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Review

Preventing MSC aging and enhancing immunomodulation: Novel strategies for cell-based therapies

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ABSTRACT

The efficacy of mesenchymal stem cells (MSCs) mediated regenerative therapies has been hindered by the senescence of them during long period cultures. Aged MSCs exhibit altered morphology, decreased stemmas, changed intercellular communication, and poor differentiation ability. Besides in physiological condition, upon transplantation of senescent MSCs, they are capable of activating both the innate and adaptive immune systems, playing a crucial role in preserving tissue homeostasis. Therefore, enhancing immunomodulation properties and preventing aging progress of MSCs to achieve successful future clinical applications seems necessary. This review delves into the current knowledge of the underlying cellular and molecular mechanisms that promote MSCs senescence as well as the developed approaches for reversing or preventing MSCs aging. These include pre-treatment of MSCs with various types of molecules to inhibit aging process and implementation of different types of three-dimensional culture systems. In addition, the recently developed strategies to improve immunomodulatory properties of MSCs have been discussed. By addressing the limitations of aged MSCs and augmenting their immunomodulation, these approaches offer a promising avenue for the future of cell therapy and provide valuable tools for maximizing the effectiveness of MSCs therapy in biomedical applications.

Contents

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1. Introduction

Mesenchymal stem/stromal cells (MSCs), have been widely utilized in the clinical field due to their immunomodulatory potency and capacity for tissue regeneration [1,2]. These highly regenerative stromal cells which are obtainable from ectoderm or mesoderm, have been utilized for the treatment of various diseases [3–5]. These cells play a central role in maintaining tissue integrity, postponing age-related illnesses, and offering potent cellular treatments [5]. MSCs were discovered in 1968 for the first time and have since been extracted from various tissue sources such as bone marrow, the thymus, tooth pulp, peripheral blood, placenta, pancreas, adipose tissue, and endometrium [6]. These cells possess extraordinary properties such as proliferation, multi-potency, immunomodulation, and paracrine effects, making them a great option for use in cell therapy [6,7]. Additionally, application of MSCs do not raise any ethical concerns joined to embryonic stem cells use [8]. However, the MSC therapy have been limited by a few challenges. One of these major obstacles is entering to senescence phenomenon of MSCs at in vitro conditions [9]. The development and culture of MSCs are associated with various senescence-related traits, such as morphological changes, reduced proliferation, and imbalanced biological activity [10]. Studies have shown that both the tissue environment and intrinsic factors contribute to the senescence of MSCs. The MSC niche, which consists of the extracellular matrix, cytokines, and surrounding cells, plays a critical role in maintaining the balance between quiescence, self-renewal, and lineage fate in MSCs [11]. The niche is influenced by the origin-dependent properties of MSCs and significant transcriptional changes associated with senescence have been observed in this environment. Senescent MSCs release senescence associated secretory profile (SASP), which triggers their own signaling pathways and promotes senescence, creating an unfavorable environment [12]. While senescence serves as a defense mechanism, the accumulation of senescent cells can lead to tissue dysfunction through SASP and induce senescence in nearby cells through paracrine mechanisms. In order to maintain the characteristics of MSCs, their favorable niche can be replicated in culture [13]. Aging can hinder MSCs critical activities so addressing the aging process of MSCs has emerged as a significant area of interest, particularly in efforts to preserve their essential immunoregulatory functions [14].

MSCs derived from late passages exhibit increased senescence, along with changes in their immunophenotype and morphology [15]. The two predominant processes associated with aging, immunosenescence and inflammaging, play a crucial role in shaping the phenotypes related to aging and age-associated diseases [14]. Improving their immuonomodulatary features is a vital approach that leads to the increased homing and adhesion of MSCs, and enhanced immune regulatory properties [16,17]. This review sheds light on the immunomodulatory characteristics of MSCs and discusses the approach that have been developed to improve these features. Additionally, considering very close association of the processes which are responsible for MSC senescence and immunomodulatory functions, aging process have been explained. Finally, the approaches that have the potential to reverse or prevent MSCs aging including pre-treatment of MSCs with various types of molecules, and implementation of three-dimensional (3D) culture systems, will also be described.

2. Immunomodulatory properties of MSCs

MSCs are crucial in cell therapy due to their ability to modulate the immune response. They can initiate an immunologic reactions when injury occurs and suppress it when is excessive. MSCs exhibit pro-inflammatory functions under low levels of IFN-γ and TNF-α by stimulating T cells and M1 macrophage proliferation. Conversely, in highly inflammatory environments, they adopt anti-inflammatory roles by producing TGF-b and HGF, along with factors like IDO,

PGE2, and NO that activate regulatory T cells (Tregs). The immunompdulatory properties of MSCs are shown in Fig. 1. Stimulation by IL-6 further enhances Treg activation and influences macrophage polarization, with M2 macrophages promoting immunosuppression by inhibiting effector T cells [18–20]. MSCs with an anti-inflammatory phenotype help restore immune balance by inhibiting T lymphocyte activation and promoting the proliferation and activation of Tregs. They primarily modulate the immune response through direct interactions with various immune cells, including monocytes, neutrophils, macrophages, dendritic cells, mast cells, natural killer (NK) cells, T cells, and B cells. MSCs regulate excessive immune responses by interacting with anti-inflammatory cells like Tregs and M2 macrophages. They produce extracellular vesicles that promote M2 macrophage and Treg proliferation while inhibiting T and B lymphocyte activity, as well as M1 macrophages. This M2 polarization is induced by the transactivation of arginase-1 via STAT3, transported through MSC-released exosomes. Additionally, miR-182 from MSCs aids in converting M1 macrophages to the M2 phenotype [21–23]. MSCs attract neutrophils by secreting IL-8 and macrophage migration inhibitory factor. When activated human neutrophils are incubated in MSC-cultured media, there is a reduction in IL-6 and macrophage inflammatory protein 2 secretion, as well as lower levels of anti-apoptotic proteins Bcl-xL and Mcl-1. MSC-released exosomes enhance the respiratory burst of neutrophils in patients with severe congenital neutropenia. MSCs also inhibit immune responses by modulating antigen presentation in dendritic cells (DCs) and promoting their differentiation into a tolerogenic phenotype through hepatocyte growth factor (HGF) via the HGF/Akt pathway. Additionally, MSCs can suppress mast cell degranulation, which is crucial for managing allergic conditions, as excessive mast cell activation can lead to severe reactions like systemic anaphylaxis [24–26]. NK cells play a vital role in cancer, organ transplants, autoimmune disorders, and other immune-related diseases, making the interaction between MSCs and NK cells important to understand. Research shows that MSCs have a dual effect on NK cell activity, inhibiting cytokine production, proliferation, and cytotoxicity in some contexts while promoting activation in others, depending on the inflammatory environment. MSCs can downregulate activating receptors on NK cells, such as 2B4 (CD244) and NKG2D, which are crucial for their function. Additionally, MSCs influence adaptive immunity by modulating B lymphocyte activation, proliferation, differentiation, and apoptosis, promoting a shift toward B regulatory cells (Bregs) that secrete IL-10 and maintain immune homeostasis. Their ability to inhibit B lymphocyte activity is partly regulated by the suppressor of cytokine signaling [27,28]. MSCs inhibit T lymphocyte proliferation through direct interactions and the release of soluble factors like IFN-γ, indoleamine 2,3-dioxygenase (IDO), and prostaglandin E2 (PGE2). They also promote a shift from proinflammatory T cells to pro-healing Tregs by secreting transforming growth factor-b (TGF-b). MSCs reduce pro-inflammatory molecules (e.g., TNF-α, IL-1, IL-6) while increasing anti-inflammatory mediators like IL-10. Mediators such as hepatocyte growth factor (HGF) and TNF-a-stimulated gene/protein 6 (TSG-6) are linked to treating immune-mediated diseases, with TSG-6 specifically inhibiting neutrophil migration.MSC migration is influenced by several factors, including the SDF-1/CXCR4 axis, osteopontin, and various growth factors. SDF-1 and CXCR4 are key regulators, with enhanced MSC migration improving tissue repair in models like myocardial infarction. Chemokines CCL2/MCP-1 and CCL3/MIP-1a also attract MSCs to injured tissues, and their external introduction boosts MSC homing efficiency. Additionally, complement components and bioactive lipids can enhance the chemotactic properties of CXCL12 [29–31]. In summary, immunomodulation is the key mechanism by which MSCs function, allowing their use in various therapies for tissue and organ regeneration, immune system regulation, and therapeutic agent delivery.

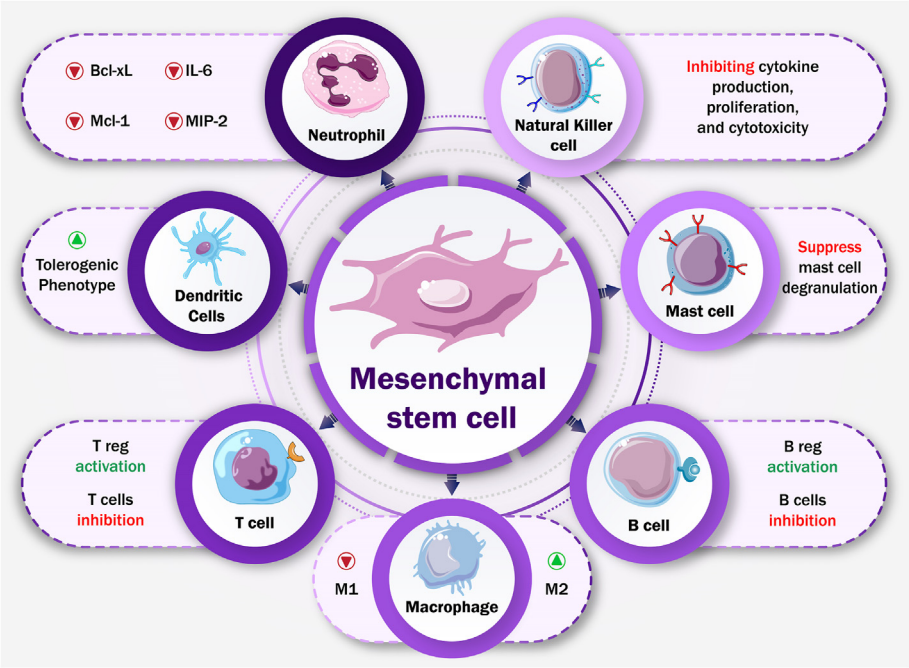

Fig. 1. Immunomodulatory properties of MSCs. MSCs inhibit T and B lymphocyte activation, mast cell degranulation, as well as M1 macrophages, and promote the activation of Tregs, as well as the differentiation of dendritic cells into a tolerogenic phenotype. MSCs attract neutrophils and reduce the levels of IL-6 Bcl-xL, macrophage inflammatory protein 2 secretion (MIP2) and Mcl-1.

2.1. MSC-mediated immunomodulation in clinical studies and trials

The encouraging findings from preclinical studies of MSCs have led to many efforts to implement them in clinical trials. MSC therapy was first evaluated in humans in 1995, and since then, the number of clinical studies examining its use has grown rapidly. Recent reports show that there are over 1000 registered clinical trials involving MSCs [32]. The trials evaluate MSC therapy for treating autoimmune diseases and infections, including COVID-19, as well as inflammatory and degenerative conditions affecting the heart, liver, lungs, skin, and nervous system. Notably, the outcomes of clinical research on MSCs have been quite promising. For example, a recent systematic review and meta-analysis showed that MSCs could lower mortality rates in patients with severe or critical COVID-19 [33]. It's important to note that many clinical trials have focused on harnessing the immunomodulatory properties of MSCs. In these studies, the immunosuppressive characteristics of MSCs were utilized to treat conditions such as graft-versus-host disease, Crohn's disease, multiple sclerosis, type 1 diabetes, gastrointestinal tumors, osteoarthritis, and atopic dermatitis [34]. Regarding the preconditioning strategies discussed in this paper, it is noteworthy that nearly all of the approaches mentioned in this review have successfully progressed to clinical application [35]. Specifically, strategies such as priming with cytokines and bioactive molecules, altering culture conditions, genetic engineering, and hypoxia preconditioning have been effectively applied in clinical trials, whereas the regulation of autophagy in MSCs is still pending clinical use. For example, in a Phase III trial (NCT01541579, registered on [2012-02-21]), modifying culture conditions, particularly the “rescue” of freshly thawed MSCs, proved effective in boosting their therapeutic potential for complex perianal fistulas in Crohn's disease patients who did not respond to standard medications and biologics [36]. In another study (Phase II, NCT02017912), patients with amyotrophic lateral sclerosis were effectively treated using MSCs that had been primed with different trophic and growth factors [35]. In two additional clinical trials (Phase I/II, NCT01849159 and Phase II, NCT04042844), hypoxia-preconditioned MSCs are being assessed for their effectiveness in treating severe pulmonary emphysema and chronic lumbar disc disease, respectively. In another study (Phase I/II, NCT02068794), genetically engineered MSCs designed to produce sodium iodine symporter are being evaluated for the treatment of cancers affecting the ovaries, fallopian tubes, and peritoneum [37]. Moreover, the function of MSCs in the treatment of autoimmune diseases, particularly T cell disorders was investigated. It reveals that bone marrow MSCs lacking ERK1 do not induce T cell apoptosis because they are unable to trigger the ETS2/AURKA/NF-kB/Fas/MCP-1 signaling pathway, in contrast to those lacking ERK2. Furthermore, ERK1-deficient MSCs do not promote the development of regulatory T cells or suppress T helper 17 cells. The substance licochalcone A (LA), which stimulates the ERK pathway, improves the effectiveness of MSC therapy in models of ulcerative colitis and collagen-induced arthritis. These findings indicate that ERK1 plays a critical role in T cell regulation within MSCs, and LA-treated MSCs may enhance the efficacy of MSC-based treatments for autoimmune diseases [38]. In another research to conclude that In comparison to CD146-MSCs, CD146+MSCs can hasten the resolution of inflammation and exhibit strong anti-inflammatory properties. They achieve this by increasing regulatory T cells, promoting macrophage phagocytosis, enhancing reparative macrophage activity, and secreting higher levels of VEGF, among other mechanisms [39]. A study investigated the therapeutic effects, mechanisms, and immune responses of human MSCs (hMSCs) versus mouse MSCs (mMSCs) in immunocompetent mice suffering from CCl4-induced acute liver failure. mMSCs facilitated the recruitment of F4/80+ hepatic macrophages to the injured liver, enhanced IL-6-dependent hepatocyte proliferation, and lowered levels of the inflammatory cytokine TNF-α. They also mitigated α-SMA$^+$ myofibroblast activation by reducing TGF-β1 levels in the affected tissue. In contrast, hMSCs decreased TNF-α and TGF-β1 by limiting the recruitment of F4/80+ macrophages, which hindered their ability to clear debris and support IL-6-mediated liver regeneration. Furthermore, hMSCs induced a significant antibody response in immunocompetent mice, rendering them unsuitable for long-term MSC studies [40]. A study investigated the immunoregulatory properties of MSCs and their extracellular vesicles (EVs) when stimulated with different cytokines. It found that MSCs treated with IFN-γ, TNF-α, and IL-1β upregulated PD-1 ligands, crucial for their immunomodulatory effects. Primed MSCs and MSC-EVs showed enhanced immunosuppressive effects on activated T cells and promoted regulatory T cell induction in a PD-1-dependent manner, compared to unstimulated MSCs. Additionally, EVs from primed MSCs improved clinical outcomes and increased survival in a graft-versus-host disease model. These effects could be reversed by neutralizing antibodies against PD-L1 and PD-L2. Overall, the study suggests a priming strategy that boosts the immunoregulatory function of MSCs and their EVs, potentially enhancing the effectiveness of MSC-based therapies [41]. Recent research highlights the critical need for precise control over the design of 3D scaffolds at the nanoscale to enhance the effectiveness of regenerative therapies. Unlike 2D environments, engineered 3D structures with specific chemical compositions and finely tuned physical nano-features significantly boost MSC secretion of immunosuppressive factors such as transforming growth factor-β1 (TGF-β1), prostaglandin E2 (PGE2), indoleamine-pyrrole 2,3-dioxygenase (IDO), and interleukin-10 (IL-10), thereby improving cartilage and osteogenic differentiation [42]. A study evaluated the immunomodulatory effects of GMP-compliant clinical-grade MSCs from four sources: bone marrow, adipose tissue, Wharton's Jelly, and decidua tissue, on allogeneic peripheral blood mononuclear cells (PBMCs). The findings revealed that WJ-MSCs were the most effective at inhibiting PBMC proliferation and enhancing the regulatory T cell population, with the highest levels of prostaglandin E2 (PGE-2). In contrast, the DS-MSC group had the highest interleukin-10 (IL-10) secretion and the lowest levels of inflammatory cytokines IL-12 and IL-17. Transcriptome analysis showed WJ-MSCs had the lowest IL-6 expression, while DS-MSCs exhibited the highest levels of immunomodulatory factors like hepatocyte growth factor (HGF) and transforming growth factor-β (TGF-β). Overall, the results indicate that Wharton's Jelly and decidua-derived MSCs have superior immunomodulatory properties due to their paracrine factors [43]. A study examined the immunomodulatory activity of canine Wharton Jelly (WJ)-derived MSCs in comparison to human MSCs from the same tissue. It found that canine MSCs were more prone to in vitro aging than human MSCs. Both types effectively inhibited the activation and proliferation of CD4$^+$ and CD8$^+$ T cells. Treatment with IFNγ increased indoleamine-2,3-dioxygenase 1 (IDO1) production in both species, with further enhancement from poly(I:C), a TLR3 ligand. Unstimulated MSCs from both species released similar levels of TGFβ, but IFNγ significantly increased TGFβ secretion, more so in human cells. Despite some differences, both canine and human MSCs similarly inhibit activated T cell proliferation and enhance anti-inflammatory activity. The findings suggest that MSC transplantation in dogs with immune-mediated diseases could offer valuable insights for human clinical trials [44]. MSCs play a crucial role in cell-based therapies and tissue regeneration because of their effective secretome that supports host cell recruitment and modulates inflammation. Compared to monodisperse cells, MSC spheroids demonstrate improved viability and greater secretion of immunomodulatory cytokines. This study examined how controlled uniaxial cyclic compression affects cytokine secretion

from human MSC spheroids and the impact of mechanical loading on gene expression related to MSC mechano responsiveness. The MSC spheroids were embedded in alginate hydrogels and exposed to three cyclic compressive regimes with different stress levels (5 and 10 kPa) and hold durations (30 and 250 s). The results showed that cytokine and chemokine expression changed depending on the loading regime, with higher stress resulting in more significant alterations. Importantly, only the L10H30 regime led to the polarization of human THP-1 macrophages into an M2 phenotype. Both static and L10H30 conditions supported a robust F-actin structure, whereas L5H30 and L10H250 disrupted it. When the actin cytoskeleton was disrupted using Y-27632, there was a downregulation of YAP-related genes and a general reduction in inflammatory cytokines. These results underscore the vital role of mechano signaling in enhancing the immunomodulatory capabilities of MSC spheroids [45]. Considering the abundance of favorable preclinical results and ongoing clinical studies, it is anticipated that there will be an increase in human trials involving MSCs optimized with growth factors, cytokine pretreatment, hypoxia preconditioning, culture modifications, and genetic engineering. However, regarding autophagy regulation, which is also addressed in this review, there is limited preclinical data available, hindering its progression to clinical research.

2.2. Improving the immunomodulatory function of MSCs

Immunomodulatory features of MSCs make them a promising tool for cell therapy. MSC-mediated immunomodulation is referred as detecting the location of injury and stimulating an immune response, particularly when the response is not very strong or as inhibiting the immune response in the damaged site when the immune cells are overactive. This means that MSCs can serve as pro-inflammatory agent or as anti-inflammatory agent based on the level of secreted factors in inflammation site [18,46]. To date several approaches have been utilized to improve the potential of MSCs including pretreatment with cytokines, pretreatment with immune receptor agonists, improving culture condition, hypoxia preconditioning, and autophagy regulation (Fig. 2).

2.2.1. Pretreatment of MSCs with cytokines

Preconditioning MSCs with cytokines has been shown to enhance the therapeutic capabilities of MSCs by influencing their ability to decrease the immune response, which includes increasing the production of anti-inflammatory chemicals and improving their ability to migrate to the site of damage [47,48]. INF-γ is one of these cytokines that have been confirmed to increase the expression of indoleamine 2, 3-dioxygenase (IDO) by stimulating JAK signal transducer and STAT1 signaling cascade [49]. IDO is, a soluble factor secreted by MSCs and its enhanced expression is associated with the suppression of T lymphocyte proliferation by MSCs [29]. Moreover, INF-γ has been found to improve the ability of MSCs to reduce tissue fibrosis. Pretreatment of BM-MSCs with INF-γ and administration of these MSCs to the rat models of acute kidney injury and renal fibrosis have reduced the fibrosis by decreasing the infiltration of pro-inflammatory immune cells to the site of injury, and increasing number of immunosuppressive macrophages in the damaged site, leading to decreased inflammation [17]. Preconditioning BM-MSCs with TNF-α, another pro-inflammatory cytokine, before the administration has been reported to decrease IL-4 and TNF-α levels, and to reduce the number of pro-inflammatory immune cells, in the mouse model of allergic conjunctivitis [50]. The pretreatment of MSCs with IL-1β has been reported to improve the immunomodulatory effect of UC-MSCs by stimulating NF-kB, and cyclooxygenase2 (COX2) pathways [51]. Furthermore, the injection of BM-MSCs to the rats with hemorrhagic shock induced tissue injury, resulted in the decreased levels of IL-1α, IL-6, and IL-10 [52].

2.2.2. Pretreatment of MSCs with agonists of immune receptors

Priming MSCs with immune receptor agonists, induce the pro-inflammatory effects of MSCs. Toll-like receptors (TLRs), that recognize pathogen-associated molecular patterns, are expressed in both immune cells and MSCs. TLRs initiate intracellular adaptor molecules activating transcription factors such as NF-kB, MAP kinases and IRF3/7, following pathogen recognition. IRF3/7 in turn, stimulates the secretion of proinflammatory cytokines leading to protection against pathogens [53–55]. MSCs have been primed with agonists of TLR3 and TLR5. Priming MSCs with TLR3 agonist has been found to inhibit the infiltration of immune cells to the injured site, decreased secretion of pro-inflammatory cytokines, and reduced proliferation of T helper lymphocytes in vivo [56,57]. While the pre-treatment of MSCs with TLR5 agonist led to increased expression of IL-10, stimulated Treg proliferation while impeding Th17 differentiation [58].

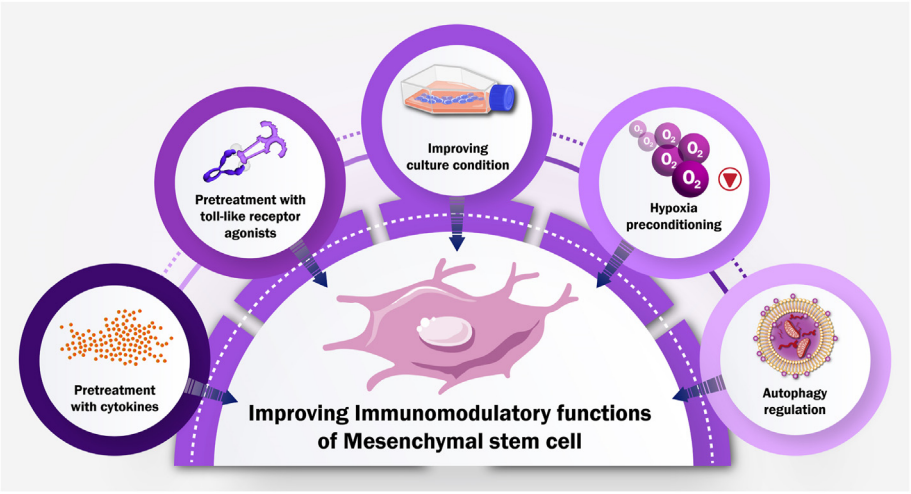

Fig. 2. Strategies to improve immunomodulatory functions of MSs. Pretreatment with cytokines or toll-like receptors, improving culture condition, hypoxia preconditioning, and autophagy regulation.

2.2.3. Improving culture condition

The implementation of a 3D environment in cell culture enhances the long-term preservation of stemness, the capability of MSCs to migrate and home, and their capacity to regulate the immune system. In a research 3D culture led to a significant increase in the expression levels of the pro- and anti-inflammatory genes at the transcriptional level, compared to 2D culture. The researchers also disclosed the significant upregulation of ZC3H12A protein, which encodes RNase and subsequently degrades the mRNAs of IL2, IL6, CXCL1, CXCL2, and CXCL3. The findings of this research suggest a potential novel mechanism for the enhanced anti-inflammatory effects of 3D-cultured MSCs. This mechanism functions at the post-transcriptional level [59]. This is in accordance with another study in which 3D spheroid-cultured MSCs were found with a high survival rate with enhanced expression of Tumor necrosis factor-inducible gene 6 protein (TSG-6), matrix metalloproteinase 2(MMP-2), and Vascular endothelial growth factor (VEGF), which are immunomodulatory factors. In addition it was reported that 3D culture increased the homing and adhesion of MSCs [16]. Hydrogel, a biocompatible and biodegradable culture scaffold, is the other type of 3D culture. Studies have indicated that the level of the release of immunosuppressive factors by MSCs, can be increased by modifying the stiffness of hydrogel in which MSCs are cultured. The expression levels of anti-inflammatory factors including COX2, TSG6, IDO, prostaglandin E2 (PGE2), as well as immune regulatory factors such as IL6 and CCL2 has been reported to be higher in the softer hydrogel scaffold compared to the stiffer one [60,61].

2.2.4. Hypoxia preconditioning

Hypoxia preconditioning of MSCs have been demonstrated favorable outcomes in enhancing their ability to regulate the immune system. MSCs are often cultured in vitro under normoxic conditions. Nevertheless, the severe lack of oxygen at the location of tissue damage tends to impede the survival of MSCs [62]. Surprisingly when hypoxia exposure is combined with other drugs, it has a magnifying impact on MSCs by improving the systems involved in anti-apoptosis, survival, proliferation, and angiogenesis. Therefore, the presence of IFN-γ during the conditioning of MSCs can enhance the expression pattern of various immunosuppressive and immunomodulatory proteins such as IDO, PD-L1, HLA-E, and HLA-G. At the same time, hypoxia triggers glycolysis in MSCs, resulting in elevated levels of lactate, which can contribute to the inhibition of T cells [63]. In the same way, exposing MSCs to hypoxic condition prior to forming 3D spheroids improves the survival of cells, increases the ability of epithelial cells to promote blood vessel formation, and stimulates the regeneration of bone tissue by activating hypoxia induced factor 1-α (HIF-1α) [64].

2.2.5. Autophagy regulation

Organelles and other molecules are delivered to the lysosome by autophagy, a crucial procedure in cells that breaks them down and recycles them. It possesses homeostatic and cytoprotective effects [65,66]. Multiple studies have indicated that the activation of autophagy has the potential to improve the immunomodulatory characteristics of MSCs, which can be beneficial for treating a range of disorders. A new investigation showed that increasing autophagy in MSCs has positive benefits in a mouse model of inflammatory bowel illness [67]. The investigators conducted experiments to evaluate the immunosuppressive effects of a new autophagy enhancer named PACER. They discovered that this protein plays a critical role in the immunomodulation performed by MSCs. Consequently, the inhibition of the Pacer gene through the use of siRNA led to a reduction in the levels of prostaglandin endoperoxide synthase 2 enzyme (PTGS), which is a key modulator of the immunomodulatory capabilities of MSCs. This, in turn, led to diminished ability of MSCs to limit T cell proliferation in culture. In other words, the overexpression of PACER in MSCs resulted in a decrease in inflammation in the colon of diseased animals. This led to a considerable improvement in the symptoms of colitis. The underlying mechanism was reported to be an increased number of Tregs and suppressed activity of Th1 cells and inflammatory cytokines by PACER. On the other hand, the suppression of autophagy through the use of inhibitors, has been shown to enhance the immunosuppressive abilities of MSCs. Wang et al. conducted an examination that found that autophagy in MSCs is the cause of their reduced ability to suppress the immune response and prevent fibrosis when used in the treatment of liver fibrosis [68]. The authors subsequently suggested that the immunosuppressive and anti-fibrotic characteristics of MSCs may be restored by inhibiting autophagy through the silencing of Becn1. The treatment with Becn1-knockdown MSCs effectively resulted in elevated amounts of PGE2, which is one of the immunosuppressive mediators released by MSCs. Furthermore, the suppression of autophagy in MSCs led to a reduction in the infiltration of CD^{4+} and CD^{8+} T cells in the fibrotic liver. One of the favorable outcomes of silencing Becn1 was a decrease in fibrosis, as seen by a smaller region of fibrotic tissue and lower collagen formation in the liver. Therefore, the results indicate that suppressing autophagy through Becn1 gene knockdown has the ability to improve the immunomodulatory function of MSCs in the therapy of hepatic fibrosis.

3. MSCs aging mechanisms

There are three stages to the intricate process of aging: molecular, cellular, and systemic [69].

It involves loss of proteostasis, telomere malfunction, DNA damage, epigenetic modifications, and mitochondrial failure at the molecular level (Fig. 3). Senescence, stem cell loss, and alterations in intercellular communication are the cellular hallmarks of aging. It affects metabolism and nutrition sensing on a systemic level. Obtaining an understanding of these factors is essential to become scientifically master with the complex molecular signaling networks involved in aging. The interaction of these features leads to the functional deterioration that comes with aging. An update on MSCs aging-causing alterations will be discussed in the sections that follow.

3.1. DNA damage

DNA damage is one of the main reasons why MSCs experience cellular senescence [70]. Over time, MSCs may experience DNA damage due to telomere attrition and replication mistakes.

Somatic mutations, chromosomal aneuploidy, copy mutations, endogenous and exogenous mutagens, among other things, can all be responsible for DNA damage [71]. One of the features of MSCs senescence, cell cycle arrest, is primarily regulated by the p21CIP1/WAF1 and p16INK4A signaling pathways; the DNA damage response (DDR) network is believed to be a major regulator of this process [72,73]. Retinoblastoma protein (RB) is less phosphorylated when p21CIP1/WAF1 is active, allowing RB to keep blocking the E2 transcription factor (E2F) [74,75]. This significant gene regulator affects the control and multiplication of cell growth. In addition, by suppressing CDK4 and CDK9 and keeping pRB phosphorylated, p16INK4A can also result in cellular growth arrest [72,76]. It's crucial to know that p21CIP1/WAF1 and p16INK4A may be engaged without the DDR, and those brief stimuli having the potential to cause the DDR can result in DNA repair activities rather than senescence by activating the p53 pathway [77]. In addition, MSCs are more resistant to genotoxic damage than cycling cells [78]. The activation of p53, which is controlled by ATM and ATR, two crucial kinases in the DDR signaling network that recognizes and reacts to DNA damage, is connected to this resistance [73].

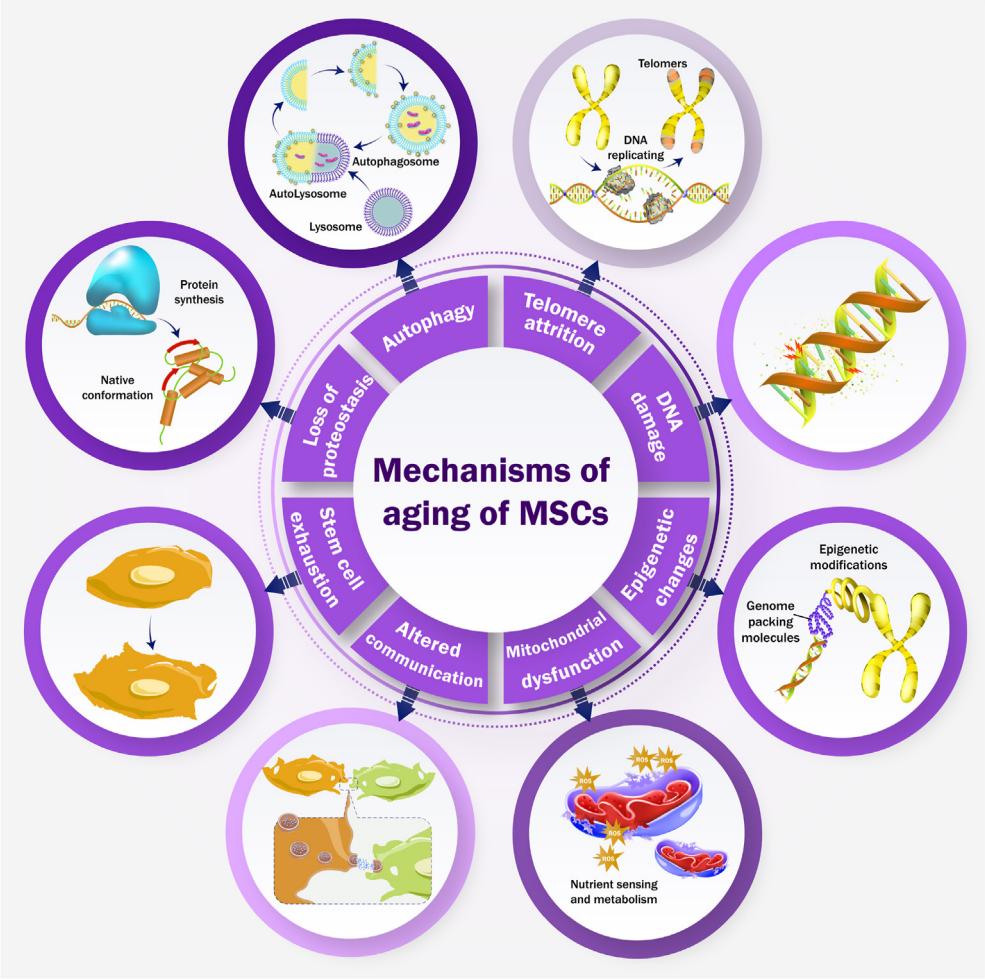

Fig. 3. Mechanisms of aging of MSCs. Telomere attrition; DNA damage; Epigenetic changes; Mitochondrial dysfunction; Altered communication; Stem cell exhaustion; Loss of proteostasis; Autophagy.

3.2. Telomere changes

One of the most important processes of cellular senescence is decrease of telomere length.

The telomere, a nucleoprotein structure located at the terminal end of linear chromosomes, is crucial for controlling cell division and preserving chromosomal integrity [79]. MSCs telomeres in the time of proliferations gradually shorten [80]. MSC senescence a result of chromosomal instability become decreased by severely short telomeres [81]. Telomerase catalysis maintaining telomere length by continuously re-introducing deleted 5'-TTAGGG sequences, which were proved to interact with the TGF-β1 and p53 signaling pathways [82]. It has recently been determined that telomerase and sirtuin 1 (SIRT1) are closely related. Cellular senescence is caused by week telomerase function, which is the outcome of blocking SIRT1 activity in MSCs [83]. On the other hand, increasing SIRT1 levels in aged MSCs has demonstrated the ability to counteract the consequences of senescence. Furthermore, defective telomeres have an impact on SIRT1, and increasing SIRT1's activity may aid in stabilizing telomeres [81]. In addition, hTERT, the human telomerase reverse transcriptase, plays a critical role in controlling cellular senescence. Its overexpression causes telomerase activity, which lengthens telomeres and gives MSCs an enhanced ability to withstand with oxidative stress [84,85].

3.3. Epigenetic changes

Main epigenetic alterations that can adjust genetic expression without shifting the sequence are histone modification and DNA methylation. Age-related disorders and premature aging may be move to higher prevalence by these epigenome alterations. These alterations are vital to aging and the body's reaction to outside stimuli. Age-related modifications to chromatin's state and regulation include promoting extra transcription levels and chromatin accessibility by RNA polymerase. Jin et al. find that monocyte chemoattractant protein-1 (MCP-1) activates C–C chemokine receptor type 2 (CCR2) in aged human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs), driving MSCs to senescence via ROS-p38-MAPK-p53/p21 signaling pathways. Diminishes in BMI1 with age lead to modifications in the epigenetic state of MCP-1, which in turn induce MCP-1 suppression and the loss of monoubiquitinated histone H2A lysine 119 (H2AK119ub) [86]. SASP and epigenetic control must be understood in order to maintain a stable senescent phenotype. Telomerase length affects chromatin alterations in MSCs, which can disturb how environmental and biophysical variables affect MSC aging [87]. Cell cycle arrest and MSCs death can result from telomere shortening that occurs during cell division. Cellular senescence takes place when telomere length reaches a crucial value [88]. Samuelraj et al. have investigated the

telomere length of human bone marrow MSCsHBM at seven separate passages from p1 to p15 and found that telomerase is shorter than its longer counterpart when it divides more frequently [88]. Similar changes have been seen in human bone marrow mesenchymal stem cells (BM-MSCs) and adipose tissue-derived mesenchymal stem cells (AD-MSCs) from older individuals [89]. Overexpression of telomerase reverse transcriptase TERT is stimulating proliferation and reducing senescence in MSCs, while downregulation of telomerase reverse transcriptase TERT is causing senescence and mortality [90].MSCs senescence can be avoided by carefully controlling telomere length and telomerase activity.

3.4. Mitochondrial dysfunction

Dysfunction of mitochondria has a major influence on the cell's senescence. Because mitochondrial DNA (mtDNA) has less efficient repair mechanism than nuclear DNA, higher concentrations of reactive oxygen species ROS can damage mtDNA more than nuclear DNA [91]. Denham Harman's free radical hypothesis of aging postulates that aging-related anomalies in mtDNA might cause a rise in ROS expression, which in turn can upset the balance between ROS and antioxidants [92]. Mitochondria are essential to the cell's regeneration and energy production. Abnormalities in the respiratory chain, such as an imbalance between ATP + ADP and NAD + NADH ratios that are associated with MSC senescence, have been shown to be caused by malfunctioning mitochondria [93]. If the ratio of ATP to ADP is out of balance, AMPK activation can result in senescens through p53 and p21 CIPWAF1 pathway. Moreover, the AMPK-induced p53 pathway has been used to connect an aberrant NAD+/NADH ratio to mitochondrial dysfunction-associated senescence (MiDAS) [94]. Although the precise biological role of MiDAS is yet unknown, it is identified that by eliminating defective mitochondria, mitophagy regulates mitochondrial quality control [94,95]. In adequate mitophagy may be a factor in senescence-related cellular damage. In the same way, senescence can result from blocking mitophagy, which permits to collect up damaged mitochondria [96]. Senescence impairs the p53-mediated translocation of PARKIN into the injured mitochondria, which results in poor mitophagy. Inadequate mitophagy may be a factor in senescence-related cellular damage. Thus, p53 inhibition can enhance the function of mitophagy [97]. Cell senescence is also caused by ROS buildup and SIRT deficiency, which impede PARKIN-mediated mitophagy (Parkin-dependent mitophagy includes the creation of linkage-specific polyubiquitin chains). An imbalance between fusion and fission events is another component that leads to mitochondrial malfunction associated with senescence [98]. Under normal circumstances, mitochondrial fission aids in the removal of damaged mitochondria. On the other hand, pro-fusion states may inhibit efficient mitophagy during senescence [99]. Fission is the process by which different stimuli or stressors cause mitochondria to split, producing tiny, spherical mitochondria that help remove damaged ones. In addition, fusion is preferred by mitochondria throughout the senescence process, resulting in an extensive and extended network of fused mitochondria that hinders the efficient execution of mitophagy [100]. Furthermore, molecules implicated in the fission process have been recognized to take a part to less fissions. These molecules include mitochondrial fission protein 1, dynamin-related protein 1, PTEN-induced putative kinase 1, and phosphoglycerate mutase 5 [101]. This exposes them as plausible subjects for the advancement of therapeutic methodologies intended to tackle the process of aging.

3.5. Loss of proteostasis

Protein synthesis, folding, trafficking, aggregation, disaggregation, and configuration are all regulated by a complex system of interconnected processes known as proteostasis. Stress from the environment or genetically misfolded proteins might disrupt this delicate balance, leading to an inequality of folding capacity, aggregation, and degradation [102]. Consequently, molecular interventions are required to promote proteostasis, including regulators that can increase its efficiency or therapeutic chaperones [102,103]. These therapies may be used in novel ways to treat various proteinopathies and disorders associated with aging. For instance, through its target, eukaryotic translation initiation factor 4E-binding protein (4E-BP), FOXO contributes to transcription in cells [104]. This protein helps break down damaged proteins that build up and interfere with proteostasis. This thereby lengthens life expectancy and lessens the signs of aging [103]. Furthermore, the proteasome is a crucial component of MSCs that maintains proteostasis; its malfunction causes undesirable biological alterations, such as MSC senescence. Kapetanou and colleagues found that the proteasome is intimately associated with the immunomodulatory function of MSCs and their aging, even if the role of the proteasome in the self-renewal ability and potency of MSCs is still not entirely understood. This group of researchers discovered that MSCs produced from aged human adipose tissues and Wharton's jelly have reduced proteasome activity [105]. Additional research has verified that the proteasome β-2/5 promoter region may exhibit elevated expression levels subsequent to its connection via oct4, potentially offering a unique approach to augment the stemness and longevity of MSCs [106]. As well, it has been suggested that the control of MSCs' stemness may be significantly impacted by the proteostasis of transcription factor 7-like 2 (TCF7L2) [107].

3.6. Autophagy

Autophagy is a very precise mechanism of degradation that helps cells retain their homeostasis by removing macromolecules and breaking up the malfunctioning organelles such as mitochondria and lysosomes. Autophagy may be caused by a number of stress signals, such as oxidative stress, starvation, hypoglycaemia, inflammation or infection [108]. The importance of autophagy for maintaining homeostasis in the MSCs is due to its control over protein and cellular quality. Aging causes a decrease in this quality control [109]. Senescence of MSCs is therefore brought on by the suppression of autophagy. For example, the autophagy inhibitor 3-MA causes immature BM-MSCs to undergo senescence [110]. Through regulating p53, optineurin, and rapamycin, autophagy controls MSC senescence. The duality of autophagy regulation accounts for the conflicting impacts on senescence. Optineurin (OPTN) links bone metabolism with autophagy. According to Liu et al.'s work, OPTN expression is downregulated in aged mice's BM-MSCs. This causes selective autophagy, which results in bone loss [111]. At passage 6, p53 expression was elevated in more than 85 % of BM-MSCs. Aged BM-MSCs showed higher expression of autophagy-related genes (LC3 and atg12) while down-regulating the mammalian target of rapamycin (mTOR) in comparison to p2 BM-MSCs [112]. One important modulator of senescence in MSCs is GATA Binding Protein 4 [113]. Senescence regulator GATA Binding Protein 4 GATA4 degradation, MSCs senescence, SASP, and selective autophagy are all caused by GATA4-induced transcription factors that activate NF-κB and MCP-1. GATA Binding Protein 4 (GATA4) is an essential regulator of cellular senescence in the aging process of MSCs. The transcription factors NF-κB and MCP-1 are activated by GATA4. Consequently, this triggers the start of SASP and causes MSC senescence. Through its facilitation of the breakdown of the senescence regulator GATA4, this pathway also promotes selective autophagy and prevents senescence [114]. Autophagy plays a crucial role in preserving MSCs during MSC senescence by breaking down large aggregates and damaged organelles. Both autophagic

activity and senescence are diminished by p53 knockdown. Furthermore, MSCs that reach a replicative senescence state exhibit increased autophagy, and p53 is a critical regulator of autophagy during the aging process [115].

3.7. Stem cell exhaustion

Stem cells are vital for regenerative therapy because of their ability to differentiate and self-renew. It has been demonstrated by several investigations that aged MSCs have reduced autophagy. Thus, autophagy activation can improve osteogenic activity and reverse aging in BM-MSCs [116,117]. According to research by Theodore T. Ho et al. both symmetric and asymmetric division events affect the stem cell pool's growth [117]. Cellular senescence can be the ultimate consequence of improper regulation of these division patterns, which can also lead to aberrant development problems or the depletion of the stem cell pool. Reduction or loss of cell polarity proteins may be associated with disorders connected to aging. Cell polarity proteins are thought to be important in regulating asymmetric cell division [118]. Senescence, for instance, has been demonstrated to adversely affect $CD8^+$ T cell asymmetric division in mice, which may have an adverse influence on T-cell lifespan and function [119]. It has been discovered, nonetheless, that mTOR inhibition can undo this impact. Furthermore, it has been determined that an organism's aging stem cell potential is influenced by cell size. It has been shown that when human and mouse stem cells initiate aging, their sizes grow. This leads to changes in metabolism, a decline in proliferation, and eventually a reduction in stem cell activity [120].

3.8. Altered intercellular communication

The exchange of information between cells occurs through the production of soluble substances that affect nearby cells. SASP causes persistent inflammation and passive senescence in healthy cells by adversely affecting surrounding cells, extracellular matrix, and structural elements in the tissue microenvironment. All cells release lipid membrane vesicles called extracellular vesicles (EVs), which act as intermediaries for intercellular communication [121]. Mesenchymal cells enhance protein translation, cell proliferation, and differentiation in granulocyte-macrophage progenitors by transferring processed tRNAs to hematopoietic progenitors via EVs [122]. An organism's status can be altered via stress-regulated communication in response to obstacles like infection [123]. Senescent cells use proximal secretory pathways or cell-to-cell communication to exchange information with nearby cells. Senescence in primary and other cells is brought on by a form of intercellular communication called cell-cell fusion [124]. Translocation of biological components like as RNA, proteins, and organelles is facilitated via cytoplasmic bridges. Senescent cells' mitochondria are communicative, utilizing tunneling nanotubes or intercellular bridges to spread to other cells in response to cues from the mTOR pathway [125]. Around 17 organs and 10 age groups of Mus musculus were subjected to bulk RNA sequencing and plasma proteomics. The study demonstrated the association between alterations in tissue gene expression and plasma protein levels, which affect systemic circulation aging. The findings pointed to an aging process that is both asynchronous and parallel within and across organs [126].

3.9. MicroRNAs in cellular senescence

Both cell cycle arrest and cell death are avoided by cellular senescence. There is evidence that it accelerates stem cell aging [127]. Age-related accumulation of the cellular senescence marker p16INK4A occurs in some stem cells; hence, blocking P16INK4A can help preserve the functionality of these aged stem cells [128]. The actions of adhacent stem cells can also be regulated by aging cells by secreting different SASPs. The activation of specific transcription factors controls the expression of SASPs. Through autocrine and paracrine processes, SASPs can disperse senescence to nearby and distant non-senescent cells [129]. As a result, senescence expands to nearby cells, altering the cell microenvironment and resulting in persistent, low-grade chronic inflammation. Cellular senescence is better explained by the paracrine impact than by proliferation arrest alone. Different environment, cell types, and stimuli result in different SASP factors. IL-1, IL-6, and IL-8 are examples of inflammatory interleukins that are among these variables [129,130]. Through inflammasome activation, IL-1 is connected to in vivo paracrine senescence, whereas IL-6 and IL-8 both promote and inhibit cellular senescence in vitro. Senescence in vitro is prevented by depleting IL-6 and IL-8 [127,130]. SASPs aid in the removal of senescent cells, such as NK cells and macrophages. Senescent cell injection on-site shows promise as a therapeutic strategy for tissue regeneration and repair. This is attributed to the significant release of exosomes by senescent cells [131]. Exosomes are small extracellular vesicles that contain particular components such proteins, lipids, DNA, RNA, and microRNAs [132]. In senescent cells, the p53/TSAP6 axis regulates the formation of exosomes and facilitates their movement [131,133]. Senescent cells produce more of these exosomes to defend themselves and get removal of dangerous substances. Senescent cells have different exosome compositions than do normal ones. Through paracrine signaling, these exosomes can affect the environment and are also implicated in SASPs. Exosomes containing different miRNAs, such as the miR-183 cluster and miR31a-5p, are released by MSCs throughout aging and have the potential to impact MSC activities. Increased amounts of miR-17, miR-34, miR-146a, miR-183 p-5p, and miR-335 p-5p are seen in senescent MSCs. These molecules regulate cellular processes and stem cell activities throughout aging, including telomere erosion and the inhibition of apoptosis [134,135].

3.10. Nutrient sensing and metabolism

Reducing calorie intake to avoid malnutrition is the most efficient way to lengthen life [136].

Among the many advantages of this strategy are the possible modifications to stem cell characteristics. It is possible to minimize cell damage and provide the ideal environment for stem cells by controlling energy metabolism pathways [137]. As a result, tissue homeostasis is maintained and functioning is enhanced. A number of mechanisms and components, including the insulin-like IGF signal, the TOR signal, AMPK, SIRT, and FOXO transcription factors, contribute to the stem cell response and lifespan that are enabled by calorie restriction. ROS and metabolic status are also important factors in stem cell senescence. High ROS levels brought on by pathological circumstances might result in oxidative stress-related damage. MSCs release more ROS as they mature and go through different phases [138]. An excess of ROS or H2O2 can hinder MSC differentiation and proliferation. However, H2O2-induced cell senescence can be somewhat mitigated by inhibiting the p38/MAPK signaling pathway [139]. On the other hand, antioxidants like melatonin promote cell division in a concentration-dependent way [140]. In addition to enabling the cell to enter the S phase, melatonin also blocks the p38/MAPK signaling pathway, which stops MSCs from entering aging phenomenon. Monocyte chemoattractant protein-1 (MCP1) is another important component involved in the senescence-related secretory phenotype regarding in vitro grown MSCs.

MCP-1 stimulates chemokine receptor 2, which in turn stimulates the ROS-p38-MAPKp53/p21 pathway and prevents the

phosphorylation of the RB protein. This leads to the deterioration of RB, which in turn encourages the senescence of MSCs [86].

4. Pre-conditioning to prevent MSCs aging

MSCs exhibit significant proliferative capacity in a laboratory setting. However, long-term growth causes detrimental change to their characteristics. They gradually lose the ability to divide and differentiate into osteogenic, adipogenic, and chondrogenic lineages as a result [10,11].

These changes, when applied, once extrapolated to a broader biological context, suggest a progression towards stem cell senescence. As a result, the negative changes that occur in MSC attributes during prolonged in vitro culture can serve as a relevant model for studying organismal aging [11]. In certain specialized settings, MSCs have the potential to regain some of their functions. The complex biological process of stem cell aging is controlled by a multitude of organized elements [141]. Therefore, researches focus on identifying strategies to neutralize factors that cause cellular senescence in order to develop anti-aging standards that can be adhered to. Even though there are several regulators to control cellular senescence, we will focus on the main methods here, each of which has numerous sub-strategies.

4.1. Drugs

MSCs are well known for having an extraordinary cellular reserve because of their capacity for pluripotent differentiation, self-renewal, and clonogenic processes. Senescence, however, has been demonstrated to adversely affect MSCs' positive suitable properties. For instance, BM-MSCs obtained from individuals suffering from Systemic Lupus Erythematosus (SLE) display indications of senescence, including modified cell shape with heightened flattening and enlargement, increased activity of senescence-associated β-galactosidase (SA-β-gal) and disturbed cytoskeleton distribution. Thus, it is vital to discover strategies to prevent MSCs from aging. Previous research has shown that rapamycin (RAPA) is a therapeutically effective therapy for SLE in humans. Notably, RAPA has been demonstrated to inhibit the mTOR pathway in MRL/lpr mice-derived BM-MSCs and SLE patients. Maximum inhibition is shown at high doses of RAPA. This inhibition is accomplished by reducing S6 phosphorylation, an important indication. Through siRNA or RAPA therapy, mTOR was removed from MSCs in order to better understand the effect of the mTOR signaling pathway on senescence and the immunoregulatory ability of MSCs [142]. According to mentioned study, MSCs generated from SLE patients showed a senescent phenotype that could be reversed by either therapy. Furthermore, it was shown that both therapies reduced the hypertrophic expansion of MSCs taken from SLE patients, with siRNA outperforming RAPA in this regard. This implies the theory that a variety of factors, both mTOR-dependent and independent, affect the size and proliferation of cells. The amount of senescence-indicating SA-β-gal-positive cells was seen to be markedly elevated in SLE MSCs. However, this was later reduced by mTOR inhibition and RAPA therapy. The immunofluorescence examination of MSCs obtained from SLE patients showed that F-actin, a protein important in cell cytoskeleton structure, was distributed irregularly. But therapy with RAPA and siRNA targeting mTOR successfully corrected this aberrant arrangement, with no change in the rate of cell division. In the second phase of their investigation, eight MRL/lpr mice received transplants of BM-MSCs obtained from SLE patients receiving RAPA treatment. Following transplantation, SLE BM-MSCs treated with RAPA (G2) had a greater survival rate than both SLE BM-MSCs (G1) and normal BM-MSCs (G3). These results demonstrated the function of the mTOR pathway and its suppression in MSC senescence in SLE patients. Additionally, MSCs from SLE patients who were previously treated with RAPA, or normal MSCs, had a significant therapeutic effect on LN in MRL/lpr mice. Senolytic medications have also been employed to moderate aged MSCs in addition to rapamycin. For instance, Grezella et al. examined the impact of four potentially senolytic medications on the rejuvenation of human mesenchymal stromal cells (hMSTCs), namely ABT-263 (navitoclax), quercetin, nicotinamide riboside, and danazol [143]. Following prolonged culture, the hMSTCs underwent treatment with senolytic medicines once they had entered replicative senescence. Next, the effect of the therapy was assessed on molecular markers linked to replicative senescence, including telomere attrition, SA-β-gal, and senescence-associated DNA methylation changes. In a co-culture study, senescent hMSTCs were the target population. PKH67 (green) was used to identify early passage hMSTCs (passage 3), while PKH26 (red) was used to identify late passage cells (at the proliferative standstill). These two groups of cells were mixed in equal parts and left to be exposed to the medications for a whole day. Next, flow cytometry and fluorescence microscopy were used to investigate the hMSTCs. Senescent hMSTCs were considerably reduced after following ABT-263 therapy. However, there was no difference in the ratio of early to late transient cells between quercetin and nicotinamide riboside. Danazol even demonstrated a marginal rise in the number of late transient cells. For hMSTCs at both the early and late passages, dose-response curves were produced, suggesting that the cytotoxic impact of ABT-263 was more noticeable in cells from the later passages. Moreover, after treatment with ABT-263, the expression of SA-β-gal in hMSTCs dropped at later stages. All things considered, ABT-263 showed a little senolytic effect on hMSTCs. It is crucial to remember that getting rid of senescent cells can also get rid of the paracrine substances that cause growth arrest, which might postpone replicative senescence while cells expand in culture. Each medication was introduced for three days during passage three or the whole culture period in order to evaluate the effect on the long-term growth patterns of hMSTCs. The maximum number of cumulative population doublings (cPDs) decreased after short-term exposure to quercetin and danazol, probably as a result of their cytotoxic effects; nicotinamide riboside and ABT-263 had no influence on the cultures' ability to expand. It was also determined how the four senolytic medications affected the molecular markers of senescence. Senescence-related genes, including plasminogen activator inhibitor-1 (PAI-1), interleukin 6 (IL-6), and cyclin-dependent kinase inhibitor 2A (CDKN2A or p16), were expressed at higher levels in later passages and somewhat elevated after treatment with senolytic medicines. Nevertheless, none of the medications improved the late passages' average telomere length. The impact of senolytic medications on senescence-related epigenetic changes was also investigated in this study Replicative senescence of hMSTCs was shown to be accompanied by consistent alterations in DNA methylation at certain CpG sites. Nevertheless, none of the drugs significantly altered these modifications. As a result of this investigation, only ABT-263, of the four medications examined, had a senolytic effect on hMSTCs. However, neither the epigenetic senescence signature nor telomere length could be reversed by therapy with this drug. For regenerative medicine to function up to the high objectives set for it, stronger senolytic drugs must be discovered. To find out how Idebenone (IDB), a yellow crystalline drug used to treat chronic cerebrovascular disorders, affected the aging process of BM-MSCs obtained from Sprague-Dawley rats, Jiahui Zhang and colleagues undertook a research [144]. The investigation examined at how IDB affected cell motility, cloning, and proliferation. After being exposed to different IDB concentrations, significant alterations were noted. At greater concentrations, BM-MSCs showed a change

in morphology from a large, flattened shape to an elongated spindle shape. IDB appeared to have an impact on the number and appearance of BM-MSC colonies, even at lower concentrations. When compared to the control group, statistical analysis showed a significant increase in cell count with higher IDB levels. The effect of IDB on the biological properties of cultivated BM-MSCs was also investigated in this research. Within the experimental groups, reticular structures and strong cellular linkages were gradually developed by BM-MSCs. Interestingly, as the drug concentration was raised, the morphology of the cells changed to resemble spindles. The protective benefits of IDB were validated by DAPI staining, which also showed slightly enhanced chromatin in the IDB group along with smaller cells as compared to the DMSO group. Notably, G1 to S phase transition was enhanced by IDB, according to cell cycle study using PI staining. Moreover, flow cytometry-based apoptosis detection revealed a decreased frequency of apoptotic cells in IDB-treated groups relative to the DMSO group, indicating a possible anti-apoptotic effect of low IDB doses. Cells were grown with raising doses of IDB for a whole day in order to evaluate the impact of IDB on MSC migration potential. As compared to the control group, the cell migration test findings showed that IDB inhibited the migration of BM-MSCs. Following IDB treatment, statistical analysis showed a substantial dose-dependent decrease in the number of cells. Furthermore, the findings of SA-β-gal staining demonstrated a decrease in the amount of senescent cells in the experimental groups, with senescence decreasing in a dose-dependent fashion. A statistical analysis verified that IDB led to a considerable reduction in the quantity of aged BM-MSCs. Proliferating cell nuclear antigen (PCNA), cell cycle-related proteins (cyclin D1, cyclin D3), signal transducer and activator of transcription (Stat-3), and protein expression of stem cell markers (Nanog, Oct-4) were all upregulated after BM-MSCs were treated with IDB. In summary, IDB greatly lowered BM-MSC migration, apoptosis, and proliferation. According to the findings, IDB may not be toxic to BM-MSCs at low concentrations. Nevertheless, more investigation is required to pinpoint the downstream genes essential for cell death and ascertain if IDB affects BM-MSCs through different pathways.

4.2. Small molecules

Multipotent hBM-MSCs can differentiate into a variety of mesenchymal tissues, including fat, cartilage, and bone. They are a major source of tissue repair and regenerative medicine. In contrast, by prolonging the culture of stem cells, they may be deprived of their ability to renew selinated and proliferation that leads to cellular senescence. It is therefore vital to inhibit this process. After comparing 1,25 vitamin D3-treated hBM-MSCs to control hBM-MSCs, the researchers discovered that there was no discernible variation in the frequency of colony-forming units (CFUs) at passages 1 and 3. Furthermore, there was no link between CFU counts at these passages and 1,25D3 therapy. Similar results have been observed in the proliferation and proliferation of hBM apoptosis MSCs stimulated with 1,25D3 and a significant decrease in both processes. Moreover, the treatment has resulted hBMMSCs to show signs of visible structural changes such as increasing cell volumes and altering their shape [145]. However, no signs of spontaneously differentiation have been observed. hBM-MSCs that were both stimulated and unstimulated were stained for SA-β-gal to assess the effect of 1,25D3 on cellular senescence. The results showed a marked decrease in staining compared to unstimulated cells when stimulating hBMMSC. After passages 1 and 3, the amount of reactive oxygen species (ROS) in 1,25D3-treated cells were also assessed in comparison to untreated hMSCs; however, there was only a little and nonsignificant decrease in the production of oxidative stress. The expression levels of particular targeted genes linked to cellular quiescence were examined in order to look into how 1,25D3 therapy affected cellular quiescence. In comparison to control cells, the results demonstrated an increase of Nanog and thioredoxin interacting protein (TxNIP) in 1,25D3-treated hBM-MSCs at passage 3. Treatment with 1,25D3 also resulted in an increase of tumor protein p53 (TP53). Also,hBM-MSCs cultivated with or without 1,25D3 for four passages displayed a substantial downregulation of P16 and a non-significant overexpression of P15 in the senescence-associated gene expression. Furthermore, in 1,25D3-treated cells at passage 4, the expression of PSG5 remained maintained while the gene expression of pregnancy-specific beta-glycoproteins (PSG1) was somewhat downregulated. In summary, the findings indicate that 1,25D3 does not cause cellular senescence or premature aging in hBM-MSCs. The impact of L-carnitine (LC) on the proliferation and survival of rADSCs generated from rat adipose tissue was assessed in a different experiment [146]. The study's objectives were to reduce aging and oxidative damage while also fostering an environment that is conducive to the culture of rADSCs. The MTT assay was utilized to test cell proliferation, and the results indicated that rADSCs treated with LC for 48 h grew noticeably more quickly. The plotted curve showed that after plating, both control and LC-treated cells started to multiply right away and plateaued after around six days. LC similarly reduced the population doubling time (PDT) in treated cells. Senescent cells were eliminated from the bottom passage of rADSCs under both culture conditions, as shown by SA-β-gal staining. Since LC reduces aging and PDT, it is a good antioxidant that can extend the life of rADSCs. MSCs from one-day and five-day sheets were compared to control in order to examine the impact of cell sheet culture on MSC aging.

According to the results, MSCs culture undergoes cell differentiation and senescence as a result of sheet culture. In comparison to control and 1-day MSC sheets, alkaline phosphatase (ALP) staining demonstrated an increase in cell differentiation in 5-day MSC sheets. Sheet culture-dependent variations in MSC proliferation were evaluated employing a cellular proliferation test based on BrdU-labeling. Comparing the 5-day sheet culture to the control and 1-day sheet cultures, MSCs showed a clearly lower rate of proliferation. It's interesting to note that, in contrast to control and 1-day cultures, cell apoptosis did not significantly increase in the 5-day sheet culture. This proves that the aging process was unrelated to apoptosis. Markers associated with the senescent phenotypes of stromal cells have been evaluated in order to gain a more complete understanding of age specific changes on cell sheet culture. In 5 days sheet cultures, the expression of cell cycle inhibitors p16 and p21 was significantly increased compared to control and 1 day culture, whereas the expression of the cell proliferation marker cyclinD1 (CCND1) was significantly decreased compared to control and 1 day culture [147]. These findings show that improving Notch signaling can reverse the aging of MSCs caused by cell sheet culture (Fig. 4). Because that Jagged1 (JAG1) effectively inhibits MSCs from aging in cell sheet culture, it would be beneficial to look into potential downstream signaling molecules of Notch that are implicated in this process. In order to investigate the critical role of hairy and enhancer of split-1(Hes1) in JAG1-mediated suppression of cell senescence, lentiviral shRNA particles were used to induce Hes1 expression reduction in MSC cocultures. It was shown that 90 % of MSCs were effectively infected by lentiviral particles during a 24-h lentivirus culture. Hes1 protein expression in MSCs infected with shRNA lentivirus, with or without JAG1 therapy, was significantly reduced, as demonstrated by Western blot analysis utilizing protein from 5-day cocultures, which further supported these knockdown results. More critically, JAG1-mediated Notch activation greatly reduced cell senescence in 5-day cultures; however, this inhibitory impact was almost eliminated when Hes1 expression in these MSCs

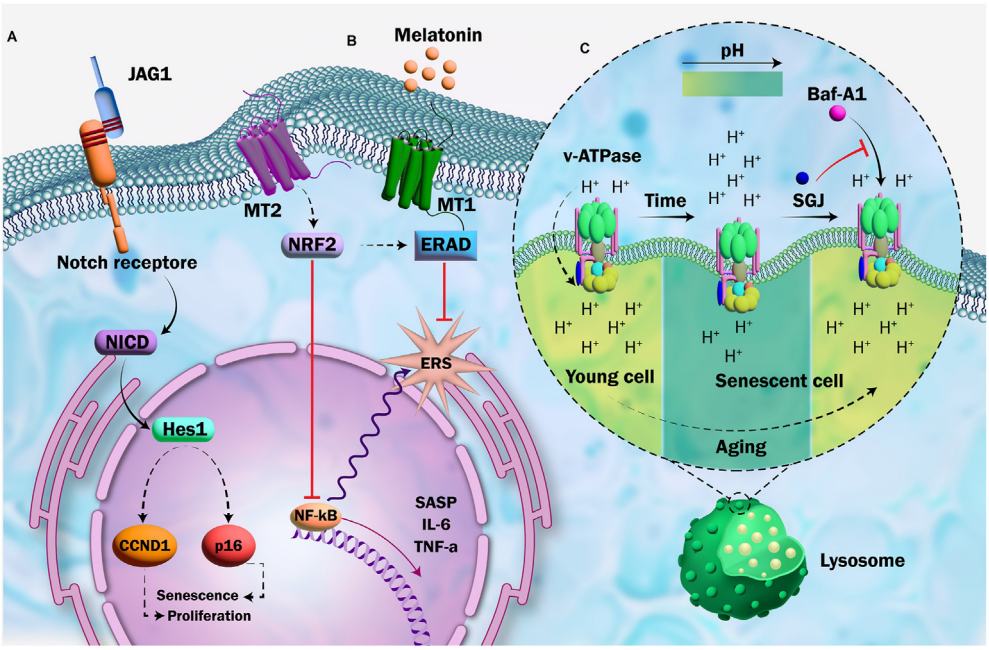

Fig. 4. A) The role of notch signaling pathway in the aging of MSCs. JAG1: Jagged1; Hes1: hairy and enhancer of split-1; CCND1: cell proliferation marker cyclinD1; NICD: Notch intracellular domain. **B)** The mechanism by which melatonin inhibits ERs. MT1/MT2: Melatonin receptor type 1/2; ERAD: Endoplasmic reticulum-associated degradation; SASP: Senescence-associated secretory phenotype; ERS: Endoplasmic reticulum stress. **C)** Representation of SGJ inhibiting the disorders of lysosomal acidification through v-ATPase in senescent BMSCs. v-ATPase: Vacuolar H + -ATPase; SGJ: 3-butyl-1-chloro imidazo [1, 5-a] pyridine-7-carboxylic acid; Bafilomycin-A1: Baf-A1.

was knocked down. Moreover, MSCs transfected with Hes1 shRNA showed a substantial increase in SA-β-gal positive cells, as measured by Image. Furthermore, Hes1 shRNA-infected MSCs exhibited elevated p16 gene expression but not p21 gene expression, according to real-time PCR results. These findings show that the target gene Hes1 and the typical Notch ligand JAG1 are required for Notch suppression of MSC senescence. Taken together, Notch signaling activation improves bone regeneration therapeutic actions and reverses aging in senescent MSCs.

The relationship between lysosomal acidification and cellular aging has been the subject of recent research since it has been demonstrated that lysosomal pH dysregulation and altered vacuolar H^+-ATPase (v-ATPase) activity occur during aging (Fig. 4) [148].Therefore, it may be possible to prevent cell ageing by increasing lysosomal acidity. It has been discovered that a novel small chemical called 3-butyl-1-chloro imidazo [1, 5-a] pyridine-7-carboxylic acid (SGJ) reacts quickly and preferentially to acidic pH. Still, it is unclear whether SGJ can raise lysosomal acidification and prevent senescence in BMSCs. The purpose of this work was to verify if SGJ elevates the H+ content in senescent lysosomes. To lower the H+ content in the lysosome, we employed Bafilomycin-A1 (Baf-A1), a recognized inhibitor of v-ATPase, which is present in the lysosomal membrane. After being subjected to SGJ and Baf-A1 for the entire day, senescent cells were cultured with LysosensorTM Green DND-189 and captured on video using a confocal microscope. Acidification of LysosensorTM Green DND-189 reagents provide a pH-dependent augmentation in fluorescence intensity. According to their findings, the red fluorescence in control cells virtually vanished, while it was noticeably enhanced in SGJ-treated cells and further decreased in Baf-A1-treated cells. Thus, in senescent MSCs, SGJ can raise the concentration of H+ in lysosomes. Additionally, they measured the amounts of lysosome-associated membrane proteins 1 and 2, or LAMP1 and LAMP2, utilizing immunoblotting. Those proteins are indicators of lysosomal activity. They reported that senescent cells had lower levels of above markers; however, SGJ therapy substantially elevated the protein production of mentioned markers, suggesting that lysosomal function had been restored. This implies that SGJ might prevent disabled lysosomes from harm. Furthermore, it was discovered that SGJ both promoted and inhibited the senescence of BMSCs in vitro. Considering the significance of autophagy in cellular aging processes, they examined at how SGJ could be affected in BMSC autophagy.

They found thatSGJ greatly reduces the protein level of p62/SQSTM1, which is broken down during autophagy, and raises the protein level of LC3B, a hallmark of autophagy. To inhibit autophagy flux Baf-A1/chloroquine (CQ) was employed which observed that SGJ enhances BMSC autophagy flux andcellular autophagy. In order to verify that the V0 proton channel of v-ATPase is the target site of SGJ, various orders of SGJ and/or Baf-A1 was added and variations in the quantities of p21 protein and SA-β-gal positive cells were detected. They stated that compared to other experimental groups, the SGJ-treated group exhibited fewer SA-β-gal positive cells and lower levels of p21 protein when Baf-A1 was present. Hence, SGJ can successfully inhibit cell senescence by preferentially occupying Baf-A1's site of action. Therefore, SGJ is a novel Baf-A1 antagonist that stimulates autophagy and prevents senescence in BMSCs. Examining the impact of fucoidan, a sulfated marine polysaccharide, on cellular senescence of MSCs has been performed in consideration of the fact that the uremic toxin p-cresol limits the therapeutic efficacy of MSCs in chronic kidney disease (CKD). Through the FAK-Akt-TWIST pathway, the study demonstrates that fucoidan can prevent p-cresol-induced cellular senescence in MSCs, indicating a possible application for fucoidan in conjunction with MSC-based therapy for the treatment of chronic kidney disease. The research evaluated cell structure, senescence, and proliferative ability following p-cresol therapy in terms of investigating the impact of the drug on cellular senescence in MSCs. The SA-β-gal test results demonstrated that p-cresol caused senescence and a considerable increase in MSCs size. Furthermore, p-cresol therapy

reduced the cells' ability to proliferate, which resulted in cell senescence. Employing western blotting, the study evaluated the expression of proteins associated with the cell cycle (cyclin-dependent kinase 2 (CDK2), (CDK4), (cyclin D1), and (cyclin E) and senescence (SMP30 and p21) to learn more about the impact of p-cresol on these signaling pathways in MSCs. The anti-senescence marker SMP30's expression was found to be lowered by p-cresol, while the pro-senescence marker p21's expression was found to be elevated in a time-dependent way.

These results imply that p-cresol decreases MSC proliferative capacity and causes senescence in MSCs. According to earlier research, the TWIST pathway is essential for reversing endothelial progenitor cells' cellular senescence. A prominent fucoidan receptor is integrin αMβ2, which when activated, results in FAK. In endothelial colony-forming cells, the FAK-Akt signaling pathway is related to protection against cellular senescence. The study evaluated the activation of the FAK-Akt-TWIST signaling pathway following fucoidan therapy in order to determine if fucoidan affects this pathway in MSCs in a time-dependent manner. The phosphorylation of FAK and Akt was shown to be enhanced by fucoidan therapy in a time-dependent manner, with the greatest impact occurring after 48 h. Additionally, TWIST expression considerably elevated with increasing treatment time. The frequency of SA-β-gal positive cells become higher and the structure of MSCs altered when Akt signaling was inhibited, indicating that p-cresol-induced MSC senescence is prevented by fucoidan-mediated Akt signaling. Additionally, prior research has demonstrated that stem cell self-renewal and proliferation depend critically on the cellular prion protein PrPC [149]. Fucoidan was added to normal MSCs to check if it had any effect on PrPC expression in senescent MSCs. Thefucoidan stimulates PrPC expression in a time-dependent way. PrPC mRNA and protein expression were markedly elevated following exposure to p-cresol; however, fucoidan therapy restored PrPC expression. TWIST siRNA transfection intensified this effect, suggesting that senescent MSCs' fucoidan-induced TWIST expression is necessary for the reduction in PrPC expression that occurs with cell senescence. Finally, by activating the FAK-Akt-TWIST pathway, fucoidan prevents p-cresol-induced cellular senescence in MSCs and raises the possibility of utilizing it in combination with functional MSC-based treatments to treat chronic kidney disease.

4.3. Proteins

One growth factor that plays a role in angiogenesis and tissue repair is fibroblast growth factor-2 (FGF-2). When hBMSCs are grown in vitro, FGF-2 keeps them in an immature state. FGF-2 promotes development and maintains the ability of hMSCs to differentiate into multiple cell types, making it a crucial mediator in tissue regeneration. To investigate the effect of TGF-b2 on senescence in hMSCs, they were grown in MSCGM media supplemented with either TGFb1 or TGFb2 [150]. SA-b-Gal staining was then performed and the amount of thymidine analog BrdU was measured. One day after treatment with TGF-b1 or TGF-b2, hMSCs showed a fibroblast-like morphology similar to the control group. However, after five days, the cells treated with TGF-b1 or TGF-b2 appeared depressed and some showed blue staining from SA-b-Gal, while the control group did not. Additionally, BrdU incorporation was lower in the TGF-b1 and TGF-b2 treated cells compared to the control group. Flow cytometry was used to analyze the cell cycle and showed that TGF-bs caused G1 cell growth arrest in hMSCs by increasing the proportion of cells in G1 phase and decreasing it in S and G2 phases. TGF-bs also increased the expression of pRB protein and three mRNA levels of G1 phase CDK inhibitors (p16INK4a, p21Cip1, and p53), but reduced the production of phosphorylated pRB (ppRB). Those outcomes suggest that TGF-b1 and TGF-b2 induce hMSC cell senescence through G1 growth arrest. To further investigate the role of FGF-2 in inhibiting cell senescence, hMSCs were cultured with or without FGF-2 and then stained with SA-b-Gal. After 10 days of growth in FGF-2(−), the morphology of MSCs resembled that of fibroblasts, with 20.5 % of the cells exhibiting blue staining by SA-b-Gal. However, after 50 days in culture, the percentage of blue-stained hMSCs increased to 57.6 % and the cells took on a depressed shape. In contrast, treatment with FGF2(+) for 10 or 50 days resulted in a fibroblast-like morphology in hMSCs, with 35.8 % or 27.3 % of the cells exhibiting blue coloration, respectively. Furthermore, after 50 days of culture, MSCs in FGF-2(−) incorporated 40 % less BrdU compared to those after 10 days, but this was not observed in FGF-2(+). Additionally, the impact of FGF-2 on the cell cycle was investigated. The number of cells in the G1 phase increased after 50 days of culture in FGF2(−), but decreased after culture in FGF-2(+). To assess the effects of FGF-2 on cell cycle regulation, the mRNA expression levels of p16INK4a, p21Cip1, and p53, as well as the expression levels of pRB, were measured in hMSCs after 10 or 50 days of culture in FGF-2(+). The results showed that after 50 days of culture in FGF-2(+), the mRNA expression levels of p16INK4a, p21Cip1, and p53 were significantly higher compared to those after 10 days of culture. However, this increase was not observed after culture in FGF-2(−). Conversely, during 50 days of culture in FGF-2(−), the expression levels of both total pRB and ppRB decreased compared to the 10-day culture period in FGF-2(+). These findings advocate that the effects of FGF-2 on hMSC cellular senescence may vary depending on the duration of culture. To examine the impact of FGF-2 on TGF-b mRNA and protein expression levels in hMSCs, cultures were conducted for 1, 10, or 50 days in the presence or absence of FGF-2. The results showed that after 50 days of culture, the expression levels of TGF-b1 mRNA were higher in both FGF-2(−) and FGF-2(+) conditions compared to cultures lasting 1 or 10 days. This indicates that FGF-2 may inhibit cellular senescence in hMSCs by down-regulating TGF-b2 expression, and this effect is dependent on the duration of culture. Furthermore, treatments using MSCs have shown potential in animal models of cardiovascular disease. However, as the age of donor increases, the number and functionality of MSCs decrease. This limits their ability to aid in the healing of injuries. To address this issue, targeted pretreatments aimed at delaying senescence and enhancing stem cell regenerative capacities can be used to improve the repair of injured tissue in the elderly. One potential approach is the use of macrophage migration inhibitory factor (MIF), a proinflammatory cytokine that has been shown to have rejuvenative effects by modifying signaling pathways associated with aging. Quantitative PCR analysis revealed that the basal level of MIF mRNA expression differs significantly between young and aged MSCs, with young cells showing nearly eight times higher expression levels. This emphasizes that the concentration of secreted MIF in the culture media of young MSCs is two to three times higher than that of elderly cells. Additionally, MSCs secrete various cytokines and growth factors that can function in an autocrine or paracrine manner [151]. To investigate if MIF can restore the trophic activity of MSCs isolated from aged bone marrow, quantitative real-time PCR was used to measure the expression of VEGF, bFGF, HGF, and IGF. Expectedly, aged cells had significantly lower mRNA levels of all four components compared to younger MSCs, under both normal and hypoxic conditions. However, this difference was eliminated when aged MSCs were treated with MIF. To further investigate this, the levels of VEGF, bFGF, HGF, and IGF were measured in the culture medium of aged MSCs with and without MIF treatment using enzyme-linked immunosorbent assays. Their data were consistent with previous findings, showing a significant increase in the secretion of all four factors in MIF-treated aged MSCs, comparable to levels found in

young MSCs. Thus, MIF not only has regenerative effects on senescent MSCs, but also has anti-apoptotic properties. In fact, MIF-treated aged MSCs showed better survival rates than untreated young MSCs. This led to the examination of CD74, also known as the MIF receptor, and its role in MIF-mediated repair. However, there were no significant differences in CD74 expression between old, MIF-treated, and young MSCs. This led to the hypothesis that MIF may activate the AMPK-FOXO3a signaling pathway in aged MSCs. To test this, siRNAs were used to silence the translation of AMPK and FOXO3a, resulting in a decrease in proliferation and paracrine signaling in aged MSCs. This also eliminated MIF's anti-apoptotic effects and decreased the production of trophic factors. These findings suggest that MIF can restore aged MSCs through its interaction with CD74 and activation of the AMPK-FOXO3a signaling pathway. Therefore, underwent potential therapeutic implications for the renewal and restoration of endogenous BMSCs in elderly patients. Heat shock proteins, including the key stress protein HSP70, play a crucial role in maintaining intracellular homeostasis and protecting against protein degradation in response to external stressors such as temperature increases and aging. Previous research has shown that long-term injection of recombinant human HSP70 in animals with Alzheimer-like neurodegeneration and during sepsis can significantly reduce the clinical symptoms of both illnesses [152]. In a study examining the activity of recombinant human HSP70, young and old mouse MSCs were used. The results indicate that HSP70 greatly enhances the development of elderly MSCs, but not young MSCs, at doses of 2 μg/ml and higher. A modest heat shock (42 °C for 5 min) had a similar effect on the cells. Interestingly, aged MSCs were more susceptible to exposure to greater heat stress than young cells, and the response of young and aged MSCs to heat shock treatment varied significantly in terms of duration. Western blotting and protein labeling experiments revealed that while mild heat shock increased the levels of HSC70 in aged MSCs, neither exogenous injection of HSP70 nor mild heat shock induced endogenous HSP70 in young or old MSCs. These findings proved that moderate heat stress and exogenous HSP70 injection may have a rejuvenating effect on MSCs and other cell types in vivo, potentially prolonging lifespan by delaying the aging process at the molecular and cellular level.

4.4. Inhibitors

To achieve high therapeutic efficiency, a sufficient amount of MSCs is necessary. This requires long-term culturing to obtain high quantities of MSCs. However, during this process, it is important to prevent spontaneous differentiation and aging. A research group used epigenetic modification to prevent spontaneous differentiation and aging in hUCMSCs [153]. It is worthy to mention that histone H3 acetylation and methylation, regulates the aging and differentiation of MSCs [154,155]. Acetylation of K9 and K14, as well as dimethylation of K4, in histone H3 is necessary for initiating the transcription of genes associated with the stemness of MSCs. Therefore, two types of histone deacetylase inhibitor (HDACI), largazole (LZO) and trichostatin (TS*A*), were used to investigate the effect of epigenetic changes on the aging of hUCMSCs. This study, confirmed that culturing hUCMSCs with a low concentration of TSA or LZO, promotes stem cell proliferation. According to the growth curve, the proliferation of hUCMSCs in the logarithmic phase was higher than that in the control group. It is worth noting that LZO induced the proliferation, better than TSA. After proliferation, the aging of hUMSCs was studied. The hUMSCs started to age at passage 8 and this was indicated by a decrease in the mRNA expression of pluripotent genes such as Octamer-binding transcription factor 4 (OCT4), and Nanog, and telomerase reverse-transcriptase (TERT) gene, in hUCMSCs. These genes are associated with the self-renewal ability of MSCs. Additionally, the mRNA expression of the migration gene C-X-C chemokine receptor 4 (CXCR4), which is vital for the migration of MSCs, also started to decrease at passage 8. At passages 12 to 16, the mRNA expression of these genes decreased even further. However, in hUMSCs treated with TSA or LZO, the expression levels of these pluripotent and migration genes increased at passage 4. From passage 8 to 12, the expression levels of each gene decreased slightly, but they were still higher in the TSA and LZO groups compared to the control group. Furthermore, the mRNA expression of ALP and OPN genes increased at passage 8 in untreated hUMSCs (control), indicating the osteogenic differentiation of hUCMSCs. However, the expression levels of these osteogenic differentiation-associated genes decreased in the TSA and LZO groups. In conclusion, in this investigation, it was observed that hUCMSCs have the inherent capability to differentiate and undergo aging during the process of expansion. Nevertheless, the application of low doses of HDACI was shown to enhance the proliferative potential of hUCMSCs and inhibit their aging progression as well as their spontaneous differentiation [153]. Finding the molecular pathways by which senescent BM-MSCs contribute to systemic lupus erythematosus (SLE) is crucial. One important factor to consider is the deacetylation inhibitor mentioned previously. For example, a study found that the Wnt/b-catenin signaling pathway may have a major effect on the senescence process in bone marrow-derived mesenchymal stem cells from patients with SLE by activating the p53/p21 pathway. This study used immunofluorescence and Western blot to investigate Wnt/β-catenin signaling in BM-MSCs of SLE patients. The results showed that the SLE group had higher levels of both nuclear and cytoplasmic b-catenin expression compared to the normal group, with nuclear b-catenin expression being significantly enhanced. As Wnt/β-catenin signaling is negatively regulated by glycogen synthase kinase 3 beta (GSK-3b), the expression levels of GSK-3b were also examined. The results showed a decrease in cytoplasmic fluorescence intensity and a considerable decrease in GSK-3b expression in the SLE group compared to the normal group. Additionally, the downregulation of Wnt/β-catenin signaling in BM-MSCs from SLE patients was confirmed by treatment with Dickkopf-related protein 1 (DKK1) and si-β-catenin, as shown by immunofluorescence examination. Treatment with high DKK1 or si-β-catenin reversed the elevated expression of β-catenin in SLE BM-MSCs, particularly in the nucleus. Furthermore, treatment with DKK1 or si-β-catenin resulted in a substantial increase in GSK-3b expression in SLE BM-MSCs. It is important to note that BM-MSCs from SLE patients also had higher levels of p53 and p21 expression due to activated Wnt/b-catenin signaling. To further explore this, Western blot and immunofluorescence were used to measure p53 and p21 expression. The results showed that the SLE group had significantly higher levels of p53 and p21 expression compared to the normal group. However, treatment with DKK1 or si-b-catenin resulted in a marked reduction in p53 and p21 levels in SLE BM-MSCs. This finding suggests a strong relationship between the target genes of p53 and p21 and the cell senescence caused by over-activation of Wnt/β-catenin signaling. In summary, this study found that Wnt/b-catenin signaling is essential for the senescence of BM-MSCs derived from SLE patients, with the p53/p21 pathway being the primary mediator of BM-MSC senescence caused by excessive Wnt/b-catenin signaling. Understanding the pathways of Wnt/b-catenin signaling implicated in the senescence of SLE BM-MSCs can enhance the effectiveness of BM-MSC transplantation in SLE patients [156]. Apart from the elements mentioned above, there is additional evidence indicating the role of Rho family GTPase, Cdc42 activity in MSC senescence and differentiation potential. It is also possible to partially rejuvenate old MSCs through pharmacological suppression of Cdc42 activity. This is an important factor to

consider when using Western blot analysis. When culturing ADMSCs with CASIN, there is a significant decrease in Cdc42-GTP levels in both the old and young groups. However, SA-b-Gal levels only decrease in the old group. This is significant because cellular senescence has been linked to increased ROS generation. CASIN has been shown to reduce this enhanced ROS generation in ADMSCs from aged rats. Another characteristic of aging cells is actin polymerization, which can be caused by Cdc42 activation and overexpression. In elderly cells, CASIN treatment decreased the intensity of F-actin, although not to the same extent as observed in ADMSCs from young rats. This treatment did not lead to a rearrangement of the cytoskeleton in the younger control group. Furthermore, CASIN's inhibition of Cdc42 greatly enhanced the ability of aged rats' ADMSCs to differentiate into adipogenic cells. Similarly, after incubation in osteogenic medium, the amount of mineralized matrix in 1-month-old rat cells was approximately tenfold higher than that of old rats' cells, as quantified by Alizarin Red S, a marker of osteogenic differentiation and calcium deposition in cells.Treatment with CASIN resulted in a more than two-fold increase in osteogenic differentiation in ADMSCs from older rats. However, this increase was not as high as that observed in ADMSCs from young rats. Furthermore, data point to the role of MAP kinases in MSC senescence and the reduction of their ability to differentiate. It has been demonstrated that Cdc42 regulates MAPK/ERK signaling pathways in response to stress. Treatment of ADMSCs from old rats with CASIN resulted in a significant reduction in the phosphorylation of ERK1/2 and JNK, two key kinases involved in differentiation. These levels were comparable to those observed in ADMSCs from young rats, indicating a decrease in kinase activity. To confirm that the effects of CASIN were due to Cdc42 inhibition and not off-target effects, the impact of two other Cdc42 inhibitors, ZCL278 and ML-141, on the expression and proliferation of SA-b-Gal in ADMSCs isolated from aged mice was assessed. The results showed that these inhibitors significantly reduced the percentage of SA-b-Gal positive cells, with the most pronounced effect seen with CASIN and ZCL278. The growth kinetics of ADMSCs were assessed using the CCK-8 test, which revealed that CASIN, ML141, and ZCL278 improved the reduced proliferative activity of ADMSCs from elderly mice. In summary, these findings suggest that Cdc42 plays a role in the aging and differentiation capacity of male rat mesenchymal stem cells, although the exact mechanisms of Cdc42 action require further research. A better understanding of the fundamental molecular pathways behind stem cell aging could have implications for regenerative medicine, stem cell therapy, and rejuvenation treatments [157].

4.5. Other molecules

It has been shown that transplantation of ADMSCs is effective in treating various illnesses. However, the normal aging process of ADMSCs during in vitro proliferation before transplantation may decrease their effectiveness. Previous studies have demonstrated that melatonin has anti-oxidant, anti-senescence, and biorhythm control properties [158,159]. In a study canine ADMSCs (cADMSCs) at a high passage were treated with either the ERS activator tunicamycin (TM) or the ERS inhibitor 4-PBA, to examine the relationship between melatonin's anti-aging effects and its ability to reduce endoplasmic reticulum stress (ERS) (Fig. 4). The results showed that 4-PBA decreased the expression of ERS markers (Grp78, Chop, Xbp1, Atf4, and Atf6) and improved the senescent phenotype of cADMSCs. Additionally, cADMSCs treated with 4-PBA exhibited reduced expression of SASP markers, a shorter population doubling time, a lower nucleus-to-cytoplasm ratio, and less SA-β-gal staining compared to untreated cells. On the other hand, exposure of ADMSCs to the ERS activator TM resulted in various changes, including increased expression of ERS markers, a decrease in the nucleus-to-cytoplasm ratio, elevated SA-β-gal staining, a prolonged population doubling time, and a decrease in both telomerase activity and the telomere length T/S ratio. These findings further support the hypothesis that reduced senescence is associated with ERSmarkers. Previous research has also shown that melatonin can lower ERS by inducing endoplasmic reticulum-associated degradation (ERAD). This is supported by the fact that cADMSCs treated with melatonin for 12 h showed significant increases in the ERAD markers Hrd1, Vcp, and Os9, while Hrd1 was reduced by the melatonin receptor inhibitor luzindole. Melatonin decreases ERS through ERAD, as evidenced by the fact that ubiquitination and the VCP-specific inhibitors MG132 and NMS-873 prevented the reduction of ERS by melatonin. Since the NF-κB pathway is known to control SASP, it was investigated how melatonin therapy affected NF-κB. Treatment with melatonin or oltipraz (an NRF2 activator) for 12 h decreased the transcript levels of p65 and p50, as well as the levels of SASP-related proteins IKK, p-P65, and p65. However, luzindole prevented melatonin's inhibitory effects on NF-κB. When melatonin and luzindole were used together, transcript levels of p65, p50, and IL6 increased, as did protein levels of IKK, p-P65, and p65. Several animal and cellular models have demonstrated that melatonin exhibits anti-aging properties. For example, it has been shown to slow down the aging of BMMSCs and the decline in telomerase activity in the retina of individuals with age-related macular degeneration. This study also shows that melatonin inhibits ERS as part of its impact on cellular aging. Additionally, melatonin has been shown to have ERS-suppressive effects in various illnesses and to decrease memory impairments and Alzheimer-like damage. These results suggest that melatonin can reduce the senescence of cADMSCs and increase the effectiveness of cADMSC transplants [160].

5. 3D culture systems to prevent MSCs aging

The dimension of culture in which MSCs are cultured, affects the functions and properties of MSCs. based on previous studies, the expression levels of some secretomes of MSCs are increased in 3D-cultured MSCs compared to 2D-cultured MSCs. VEGF [161,162], hepatocyte growth factor (HGF) [163], IL-1 receptor antagonist (IL-1Ra), granulocyte-colony stimulating factor (G-CSF) [164], Interleukin-6 (IL-6), IL-8 [165] are some of these secretomes which take part in the vital features of MSCs. Moreover, the therapeutic efficiency of MSCs, and the regenerative potential of their secretomes, increase when the cells are cultured in 3D condition [166,167]. One other difference between 2D and 3D culturing of MSCs is aging related markers. Cell cycle arrest, is an indicator of replicative senescence [168]. An increase in the percentage of cells which are in the G0/G1 phase of cell cycle, is marker of the quiescence [14,169]. In a research, after 14–16 passages of 2D-culture, 90 % of MSCs were in the G0/G1 phase and only 5 % of MSCs were in the S-phase. Conversely, when the same cells were cultured in 3D-spheroids, and transited back to the 2D-culture, 50 % of MSCs were in G0/G1 phase and 27 % of MSCs were in the S-phase. Thus, 3D-culturing, results in lower MSCs senescence. In the same research, the activity of SA-β-Gal, another marker of senescence, was compared between 2D and 3D cultured MSCs. After 14–16 passages in 2D culture 75 % of MSCs were SA-β-Gal positive. Conversely, when these SA-β-Gal positive MSCs were used to form 3D spheroids and put back to the 2D culture, only 1 % of MSCs were positive for the SA-β-Gal. These results, showed that 3D-culture reverses the aging of MSCs and this is confirmed by the loss of senescence associated markers [170]. Hence, various types of 3D platforms have been developed for culturing MSCs, in order to achieve MSCs with higher therapeutic efficiency, higher levels of beneficial

secretomes, delayed senescence, increased stem cell properties, and higher stemness genes. Scaffolds, hydrogels, extra cellular matrix (ECM), are static 3D culturing platforms. While, spheroids and micro-carriers are dynamic 3D culturing platforms (Fig. 5).

5.1. 3D-culture with scaffolds

Polycaprolactone (PCL) is a biodegradable and biocompatible polyester that is commonly used for creating scaffolds for tissue regeneration due to its desirable mechanical properties [171]. PCL has been combined with hydroxyapatite (HA) to fabricate porous 3D scaffolds for bone regeneration using 3D printing methods [172]. Heo and co-workers used PCL 3D scaffolds to study the impact of the scaffold on the stemness and differentiation potential of BM-MSCs. The 3D scaffold was created through electrospinning, resulting in a nano fibrous structure that mimics the native ECM in terms of mechanical and physical properties [173]. In this study, BM-MSCs were cultured on tissue culture plastic (TCP) or the 3D scaffold. After the first passage, the expression of Nanog, a stemness marker, was lower in the BM-MSCs cultured on TCP compared to those on the 3D scaffold. Additionally, the expression of SA-β-Gal, an indicator of early senescence, was significantly lower in the MSCs cultured on the 3D scaffold compared to those on TCP. Furthermore, the higher amount of collagen II and lower amount of collagen I in TCP-cultured BM-MSCs compared to 3D scaffold-cultured BM-MSCs suggests a shift towards fibrotic differentiation. These findings suggest that 3D culture of MSCs may be more beneficial than 2D culture. When MSCs are cultured in PCL-based 3D scaffolds, their stemness increases and early senescence decreases, while TCP may cause MSCs to shift towards fibrotic differentiation. In another study, the effect of 3D culture on the long-term expansion of UC-MSCs was investigated [174]. A porous nano-hydroxyapatite/chitosan/poly lactide-co-glycolide (nHA/CS/PLGA) scaffold, was produced by particle leaching method. This 3D scaffold, resembles the natural bone matrix. The expression level of P53, a senescence marker, was increased by 10 folds at passage 27 (P27), compared to passage 3 (P3) in hUCMSCs cultured on conventional culture dishes (hUCMSCs/CD). When the hUMSCS were cultured on nHA/CS/PLGA scaffold (hUCMSCs/NS), mRNA and protein levels of P53 was reduced significantly. Furthermore, the expression level of proliferation genes (EGF, bFGF), osteogenesis gene BMP2, migration gene CXCL5 and angiogenesis gene VEGF, were higher in P27 hUCMSCs/NS than those in P27 hUCMSCs/CD. Correspondingly, the proliferation rate of hUCMSCs/NS at P3 was similar to that at P27. Finally, the effect of developed nHA/CS/PLGA scaffold on osteogenic differentiation was studied both in vitro and in vivo. The expression levels of osteogenesis factors (Col1, OCN, and Runx2) was amplified noticeably in hUCMSCs/NS, compared with hUCMSCs/CD. Likewise, after subcutaneous transplantation of P3 hUCMSCs/NS, P27 hUCMSCs/NS, P3 hUCMSCs/CD and P27 hUCMSCs/CD into nude mice, microradiography assay revealed the efficacious osteoid formation after 3 months, in all of the mice except those which were treated with P27 hUCMSCs/CD. Through this study, it was observed that the use of a nHA/CS/PLGA nano-scaffold provided a beneficial microenvironment which could efficiently prevent the replicative senescence of hUCMSCs. Furthermore, P27 hUCMSCs/NS maintained their proliferation and differentiation abilities. The potential for the naturally based matrix to prevent replicative senescence of MSCs in long term is illustrated by these findings. Collectively, these researches offer the ability of 3D scaffolds to promote less fibrotic differentiation, decreased early and replicative senescence, enhanced cell migration, superior therapeutic efficiency, and higher cell proliferation.

5.2. 3D-culture with hydrogels

Previous experiments have shown that differentiation ability, and other functions of MSCs depend on the mechanical properties of the material on which MSCs are cultured [175,176]. Culturing on the rigid materials results in osteogenic differentiation, and decreased viability, while, culturing on the soft materials leads to the neurogenic differentiation, the enhancement of the MSCs viability and maintenance of their stemness [177–179]. Hydrogels are 3D networks, composed of cross-linked polymers, which can absorb high amounts of water [180]. Hodge eta l. utilized a 3D hydrogel which was made of a porous structure known as Tissue-Block (T-block) [181]. This polyethylene glycol (PEG) - based hydrogel is suitable for studying cellular function in tissue culture. They coated the surface of both TCP and T-block with fibronectin, a naturally secreted protein by ADMSCs, in order to promote cell-attachment. The researchers confirmed that T-block could efficiently mimic the native adipose soft tissue in terms of mechanical properties. Also, the expression levels of stem cell markers including CD73, CD 90, and CD105 were compared between ADMSCs cultured on TCP and those cultured on T-block. The population of the ADMSCs which were positive for the mentioned markers decreased at P6 and P10 compared to P2, in both groups. However, this was less significant in ADMSCs which were cultured

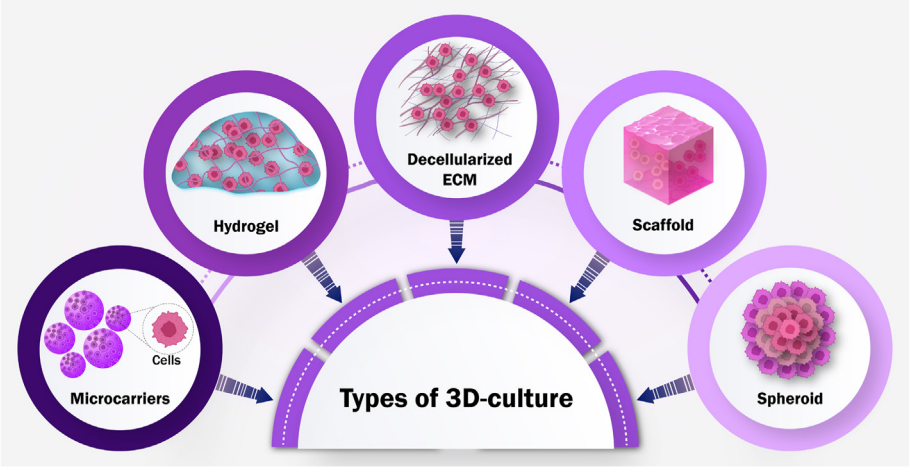

Fig. 5. Various types of 3D culture platforms. Hydrogels, microcarriers, scaffolds, decellularized extracellular matrix, and spheroids.

on T-block. The expression level of SA-β-gal was 5 % for both TCP-cultured ADMSCs and T-block- cultured ADMSCs. The expression level of SA-β-gal was increased to 11.7 % and 22.5 % at P6and P10, respectively, in ADMSCs cultured in 2D environment. In contrast, no increase in the level of SA-β-gal was observed in the ADMSCs which were cultured in 3D environment, until P10. Moreover, the population of ADMSCs with flattened morphology was higher in the TCP-cultured cells compared to T-block- cultured cells. These findings prove the priority of T-block as a culturing substrate in delaying the senescence of ADMSCs. In the last part of the study, they investigated the ability of ADMSCs to promote wound healing. Keratinocytes were treated with TCP-cultured ADMSCs or T-block-cultured ADMSCs. Based on scratch assay, the percentage of wound recovery was decreased significantly from P2 till P10 in case of 2D-culture. But in case of 3D-culture, there was no significant difference in the percentage of wound recovery from P2 till P10. This was probably due to the higher population of stem-like cells and lower senescence associated with the T-block- cultured ADMSCs.

In other researches the effect of hydrogel stiffness on delaying the aging process of MSCs has been examined. In these studies, hydrogels with lower stiffness compared to TCP were used as culture substrates. This is possibly because of the fact that stiffness of TCP is 10^6 kPa, which is much higher than that in most of the body tissues (ranging from 1 to 100 kPa) [182,183]. In this regard, Kureel et al. prepared polyacrylamide (PAA) gels with various stiffness ranging 0.5–20 kPa, were prepared [184]. They found that when hUMSCs were cultured on soft gels ($E \leq 5$ kPa) for a long time, the cells were less spread out. This is probably because the soft gels are able to cause reversible cell cycle arrest or senescence [185]. When hUMSCs were cultured on PAA gels with just 1 or 2 kPa stiffness, the amount of replicating DNA, a marker of cell division, was low. Conversely, when hUMSCs were cultured on TCP or gels with a stiffness of more than 5 kPa, the percentage of dividing cells increased up to more than 30 %. Therefore, the PAA gel with a Young's modulus of 5 kPa was chosen for the rest of the experiments. Microscopic images revealed that UCMSCs preserved their spindle-like morphology until late passages, when they were cultured on PAA, but the hUCMSCs could not maintain their morphology, when they were cultured on TCP. The doubling time (DT) of hUCMSCs, doubled at late passages, when the cells were cultured on TCP, but remained constant, when the cells were cultured on PAA gel. The cumulative population doubling (CPD) was increased until P9 and then slowed down until P18 for TCP-cultured hUCMSCs. However, the CPD was increased linearly, until |P18 for PAA-cultured hUCMSCs. Consequently 512 times more cells were obtained by using PAA as culture substrate. After long-term culture of hUCMSCs on TCP, the percentage of aged hUCMSCs, as indicated by the expression levels of SA-β-gal, reached 20 %. In contrast, after long-term culture of hUCMSCs on PAA gel, the percentage of senescent cells was only 10 %. An increase in the expression of four times vimentin, which is overexpressed in senescence fibroblasts, was seen in TCP-cultured hUCMSCs compared to those which were cultured on PAA, shown by the immunofluorescence analysis. Moreover, the accumulation of the two of nuclear envelope-associated proteins, including lamin A and lamin B1, was analyzed. As it was expected the expression of lamin B was decreased and the expression of lamin A was increased for TCP-cultured hUCMSCs compared to PAA-cultured hUCMSCs. These findings recommend that culturing on PAA with low stiffness, results in delayed senescence, increased DT, and enhanced CPD.More recently, another research group conducted a study the impact of culture substrate stiffness on the aging of BMMSCs [186]. They prepared PEG hydrogels with 30 kPa and 100 kPa stiffness. When BMSCs were cultured on the 30 kPa PEG hydrogel, there was a higher secretion of regenerative factors compared to when they were cultured on the 100 kPa PEG hydrogel or TCP. However, the microscopic images showed that the cell yield was the lowest on 30 kPa PEG hydrogel and was the highest on the 100 kPa PEG hydrogel. For that reason, the 100 kPa PEG hydrogel and TCP were chosen to further study on the impact of culture substrate stiffness in delaying the aging on BM-MSCs. In addition, SA-β-gal staining results indicated that after the continuous culturing of BM-MSCs on TCP and transferring the cells to either TCP or 100 kPa PEG hydrogel, the percentage of senescent cells increased from 5 % to 25 %, from P1 to P3. However, when the cells were cultured on the 100 kPa PEG hydrogel, this percentage remained lower than 5 % from P1 to P3. It can be concluded that culturing the MSCs on the hydrogels, which have the lower stiffness than TCP, results in more differentiation property, more preserved self-renewal capacity, higher therapeutic efficiency, and delayed senescence.

5.3. 3D-culture with decellularized ECM (dECM)

ECM, a 3D network of glycoproteins and proteoglycans, plays a crucial role in regulating various cellular processes such as cell proliferation, differentiation, migration, autophagy, and angiogenesis. This network can be derived from native tissues or organs, as well as regenerated tissues or organs constructed from cultured cells. Previous studies have shown that culturing MSCs on dECM can enhance their differentiation [187], increase their proliferation [188], and promote their migration [189]. In a recent study, a research group investigated the effects of culturing BM-MSCs on dECM derived from cultured femoral marrow cells on the aging process of MSCs [190]. BM-MSCs were obtained from young and old mice (Y-MSCs and O-MSCs) and cultured on plastic. After 7 days, the colony-forming unit of osteoblasts (CFUOB) in O-MSCs was lower than that in Y-MSCs. While, when O-MSCs were cultured on dECM, the CFUOB was restored. Additionally, the telomerase activity was higher in Y-MSCs and O-MSCs which were cultured on dECM compared to those which were cultured on plastic. This trend was also observed in ATP levels. Since telomerase activity is associated with the lifespan of MSCs and ATP levels are correlated with the number of highly functional stem cells [191,192], it can be concluded that culturing aged MSCs on dECM can reverse their aging process. In vivo experiments showed that after subcutaneous injection of BM-MSCs to into the dorsal surface of immune-deficient mic, those injected with O-MSCs cultured on plastic showed little to no bone generation. However, O-MSCs and Y-MSCs cultured on dECM formed 2–3 times more bone compared to Y-MSCs cultured on plastic. These results suggest that dECM has the ability to rejuvenate aged MSCs. Therefore, Zhou et al. studied the underlying mechanism by which dECM exhibits its anti-aging effect [193]. First dECM was derived from UCMSCs. UCMSCs were cultured on dECM or standard tissue culture polystyrene (TCPS). Cell density was higher in UCMSCs which were cultured on dECM than that in UCMSCs which were cultures on TCPS. In addition, dECM cultured UCMSCs exhibited spindle like morphology, while TCPS cultured UCMSCs exhibited flattened shape. Then after, the premature senescence was induced by H_2O_2 and cell proliferation was decreased in a dose-dependent manner. Furthermore, level of SA-β-Gal positive cells were lower in dECM group compared to TCPS group. In order to investigate the underlying mechanism by which culturing UCMSCs on dECM, reverses the aging of MSCs, silent information regulator type1 (SIRT1)-dependent signaling pathway, the levels of $p16^{INK4\alpha}$, and p53 level were examined. SIRT1 has been reported to delay the premature oxidative stress induced cellular senescence [139]. In this study after exposure to H_2O_2, the level of SIRT1 was decreased. But culturing UCMSCs on dECM kept the level of SIRT1, unchanged. Moreover, after inducing senescence, the UCMSCs were treated with nicotinamide which is an inhibitor

of SIRT1. This treatment led to enhancement of SA-β-Gal positive cells in UCMSCs which were cultured on dECM. These observations confirm that dECM reversed the aging by activation of the SIRT1 signaling pathway. Also, p53, which is a stress induced protein kinase, was attenuated in dECM group, but not in TCPS group. The level of $p16^{INK4\alpha}$, a cell cycle regulator, was increased after H_2O_2 treatment, and decreased in the dECM cultured UCMSCs. All together, these studies suggest that dECM can reverse the aging process in the MSCs and stimulate their differentiation. SIRT1 pathway, p53, and $p16^{INK4\alpha}$ are likely to play significant role in reversing the premature aging by dECM.

5.4. 3D-culture with micro carriers

Micro carriers (MC) are 3D microspheres which possess high surface area due to their small size. The large surface area of MC makes them a potential choice for the growth of anchorage-dependent cells. Moreover, the cell yield is higher than 2D-culture because of the larger culture area and better control of the environmental conditions in MC culture. There is no need to trypsinization, when the cells are cultured on MC, making them more cost-effective compared to 2D-culture [194,195]. Also, culturing the cells on MC has improved cell attachment [196], and cell expansion [195]. Moreover, MC culture offers a beneficial process for the large-scale production of MSCs under adherent conditions [197]. In a study the effect of MC culturing on the senescence of hUCMSCs was investigated [198]. hUCMSCs were cultured in the serum free medium (planar culture) or in the mixture of bioreactor, serum free medium and MC (MC–bioreactor culture). Cell population-doubling level was 1.44 times higher in the MC–bioreactor culture compared to planar culture, indicating the positive effect of MC on the cell proliferation. The hUMSCs amplified by MC–bioreactor exhibited good cell adhesion. The expression level of SA-β-Gal and the level of ROS in the hUMSCs which were cultured in the MC–bioreactor condition, were lower compared to those which were cultured in planar condition. This indicates that MC culturing delays the senescence of hUMSCs.

5.5. 3D-culture with spheroids

Spheroid is a type of 3D cell culture system which self-aggregate into sphere-like constructions during cell proliferation. This allows for better cell interaction with other cells and the ECM, creating an environment that closely resembles the natural physiological environment [199]. A research group, prepared MSCs spheroids by hanging drop method [200]. Their results showed that MSCs which were cultured in 3D spheroid systems, exhibited twice higher colony formation efficiency compared to 2D culture. Moreover, the differentiation property of MSCs was increased when they were cultured in 3D spheroid culture system. The expression levels of Oct4, Sox2, Nanog and TERT, which are pluripotent genes, were increased in the MSCs which were cultured in the spheroid system, compared to 2D culture. In a more detailed study, the 3D spheroid culture system and 2D culture were compared in terms of morphology, stemness related genes, and aging related genes [201]. After 28 days of culture, the cell viability of ADMSCs were significantly higher in 3D spheroid culture system compared to 2D culture. The SOX2, a stemness associated protein, was over expressed up to 28 days, in 3D cultured-ADMSCs compared to the 2D-cultured ADMSCs. Additionally, the expression levels of stemness related genes such as SOX2, POU5F1 and NANOG, were elevated at day 14 and day 28, in the 3D cultured-ADMSCs compared to the 2D-cultured ADMSCs. Sirtunin1, a marker of anti-aging, was over expressed in the 3D-cultured cells at day 14 and day 28. This was in consistent with the down regulation of p16INK4a, which is a marker of senescence. High levels of ROS in the stem cells lead to the apoptosis [202]. ROS also promotes lipid peroxidation which in turns lead to formation of 4-hydroxynoneal (HNE) and

Table 1
Clinical trials performed in terms of aging prevention.

Study title	Objective	Participants	Methodology	Outcomes	National Clinical Trial number
Micro needle versus fractional CO_2 laser for skin aging treatment	Compare micro needling and fractional CO_2 laser methods for administering ADMSCs secretome	30 females aged 35–59 with facial aging symptoms	Single-blind, randomized clinical trial; initial skin evaluation; treatments administered in a split-face mode; topical application of ADMSCs secretome; final evaluation after 6 weeks.	Skin condition, satisfaction, adverse events, and treatment preference are assessed.	NCT05508191
Mesenchymal stem cell-derived exosomes in skin rejuvenation	Explore the role of exosomes in skin rejuvenation	Not specified	Examination of the impact of exosomes on collagen production and oxidative stress inhibition; focus on WNT/β-catenin signaling pathway for skin regeneration.	Aim to slow down skin aging through the use of exosomes.	NCT05813379
Safety study of bone marrow derived stem cells on patients with cutaneous photo aging	Consider safety and efficacy of allogeneic BM-MSCs for skin photo damage treatment	Not specified	Phase I/II open-label study; MSCs derived from healthy donors; intravenous injection to patients with significant photo damage; assessment the safety and the efficacy.	The safety and advantages of MSCs in skin rejuvenation will be determined.	NCT01771679
Skin rejuvenation using stem cell secretome	Explore the impact of umbilical cord explants and MSC secretome on skin rejuvenation	Healthy women participants	Pilot clinical trial; secretome injections in hands, face, and neck; outcomes measured using various rating scales and histologic examination; photographs taken for assessment; SPSS software used for analysis.	Assessment of skin rejuvenation effects after 12 months.	NCT06217627

malondialdehyde (MDA). These two by products cause cell death [203]. However class 3 aldehyde dehydrogenases (ALDH3) convert MDA and HNE, to non-toxic carboxylic acids [14]. In this study, when ADMSCs were cultured in spheroid system, the amount of endogenous ROS was up to 4 folds lower than 2D-cultured ADMSCs on day 28. on the other hand, the amount of ALDH3 was increased in the 3D-cultured ADMSCs compared to 2D-cultured ADMSCs.-Collectively, these experiments demonstrate that utilizing 3D spheroid system as culture substrate for MSCs, leads to higher colony formation, better differentiation ability, increased expression of pluripotency genes and stemness associated genes, enhanced cell viability, decreased expression of aging markers, and lower levels of endogen ROS and lipid peroxidation.

6. Clinical trials

Skin aging, is a consequence of oxidative stress which results in ROS accumulation [204]. On the other hand as mentioned in previous parts, WNT/β-catenin signaling pathway induces ROS production and consequent MSCs senescence [205]. This signaling pathway is included in the skin aging [206]. Accordingly, any procedure which can prevent or treat the skin aging is a potential approach for inhibition or reversing the MSCs aging. Table 1 indicates the clinical trials in which MSCS or their secretome have been used for skin aging treatments.

7. Challenges and limitations

Despite noteworthy advances in optimizing MSC immunomodulation and mitigating aging, several limitations hinder their clinical translation. MSCs derived from various tissues differ in terms of function, aging profile, differentiation ability, and immunomodulatory properties [207–209]. This variability complicates the standardization. On the other hand, age, gender, breed, and genetic characteristics of donors impact MSC quality and functionality, raising reproducibility concerns [210,211]. Moreover, autologous MSCs induce minimum immune response but are costly and time-consuming and allogeneic MSCs induce high immune responses which leads to their disappearance after transplantation [212,213].

One major obstacle hindering the clinical use of MSCs is their senescence during long cultures. Furthermore, strategies aimed at impeding the aging process in MSCs, such as 3D culture systems and pretreatment with small molecules or proteins, can significantly increase production costs and create logistical hurdles for large-scale clinical use. In addition, while preconditioning may prevent MSC aging, its potential long-term effects on genomic stability and tumorigenicity have not yet been studied. An other challenge is that inflammation micro environment has been reported to induce MSC aging [214]. Therefore, pretreatment of MSCs with pro-inflammatory cytokines enhance immunomodulation but may accelerate senescence.

Accordingly, most of the studies are performed in vitro, with no clinical trial utilizing MSC aging-delaying strategies in vivo. Thus, while novel approaches have shown promise in enhancing MSC function and longevity, overcoming the mentioned issues remains critical to harnessing their full therapeutic potential in clinical settings.

8. Conclusion

MSCs have been utilized for the treatment of various types of diseases as well as for tissue regeneration. However, their aging during long cultures is a bottleneck for cell therapy, due to the development of SASP in aged MSCs which leads to changes in their features and therapeutic efficiency. Researchers have recognized several factors that contribute to MSCs aging including loss of proteostasis, telomere malfunction, DNA damage, epigenetic modifications, mitochondrial failure, ROS accumulation, and various signaling path ways such as mTOR, p38/MAPK, and WNT/β-catenin. Conversely, they have identified anti-aging signaling pathways such as Notch, SIRT1, and FAK-Akt. To combat MSsC aging pre-conditioning with drugs, small molecules, proteins, and inhibitors, has been found to inhibit the signaling pathways that promote MSC senescence or stimulate the activation of anti-aging signaling pathways. Furthermore, culturing MSCs in 3D culture systems such as scaffolds, hydrogels, micro carriers, spheroids, and decellularized extracellular matrix, has been found to reverse the aging process of MSCs. On the other hand, MSCs are a potential candidate for autoimmune diseases, degenerative diseases, and other aging-related diseases, due to their immunomodulatory properties. Pre-treatment of MSCs with cytokines and immune receptors, improved culture condition, hypoxia preconditioning, and autophagy regulation have been reported to decrease pro-inflammatory cytokines, and increase immune suppressive proteins leading to improved immunomodulatory effects of MSCs which in turn results in more efficient treatment of tissue fibrosis, allergic diseases and other immune-related diseases. Looking at it in its entirety, optimizing on culture conditions augment immunomodulation properties of MSCS and overcome their aging process, leading to more potent cell-therapy approaches for clinical use.

Ethical statement

This study was conducted under the ethics code 68978 from Tabriz University of Medical Sciences.

Authors' contributions

Author contributions are as follows: **Faranak Elmi**: Literature review and Writing the original draft. **Fatemeh Soltanmohammadi:** Literature review and writing the original draft, **Tahura Fayeghi:** Reviewing the original draft and depicting the figures. **Safar Farajnia:** Reviewing and editing original draft. **Effat Alizadeh** Conceptualization, Writing-reviewing and editing the original and final draft. All authors have read the journal's authorship agreement and the manuscript has been reviewed by and approved by all named authors.

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Declaration of competing interest

The authors declare no conflict of interest.

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