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**Chapter**

# Activation and Metabolic Shifting: An Essential Process to Mesenchymal Stromal Cells Function

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## Abstract

To elucidate the basal metabolism of Mesenchymal Stromal Cells (MSCs), as well as knowing how they are activated, can bring important clues to a successful cell-based therapy. Naive MSCs, in their niche, mainly keep the local homeostasis and the pool of tissue stem cells. Once activated, by an injury, MSCs' response leads to a lot of physiological differences in its metabolism that are responsible for its healing process. Since endogenous MSC seems to be ineffective in pathologic and aging conditions, cell-based therapy using MSC is focused on administration of exogenous MSC in patients to exert its healing functions. From quiescent to activated state, this "Metabolic Shifting" of MSC interferes directly in its secretion and cellular-derived particle generation. We will address here the differences between the MSCs activation phases and how they can modify the MSCs metabolism and its function. Moreover, understanding MSC in their niche and its damped function in pathologic and aging processes can improve stem cell-based therapies.

**Keywords:** mesenchymal stromal cell, metabolism, MSC activation, MSC niche, cell therapy

## 1. Introduction

Stem cells research has brought great insight in regenerative medicine. Currently, over 1700 clinical trials are registered at Clinicaltrials.gov (clinicaltrials.gov "mesenchymal stem cell OR mesenchymal stromal cell", August 2022), with ten approved MSC therapies worldwide [1].

Besides efforts to promote standardization of procedures and classifications for MSCs, the translation of promising preclinical results to human clinical trials has not matched full desired effects. Such variability may come from differences among species or source-tissues of MSCs in both *in vivo* or *in vitro* preclinical studies [1].

Initially, MSCs therapeutic potential was associated with engraftment of MSCs into tissues and to a contact-dependent cell communication. Advances in the field

now confirm that paracrine mechanisms are the primary effector of MSCs for tissue regeneration, angiogenesis and modulatory effects on inflammation, apoptosis and fibrosis. These effects may be achieved by the secretion of biologically active molecules by MSC, such as cytokines and chemokines, growth factors, extracellular matrix and extracellular vesicles. Indeed, the use of secreted factors in the medicine and research fields lead to a cell-free approach, which can overcome major adversities found in the use of allogeneic or even autologous MSCs therapy [2, 3].

In fact, for some regenerative approaches, no additional cell is necessary, and nowadays, beyond adult stem cells; there are other stem cells-based products such as: (i) conditioned medium, (ii) concentrated supernatant, (iii) lyophilized secretome, (iv) cellular particles (i.e. exosomes, microvesicles, small body particles), and (v) small regulatory molecules (i.e. lncRNAs, microRNAs, ceRNAs, circRNAs). All together, these approaches are new fields to be explored in stem cell technologies and cellular-based therapies [4, 5].

Several questions can be formulated regarding the MSC paracrine mechanism of repair: How did MSC become so secretive? How is MSC activated to secrete those molecules responsible for its regenerative mechanism? How is MSC in its quiescent state?

Currently, the metabolism and cell activation of MSC has been the focus of study of many researchers worldwide. Recent reports have provided evidence that stem cells have a metabolic/activation signature which is distinct and specific to each tissue to maintain the homeostasis. Regarding therapy, the choice of the MSC origin and thus how it is MSC activated directly regulates therapy performance, since MSC metabolism is crucial to the paracrine effect. MSC activation is also controlled by the microenvironment, for instance, a metabolically activated MSC can interact with other cells in their niches and they are able to sense and to adapt to dietary changes, exercise, aging, epigenetics changes etc. [5, 6].

Thus, in this chapter, we will attempt to elucidate the importance of MSCs activation/metabolism in its therapeutic function. More specifically, we will describe here the impact of MSCs activation in its metabolism and function. In addition, we will discuss how this "Metabolic/activation Shifting" can interfere directly in the MSC secretory function and in its cellular-derived particle generation. Moreover, stem cell dysfunction and disabilities will also be discussed. Hence, understanding these basic steps about naïve and activated MSCs should improve the establishment of new stem cell-based therapies and other associated approaches around MSC technologies, expanding its use and resources for future implementation as a translational and effective therapy.

## 2. Mesenchymal stromal cells definitions

Isolated from a huge number of tissues, MSC has been used in several clinicals trials, despite its basic studies are still ongoing. Since MSC therapy leads to amelioration of pathologic state, it causes a frenesi in clinicals trials and cell-based therapies. This frenesi creates inconsistencies, for instance at MSC's characterization, nomenclature, culture parameters, etc. Lacking the principle of reproductibile and quality control, several works and clinical trials have still been done improperly [7, 8]. How are the MSCs defined? A brief historic event of its discovery may help to elucidate it.

Described in the early 1970 by Friedenstein and colleagues [9], they observed that bone marrow cells, in a cell culture condition, generated attached cells in culture

plates. These cells showed fibroblastic shape that started growing in this condition. Moreover, they observed that these cells induce osteogenesis in an experimental model. In 1990, Caplan first used the name Mesenchymal Stem Cell to describe these cells with differentiation properties, depending on local (niche concept) and genetics factors [10]. In 1999, Pittenger studies had flourished in the MSC area. Pittenger et al. showed the isolation of MSC from human bone marrow, listing some criteria to define them, such as (i) adherent culture cells and (ii) differentiation capabilities under specific stimulation [11].

Then, the mess comes ... everybody, everywhere, every tissue could generate MSC. However each culture condition was different from each other, with different techniques of isolation, with different patterns of characterization [12]. And this confusion is used indiscriminately by some clinicals to sell cell therapies treatment [13]. Placing order to it, in 2006, the International Society for Cellular Therapy (ISCT) defined the minimal criteria to MSC [14]. Those criteria were upgraded in 2019 [15], where ISCT defines:

1. Terminology: MSC means Mesenchymal Stromal Cells. The terms Mesenchymal Stem Cell, Medicinal Signaling Cells, Multipotent Stromal Cells are not recommended. The term "stem" can be used if there is evidence for self-renewal and differentiation properties.

2. Tissue of origin must be described. If the cell is from bone marrow, it will be called bone marrow mesenchymal stromal cell.

3. Mesenchymal stromal cells are used to describe the heterogeneous populations of adherent cells. Characterization using several functional assays to define and exclude some cells must be done. It enrolls RNA analyses of selected genes, immunophenotyping assay, protein analysis of MSC secretome and IFNg activation assays. It requires attention that those assays are to be informed by the intended therapeutic mode of actions.

The most cited MSCs are the Bone marrow-MSC (BM-MSC) and adipose tissue-MSC (AT-MSC). Other sites are also well known such as Umbilical cord MSC (UC-MSC) and Wharton's Jelly MSC (WJ-MSC). Each one has different characterization patterns but all have paracrine effects and immunomodulatory properties, however with different amounts of molecules secreted by each one. For instance, AT-MSC shows a higher pro-angiogenic pattern than BM-MSC and WJ-MSC. WJ-MSC shows an increased expression of inflammatory cytokines and chemokines than BM and AT-MSC [16]. MSC secretome not only includes molecules secreted by them but also extracellular vesicles (EV) productions that reflect in its internal content the same pattern of MSC from origin. For clinicals trials, it's a quite exciting way to treat with MSC without MSC *per se*. In this sense, there are several clinicals trials ongoing using EV from MSC.

But again, regarding the use of EV at clinicals trials, the cell culture protocol standardization, as well as detailed description of isolating methods, requires more attention [13, 17]. For immune regulation capabilities of MSC, ISCT describes assays to standardize the protocols for clinicals trials. Several researchers and groups summarize three assays that must be followed by all clinicals trials: real time PCR of selected gene products, immunophenotyping assays by flow cytometry and secretome assays [18]. In addition, clinicals parameters such as time to administer MSC, dosage,

delivery, homing, fresh or frozen MSC, autologous or allogeneic transplantation, etc., all these can generate different responses for patient's' treatment. Thus, more quality control to clinicals trials must be done [19].

## 3. Healing mechanisms of mesenchymal stromal cells

The physiological and clinical properties of MSCs include not only differentiation potential but also maintenance of tissue homeostasis, immunomodulation, secretion of particles and molecules, and of course, tissue regeneration/healing [20].

Initially, it was believed that MSCs could act directly in the tissue repair and regeneration through migration and engraftment to the site of injury, differentiating into functional local cells and promoting regeneration to the damaged tissue. However, it is now understood that MSCs major effects are promoted largely through secretion of modulatory factors (paracrine activity) and less due to its tissue replacement [21].

In this sense, the ability of regeneration and healing of tissue depends on multiple factors. In the aspect of wound healing, for example, different cell types are involved, including platelets, macrophages, fibroblasts and MSCs. Thus, the balance among proinflammatory M1 macrophages, transformation to anti-inflammatory M2 macrophages and fibroblast extracellular matrix production are crucial to the process of healing. For instance, Adipose-tissue derived MSCs (AT-MSC), as well as its derived exosomes, have been reported to induce M2 macrophage phenotype, modulating the inflammatory process and to enhance the proliferation and migration of fibroblast, contributing to the wound healing process [22].

MSC paracrine signaling can act as anti-inflammatory, anti-fibrotic and pro-angiogenic effects leading to tissue healing and regeneration. In this case, MSCs have been shown to promote accelerated peptic ulcer healing leading to higher proliferative cells population over the ulcer margin, by increasing vascularity in the site of lesion with increased expression of interleukin-10, an anti-inflammatory cytokine, resulting in ulcer healing, such as reepithelization, angiogenesis, and reduced inflammation [23].

Furthermore, this triad process of healing of MSCs based on its anti-inflammatory, anti-fibrotic and pro-angiogenic effects was observed in many studies confirming the pleiotropic effect of these cells during therapeutic process. Briefly, the use of MSCs in ischemic diseases have also been explored. In this scenario, transplantation of MSCs induced angiogenesis with reported differentiation of MSCs into endothelial cells to compose new blood vessels in the infarcted cardiac tissue. Classically, MSCs have been used in Graft versus host disease (GVHD) and autoimmune diseases and have presented decreasing of global inflammatory process with modulation of inflammatory cells (lymphocytes, NK cells, macrophages) and expanded survival or reduced the use of corticoids by transplanted patients [24]. MSCs paracrine secretion of extracellular vesicles or soluble factors may also contribute to angiogenic or immunomodulatory activity in the ischemic heart and brain, even leading to activation of endogenous cardiac stem cells responsible for myocardial regeneration [25].

## 4. Mesenchymal stromal cells at niche

Cellular turnover varies immensely among the human body tissues. Skin and gut epithelia are replenished every 3–5 days. On the other hand, a neuron's lifespan is

huge [26]. This turnover is regulated by stem cells in adult tissues. How these stem cells are *in vivo* and how they keep the homeostasis of tissue is a challenging subject. Despite this, it is known that the MSC and the stem cells live together in specific areas called niches.

A niche is an area of a tissue that provides a specific microenvironment, in which stem cells are present in an undifferentiated, quiescent and self-renewable state. The niche is composed of: (1) a population of stem cells; (2) a population of stromal cells, mainly MSC; (3) an extracellular matrix in which stem cells, stromal cells and molecular cues are embedded; (4) blood vessels support; and (5) neural inputs [27].

The niche is the place where humoral, neuronal, local (paracrine), positional (physical) and metabolic cues interact with each other to regulate stem cell fate [28]. MSC also lives in this environment and has a crucial role in the niche. The cross-talk between stem cells and MSC is very important to both cells. Cells of the niche, mainly MSC, interact with the stem cells to maintain them or promote their differentiation. And tissue homeostasis depends on this balance [27, 29].

The role of MSC in the niche has been studied in recent years. MSC may be the cell that sustains the niche and the cell that keeps the tissue stem cell in the quiescent state. MSC can secrete soluble factors, produce extracellular matrices due to its sensing of the extracellular signals and thus regulate stem cell fate [30].

MSC can be found in every vascularized tissue. Several studies have demonstrated a population of MSC in different tissues, mainly the ones highly vascularized. Following the minimal criteria defined by ISCT, several studies have demonstrated that MSC are the perivascular cells in tissues. Crisan et al. have isolated cells phenotypically positive for pericytes markers (CD146, NG2 and PDGF-Rβ2) from placenta, adipose tissue, pancreas and skeletal muscle and when cultured these cells shown MSC patterns [31]. Not only microvascular pericytes have been described to be the MSC origin cell but also adventitial perivascular cells [32].

Are the *in vitro* MSC the *in vivo* pericytes? Some authors state that MSCs are cell culture artifacts [33]. They disagreed that MSCs are pericyte because since our body is extremely vascularized thus the MSC population should be huge enough to guarantee efficient repair after injury. However our regeneration is not so efficient. In this sense, it has been shown that pericytes *in vitro* generate a cell similar to MSC, but *in vivo* all the pericyte functions may not release them to act as MSC [17]. In addition, if all MSC should be pericytes *in vivo*, then all MSC *in vitro* should be the same, and they are not. MSC from adipose tissue differs phenotypically (CD markers, secretion of molecules, etc.) from bone marrow-MSC, that differs from Wharton Jelly MSC, that differs from cord blood MSC etc. However, there is a hypothesis of an imprinting of tissue source on MSC properties that make tissue-MSC differs from each other [34]. All these opposite points of view show that the search for MSC *in vivo* continues.

Of note, all the knowledge on the MSC field achieved until now is obtained from cultured cells, expanded ex-vivo. In addition, in a plastic dish, MSC is not a pure population. The isolation methods and expansion in culture conditions did not exclude other cells from rising together. They are a heterogenous population in these conditions. Single cell RNA sequencing studies demonstrate that MSCs are heterogeneous and moreover MSC from different sites differs from each other [34–36].

Since most clinicals trials have been using ex-vivo expanded MSC and showing mild positive results *in vivo*, some researchers claim that the stimulation of the niche and their endogenous MSC should be a better option than administered exogenous MSC [30, 37, 38]. Thus, knowing how a niche works and how to properly stimulate it may result in better clinicals outcomes.

Hypoxic areas in the niches are common. At the bone marrow niche, the concentration of O2 is near 3%. Indeed, tissue O2 concentration may vary from 1 to 5% [39]. Several studies in rodents models as well as with human BM-MSC have demonstrated that a hypoxic condition increased osteogenic capabilities [40], increase the expression of pro-angiogenic factors [41], enhance MSC immunosuppression profile [42], maintain genomic stability [43], etc.

Since hypoxia has a huge effect on MSC metabolism, it is clear that energy metabolism can also be linked to MSC cross-talk to stem cells or its stemness. Several works have been studying the energy metabolic process at MSC. The homeostasis state of MSC can be regulated by metabolic signals leading to its stemness of MSC as described by Sun et al. [44]. They show that low levels of sodium lactate, upregulation of glycolysis, both induced by lysine demethylase 6B (KDM6B), can maintain MSC stemness. Indeed, energy metabolism is extremely important in the activation/differentiation of MSC [45]. At the pathological stage, glucose, fatty acid, and amino acid metabolism are altered at MSC. If those pathways could be restored, tissular homeostasis can also be restored [46].

Extracellular matrices (ECM) can also be regulated by MSC. Beyond the structural scaffold, ECM is an acellular 3D structure that is in close contact with the cells. ECM is composed of several proteins (mainly collagen and elastin), glycosaminoglycans and proteoglycans. ECM participates in cell adhesion and in signaling through mimicking several receptors. In addition, mechanical patterns of ECM can also interfere in cell response, such as stiffness [47, 48]. During injury, ECM can be remodeled. Stromal cells, including MSC, secrete more ECM to reconstruction, helping other cells to migrate to this injury site. We will exploit it below regarding MSC secretome.

## 5. Metabolically activated mesenchymal stromal cells

### 5.1 The MSC secretome

The MSC secretome is composed of a soluble fraction of bioactive molecules (cytokines, chemokines and growth factors) and particles (extracellular vesicles and exosomes, responsible for the delivery of microRNAs and proteins) with several regulatory effects such as (1) anti-inflammatory; (2) pro-angiogenic; (3) stimulation of endogenous progenitor cells; (4) anti-apoptotic; (5) anti-fibrotic; and (6) anti-oxidant [49]. In addition, there is secretion of extracellular vesicles (exosomes, microvesicles and apoptotic bodies). Inside these vesicles, there are a pool of active molecules (enzymes, receptors, cytokines, chemokines, miRNA, DNA) that can perform the same function of its mother cells (See **Figure 1**) [50, 51].

The whole MSC secretome, which is composed of proteins, nucleic acids, lipids, carbohydrates and extracellular vesicles can also be obtained from MSC-derived conditioned medium (MSC-CM). The soluble component of the secretome and their extracellular vesicles may be then separated with the use of specific methodologies as centrifugation, filtration and chromatography [2]. MSC-CM and extracellular vesicles are enriched with various regulatory components, including transforming factor-β (TGF- β), hepatic growth factor (HGF), indoleamine 2,3-dioxygenase-1 (IDO-1), prostaglandin E2 (PGE2), interleukin (IL)-10, IL-1 receptor agonist (IL-1Ra) and others. Thus, the exposure of different cells to MSC-CM or extracellular vesicles induces different responses depending on the secreted factor available [52].
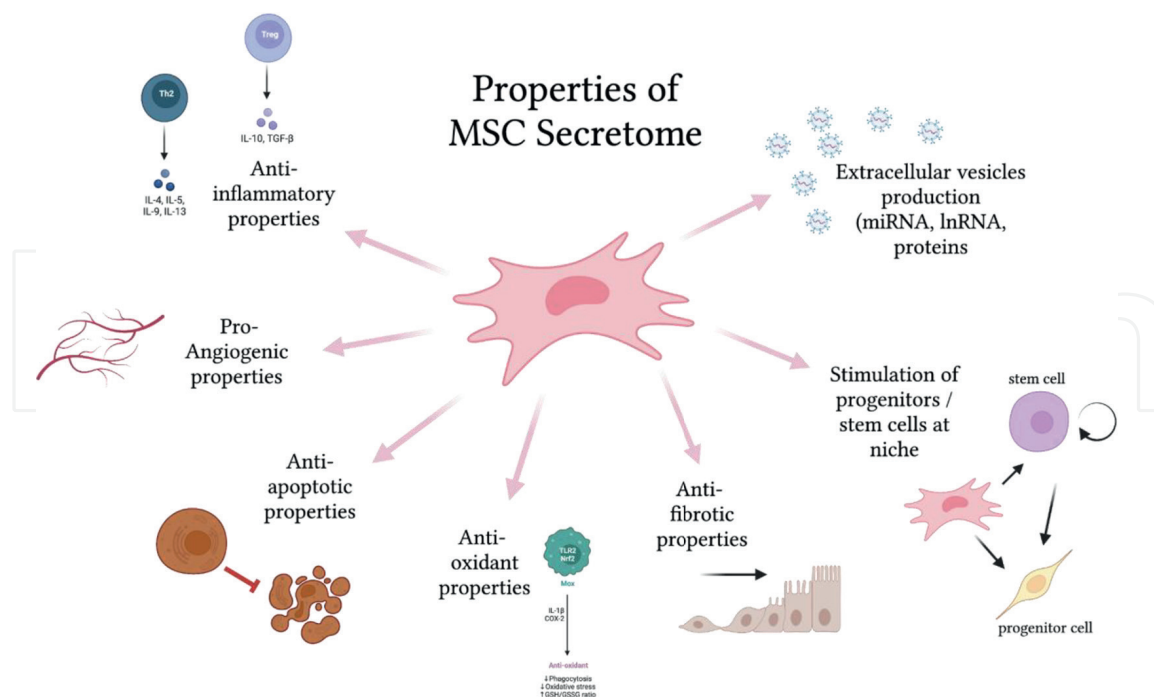
**Figure 1.**
*The MSC secretome. Different molecules are secreted by MSCs. Some of them may induce modulation of immune response, by the expression of cytokines and chemokines to act as anti-inflammatory. It mainly occurs by induction of lymphocytes T helper 2 (TH2) and T regulatory cells (Treg). Molecules secreted by MSC can also be pro-angiogenic, anti-apoptotic, antioxidant and anti-fibrotic. Furthermore, MSCs secretome promotes the stimulation of other stem cells at niche. Extracellular vesicles, also considered part of MSC secretome, contain several molecules and RNA (miRNA, lnRNA) and proteins that may act over other cells through interaction with surface receptors or entering the contact with neighboring cells. IL, interleukin; TGF- β, transforming factor-β; COX-2, Cyclooxygenase-2; GSH, glutathione; GSSG, glutathione disulfide; miRNA, MicroRNA; lnRNA, long noncoding RNA; Th2, T helper 2; Treg, regulatory T cells; TLR2, toll-like receptor 2; Nrf2, nuclear factor erythroid 2-related factor 2; and Mox, oxidized phospholipids-activated macrophages phenotype. Created with BioRender.com.*

As appointed by Filidou et al., the anti-inflammatory, anti-fibrotic and tissue regeneration properties of MSC-CM promote *in vivo* and *in vitro* beneficial effects on different disease models that, in general, damages the tissues. Specifically, the use of AdMSC-CM resulted in reduced expression of inflammatory chemokines and cytokines in human pulmonary subepithelial myofibroblasts in response to exposure to IL-1α and Tumor Necrosis Factor-α (TNF-α) and also to TGF-β-induced fibrotic responses in these cells. In addition, the authors showed a reduction in chemotaxis (CCL and CXCL), inflammatory (IL-1α) and fibrotic (collagen Type III) molecules mRNA and protein expression by CM derived from human AT-MSC [53]. Furthermore, an experimental model of preeclampsia induced by bacterial lipopolysaccharide (LPS) showed that human placenta-derived MSC-CM reduced expression levels of TNF-α and IL-6 in the mice placenta, while also reducing the expression of the anti-angiogenic factor sFlt-1 [54].

## 5.2 MSCs extracellular vesicles

The use of MSC of extracellular vesicles (MSC-EVs) has attracted attention for its ability to promote beneficial effects even when MSC itself is not present [55]. MSC biological characteristics may compromise its use as a therapeutic agent. MSCs proliferation decreases over culture passages, studies report concerns about increased tumorigenicity and the uncertainty of MSCs fate after venous injection calls attention to weak points of such therapeutic strategy [56].

MSC-EVs are classified according to their size, which ranges from apoptotic bodies (> 1000 nm), to microvesicles (100–1000 nm) and exosomes (30–200) [57]. Up to date, 45 MSC-EVs clinical trials are registered in Clinicaltrials.gov [clinical-trials.gov "(mesenchymal stromal cells OR mesenchymal stem cells) AND (extra-cellular vesicle OR exosome OR microvesicle)", October 2022] of which 5 studies are currently at phase 3, including therapeutic approaches to rhinitis pigmentosa (NCT05413148), SARS-CoV-2 infection and acute respiratory distress syndrome (NCT05216562, NCT05354141), diabetes mellitus type 1 (NCT02138331) and stroke (NCT01716481).

The MSC-EVs content vary depending on the derived cell, microenvironment and physiological conditions, thus can be modulated by preconditioning methods, but are known to contain molecules such as messenger RNA, microRNAs, others regulatory RNAs (i.e., lncRNAs, microRNAs, ceRNAs, and circRNAs), enzymes, receptors, cytokines, chemokines and growth factors. Once released to the extracellular environment from the donor cell, MSC-EVs can be internalized by another cell via endocytosis or trigger responses through receptor-ligand interaction acting as a paracrine and endocrine agent. Furthermore, these MSC-derived EVs are capable of homing to injured tissue, having immunosuppressive effects or others similar to those promoted by transplanted MSCs [57].

MSC-EVs can be used in almost all therapy conditions that native MSCs are used or predicted to be; for instance, the MSC-derived exosomes were utilized in wound healing and was verified the promotion of collagen synthesis and proliferation and migration of fibroblasts and keratinocytes, important cells in the mechanisms of wound regeneration. Furthermore, it was detected that these effects are, greatly in part, promoted by microRNA in the exosomes. The therapeutic effects of microRNA derived from MSC-exosomes was widely reported in several studies showing benefits in the treatment of chronic skin ulcers, bone repair, promoting the immunomodulation in favor of inflammation resolution, improving angiogenesis, neurogenesis, macrophage polarization and limiting cardiac fibroblast proliferation, and improving tissue function after ischemia-reperfusion injury [55].

Using AT-MSC-derived exosomes Heo and Kim [58] reported a reduction in the gene expression of pro-inflammatory molecules as TNF-α, IL-6 and IL-8 which were induced by LPS in the THP-1 cell line, while the expression levels of anti-inflammatory CD163, ARG1, CD206, TGF-β1 and IL-10 were shown to be increased in the LPS + exosomes group. The treatment of human umbilical vein endothelial cells (HUVECs) with AT-MSC-derived exosomes increased the proliferation of HUVECs and gene expression level of pro-angiogenic genes like angiopoietin1 and flk1, while reducing the expression of those with detrimental vascular function as vasohibin-1 and thrombospondin-1. Remarkably, the expression of miR-132 and miR-146a were found increased in exosome-treated HUVECs, and these microRNAs bound to the anti-angiogenic genes thrombospondin-1 and vasohibin-1, respectively [58].

Furthermore, a study aiming to elucidate the role of MSC-EVs in mitochondrial damage showed a reversion of mitochondrial DNA deletion to the treated group that was not observed in injured renal tubular cells. Utilizing an *in vivo* model of acute kidney injury, the authors observed the same effect through up-regulation of mitochondrial factor A pathway activity. These findings suggest that MSC-EVs therapeutic effects can also be related to improvement of mitochondrial function in diverse diseases in addition to its role as anti-inflammatory, antioxidant and anti-apoptotic as observed in many other injury models [59].

Finally, use of MSC-EV are promisor therapies that comprehends the major effects attributed to MSC secretome, promoting desired improvements in regeneration and immunomodulation as that offered by paracrine effects credited to MSCs.

## 5.3 Activation signaling and pre-conditioning

The paracrine effect of MSC is highly dependent on the microenvironment around MSCs. The MSCs have some sensors receptors (i.e., TLRs, AhRs, TNFRs, and IFNRs) which act as an "antenna" that captures external signals that drive a special cellular effect. In contrast, in the absence of stimuli the MSCs show little to no expression of molecules responsible for their function, for instance, the immunomodulatory profile, such as the expression of human leukocyte antigen (HLA)-I and intercellular adhesion molecule-1 (ICAM-1).

The production of molecules from MSC secretome can be stimulated by the presence of inflammatory components that induce an immunomodulatory pheno-type on MSCs [60]. The MSCs preconditioning with inflammatory factors such as IL-1β and interferon gamma (IFN-γ) result in augmented production of modulatory components by MSCs which can influence and regulate other cell types, such as macrophages, to acquire a regulatory phenotype [61]. Hence, exposure of MSCs to an inflammatory environment, containing for example IFN-γ and TNF-α cytokines, induces MSCs to start the production of specific molecules which will play a role as immunoregulators [62].

TNF-α is one of the first secreted cytokines during an inflammatory event. TNF-α binds to two distinct receptors, TNFR1 and TNFR2. While TNFR1 is expressed ubiquitously, few cellular populations express TNFR2, including immune cells and MSCs. In MSCs, TNFα/TNFR2 interaction promotes the expression or secretion of pro-angiogenic and cytoprotective mediators. Beldi et al. investigated the role of TNFR2 in MSCs and found that in comparison to TNFR2+ wild type MSCs, MSCs lacking TNFR2 were less immunosuppressive to CD4 and CD8 T cells when reducing cellular proliferation and cytokines production in T cells. Furthermore, while TNF-α stimuli did not result in increased expression of early HLA-I, MSC exposure to IFN-γ increased expression of HLA-I, an indicator of MSC activation [63].

Regarding the MSCs-EV, preconditioning may also be expected to happen. In fact, cultures of PBMCs in presence of MSC-derived exosomes preconditioned with TNF-α and IFN-γ, resulted in cytokines shifting: 34 inflammatory cytokines and chemokines were found to be downregulated and several anti-inflammatory, as IL-10, were upregulated. Moreover, preconditioning of MSC-exosomes with atorvastatin enhanced angiogenesis when compared to non-pretreated MSCs in myocardial infarction injury; and also TNF-α preconditioning of adipose tissue MSCs promoted higher osteoblast differentiation upon exosome treatment [64, 65].

Although showing interesting results during preclinical in *vivo/in vitro* studies, the preconditioning of MSCs is still performed with human and non-human recombinant factors with lack of consistency at human clinical trials. To overcome this, the use of fresh human derived products can be an effective resource when we take in mind the use of preconditioning on the clinical scale. Thus, platelets or platelet-rich plasma have been proposed as a beneficial enhancer to therapeutic properties of MSCs. These platelets or platelet-rich plasma medium stimulates proliferation of MSCs and offer protection against oxidative stress, mainly due to the release of growth factors that exerts beneficial effects on MSCs. Further, the transfer of platelets mitochondria

to MSCs stimulates wound-healing activity. And, the incubation of MSCs with full functional platelets, but not with dysfunctional mitochondria platelets, resulted in increased expression of pro-angiogenic genes [66].

Moreover, other similar approaches aiming to control extrinsic factors in MSCs modulation are available. Considering these aspects, some MSCs variability to its activity is found in response to (i) source or location, that is, Bone marrow-derived MSCs or Adipose tissue-derived MSCs, (ii) passage number in culture, and (iii) oxygen concentration and presence of different compounds in the environment, such as pharmacological agents. These extrinsic factors are useful methods of preconditioning MSCs and can be used to improve its therapeutic potential regulating the secretory MSCs profile. These effects can be reached using the hypoxic environment of cell culture, inflammatory cytokines, pharmacological compounds, and 3D cell culture models [60].

Finally, an interesting cell culture method of 3-dimensional culture can be used as a preconditioning factor as well. In this culture method, the physiological conditions seen as in the *in vivo* cell environment are replicated, as the spheroid 3D culture promotes a physiological-like environment, like those found in MSCs niche. In this spheroid culture, internal cells receive lower oxygen levels than MSCs in the surface of the 3D structure, creating a hypoxic environment. These spheroid cultured MSCs presented an increase in cytoprotective factors and enhanced proliferation, with increased immunomodulatory factors expression, along with elevated angiogenic, anti-fibrotic and anti-apoptotic activity [67].

## 6. Mesenchymal stromal cell dysfunction

Our knowledge on MSC is focused on how healthy MSC responds to an injury, by secreting several molecules, trying to rebuild the tissue homeostasis. At cellular therapy, healthy exogenous MSCs are administered to patients and in response to the injury, this exerts its regenerative role and helps heal the damage.

However, there are several conditions that can damp MSC capabilities of healing *in situ*. Autologous transplantations of MSC have mild results in clinical trials despite animal models generated great results [68]. Allogeneic transplantation of MSCs seems to have better outcomes. Moreover, the functional decline of MSCs has been associated with a pathophysiological driver of several diseases and aging [69].

Regenerative properties of endogenous MSC can be decreased *in vivo* and *in vitro*. Aging, metabolic changes due to pathologies and epigenetics changes can interfere at MSC *in vivo*. *In vitro*, MSC can be altered by cell culture passages (senescence), by storage at cryogenic conditions, by culture conditions (such as serum deprivation), by cell contact loss, by normoxia, etc. [69–71]. We will further exploit some of the MSC disabilities below. See **Figure 2**.

### 6.1 Aging: epigenetic and PMT at MSC disruption

Aging is a settled multifactorial process. Lopez-Otin has described 9 hallmarks that represent common denominators of aging: (1) genomic instability, (2) telomere attrition, (3) epigenetic alterations, (4) loss of proteostasis, (5) deregulated nutrient-sensing, (6) mitochondrial dysfunction, (7) cellular senescence, (8) stem cell exhaustion, and (9) altered intercellular communication [72]. Herein, we will focus on some of these hallmarks and its impact on MSC.
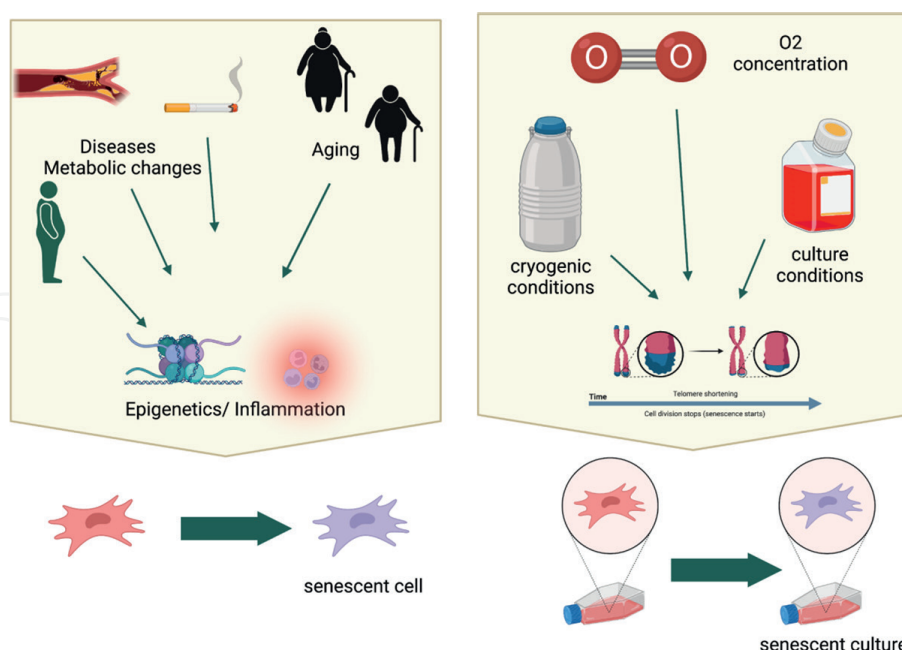
**Figure 2.**
*Senescence In vivo and in vitro: Conditions that lead to MSC senescence. Several factors are correlated with MSC senescence. In vivo, pathologies, metabolic diseases and aging may interfere at epigenetic levels and/or generate chronic inflammation responses that can cause MSC senescence. In vitro, other factors contribute to a senescence culture such as $O_2$ concentration, culture and cryogenic conditions etc. mainly due to telomerase disruption. Created with BioRender.com.*

Aging and age-related diseases have been associated with the higher number of senescent cells in the tissue [73]. In 1995, Dmiri et al. have described the quantification of the amount of beta-galactosidase as a biomarker of senescence. He demonstrated that a higher amount of beta-galactosidase is present at senescent cultured fibroblasts in human cells [74]. Since then, several works have been demonstrating that beta-galactosidase is not a reliable marker, so the search for a biomarker for senescence is still ongoing. It has been demonstrated that p16Ink4a-positive senescent cells accumulate with age in multiple tissues [75]. DNA damage response (DDR) is induced in healthy MSCs leading to the activation of the two main signaling pathways p19*ARF* and p16*INK4A* [73].

Evidence suggests that MSC senescence is a dynamic process driven by epigenetic and genetic changes. Moreover, aging can be impacted by both environmental and inherent factors. Genetics factors are associated with long term mutations in DNA that lead to failure of the replicative state of the cell. Environmental factors that do not change DNA, also affect cell cycle. To date, epigenetics refers to the study of heritable phenotypic alterations linked to differential gene expression when the same DNA sequence is maintained [76]. Epigenetic dysregulation is associated with (1) DNA-based mechanisms: DNA methylation and histone modifications (2) RNA-based mechanisms: noncoding RNAs and RNA modifications [69].

These genetics and epigenetic modifications can interfere directly with MSC by inducing the arrest of cell cycle, by producing a defective ECM niche production and by disrupting the MSC differentiation leading to tissue aberrations evidentiated at aging and disease [69, 76]. Several articles described differential methylation patterns at MSC isolated from young X olders patients. Moreover, these differential methylation patterns were also observed at long term cultures *in vitro* of MSC [77, 78].

Single-cell sequencing analysis of young X elderly BM-MSC have shown that young MSC have higher expression of genes related to tissue regeneration. Moreover, at young BM-MSC there is a cluster of cells that have a lower expression of genes of proliferation, that characterize them as quiescent cells, so stem cells. And these clusters of cells were not observed at elderly BM-MSC [79].

Not only epigenetic modification in DNA but also modifications in protein has huge importance in the differentiation processes. Protein post-transductional modifications (PTM) are protein modifications caused by adding groups of phosphates, acetyl, methyl, etc. in one or multiple amino acids and/or caused by proteolytic cleavage by ubiquitin [80]. These modifications can determine its activity state, localization, turnover, and interactions with other proteins. At MSC, PMT has been associated with differentiation to osteogenic lineage [81] Osteogenic differentiation of BM-MSC has been linked to *O*-GlcNAc cycling to the Runx2-dependent regulation of the early ALP marker [82].

In addition, aging decreases the number of stem cells in the niche, but not only it, aging also affects MSC and stem cell response due to metabolic and epigenetics changes [83]. Muscle stem cells (satellite cells) in aging tend to be converted to a fibroblast lineage instead of myogenic lineage [84, 85]. Several authors have been demonstrating that niche ECM stiffness leads to the aging process, dampening regeneration of the tissue and its homeostasis and moreover, leading to stem cell aging. In central nervous systems (CNS), ECM niche stiffness of oligodendrocyte progenitor cells (OPCs) have been related to aging processes mainly through the mechanoresponsive ion channel Piezo1 [83, 86].

Immunophenotypic profile of MSC can also be affected by senescence. Laschober et al. described that CD295 (leptin receptor or LEPR) have been found to increase during MSC senescence and it correlates with reduced proliferation capacities of MSC [87].

## 6.2 MSC senescence in culture conditions

In culture conditions, long term cultures are not welcome to be used in therapy due to its altered therapeutic profile. These cells became large and flattened ("sunny side up egg" morphology), less proliferative and less responsive. Senescence in culture characterized by the arrest of cell cycle. It is a known issue, as described by Hayclifk in fibroblast cultures [88]. Four types of senescence have been distinguished: replicative senescence (RS), oncogene-induced senescence (OIS), stress-induced premature senescence (SIPS), and developmental senescence [29, 89, 90].

Stress conditions at culture, such as adaptations to 2D culture, O2 concentration, confluency condition, the amount of nutrients even though the exposure to light lead to modifications that cause its arrest in the G0 phase of cell cycle of MSC [91, 92].

Cryopreservation is also a concern regarding MSC stability. Dimethyl sulfoxide (DMSO) has been the gold standard agent for cryobiology. However, the use of DMSO has been associated with *in vitro* toxicity. Since it has been associated with DNA methylation processes, DMSO affects many cellular processes and dysregulation of gene expression [93]. Mol et al. described that fresh culture MSC have a trend to have better outcomes for acute graft versus host disease (GvHD) and tissue injury in hemorrhagic cystitis than freeze-thawed MSC. Fresh MSCs have higher mRNA expression of IDO after 24 h IFNg priming, showing higher immunomodulatory properties than cryopreserved MSC [94].

### 6.3 Inflammation and senescence of MSC

Cycle arrest occurs due to a persistent DNA damage response (DDR) caused by either intrinsic (oxidative damage, telomere attrition, hyperproliferation) or external insults (ultraviolet, γ-irradiation, chemotherapeutic drugs) [95]. The more DNA damage, the more cell death, senescence and tissue dysfunction contributing to aging. Growing evidence has been describing that inflammation can also lead to DNA damage [96].

DNA damage induces the expression of type I interferons and other inflammatory factors [97]. The connection between DNA damage and inflammation is through the cytoplasmic DNA sensing pathway. Micronuclei formations (formed due to DNA damage during mitosis) can stimulate the cell senescence throughout cyclic GMP–AMP synthase (cGAS), a DNA sensor that stimulates STING (stimulator of interferon genes). To prevent undesired inflammation, besides cGAS-STING pathway, there are also the deoxyribonucleases (DNases) in the cytoplasm, digesting excessive DNA, serving as a negative regulator of cytoplasmic DNA. There are two major DNases in the cytoplasm: DNase2α (encoded by *DNaseII*) and TREX1 (originally designated DNaseIII). Intriguingly, both DNases are downregulated in senescent cells, contributing to aberrant cytoplasmic DNA sensing and inflammation [98].

Inflammaging, a term to define a chronic, low-grade sterile inflammation frequently observed during aging [99]. It is a macrophage centered process, involves several tissues and organs, including the gut microbiota, and is characterized by a complex balance between pro- and anti-inflammatory responses [100]. In elderly, the chronic inflammation observed is due to cells in tissue expressing pro-inflammatory cytokines, such as IL-1α, IL-6, TNF, and NF-κB activity and other inflammatory factors [101]. Chronic inflammation during aging and its negative outcome is supported by clinical data in kidney [102], liver [103], lung [104] etc.

Since MSC are perivascular cells and that they have a close connection with circulant factors in blood, it is possible to consider MSC with a central role in inflammaging, together with macrophages [105]. Rejuvenation strategies, such as culturing MSC with serum from older rats and parabiosis, showed a lower proliferation rate and survival of MSC exposed to serum from elderly subjects [106]. Thus, there are circulant molecules/cytokines that can impair MSC functions in aged individuals. Higher amounts of circulant beta-catenin and SMAD3 have been associated with senescence MSC profile [29]. More basic research must be done in this area.

Senescent cells are functional cells. Senescent cells were shown to secrete a range of inflammatory factors, which was termed the 'senescence-associated secretory phenotype' (SASP) [107]. The SASP mediates many of the cell-extrinsic functions of senescent cells. The SASP has its physiologic role: (1) by maintaining the SASP profile of the senescent cell (maintaining cell cycle arrest and SASP expression), (2) by eliciting immune response to generate a senescent cell clearance and (3) by secreting ECM and angiogenic factors leading to tissue regeneration [90, 108]. However, SASP has also deleterious effects by promoting inflammation (leading to inflammaging) and, potentially, tumor progression in neighboring cells. The correlation of SASP and inflammaging is beginning to be investigated using models to detect and eliminate the senescent cell (the INK-ATTAC model) [90, 108]. SAPS at MSC a is also related to higher secretion of extracellular microvesicles in aged subjects, as well its higher amount of microRNA content [109, 110] .

Interestingly, as it was described early in this chapter, MSCs have potent anti-inflammatory functions, whereas senescent MSCs play a pro-inflammatory role

due to SASP, which has been considered a major cause of aged MSCs' detrimental effects [111]. In accordance with this, HMGB1 secreted by senescent fibroblasts is recognized by TLR4, followed by increase in SASP secretion [112]. These findings establish the critical role played by innate immune sensing mechanisms in regulating senescence [91].

### 6.4 Diseases and MSC

At MSC therapy, attention must be done regarding the pathological state of the patients at the harvest of MSC, since aging and pathological diseases can interfere at this isolated MSC. Moreover, when treating the patient, the pathogenic milieu where exogenous MSC is administered requires attention, because it may interfere with the MSC mechanism of action.

Obesity can impact BM-MSC. Ulum et al. described BM-MSC from patients with high body mass index (BMI) are more senescent, have disrupted differentiation to osteogenic and adipogenic cells, and highly expressed endoplasmic reticulum genes related to stress [113]. Diabetes can regulate AT-MSC as described by Abu-Shahba et al. They isolated AT-MSC from diabetic and non-diabetic patients and demonstrated that IL-1b is highly expressed in AT-MSC from diabetic patients [114].

## 7. Conclusion and new perspectives

The knowledge of MSC still requires much more research to elucidate its regenerative properties. More than 30 years of research and yet there is a lot to understand. The search for a better performance in MSCs cultures, the secretome profile, how to stimulate MSC to secrete higher amounts of such molecules using preconditioning techniques or niche stimulation, how MSC acts *in vivo*: a lot of questions with some clues, but far from the right answer.

## Conflict of interest

The authors declare no conflict of interest.

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