Mesenchymal Stromal Cell Aging

Mesenchymal stem/stromal cells (MSCs) are a reservoir for tissue homeostasis and repair that age during organismal aging. Beside the fundamental in vivo role of MSCs, they have also emerged in the last years as extremely promising therapeutic agents for a wide variety of clinical conditions. MSC use frequently requires in vitro expansion, thus exposing cells to replicative senescence. Aging of MSCs (both in vivo and in vitro) can affect not only their replicative potential, but also their properties, like immunomodulation and secretory profile, thus possibly compromising their therapeutic effect. It is therefore of critical importance to unveil the underlying mechanisms of MSC senescence and to define shared methods to assess MSC aging status.

1. Introduction

Mesenchymal stem/stromal cells (MSCs) or adult stem cells are tissue-specific pools of progenitor cells with long-term self-renewal ability and differentiation potential, with a fundamental role in tissue and organ homeostasis [1][2][3][4][5][6]. These cells possess both differentiation plasticity (stemness) and tissue supportive functions (stromalness) that can coexist and overlap, with differences depending on tissue source, donor characteristics, culture conditions and delivery strategies, leading to alternative best fittings for the term "stem" or "stromal" [7]. MSCs represent a lifelong reservoir for the generation of somatic cells and for cell replacement. They emerged in the last years as powerful tools in regenerative medicine and tissue engineering, mostly for their easy of isolation and expansion, for their immunomodulatory [8][9][10][11] and paracrine activities, exerted through the release of soluble factors [12], exosomes and microvesicles [13], thus modulating tissue microenvironment, counteracting inflammation and stimulating repair [2][8][14].

During aging of the organism, MSCs also age, and this implies an impairment of stem cell functions contributing to the progressive decrease in tissue maintenance and repair, a characteristic of the aging process. Actually, stem cell exhaustion is considered one of the promoters of aging. One example is the delay in fracture healing with advanced age, attributed to a decreased number and function of MSCs [15].

Due to the low abundance of MSCs in human adult tissues (about $1/10^6$ cells in adult bone marrow and $1/10^{3-4}$ cells in adipose tissue and umbilical cord) [16], frequently ex-vivo expansion precedes therapeutic administration, to obtain a clinically relevant number of cells. However, in vitro expansion drives senescence and reduces DNA synthesis and repair efficiency, finally leading to DNA damage accumulation [2][17][18][19][20][21][22][23][24]. Persistent DNA damage can block transcription and replication and drives the aging process [25].

The most relevant effect of in vitro expansion is progression to replicative senescence [21][22][24] accompanied by morphological and functional alterations, and modification of the secretory phenotype. With aging, MSCs show reduced immunomodulation [26][27], proliferation, differentiation potential and homing ability [28][29][30][31]. They develop the so-called senescence-associated secretory phenotype, SASP [21][24][32][33], and modify their transcriptional profile [34]. All these modifications can impair MSC functional properties and are critical for the efficacy and safety of MSC treatments, since they can cause not only therapeutic failure, but also unwanted effects, such as the exacerbation of the inflammatory response and the tumor-promoting activity mediated by the SASP, thus raising a safety issue [2]. It appears therefore of critical importance to evaluate the impact of senescence on MSC biological properties and to monitor senescence of MSC preparations before application [35].

2. MSC Senescence

MSC senescence represents a relevant driver of organismal aging and contributes to the pathogenesis of age-related diseases. Accordingly, senescent cells accumulate in aged tissues [36]. Key regulators of MSC aging are still incompletely identified and can represent therapeutic targets to counteract age-associated diseases and organismal aging. MSC

senescence can arise from different stimuli, including oxidative stress, irradiation, chemicals (inducing acute senescence) or replicative exhaustion (inducing chronic senescence) [37].

As for other cellular types, aging of MSCs is a dynamic process taking place through a series of steps that follow the initial growth decline [31][38]. It is accompanied by functional alterations due to metabolic, genetic, epigenetic, transcriptional and translational changes [31][39][40][41] (Figure 1).
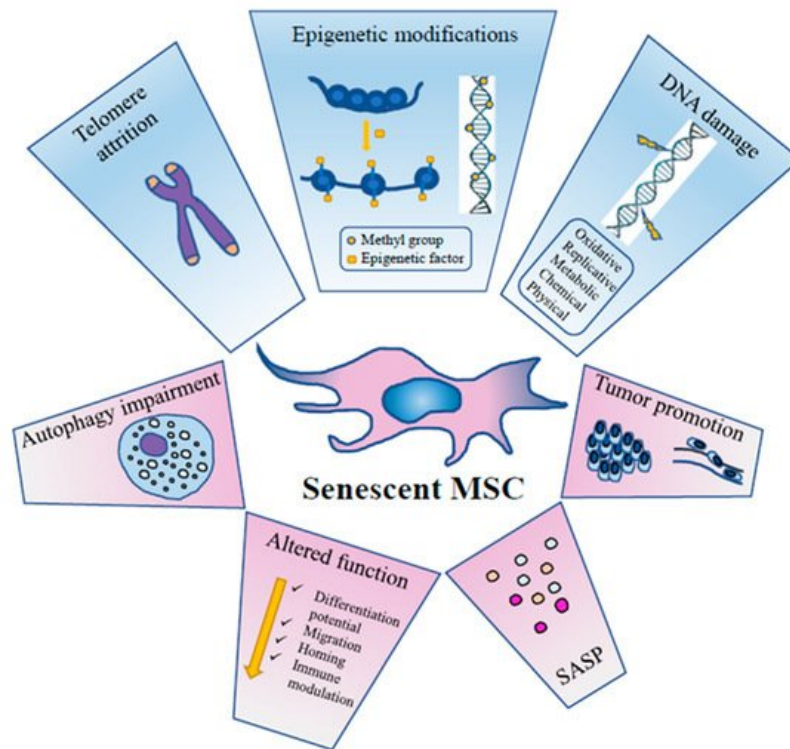

Figure 1. The principal events inducing MSC senescence and their effects on MSC activity, metabolism and function.

Senescent MSCs loose metabolic flexibility, they show an impairment of the autophagic flux with reduced active vacuoles, and an impairment of the ubiquitin-proteasome pathway, the main proteolytic cellular system to degrade damaged proteins [37].

MSC senescence involves multiple signaling pathways such as p53/p21, retinoblastoma (p16/RB), protein kinase B (Akt)/mTOR, mitogen activated protein (p38MAP) kinase and signal transducer and activator of transcription 3 (STAT3), finally causing a permanent cell cycle arrest and irreversible damage [38][42][43][44].

Redox control has a central role in MSC senescence development. At a steady state, MSCs prevalently perform glycolysis and have low ROS levels [45]. However, with progressive replications, a marked production of ROS occurs, inducing damage to cellular macromolecules, with the involvement of several cellular pathways including p53, forkhead box transcription factors of the class O (FOXO1), nuclear factor erythroid 2-related factor (2Nrf2), micro RNAs (miRNAs) and long non-coding RNAs (lncRNAs) (for a comprehensive review, see [46]). Toll-like receptor 3 (TLR3) is upregulated in senescent human umbilical cord MSCs (UC-MSCs) and can drive senescence through the Janus kinase 1 (JAK1) pathway, a key regulator factor in senescent cells. Interestingly, the process is driven by a toll-like receptor 3 (TLR3)-dependent SASP autocrine loop where interferon β (IFN-β) upregulates in turn TLR3 [47]. The conserved small nuclear non-coding RNA Rn7SK is overexpressed in senescent adipose-derived MSCs (ASCs) and, conversely, its transient knockdown leads to delayed senescence [48].

2.1. DNA Damage

DNA damage is one of the key drivers of aging and there is strong evidence that age-related accumulation of DNA damage in MSCs is partly responsible for age-related cellular dysfunctions [23]. Signals generated downstream DNA damage are mostly addressed to counteract transformation but can also promote tissue dysfunction. A variety of mechanisms can contribute to DNA damage accumulation in MSC aging: telomere shortening, replicative stress and reduced DNA repair system efficiency [18][49]. Accumulation of DNA damage can lead to genomic instability and ultimately affect differentiation ability, self-renewal ability or induce cell transformation [2]. DNA lesions trigger ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related protein (ATR) pathways and checkpoint activation, inducing in turn cell cycle arrest (to allow for damage repair) or, conversely, senescence and apoptosis (to avoid replication of

damaged cells in case of too high levels of DNA damage). While the ATM pathway is mostly involved in double strand break (DSB) repair [50], the ATR one is prevalently involved in single strand break (SSB) and replicative stress-derived damage repair [51]. Murine bone marrow MSCs (BM-MSCs) in long-term expansion progressively lose their ability to recognize DSBs (as detected by phosphorylated histone H2AX-γH2AX- and 53BP1 foci); they show a slower repair kinetics and a consequent increased number of residual DSBs, due to an impaired ATM-mediated DNA damage response [49].

2.2. Chromatin Remodelling

One of the characteristics of aged MSCs is a general chromatin reorganization. MSCs have a peculiar chromatin architecture correlated with their uncommitment, multipotency and self-renewal. Heterochromatin domains (chromatin regions structurally inaccessible and usually transcriptionally inactive) are critical for gene expression regulation, DNA repair and maintenance of genomic stability. They are progressively lost during aging, whereas heterochromatin reestablishment promotes longevity [52][53]. Interestingly, DiGeorge syndrome critical region 8 (DGCR8, a critical component of the canonical microprocessor complex for miRNA biogenesis, downregulated in naturally and pathologically aged MSCs) was found to play a role in heterochromatin organization maintenance and aging attenuation, thus emerging as a possible therapeutic target for age-related disorders. This DGCR8 activity is miRNA processing-independent and it is played through interaction with nuclear Lamin B1 and other heterochromatin-associated proteins [54]. A role in heterochromatin reorganization in relationship to MSC aging was also described for lysine-K- specific demethylase 3A (KDM3A) and lysine-K- specific demethylase 4C (KDM4C) enzymes, two H3K9 demethylases. They activate condensing components during MSC senescence and inversely correlated with aging. Their suppression induces a strong DNA damage response and aggravates cellular senescence, whereas their upregulation blocks DNA damage response, thus highlighting a protective role of these enzymes as a surveillance mechanism to limit DNA damage accumulation [55]. As multifunctional modulator of gene expression with the ability to regulate chromatin architecture, methyl-CpG binding protein 2 (MECP2) has a key role in stem cell function and aging. In a mouse model of Rett Syndrome, impaired MECP2 expression is associated to increased senescence and accumulation of DNA damage, through RB2/P130-p16 pathway activation and without impairment of the autophagic process [41].

Deregulation of the chromatin remodeling complex switch/sucrose non-fermentable (SWI/SNF) through Brahma-related gene 1 (BRG1, an ATPase subunit of the complex) can trigger MSC senescence by suppressing homeobox transcription factor (NANOG) transcription and by modulating the expression of proteins involved in nuclear architecture, suggesting a role of BRG1 as a global regulator of gene expression [56].

2.3. Epigenetics

Epigenetics (heritable changes in gene expression without DNA sequence alterations) include DNA methylation, histone modifications, chromatin remodelling, all influencing chromatin architecture and in turn gene expression. Epigenetics has a fundamental role in stem cell biology (reviewed by [57]), including stem cell senescence. Recent evidences highlighted that epigenetic regulation is involved in MSC senescence [2][57][58][59]. A comprehensive evaluation of the dynamic of epigenetic changes and its effects on stemness and senescence is still missing [57]. Lu et al. [60] showed that octamer-binding transcription factor 4 (OCT4) maintains the self-renewal ability of human hair follicle MSCs and reverses senescence by suppressing the expression of p21 through the upregulation of DNMTs (DNA methyltransferases) leading to an overmethylation of p21 promoter and thus inhibiting its transcription and activity. Epigenetic dysregulation of histone H3 acetylation in K9 and K14 in the promoter regions of stemness genes OCT4 and sex determining region Y-box 2 (SOX2) plays a key role in MSC aging [59]. Moreover, inhibition of histone deacetylase (HDAC) function promotes apoptosis and senescence in human MSCs [57]. General DNA methylation levels appear to decrease with MSC aging and this is associated to DNMT downregulation [57]. DNMT1 and 3b inhibition with specific siRNA (short interfering RNA) or 5-azacytidine, induced senescence in UC-MSCs and p16 and p21 upregulation. Interestingly, DNMT inhibition changed histone marks in active form and induced CpG island demethylation in p16 and p21 promoter regions. It also downregulated polycomb proteins and upregulated miRNAs targeting polycomb proteins expression levels [61][62]. All these data point to a critical role of DNMTs and HDACs in regulating MSC senescence. Through molecular profiling, the role of epigenetic factors (including miRNAs) in cellular senescence has been studied [63][64][65][66]. One of the most interesting recent findings is the association of aging to very reproducible DNA methylation patterns. A specific CpG methylation pattern can therefore represent a biomarker to estimate chronological age, thus functioning as an "epigenetic clock" [67]. In a similar way, regulated and reproducible DNA methylation changes occur during in vitro replicative senescence and can be used to monitor senescence of cell preparations [65][66][68]. Another epigenetic modification of DNA bases is represented by 5-hydroxymethylcytosine (5hmC), recently proposed as additional epigenetic age-related biomarker, associated to 5 methylcytosine (5mC) loss [69].

2.4. Autophagy

Recent studies indicated that autophagy is required to maintain stemness and differentiation capacity of stem cells and that autophagy is impaired in stem cell aging (reviewed by [70]). This deficiency can be restored by rapamycin and spermidine treatment [71][72]. Correct functioning of autophagy has also the potential to preserve the anti-inflammatory properties of MSCs, that are mainly exerted through their secretome [73], particularly through the released exosomes and microvesicles [74]. Conversely, evidence is accumulating that vesicles released from MSCs have the ability to rescue autophagy in the target cells [75].

It was demonstrated that autophagy plays a role in the maintenance of BM-MSCs during aging: autophagy declines in BM-MSCs and bone cells with age; its activation could alleviate aging of BM-MSCs and restore aged BM-MSC osteogenic differentiation and proliferation. Autophagy activation also partially restores bone loss in mice, via regulating ROS levels and p53 expression. Conversely, autophagy inhibition could cause senescence of young BM-MSCs [76]. Young quiescent MSCs display a constitutive autophagy activity decreasing with aging. The impairment occurring in old cells is related to their lack in autophagosome formation capacity. Moreover, genetic impairment of autophagy in young MSCs promotes the entry into premature senescence [71].

Available data on the relationship between autophagy and senescence are partly contradictory: actually, autophagy may promote or counteract senescence depending on the cellular context and on the kind of stress [37]. Autophagy can protect BM-MSCs from oxidative stress [77] and, conversely, it has been proven to be a requirement for maintenance of replicative senescence of MSCs [78]. A possible interpretation of these contradictory results is that MSCs try to lead with stress by inducing autophagy to remove damaged components thus counteracting aging; however, in case of impaired autophagic flux and therefore of persistent cell damage, autophagy could worsen senescence.

2.5. SASP

Cell senescence is a dynamic process that can be influenced by cell to cell communication and signals from the microenvironment, including paracrine/autocrine soluble factors, gap junctions, extracellular vesicles (EVs). EVs include exosomes and microvesicles (MVs) transporting lipids, proteins, miRNAs, delivering signals to other cells via endocytosis and membrane fusion. They are fundamental mediators of MSC activity. Recently, MSC-derived EVs gained much attention for MSC-based therapies as responsible for MSC activity and as possible cell-free alternative to MSC cellular treatments [79]. EV content is not static, but highly influenced by the niche/microenvironment the MSCs are imbedded in [13]. In fact, EVs from BM-MSCs of differently aged donors show significant age-dependent differences in their content and consequently immune profile [80]. MVs from young or old MSCs contain youth or senescence signals, respectively. In a murine model, old hematopoietic stem cells (HSCs) showed restored functionality and rejuvenation after intercellular transfer of MVs from young MSCs, containing higher levels of autophagy-related mRNAs and sirtuins [81].

In an attempt to define common and specific SASP components, Ozcan et al. [32] exposed human ASCs and BM-MSCs to different genotoxic stresses (oxidative, chemical, physical and replicative) and analyzed the resulting cell secretome. By mass spectrometry followed by gene ontology analysis they were able to distinguish four SASP classes (extracellular matrix; cytoskeleton; cell junctions, metabolic processes, ox-redox factors; regulators of gene expression) common among the different stressor conditions. They also found 11 proteins exclusively expressed in all conditions possibly representing a senescence signature [32].
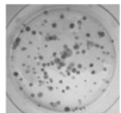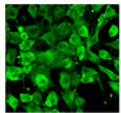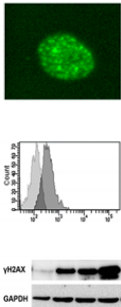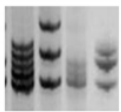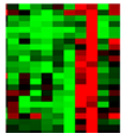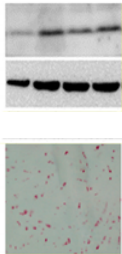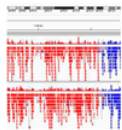
3. Detection of Senescent MSCs

Different molecules and biological processes have been proposed for senescence state determination and monitoring: proliferation arrest, telomere attrition, DNA damage, epigenetic, transcriptional and metabolic changes. Features to identify senescent cells: flattened and enlarged morphology; cell cycle arrest; increased SA-β-gal activity; senescence-associated heterochromatin foci (SAHF, specialized heterochromatin domains contributing to silencing of proliferation-promoting genes); altered gene expression (p53, p21, p16 increased expression); shortened or dysfunctional telomeres; SASP and DNA-scars (DNA segments with chromatin alterations reinforcing senescence) [37].

However, specific and univocal markers are still missing and, to be reliable, the evaluation of cell senescence should rely on the in vitro assessment of several markers. Since senescence can affect cell functions, it appears essential to evaluate the percentage of senescent cells in each MSC preparation to be delivered to patients. The identification of reliable senescence markers is therefore a critical, pivotal issue for quality assessment and for in vitro monitoring of MSC preparations before clinical application. Several papers focused on this topic, as reviewed in [82]. Here we only present a

summary (Table 1) including the most frequently used analyses to assess senescence. For each technique the relevant use, i.e., which senescence feature it addresses, as well as specific advantages and disadvantages, are indicated.

Table 1. Markers of MSC senescence and techniques most frequently used for their detection.

Senescence Marker		Techniques for Detection	Senescent Features, Pros and Cons
Cell morphology		Microscopy [83] Flow cytometry [84] (FSC for size, SSC for granularity)	Senescent MSCs show enlarged and granular cell morphology. Microscopic assessment is easy but only qualitative. Flow cytometric assessment is quantitative
CFU		Colony formation in agar culture [85]	The CFU number is a measure of cell clonogeneity and decreases with MSC age. CFU assessment requires careful plating at low density.
Sa-β-gal		Microscopy (colorimetric activity assay with X-gal) [86] Flow cytometry (fluorimetric activity assay with $C_{12}FDG$) [87] IHC, IF or WB with specific Abs (protein expression) [88]	Senescent cells at low density express a lysosomal β-galactosidase active at pH 6.0, detectable either with activity assays or with a specific antibody. The activity assays can give altered results on high density cultures [89]
8-oxo-dG		IHC, IF, ELISA [90], HPLC [90]-MS/MS	8-oxodG is a DNA base derivative, robust marker of oxidative DNA and RNA damage
γH2AX		IF Flow cytometry [91] WB	Histone H2AX phosphorylation is an indirect measure of DNA double strand breaks due to physical, chemical, oxidative stress. It indicates that cells organize a DNA damage response, but its persistence sustains senescence of the cells.
Telomeres		Southern blotting [92] Flow FISH [93] Real-time PCR [93] STELA [94]	Telomere attrition is directly correlated to replicative senescence, but it also occurs after exposure to oxidative damage. The subpopulation heterogeneity must be taken into account and may be addressed with emerging techniques such as STELA, detecting individual telomere length.
MSI		PCR followed by gel or capillary electrophoresis [95]	Repeated sequences variations are an indirect indication of genomic instability and deficient DNA repair due to replicative or oxidative stress. They increase with cell aging.
Gene expression of senescent markers at mRNA level		Real-time RT-PCR [33] Microarray RNAseq	Expression of genes related to senescence. Several pathways can be analyzed, but gene expression analysis prevalently focuses on p53 and cyclin dependent kinase inhibitors (p16 and p21)

Senescence Marker	Techniques for Detection	Senescent Features, Pros and Cons
Expression of senescent markers at protein level	WB [96] IHC IF Flow cytometry	Evaluation of the expression levels of proteins related to senescence (p16, p21, p53, etc.)
Global methylation	NGS after bisulfite treatment [97]	Genome wide analysis of methylated cytosines.

FSC: forward scatter; SSC: side scatter; CFU: colony forming unit; X-gal: galactosidase substrate; C_{12}FDG: fluorogenic galactosidase substrate; IHC: immunohistochemistry; IF: immunofluorescence; WB: western blotting; ELISA: enzyme-linked immunosorbent assay; HPLC-MS: high performance liquid chromatography-mass spectrometry; 8-oxo-gG: 8-Dihydro-8-oxo-2'-deoxyguanosine; γH2AX: phosphorylated H2A histone family member X; FISH: fluorescence in situ hybridization; STELA: single telomere length analysis; PCR: polymerase chain reaction; RT-PCR: reverse transcriptase PCR; NGS: next generation sequencing.

Beside the known markers indicated in Table 1, a series of new senescence markers has been proposed. The TRAIL (TNF-related apoptosis-inducing ligand) receptor CD264 was proposed as a marker of BM-MSC cellular age (but not of donor chronological age) since it negatively correlates with proliferation and differentiation potential and parallels p21 expression profile [98].

The angiotensin converting enzyme CD143 was found to be expressed only in adult MSCs [99]. Biran et al. [83] proposed a method to detect senescent cells in tissues by combining flow cytometry and image analysis in order to simultaneously acquire information about beta-galactosidase activity and cellular identity.

Endogenous autofluorescence measured by label-free flow cytometric analysis positively correlates with traditional senescence markers and was proposed as fast and non-invasive tool for real-time quantification of in vitro MSC senescence [100].

F-actin turnover, studied by real-time labelling with a fluorogenic probe, was demonstrated to be age-dependent and to decrease in in vitro aged-MSCs [101].

Exploiting high-throughput screening, Ang et al. [102] identified a senescence-specific fluorescent probe (CyBC9) that accumulates in cell mitochondria, thus allowing for rapid, non-toxic and early detection of senescent MSCs as useful tool to screen clinically intended cell preparations.

For clinical use, potency assays are useful to monitor cell properties predictive of therapeutic efficacy, including evaluation of the effects of aging. An in vitro model of low-density MSC growth was for example recently used to evaluate the effect of age on a series of MSC biophysical properties used as predictors of bioactivity. The findings of the study indicated that MSC age is a predictor of adipogenesis, while cell and nuclear shape are strongly associated to hematopoietic-supportive potency [103].

References

1. Li, C.Y.; Wu, X.Y.; Tong, J.B.; Yang, X.X.; Zhao, J.L.; Zheng, Q.F.; Zhao, G.B.; Ma, Z.J. Comparative analysis of human mesenchymal stem cells from bone marrow and adipose tissue under xeno-free conditions for cell therapy. Stem Cell Res. Ther. 2015, 6, 55.

2. Neri, S. Genetic stability of mesenchymal stromal cells for regenerative medicine applications: A fundamental biosafety aspect. Int J. Mol. Sci. 2019, 20, 2406.

3. Lv, F.J.; Tuan, R.S.; Cheung, K.M.; Leung, V.Y. Concise review: The surface markers and identity of human mesenchymal stem cells. Stem Cells 2014, 32, 1408–1419.

4. Dominici, M.; Le Blanc, K.; Mueller, I.; Slaper-Cortenbach, I.; Marini, F.; Krause, D.; Deans, R.; Keating, A.; Prockop, D.; Horwitz, E. Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. Cytotherapy 2006, 8, 315–317.

5. Graf, T. Covering the stem cell explosion at the 2017 isscr conference in boston. Stem Cell Rep. 2017, 9, 1017–1023.

6. Bianco, P.; Cao, X.; Frenette, P.S.; Mao, J.J.; Robey, P.G.; Simmons, P.J.; Wang, C.Y. The meaning, the sense and the significance: Translating the science of mesenchymal stem cells into medicine. Nat. Med. 2013, 19, 35–42.

7. Mastrolia, I.; Foppiani, E.M.; Murgia, A.; Candini, O.; Samarelli, A.V.; Grisendi, G.; Veronesi, E.; Horwitz, E.M.; Dominici, M. Challenges in clinical development of mesenchymal stromal/stem cells: Concise review. Stem Cells Transl. Med. 2019, 8, 1135–1148.

8. Uccelli, A.; Moretta, L.; Pistoia, V. Mesenchymal stem cells in health and disease. Nat. Rev. Immunol. 2008, 8, 726–736.

9. Moretta, L.; Uccelli, A.; Pistoia, V. Mesenchymal stromal cells and immunity: Introductory overview. Immunol. Lett. 2015, 168, 127–128.

10. English, K. Mechanisms of mesenchymal stromal cell immunomodulation. Immunol. Cell Biol. 2013, 91, 19–26.

11. Zhao, K.; Lou, R.; Huang, F.; Peng, Y.; Jiang, Z.; Huang, K.; Wu, X.; Zhang, Y.; Fan, Z.; Zhou, H.; et al. Immunomodulation effects of mesenchymal stromal cells on acute graft-versus-host disease after hematopoietic stem cell transplantation. Biol. Blood Marrow Transplant. 2015, 21, 97–104.

12. Kalinina, N.; Kharlampieva, D.; Loguinova, M.; Butenko, I.; Pobeguts, O.; Efimenko, A.; Ageeva, L.; Sharonov, G.; Ischenko, D.; Alekseev, D.; et al. Characterization of secretomes provides evidence for adipose-derived mesenchymal stromal cells subtypes. Stem Cell Res. Ther. 2015, 6, 221.

13. Phinney, D.G.; Pittenger, M.F. Concise review: Msc-derived exosomes for cell-free therapy. Stem Cells 2017, 35, 851–858.

14. Mun, C.H.; Kang, M.I.; Shin, Y.D.; Kim, Y.; Park, Y.B. The expression of immunomodulation-related cytokines and genes of adipose- and bone marrow-derived human mesenchymal stromal cells from early to late passages. Tissue Eng. Regen. Med. 2018, 15, 771–779.

15. Wagner, D.R.; Karnik, S.; Gunderson, Z.J.; Nielsen, J.J.; Fennimore, A.; Promer, H.J.; Lowery, J.W.; Loghmani, M.T.; Low, P.S.; McKinley, T.O.; et al. Dysfunctional stem and progenitor cells impair fracture healing with age. World J. Stem Cells 2019, 11, 281–296.

16. Oberbauer, E.; Steffenhagen, C.; Wurzer, C.; Gabriel, C.; Redl, H.; Wolbank, S. Enzymatic and non-enzymatic isolation systems for adipose tissue-derived cells: Current state of the art. Cell Regen. 2015, 4, 7.

17. Neri, S.; Bourin, P.; Peyrafitte, J.A.; Cattini, L.; Facchini, A.; Mariani, E. Human adipose stromal cells (asc) for the regeneration of injured cartilage display genetic stability after in vitro culture expansion. PLoS ONE 2013, 8, e77895.

18. Neri, S.; Cattini, L.; Facchini, A.; Pawelec, G.; Mariani, E. Microsatellite instability in in vitro ageing of t lymphocyte clones. Exp. Gerontol. 2004, 39, 499–505.

19. Neri, S.; Pawelec, G.; Facchini, A.; Ferrari, C.; Mariani, E. Altered expression of mismatch repair proteins associated with acquisition of microsatellite instability in a clonal model of human t lymphocyte aging. Rejuvenation Res. 2008, 11, 565–572.

20. Neri, S.; Pawelec, G.; Facchini, A.; Mariani, E. Microsatellite instability and compromised mismatch repair gene expression during in vitro passaging of monoclonal human t lymphocytes. Rejuvenation Res. 2007, 10, 145–156.

21. Shibata, K.R.; Aoyama, T.; Shima, Y.; Fukiage, K.; Otsuka, S.; Furu, M.; Kohno, Y.; Ito, K.; Fujibayashi, S.; Neo, M.; et al. Expression of the p16ink4a gene is associated closely with senescence of human mesenchymal stem cells and is potentially silenced by DNA methylation during in vitro expansion. Stem Cells 2007, 25, 2371–2382.

22. Cai, J.; Miao, X.; Li, Y.; Smith, C.; Tsang, K.; Cheng, L.; Wang, Q.F. Whole-genome sequencing identifies genetic variances in culture-expanded human mesenchymal stem cells. Stem Cell Rep. 2014, 3, 227–233.

23. Behrens, A.; van Deursen, J.M.; Rudolph, K.L.; Schumacher, B. Impact of genomic damage and ageing on stem cell function. Nat. Cell Biol. 2014, 16, 201–207.

24. Bonab, M.M.; Alimoghaddam, K.; Talebian, F.; Ghaffari, S.H.; Ghavamzadeh, A.; Nikbin, B. Aging of mesenchymal stem cell in vitro. BMC Cell Biol. 2006, 7, 14.

25. Ou, H.L.; Schumacher, B. DNA damage responses and p53 in the aging process. Blood 2018, 131, 488–495.

26. de Witte, S.F.H.; Lambert, E.E.; Merino, A.; Strini, T.; Douben, H.; O'Flynn, L.; Elliman, S.J.; de Klein, A.; Newsome, P.N.; Baan, C.C.; et al. Aging of bone marrow- and umbilical cord-derived mesenchymal stromal cells during expansion. Cytotherapy 2017, 19, 798–807.

27. Loisel, S.; Dulong, J.; Menard, C.; Renoud, M.L.; Meziere, N.; Isabelle, B.; Latour, M.; Bescher, N.; Pedeux, R.; Bertheuil, N.; et al. Brief report: Proteasomal indoleamine 2,3-dioxygenase degradation reduces the immunosuppressive potential of clinical grade-mesenchymal stromal cells undergoing replicative senescence. Stem Cells 2017, 35, 1431–1436.

28. Ganguly, P.; El-Jawhari, J.J.; Giannoudis, P.V.; Burska, A.N.; Ponchel, F.; Jones, E.A. Age-related changes in bone marrow mesenchymal stromal cells: A potential impact on osteoporosis and osteoarthritis development. Cell Transplant. 2017, 26, 1520–1529.

29. Li, Y.; Wu, Q.; Wang, Y.; Li, L.; Bu, H.; Bao, J. Senescence of mesenchymal stem cells (review). Int. J. Mol. Med. 2017, 39, 775–782.

30. Liu, M.; Lei, H.; Dong, P.; Fu, X.; Yang, Z.; Yang, Y.; Ma, J.; Liu, X.; Cao, Y.; Xiao, R. Adipose-derived mesenchymal stem cells from the elderly exhibit decreased migration and differentiation abilities with senescent properties. Cell Transplant. 2017, 26, 1505–1519.

31. Turinetto, V.; Vitale, E.; Giachino, C. Senescence in human mesenchymal stem cells: Functional changes and implications in stem cell-based therapy. Int. J. Mol. Sci. 2016, 17, 1164.

32. Ozcan, S.; Alessio, N.; Acar, M.B.; Mert, E.; Omerli, F.; Peluso, G.; Galderisi, U. Unbiased analysis of senescence associated secretory phenotype (sasp) to identify common components following different genotoxic stresses. Aging 2016, 8, 1316–1329.

33. Cheng, H.; Qiu, L.; Ma, J.; Zhang, H.; Cheng, M.; Li, W.; Zhao, X.; Liu, K. Replicative senescence of human bone marrow and umbilical cord derived mesenchymal stem cells and their differentiation to adipocytes and osteoblasts. Mol. Biol. Rep. 2011, 38, 5161–5168.

34. Mieczkowska, A.; Schumacher, A.; Filipowicz, N.; Wardowska, A.; Zielinski, M.; Madanecki, P.; Nowicka, E.; Langa, P.; Deptula, M.; Zielinski, J.; et al. Immunophenotyping and transcriptional profiling of in vitro cultured human adipose tissue derived stem cells. Sci. Rep. 2018, 8.

35. Szychlinska, M.A.; Stoddart, M.J.; D'Amora, U.; Ambrosio, L.; Alini, M.; Musumeci, G. Mesenchymal stem cell-based cartilage regeneration approach and cell senescence: Can we manipulate cell aging and function? Tissue Eng. Part. B. Rev. 2017, 23, 529–539.

36. Lopez-Otin, C.; Blasco, M.A.; Partridge, L.; Serrano, M.; Kroemer, G. The hallmarks of aging. Cell 2013, 153, 1194–1217.

37. Capasso, S.; Alessio, N.; Squillaro, T.; Di Bernardo, G.; Melone, M.A.; Cipollaro, M.; Peluso, G.; Galderisi, U. Changes in autophagy, proteasome activity and metabolism to determine a specific signature for acute and chronic senescent mesenchymal stromal cells. Oncotarget. 2015, 6, 39457–39468.

38. Yu, K.R.; Kang, K.S. Aging-related genes in mesenchymal stem cells: A mini-review. Gerontology 2013, 59, 557–563.

39. Mani, C.; Reddy, P.H.; Palle, K. DNA repair fidelity in stem cell maintenance, health, and disease. Biochem. Biophys. Acta Mol. Basis Dis. 2019, 1866, 165444.

40. Ren, R.; Ocampo, A.; Liu, G.H.; Izpisua Belmonte, J.C. Regulation of stem cell aging by metabolism and epigenetics. Cell Metab. 2017, 26, 460–474.

41. Squillaro, T.; Alessio, N.; Capasso, S.; Di Bernardo, G.; Melone, M.A.B.; Peluso, G.; Galderisi, U. Senescence phenomena and metabolic alteration in mesenchymal stromal cells from a mouse model of rett syndrome. Int J. Mol. Sci. 2019, 20, 2508.

42. Gharibi, B.; Farzadi, S.; Ghuman, M.; Hughes, F.J. Inhibition of akt/mtor attenuates age-related changes in mesenchymal stem cells. Stem Cells 2014, 32, 2256–2266.

43. Lin, S.P.; Chiu, F.Y.; Wang, Y.; Yen, M.L.; Kao, S.Y.; Hung, S.C. Rb maintains quiescence and prevents premature senescence through upregulation of dnmt1 in mesenchymal stromal cells. Stem Cell Rep. 2014, 3, 975–986.

44. Wong, T.Y.; Solis, M.A.; Chen, Y.H.; Huang, L.L. Molecular mechanism of extrinsic factors affecting anti-aging of stem cells. World J. Stem Cells 2015, 7, 512–520.

45. Sart, S.; Song, L.; Li, Y. Controlling redox status for stem cell survival, expansion, and differentiation. Oxid. Med. Cell Longev. 2015, 2015, 105135.

46. Vono, R.; Jover Garcia, E.; Spinetti, G.; Madeddu, P. Oxidative stress in mesenchymal stem cell senescence: Regulation by coding and noncoding rnas. Antioxid. Redox Signal. 2018, 29, 864–879.

47. Lee, H.J.; Choi, B.; Kim, Y.; Lee, S.E.; Jin, H.J.; Lee, H.S.; Chang, E.J.; Kim, S.W. The upregulation of toll-like receptor 3 via autocrine ifn-beta signaling drives the senescence of human umbilical cord blood-derived mesenchymal stem cells through jak1. Front. Immunol. 2019, 10, 1659.

48. Musavi, M.; Kohram, F.; Abasi, M.; Bolandi, Z.; Ajoudanian, M.; Mohammadi-Yeganeh, S.; Hashemi, S.M.; Sharifi, K.; Fathi, H.R.; Ghanbarian, H. Rn7sk small nuclear rna is involved in cellular senescence. J. Cell Physiol. 2019, 234, 14234–14245.

49. Hladik, D.; Hofig, I.; Oestreicher, U.; Beckers, J.; Matjanovski, M.; Bao, X.; Scherthan, H.; Atkinson, M.J.; Rosemann, M. Long-term culture of mesenchymal stem cells impairs atm-dependent recognition of DNA breaks and increases genetic instability. Stem Cell Res. Ther. 2019, 10, 218.

50. Shiloh, Y.; Ziv, Y. The atm protein kinase: Regulating the cellular response to genotoxic stress, and more. Nat. Rev. Mol. Cell Biol. 2013, 14, 197–210.

51. Cimprich, K.A.; Cortez, D. Atr: An essential regulator of genome integrity. Nat. Rev. Mol. Cell Biol. 2008, 9, 616–627.

52. Li, H.; Ghazanfari, R.; Zacharaki, D.; Lim, H.C.; Scheding, S. Isolation and characterization of primary bone marrow mesenchymal stromal cells. Ann. N. Y. Acad. Sci. 2016, 1370, 109–118.

53. Larson, K.; Yan, S.J.; Tsurumi, A.; Liu, J.; Zhou, J.; Gaur, K.; Guo, D.; Eickbush, T.H.; Li, W.X. Heterochromatin formation promotes longevity and represses ribosomal rna synthesis. PLoS Genet. 2012, 8, e1002473.

54. Deng, L.; Ren, R.; Liu, Z.; Song, M.; Li, J.; Wu, Z.; Ren, X.; Fu, L.; Li, W.; Zhang, W.; et al. Stabilizing heterochromatin by dgcr8 alleviates senescence and osteoarthritis. Nat. Commun. 2019, 10, 1–16.

55. Huang, B.; Wang, B.; Yuk-Wai Lee, W.; Pong, U.K.; Leung, K.T.; Li, X.; Liu, Z.; Chen, R.; Lin, J.C.; Tsang, L.L.; et al. Kdm3a and kdm4c regulate mesenchymal stromal cell senescence and bone aging via condensin-mediated heterochromatin reorganization. iScience 2019, 21, 375–390.

56. Squillaro, T.; Severino, V.; Alessio, N.; Farina, A.; Di Bernardo, G.; Cipollaro, M.; Peluso, G.; Chambery, A.; Galderisi, U. De-regulated expression of the brg1 chromatin remodeling factor in bone marrow mesenchymal stromal cells induces senescence associated with the silencing of nanog and changes in the levels of chromatin proteins. Cell Cycle 2015, 14, 1315–1326.

57. Ozkul, Y.; Galderisi, U. The impact of epigenetics on mesenchymal stem cell biology. J. Cell Physiol. 2016, 231, 2393–2401.

58. Cakouros, D.; Gronthos, S. Epigenetic regulation of bone marrow stem cell aging: Revealing epigenetic signatures associated with hematopoietic and mesenchymal stem cell aging. Aging Dis. 2019, 10, 174–189.

59. Li, Z.; Liu, C.; Xie, Z.; Song, P.; Zhao, R.C.; Guo, L.; Liu, Z.; Wu, Y. Epigenetic dysregulation in mesenchymal stem cell aging and spontaneous differentiation. PLoS ONE 2011, 6, e20526.

60. Lu, Y.; Qu, H.; Qi, D.; Xu, W.; Liu, S.; Jin, X.; Song, P.; Guo, Y.; Jia, Y.; Wang, X.; et al. Oct4 maintains self-renewal and reverses senescence in human hair follicle mesenchymal stem cells through the downregulation of p21 by DNA methyltransferases. Stem Cell Res. Ther. 2019, 10, 28.

61. Li, H.; Liu, P.; Xu, S.; Li, Y.; Dekker, J.D.; Li, B.; Fan, Y.; Zhang, Z.; Hong, Y.; Yang, G.; et al. Foxp1 controls mesenchymal stem cell commitment and senescence during skeletal aging. J. Clin. Invest. 2017, 127, 1241–1253.

62. So, A.Y.; Jung, J.W.; Lee, S.; Kim, H.S.; Kang, K.S. DNA methyltransferase controls stem cell aging by regulating bmi1 and ezh2 through micrornas. PLoS ONE 2011, 6, e19503.

63. Bork, S.; Pfister, S.; Witt, H.; Horn, P.; Korn, B.; Ho, A.D.; Wagner, W. DNA methylation pattern changes upon long-term culture and aging of human mesenchymal stromal cells. Aging Cell 2010, 9, 54–63.

64. Koch, C.M.; Reck, K.; Shao, K.; Lin, Q.; Joussen, S.; Ziegler, P.; Walenda, G.; Drescher, W.; Opalka, B.; May, T.; et al. Pluripotent stem cells escape from senescence-associated DNA methylation changes. Genome Res. 2013, 23, 248–259.

65. Koch, C.M.; Joussen, S.; Schellenberg, A.; Lin, Q.; Zenke, M.; Wagner, W. Monitoring of cellular senescence by DNA-methylation at specific cpg sites. Aging Cell 2012, 11, 366–369.

66. Schellenberg, A.; Lin, Q.; Schuler, H.; Koch, C.M.; Joussen, S.; Denecke, B.; Walenda, G.; Pallua, N.; Suschek, C.V.; Zenke, M.; et al. Replicative senescence of mesenchymal stem cells causes DNA-methylation changes which correlate with repressive histone marks. Aging 2011, 3, 873–888.

67. Wagner, W. Epigenetic aging clocks in mice and men. Genome Biol. 2017, 18, 107.

68. Bentivegna, A.; Miloso, M.; Riva, G.; Foudah, D.; Butta, V.; Dalpra, L.; Tredici, G. DNA methylation changes during in vitro propagation of human mesenchymal stem cells: Implications for their genomic stability? Stem Cells Int. 2013, 2013, 192425.

69. Torano, E.G.; Bayon, G.F.; Del Real, A.; Sierra, M.I.; Garcia, M.G.; Carella, A.; Belmonte, T.; Urdinguio, R.G.; Cubillo, I.; Garcia-Castro, J.; et al. Age-associated hydroxymethylation in human bone-marrow mesenchymal stem cells. J. Transl. Med. 2016, 14, 207.

70. Revuelta, M.; Matheu, A. Autophagy in stem cell aging. Aging Cell 2017, 16, 912–915.

71. Garcia-Prat, L.; Martinez-Vicente, M.; Perdiguero, E.; Ortet, L.; Rodriguez-Ubreva, J.; Rebollo, E.; Ruiz-Bonilla, V.; Gutarra, S.; Ballestar, E.; Serrano, A.L.; et al. Autophagy maintains stemness by preventing senescence. Nature 2016, 529, 37–42.

72. Ho, T.T.; Warr, M.R.; Adelman, E.R.; Lansinger, O.M.; Flach, J.; Verovskaya, E.V.; Figueroa, M.E.; Passegue, E. Autophagy maintains the metabolism and function of young and old stem cells. Nature 2017, 543, 205–210.

73. Xu, J.; Camfield, R.; Gorski, S.M. The interplay between exosomes and autophagy—Partners in crime. J. Cell Sci. 2018, 131, jcs215210.

74. D'Arrigo, D.; Roffi, A.; Cucchiarini, M.; Moretti, M.; Candrian, C.; Filardo, G. Secretome and extracellular vesicles as new biological therapies for knee osteoarthritis: A systematic review. J. Clin. Med. 2019, 8, 1867.

75. Liu, L.; Jin, X.; Hu, C.F.; Li, R.; Zhou, Z.; Shen, C.X. Exosomes derived from mesenchymal stem cells rescue myocardial ischaemia/reperfusion injury by inducing cardiomyocyte autophagy via ampk and akt pathways. Cell Physiol. Biochem. 2017, 43, 52–68.

76. Ma, Y.; Qi, M.; An, Y.; Zhang, L.; Yang, R.; Doro, D.H.; Liu, W.; Jin, Y. Autophagy controls mesenchymal stem cell properties and senescence during bone aging. Aging Cell 2018, 17, e12709.

77. Song, C.; Tong, F. Autophagy induction is a survival response against oxidative stress in bone marrow-derived mesenchymal stromal cells. Cytotherapy 2014, 16, 1361–1370.

78. Zheng, Y.; Hu, C.J.; Zhuo, R.H.; Lei, Y.S.; Han, N.N.; He, L. Inhibition of autophagy alleviates the senescent state of rat mesenchymal stem cells during long-term culture. Mol. Med. Rep. 2014, 10, 3003–3008.

79. Najar, M.; Bouhtit, F.; Melki, R.; Afif, H.; Hamal, A.; Fahmi, H.; Merimi, M.; Lagneaux, L. Mesenchymal stromal cell-based therapy: New perspectives and challenges. J. Clin. Med. 2019, 8, 626.

80. Fafian-Labora, J.; Lesende-Rodriguez, I.; Fernandez-Pernas, P.; Sangiao-Alvarellos, S.; Monserrat, L.; Arntz, O.J.; van de Loo, F.J.; Mateos, J.; Arufe, M.C. Effect of age on pro-inflammatory mirnas contained in mesenchymal stem cell-derived extracellular vesicles. Sci. Rep. 2017, 7, 1–12.

81. Kulkarni, R.; Bajaj, M.; Ghode, S.; Jalnapurkar, S.; Limaye, L.; Kale, V.P. Intercellular transfer of microvesicles from young mesenchymal stromal cells rejuvenates aged murine hematopoietic stem cells. Stem Cells 2018, 36, 420–433.

82. Zhai, W.; Yong, D.; El-Jawhari, J.J.; Cuthbert, R.; McGonagle, D.; Win Naing, M.; Jones, E. Identification of senescent cells in multipotent mesenchymal stromal cell cultures: Current methods and future directions. Cytotherapy 2019, 21, 803–819.

83. Biran, A.; Zada, L.; Abou Karam, P.; Vadai, E.; Roitman, L.; Ovadya, Y.; Porat, Z.; Krizhanovsky, V. Quantitative identification of senescent cells in aging and disease. Aging Cell 2017, 16, 661–671.

84. Guidotti, S.; Minguzzi, M.; Platano, D.; Cattini, L.; Trisolino, G.; Mariani, E.; Borzi, R.M. Lithium chloride dependent glycogen synthase kinase 3 inactivation links oxidative DNA damage, hypertrophy and senescence in human articular chondrocytes and reproduces chondrocyte phenotype of obese osteoarthritis patients. PLoS ONE 2015, 10, e0143865.

85. Friedenstein, A.J.; Chailakhjan, R.K.; Lalykina, K.S. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet. 1970, 3, 393–403.

86. Dimri, G.P.; Lee, X.; Basile, G.; Acosta, M.; Scott, G.; Roskelley, C.; Medrano, E.E.; Linskens, M.; Rubelj, I.; Pereira-Smith, O.; et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc. Natl. Acad. Sci. USA 1995, 92, 9363–9367.

87. Noppe, G.; Dekker, P.; de Koning-Treurniet, C.; Blom, J.; van Heemst, D.; Dirks, R.W.; Tanke, H.J.; Westendorp, R.G.; Maier, A.B. Rapid flow cytometric method for measuring senescence associated beta-galactosidase activity in human fibroblasts. Cytometry A. 2009, 75, 910–916.

88. Shimada, H.; Sakakima, H.; Tsuchimochi, K.; Matsuda, F.; Komiya, S.; Goldring, M.B.; Ijiri, K. Senescence of chondrocytes in aging articular cartilage: Gadd45beta mediates p21 expression in association with c/ebpbeta in senescence-accelerated mice. Pathol. Res. Pract. 2011, 207, 225–231.

89. Severino, J.; Allen, R.G.; Balin, S.; Balin, A.; Cristofalo, V.J. Is beta-galactosidase staining a marker of senescence in vitro and in vivo? Exp. Cell Res. 2000, 257, 162–171.

90. Wu, L.L.; Chiou, C.C.; Chang, P.Y.; Wu, J.T. Urinary 8-ohdg: A marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics. Clin. Chim. Acta 2004, 339, 1–9.

91. Johansson, P.; Fasth, A.; Ek, T.; Hammarsten, O. Validation of a flow cytometry-based detection of gamma-h2ax, to measure DNA damage for clinical applications. Cytometry B. Clin. Cytom. 2017, 92, 534–540.

92. Baxter, M.A.; Wynn, R.F.; Jowitt, S.N.; Wraith, J.E.; Fairbairn, L.J.; Bellantuono, I. Study of telomere length reveals rapid aging of human marrow stromal cells following in vitro expansion. Stem Cells 2004, 22, 675–682.

93. Ferreira, M.S.V.; Kirschner, M.; Halfmeyer, I.; Estrada, N.; Xicoy, B.; Isfort, S.; Vieri, M.; Zamora, L.; Abels, A.; Bouillon, A.S.; et al. Comparison of flow-fish and mm-qpcr telomere length assessment techniques for the screening of telomeropathies. Ann. N. Y. Acad. Sci. 2019.

94. Serakinci, N.; Cagsin, H.; Mavis, M. Use of u-stela for accurate measurement of extremely short telomeres. Methods Mol. Biol. 2019, 2045, 217–224.

95. Neri, S.; Mariani, E.; Cattini, L.; Facchini, A. Long-term in vitro expansion of osteoarthritic human articular chondrocytes do not alter genetic stability: A microsatellite instability analysis. J. Cell Physiol. 2011, 226, 2579–2585.

96. Lamichane, S.; Baek, S.H.; Kim, Y.J.; Park, J.H.; Dahal Lamichane, B.; Jang, W.B.; Ji, S.; Lee, N.K.; Dehua, L.; Kim, D.Y.; et al. Mhy2233 attenuates replicative cellular senescence in human endothelial progenitor cells via sirt1 signaling. Oxid. Med. Cell Longev. 2019, 2019, 6492029.

97. Zhou, J.; Wu, Y.C.; Xiao, B.J.; Guo, X.D.; Zheng, Q.X.; Wu, B. Age-related changes in the global DNA methylation profile of oligodendrocyte progenitor cells derived from rat spinal cords. Curr. Med. Sci. 2019, 39, 67–74.

98. Madsen, S.D.; Russell, K.C.; Tucker, H.A.; Glowacki, J.; Bunnell, B.A.; O'Connor, K.C. Decoy trail receptor cd264: A cell surface marker of cellular aging for human bone marrow-derived mesenchymal stem cells. Stem Cell Res. Ther. 2017, 8, 201.

99. Amati, E.; Perbellini, O.; Rotta, G.; Bernardi, M.; Chieregato, K.; Sella, S.; Rodeghiero, F.; Ruggeri, M.; Astori, G. High-throughput immunophenotypic characterization of bone marrow- and cord blood-derived mesenchymal stromal cells reveals common and differentially expressed markers: Identification of angiotensin-converting enzyme (cd143) as a marker differentially expressed between adult and perinatal tissue sources. Stem Cell Res. Ther. 2018, 9, 10.

100. Bertolo, A.; Baur, M.; Guerrero, J.; Potzel, T.; Stoyanov, J. Autofluorescence is a reliable in vitro marker of cellular senescence in human mesenchymal stromal cells. Sci. Rep. 2019, 9, 2074.

101. Mishra, P.; Martin, D.C.; Androulakis, I.P.; Moghe, P.V. Fluorescence imaging of actin turnover parses early stem cell lineage divergence and senescence. Sci. Rep. 2019, 9, 1–12.

102. Ang, J.; Lee, Y.A.; Raghothaman, D.; Jayaraman, P.; Teo, K.L.; Khan, F.J.; Reuveny, S.; Chang, Y.T.; Kang, N.Y.; Oh, S. Rapid detection of senescent mesenchymal stromal cells by a fluorescent probe. Biotechnol. J. 2019, 14, e1800691.

103. Khong, D.; Li, M.; Singleton, A.; Chin, L.Y.; Mukundan, S.; Parekkadan, B. Orthogonal potency analysis of mesenchymal stromal cell function during ex vivo expansion. Exp. Cell Res. 2018, 362, 102–110.