. Electroporation is another technology to promote miRNA entry, but electroporation may trigger EV aggregation and change its morphological characteristics, thus affecting the effect. The existing limitations still need to be improved.

Conclusions

Cell senescence is a dynamic process evolving with time, and its specific regulation remains unknown. Analyzing the senescence properties of MSCs is very important for developing methods to assess MSC senescence, as well as for understanding how senescence affects the quality and efficacy of MSCs. A comprehensive analysis of miRNAs provides a more detailed and in-depth insight into how senescence influences MSCs. Advances in understanding the role of miRNAs in aging may provide new ways to alleviate MSC senescence. Undoubtedly, continued in-depth studies of miRNAs within MSC senescence will shed light on their mechanisms of action during senescence and may reveal clues for the potential roles in the extracellular environment.

Senescence may influence the production rate and cargo type of MSCs and their EVs. Systematic analysis and comparison of miRNAs related to MSC senescence and those contained in MSC-EVs will help to discover universal senescence markers to identify senescent cells. Translating preclinical results into the clinic faces different challenges related to EV dynamics and biology. Effective MSC-EV therapy may depend on the physiological function and state of the parental cells, as senescent may deprive cells of reverse/reduce disease efficacy. A correct understanding of the detailed mechanisms involved in miRNAs and EV-miRNAs during senescence may contribute to the regulation of MSC efficacy, as well as the development of MSC-EVs to improve tissue regeneration and aging-related diseases.

Declarations

Authors' contributions: Conceptualization, Liangge He and Guangqian Zhou; Investigation, Arshad Ahmed Padhiar, and Zhen Liu; Writing-Original Draft Preparation, Liangge He and Mingzhu Li; Writing-Review & Editing, Liangge He and Guangqian Zhou; Supervision, Guangqian Zhou; Project Administration, Guangqian Zhou; Funding Acquisition, Guangqian Zhou. All authors were involved in approving the final manuscript.

Availability of data and materials: Not applicable.

Financial support and sponsorship: This work is supported, in part, by the National Natural Science Foundation of China (2072480 and 32100603), and Shenzhen Commission of Development Reform (Funding for Shenzhen Engineering Laboratory for Orthopedic Diseases and Regenerative Technologies).

Conflicts of interest: The authors declare no conflict of interest.

Ethical approval and informed consent statement: Not applicable.

Consent for publication: Not applicable.

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Cite this article as: He LG, Li MZ, Liu Z, Padhiar AA, & Zhou GQ. Senescence of mesenchymal stem cells: implications in extracellular vesicles, miRNAs and their functional and therapeutic potentials. *Aging Pathobiol Ther*, 2023, 5(1): 03-17. doi: 10.31491/APT.2023.03.107

Aging Pathobiology and Therapeutics

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JOURNAL INFORMATION

- Official Publication: Geropathology Research Network, Geriatric Department of Guangzhou First People's Hosipital (CN)

- Funded: APT is funded by ANT Publishing Corporation
- Ownership: APT is owned by Geropathology Research Network and published by ANT Publishing Corporation

JOURNAL STATISTICS

- Launch date: 11 November 2019
- Publication model: Open access
- ISSN: 2690-1803 (Online)
- Digital archive: Portico
- Frequency: Quarterly published
- DOI: 10.31491/APT

SUBMISSION TURNAROUND TIME

- Initial review: 3 days
- External peer review: 2–3 weeks
- Publication ahead of print: within 2 weeks after being accepted
- Formal publication: within 1-2 months after being accepted

INDEXING/ABSTRACTING

- Scopus
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