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REVIEW

Functional heterogeneity of mesenchymal stem cells from natural niches to culture conditions: implications for further clinical uses

Luis A. Costa[1] · Noemi Eiro[1] · María Fraile[1] · Luis O. Gonzalez[1,2] · Jorge Saá[1] · Pablo Garcia-Portabella[1] · Belén Vega[1] · José Schneider[3] · Francisco J. Vizoso[1]

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Abstract

Mesenchymal stem cells (MSC) are present in all organs and tissues. Several studies have shown the therapeutic potential effect of MSC or their derived products. However, the functional heterogeneity of MSC constitutes an important barrier for transferring these capabilities to the clinic. MSC heterogeneity depends on their origin (biological niche) or the conditions of potential donors (age, diseases or unknown factors). It is accepted that many culture conditions of the artificial niche to which they are subjected, such as O_2 tension, substrate and extracellular matrix cues, inflammatory stimuli or genetic manipulations can influence their resulting phenotype. Therefore, to attain a more personalized and precise medicine, a correct selection of MSC is mandatory, based on their functional potential, as well as the need to integrate all the existing information to achieve an optimal improvement of MSC features in the artificial niche.

Keywords Regenerative medicine · Aging diseases · Diabetes · Lupus · Secretome · Conditioned medium · Extracellular vesicles · Exosomes

Abbreviations

2D	Two-dimensional
3D	Three-dimensional
AD	Adipose-derived
AD-MSC	Adipose-derived mesenchymal stem cell
Ad-FKN	Adenoviral vector fractalkine gene
BM	Bone marrow
BM-MSC	Bone marrow-derived mesenchymal stem cell
bBM-MSC	Bovine bone marrow-derived mesenchymal stem cell
BNDF	Brain-derived neurotrophic factor
CD	Cluster of differentiation
cGMP	Current good manufacturing practice
CIN	Cervical intraepithelial neoplasia
CM	Conditioned medium
CTL	Cytotoxic T lymphocyte
CXCR	C-X-C chemokine receptor
DAMPs	Damage-associated molecular patterns
DNA	Deoxyribonucleic acid
DPSC	Dental pulp stem cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
EVs	Extracellular vesicles
EMA	European Medicines Agency
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
GVHD	Graft-versus-host disease
hAFMSC	Human amniotic fluid mesenchymal stem cell
HGF	Hepatocyte growth factor
hMESC	Human endometrium-derived mesenchymal stem cell
hUCESC	Human uterine cervical stem cells
IDO	Indoleamin2,3-dioxygenase
IFNα	Interferon alpha
IFNβ	Interferon beta
IFNγ	Interferon gamma
IGF	Insulin-like growth factor

Luis A. Costa and Noemi Eiro contributed equally to this work.

✉ Francisco J. Vizoso
investigacion@hospitaldejove.com

[1] Unidad de Investigación, Fundación Hospital de Jove, Avda. Eduardo Castro 161, 33920 Gijón, Asturias, Spain

[2] Department of Anatomical Pathology, Fundación Hospital de Jove, Gijón, Spain

[3] Department of Obstetrics and Gynecology, University of Valladolid, Valladolid, Spain

IL	Interleukin
iNOS	Inducible nitric oxide synthase
KLF4	Kruppel-like Factor 4
LAP	Liver-enriched transcriptional activator protein
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
NK	Natural killer cell
NO	Nitric oxide
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PGE2	Prostaglandin E2
PGN	Peptidoglycan
PIGF	Placenta growth factor
PDLSC	Periodontal ligament stem cell
O_2	Oxygen
RNA	Ribonucleic acid
SCAP	Stem cells from the apical papilla
SHED	Stem cell from human exfoliated deciduous teeth
SWAT	Subcutaneous white adipose tissue
TGFα	Transforming growth factor alpha
TGFβ	Transforming growth factor beta
TIMP	Tissular inhibitor of metalloproteases
TLR	Toll-like receptor
TNF α	Tumor necrosis factor alpha
TRAIL	Tumor necrosis-factor-related apoptosis-inducing ligand
UC	Umbilical cord
UC-MSC	Umbilical cord-derived mesenchymal stem cell
VEGF	Vascular endothelial growth factor
VLC	Vascular leukocytes
VWAT	Visceral white adipose tissue

Introduction

Among the various stem cell types, i.e., hematopoietic, embryonic, mesenchymal, and induced pluripotent, mesenchymal stem cells (MSC) are awakening an extraordinary interest.

The number of studies devoted to them has been increasing exponentially during the last decade [1]. MSC are implicated in basic functions, such as cell differentiation and proliferation, angiogenesis/vasculogenesis, regulation of the inflammatory process or control of oxidative stress [2]. Numerous pre-clinical studies, testing MSC or their secretome-derived products in animal models, have shown to have therapeutic effects on key pathological processes that are associated with alterations of the internal homeostasis [3–7]. In addition, they also exert antimicrobial effects, indicating that MSC possess an immune function independent of the host's immune system [8]. Likewise, mostly human clinical trials in phase I and phase II have confirmed a positive safety profile in a variety of indications including immunological, bone, heart, or neurodegenerative disorders [9] and we have even results from phase III clinical trials in the case of graft-versus-host disease (GVHD), Crohn's disease, myocardial infarction and liver cirrhosis [1]. A remarkable fact in this context is that no serious adverse effects have been reported following MSC transplantation, whereas the contrary has been the case after allogeneic hematopoietic stem cell transplantation, i.e., allergic reactions to cyclophosphamide, fever, infection, nausea, vomiting, elevation of liver enzymes, macrophage activation syndrome and GVHD [10].

2018 was a milestone in the field of MSC therapy with the first European Medical Agency (EMA) marketing approval of an MSC product. The TiGenix-sponsored phase III clinical trial NCT01541579, reported statistically significant improvement of intra-lesional administration of allogeneic expanded adipose (AD) MSC (darvadstrocel, formerly Cx601) in the treatment of complex perianal fistulas in Crohn's disease patients [11]. In September 2018, Mesoblast announced the positive results of its phase III trial (NCT02336230) with this treatment with allogeneic bone marrow (BM) MSC (remestemcel-L) in children with steroid-refractory acute GVHD, which resulted in the preparation of a licence application for use to the Food and Drug Administration (FDA) in the United States.

For all these reasons, 2019 could be the start of the therapeutic era of MSC [12], such as for example therapies based on MSC for acute respiratory distress syndrome (ARDS) associated to COVID-19 [13]. Nevertheless, many questions should be assessed with regard to this novel technology, including donor selection, cell harvesting, expansion and storage. The identification of predictive efficacy stratification biomarkers, the appropriate posology and route of administration for each indication still need to be determined. To consider all these technological aspects, we have to assume the concept of MSC heterogeneity.

This review addresses some aspects of MSC heterogeneity, as well in their natural niches as under culture conditions, one key aspect to keep in mind for future clinical applications (Fig. 1).

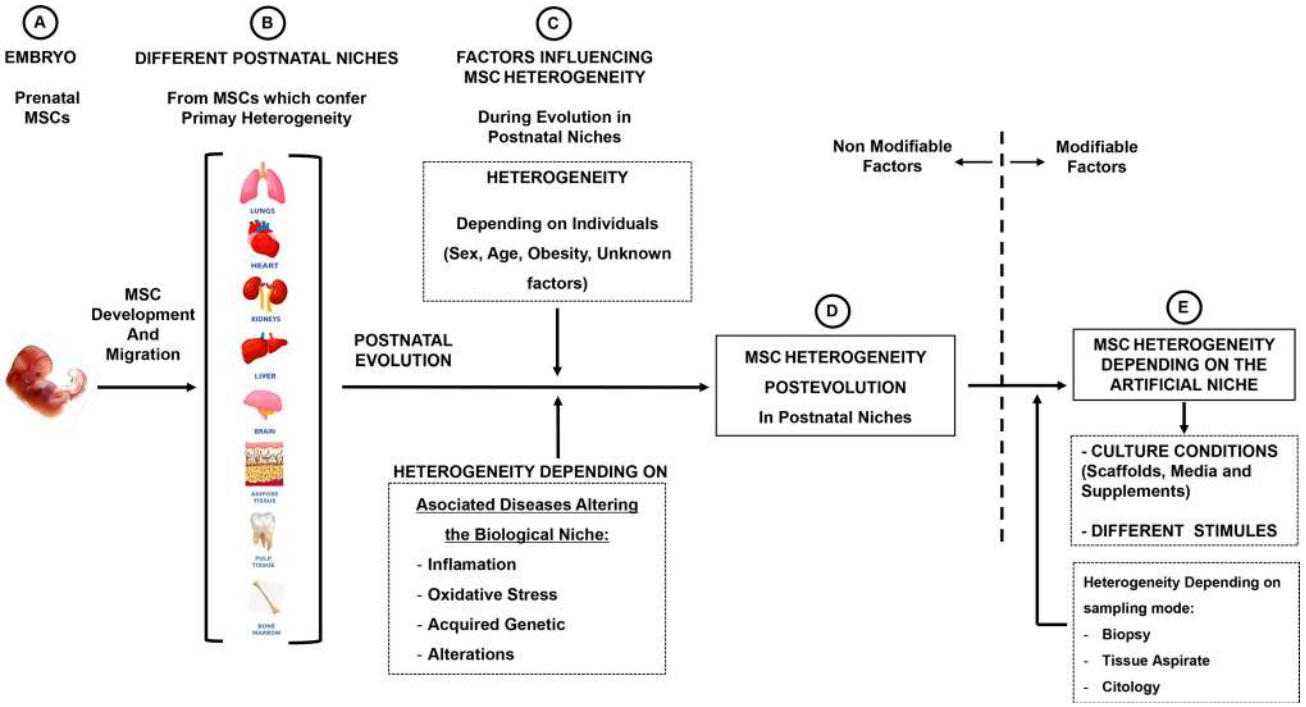

Fig. 1 Schematic representation of MSC heterogeneity in their different niches and factors that could influence their fate

MSC nomenclatures and definition

MSC were first described in the 1950s by the Russian haematologist Friedenstein, as a rare population in the bone marrow [14]. Although in small amounts, MSC have been isolated from numerous organs and tissues [15]. Adipose-derived MSC (AD-MSC) have been the most widely used ones in regenerative medicine, perhaps because of the ease in obtaining them [16, 17]. Compared with the relatively low yield in BM-MSC (from 0.001 to 0.1% in the mononuclear fraction) [18], AD tissue can provide up to 500-fold more MSC than from an equivalent amount of BM aspirates [19].

Morphologically MSC akin to fibroblasts and are capable of differentiating into mesenchymal lineages [20–22]. To refer to mesenchymal-like cells, various nomenclatures are used, such as "mesenchymal stem cells", "mesenchymal stromal cells", "multipotent stromal cells", "marrow stromal cells" and "medicinal signalling cells [23], but the acronym MSC is now generally used to identify this class of cells. The "International Society for Cellular Therapy" established the minimum criteria required for MSC definition in 2006 as follows: (a) plastic-adherent cells when maintained in standard culture conditions; (b) simultaneous expression of CD105, CD73 and CD90, and lack of expression of CD45, CD34, CD14 or CD19, CD79a or CD11b, and HLA-DR surface molecules and (c) capacity to differentiate into osteoblasts, adipocytes and chondroblasts in vitro [22].

Fetal mesenchymal stem cells

Platelet-derived growth factor receptor alpha (PDGFRA) is a main marker of MSC in bone marrow [24, 25]. The expression of PDGFRA is critically important for fetal development, and PDGFRA knockout led to embryonic lethality in mouse [26]. Therefore, there is a large number of PDGFRA positive circulating cells in the embryo that decrease immediately after birth. Circulating MSC have rarely been found in the adult circulatory system [27, 28]. However, MSC are present in umbilical cord blood, indicating that circulating MSC/stromal cells in the fetus originate from fetal blood cells [29–31], which exhibit high differentiation potential in mesenchymal lineages (adipocytes, osteocytes, and chondrocytes) [32]. The importance of the original niche on the potentiality of MSC is reflected by the fact that adipose stem cells of embryonic origin show great multi-lineage potential [33]. However, ethical aspects derived from obtaining them do not allow to exploit their potential.

All these data suggest the existence of a migration, dissemination and functional specification of MSC to different niches during fetal development, which seems to represent the start of progressive MSC heterogeneity along the postnatal lifetime (Fig. 1).

Heterogeneity among MSC from different postnatal niches

Although investigations into the lineage of these cells strongly suggest that progenitor cells of cultured MSC are of perivascular origin [34, 35], it has been also reported that MSC obtained from various sources differ in their biological features [36, 37]. This suggests that MSC heterogeneity mirrors the diversity of environments present in the natural stem cell niches, which are a consequence of the broad cellular communities that have variable chemical and mechanical conditions. In addition, there is evidence that cultured cells retain biological behaviors related to their previous environments [38] (Fig. 1).

The heterogeneity of MSC is reflected through their different modes of action, such as proliferation capacity, transdifferentiation, immunophenotype or by both paracrine and microvesicle mechanisms via secretome-derived products. Differences according to each one of these mechanisms have been report among MSC from different origins.

Proliferation and differentiation

There are many examples of differences in proliferation and differentiation among MSC according to their origin. AD-MSC exhibit greater proliferative capacity than BM-MSC, but are similar in colony-forming efficiency [39]. Cell proliferation is higher for umbilical cord (UC) MSC compared with BM-MSC or AD-MSC [40, 41], or for human uterine cervical stem cells (hUCESC) compared with AD-MSC [42].

With regard to differentiation, greater tendency of BM-MSC and AD-MSC to differentiate toward osteoblasts have been observed, whereas there is a lack of differentiation of UC-MSC toward adipocytes [43, 44]. AD-MSC possess also a higher potential for angiogenesis and vasculogenesis [45], as well as a more powerful immunomodulatory potential than BM-MSC [39]. Instead, UC-MSC have the highest potential for chondrogenic differentiation, with a resulting potential for use in tissue engineering [46]. UC-MSC have a higher percentage of neuron-specific enolase-positive cells than BM-MSC after neuronal induction [41]. Placental-MSC have superior migratory capacity but less adipogenic potential [47–49]. MSC from dental origin such as dental pulp (DPSC), deciduous exfoliated teeth (SHED), apical papilla (SCAP) or periodontal ligament (PDLSC) can express several cell markers and differentiate into functionally active neurons, osteoblasts, chondrocytes or vascular cells [50].

Paracrine mechanisms

MSC secrete a wide range of paracrine factors collectively named as secretome, responsible for up to 80% of their therapeutic effect. This biological pool includes different bioactive factors such as soluble molecules (cytokines, chemokines and growth factors), but also membrane-bound vesicles that contain biomolecules. These extracellular vesicles (EVs) may be classified as: (1) exosomes (40–150 nm in diameter), originating in the endocytic pathway, (2) microparticles (50–1000 nm in diameter), formed by the outward blebbing of the plasma membrane and subsequent release after the proteolytic cleavage of the cytoskeleton, and (3) apoptotic bodies (500–2000 nm in diameter), which are released during the process of programmed cell death. EVs are phospholipid membrane-bound particles secreted from cells that contain biomolecules including growth factors, cytokines, lipids, DNA and various forms of RNAs. EVs represent an intercellular communication pathway which plays major roles in mammalian cells in mechanisms such as the exchange of genetic material and the transfer of biologically active molecules as well as in the defense against viral attacks [51]. EVs interact with recipient cells by way of mechanisms which resemble those involved in viral entry. These include binding to surface receptors in order to trigger signal cascades, internalization of surface-bound EVs, and fusion with the cell to deliver material directly to the cytoplasmic membrane and cytosol [52].

Due to all these bioactive factors, MSC have several biological effects, such as regenerative, proliferative, anti-apoptotic, anti-inflammatory, anti-oxidative stress, pro-angiogenic anti-fibrotic, anti-tumor or anti-microbial activities [15, 50, 53–60].

In particular, MSC-derived exosomes, which are complex vesicles containing a large number of proteins and RNA molecules, have been shown to have beneficial therapeutic effects in various models, including those for cutaneous wound healing, diabetic wound healing, atopic dermatitis, corneal epithelial wound healing, traumatic and degenerative ocular disease, autistic-like behaviors, liver fibrosis, hepatic injury, endotoxin-induced acute lung injury, bronchopulmonary dysplasia, *E. coli*-pneumonia, unspecific lung injury, silica-induced lung fibrosis, osteonecrosis, myocardial infarction, periodontitis or muscle injury [15].

Heterogeneity of secretomes from MSC isolated from different tissues has also been shown. Thus, for example, it has been shown that AD-MSC secrete higher amounts of pro-angiogenic molecules, such as extracellular matrix components and metalloproteinases (MMPs) [40] or vascular endothelial growth factor (VEGF) [61], compared with other MSC such as BM-MSC. This suggests that AD-MSC may be preferred over other MSC populations for augmenting therapeutic approaches dependent upon angiogenesis. Instead, UC-MSC secrete the highest amount of immunomodulatory factors, such as IL-6, -7 and -10 as well as PDGF-AA and TGF-α [40].

Antitumor effects

One of the most heterogeneous effects from MSC according to their origin are those on tumors [62, 63]. Thus, the effects of MSC on tumors are still controversial [64–66]. While it has been shown that MSC are capable of enhancing progression and metastasis of some types of tumor, such as breast cancer and colon cancer [67–71], other studies show that MSC have tumoricidal effects on liver, lung cancer cell lines, and pancreatic tumors in vitro and in vivo [72–75]. Thus, for example, there are many data in the literature suggesting that the effect of MSC on tumors depends on the origin of MSC and the type of tumor. However, we cannot exclude other influences such as growth media, cancer models, host animal model and laboratory bias.

It has been reported that human BM-MSC promoted tumor growth of pancreatic cancer cells [76], osteosarcoma and gastric cancer cell lines [77], and human AD-MSC-induced metastasis of breast cancer cells [78]. Several mechanisms have been proposed for these actions, such as MSC recruitment into tumor sites, promoting tumor growth and angiogenesis through the secretion of cytokines [79–81], or protecting cancer cells from immune clearance through modulating regulatory T cells and inhibiting natural killer (NK) cells and cytotoxic T lymphocyte (CTL) functions [82].

However, it has been shown that the secretome of BM-MSC is able to reduce the proliferation, viability and migration of certain types of cancer cells, such as non-small-cell lung carcinoma [83]. It has ben also reported that EVs obtained from BM-MSC inhibit proliferation and promote apoptosis in liver carcinoma, Kaposi's sarcoma, and ovarian tumor cell lines [84]. The same effect was reported for EVs from AD-MSC, that inhibit prostate cancer [84], ovarian cancer cells [85], or glioblastoma [86].

On the other hand, it has been shown that certain MSC, such as those derived from reproductive tissues, have antitumor effects. UC-MSC have a high tendency to move towards the tumor and to inhibit the growth of solid tumors such as breast [87–89] or HeLa cells [90]. The unique features of these cells lead to the hypothesis that UC-MSC act as a natural defense against the migration of cancer cells from mother to fetus, and thus explains why tumors are very rare in the fetus [91]. Endometrial MSC show anti-cancer effects on human epithelial ovarian cancer cells in vitro and in vivo through paracrine factors [92]. Human amniotic fluid MSC (hAFMSC), which can be obtained by amniocentesis in the second trimester or at the end of pregnancy [93], have natural tumor tropism towards ovarian cancer cells, and by releasing soluble factors have an efficient anticancer effect on them [94]. More recently, human uterine cervical stem cells (hUCESC) or their secretome have shown a potent antitumor effect on HeLa cells, highly proliferating breast cancer cells and cancer-associated fibroblasts [42].

It has been shown that MSC may secrete high amounts of cytokines which induce the inhibition of tumor growth, such as IFNα [95], IFNβ [96], IFN-γ, DKK-1/3 [97], IL12 [98], TRAIL (Tumor Necrosis- Factor-Related Apoptosis-Inducing Ligand [99], tumor necrosis factor superfamily member 14 (TNFSF14) also known as LIGHT, Fms-related tyrosine kinase 3 (FLT-3) ligand, C-X-C motif chemokine 10 (CXCL10) and liver-enriched transcriptional activator protein (LAP) [42]. It has been also reported that the antitumor effect of MCS may be partly related to the activity of tissular inhibitors of the matrix metalloproteinases TIMP-1 and TIMP-2 present in their secretome [100, 101], the inhibition of MMPs being associated with the inhibition of migration and invasion of cancer cells.

On the other hand, cancer cells have been shown to internalize a greater percentage of exosomes when compared to normal cells [102, 103]. These EV produced by MSC may be responsible for many of their antitumor effects. Accordingly, it has been reported that EVs from human UC-MSC reverse the development of bladder carcinoma cells, possibly by down-regulating the phosphorylation of Akt protein kinase and up-regulating cleaved caspase-3 [104]. Human AD-MSC suppress the proliferation of ovarian cancer cells through exosomal miRNA in vitro [85] and inhibit glioblastoma brain xenografts [86]. Likewise, intratumoral injection of miR-146b-expressing MSC-derived exosomes resulted in considerable reduction in glioma xenograft development in a rat brain tumor model and decreased the growth, migration, and invasion of tumor cells [105].

Heterogeneity of MSC in the same postnatal niche in the same individual

The situation of MSC heterogeneity is even more complex if we consider several findings inside the same postnatal niche. There are some individual factors that have been identified as responsible for this heterogeneity, such as sampling location in the same niche or between individuals, among others.

Depending on sampling location

It has been found that subcutaneous white adipose tissue (SWAT)-derived MSC show greater proliferation, and are also more easily differentiated to adipose or osteogenic lineages than MSC obtained from visceral white adipose tissue (VWAT) [106]. In addition, AD-MSC isolated from subcutaneous regions show more osteogenic potential than those obtained from deep-layer adipose tissue [107].

Depending on individuals

It is known that when comparing theoretically identical MSC populations from different individuals, they may display different secretome properties, depending on factors including age or health status of the individual [108].

Donor age

It is known that capabilities from MSC, such as their multilineage differentiation, homing, immunomodulatory and wound-healing capacities, role of oxidative stress regulation or intrinsic changes in telomere shortening, gradually disappear [109–112]. In fact, the properties of BM-MSC are strongly associated with the age of the donor. These cells collected from older donors are characterized by an increased percentage of apoptotic cells and slower proliferation rate, associated with an increased population doubling time. It has also been reported that BM-MSC from older donors have weakened ability to differentiate toward osteoblasts [113] and less reparative capacity [114]. In addition, BM- and AD-MSC obtained from aged individuals possess reduced immunomodulatory properties [115, 116] and reduced capacity to handle oxidative stress [117], compared to those from younger ones.

Recently, interesting data on MSC from cervical intraepithelial neoplasia (CIN) were reported [118]. CIN is a precancerous lesion of the uterine cervix that can regress or progress to cervical cancer, and where inflammation seems to play a pivotal role in CIN fate. Patients from a younger age group (mean age 28 ± 2) express a higher level of cytokines related to acute inflammation than older groups of patients (mean age 45 ± 3). The mechanisms to explain these differences between MSC are not completely known. Nevertheless, analysis of gene expression related to stemness, such as OCT4, SOX2, NANOG, and KLF4, revealed one discrepancy between young and old MSC. MSC from young women express a higher value of KLF4 than those from old patients. It is known that KLF4 directly binds to the promoter of NANOG to help OCT4 and SOX2 in regulating the expression of NANOG [119]. Considering the critical role of KLF4 in stem cell self-renewal as well as pluripotency, the expression of this factor may contribute to explain the functional differences of MSC depending on women's age.

Obesity

Adipose tissue is considered an important reservoir for stem cells. However, their function and "stemcellness" has been questioned. This may be, in part, because the influence of obesity as a chronic pathological condition, is a risk factor for cardiovascular disease. In fact, several studies identified changes due to obesity in isolated stem cells from adipose tissue niches. Certainly AD-MSC from obese patients show less differentiation potential and less proangiogenic capacities than those from non-obese individuals [120]. In addition, it was reported that the transcriptomic profile of the stem cells reservoir in obese subcutaneous adipose tissue is highly modified, with significant changes in genes regulating stemcellness, lineage commitment and inflammation [121]. However, the mechanisms of how obesity affects MSC functionality remain unclear. Therefore, functional analysis of MSC from this origin should be performed before clinical application.

Depending on diseases

A large amount of evidence supports the importance of age on the deterioration of stem cells in adulthood. They can become an important player in the onset of various diseases during aging, such as the metabolic syndrome [122], diabetes [123, 124], rheumatoid arthritis [125], systemic lupus erythematosus [126] or ageing syndromes [127, 128]. Thus, these clinical situations, by perpetuation of inflammatory states, constant emission of "alarm signals," proliferation, mobilization, and finally an endless sequestration of MSC into the damaged tissues, could lead to a decrease in the endogenous pools of progenitor cells, especially MSC, which are probably the most important specialized repairing cells [129, 130]. Nevertheless, there are also evidences indicating acquired MSC dysfunctions in systemic diseases. This concept arose after the observation of patients with autoimmune diseases such as systemic lupus erythematosus, diabetes, or rheumatoid arthritis, who entered disease remission when treated with mesenchymal or hematopoietic stem cells after allogenic transplants, but not after autologous transplants. Today, MSC dysfunctions are found in patients with diseases such as lupus, diabetes, rheumatoid arthritis, Parkinson disease, amyotrophic lateral sclerosis, psoriasis, idiopathic pulmonary fibrosis or myelodysplastic syndromes. In these diseases, dysfunctional MSC from bone marrow, adipose tissue, umbilical cord or dermis were found [2]. These MSC dysfunctions include multiple alterations such as cytoskeleton-related defects, decreased proliferation and capacity for pluripotency, lower expression of trophic factors, increased cell senescence, apoptosis, activation of the p53/p21 and p16INK4a pathways, reactive oxygen radicals, pro-inflammatory cytokines, impaired potential for differentiation and migration, angiogenesis/vasculogenesis, mitochondrial dysfunction or alterations in metalloproteases [2].

Unknown factors

Even MSC isolated from young and healthy donors exhibit stark differences in their proliferation rate, differentiation

capacity, and clinical utility. Thus, for example, in marrow aspirates of 17 healthy donors, it was found that MSC populations showed significant differences in growth rates, alkaline phosphatase enzyme activity or bone-specific gene induction [131]. In addition, it was reported different proliferative rates in MSC cultures from multiple bone marrow aspirates isolated from the same donor over different periods, or bilaterally from a donor at a single time point, yield MSC [131].

All these data on MSC heterogeneity should be considered in the development and application of MSC or their secretome-derived products, possibly using specific functional tests to ensure homogeneity of action.

Heterogeneity of MSC depending on sampling mode

AD-MSC are an example of heterogeneity of MSC depending on a specific sampling mode. In general, comparison of procedures indicates that a higher surface-to-volume ratio is the most efficient method. Thus, a higher yield of viable AD-MSC is obtained through lipoaspiration, in comparison with those obtained through tissue block resection [132, 133]. In addition, power-assisted liposuction methodologies show higher proliferative potential and resistance to senescence in isolated AD-MSC than laser-assisted liposuction and surgical biopsy [134]. Also, microaspiration of fat with micro-cannulas has been reported to be more efficient than the usual procedures, as expressed in higher yields, greater viability, better adhesion rates, and greater secretion of growth factors, such as insulin-like growth factor (IGF) and platelet-derived growth factor (PDGF) [135].

Interestingly, other MSC types which show differences with regard to mode sampling are UC-MSC. Thus, it was recently reported that MSC isolated from the UC of babies born vaginally had higher proliferative potential than those obtained from the cord of babies born by Caesarean section, although the reasons are not perfectly clear yet and more studies are underway for clarification [87].

Heterogeneity of MSC depending on the artificial niche

The possible modifications of MSC culture and their effects are summarized in Table 1. When MSC are placed in an artificial niche, there are several factors which influence their heterogeneity, both under basal conditions and after manipulation.

Basal conditions

Theoretically, just obtained MSC have morphologic homogeneity and uniform expression of certain surface antigens which are conserved on high-density culture for several passages [22, 136]. However, obtained MSC often represent a mixture of phenotypically, functionally and biochemically diverse cells [137, 138]. In parallel, from passage to passage, the clonogenicity of MSC decreases and proliferation slows down [139, 140].

Several studies have shown that single-cell-derived colonies of human MSC contain at least three morphologically disparate cell types: (1) extremely small and rapidly self-renewing cells; (2) elongated spindle-shaped fibroblast-like cells; and (3) large, cuboidal and slowly replicating cells, [141, 142]. In addition, there exists a clear clonal variability in cell differentiation, proteomic, transcriptomic and epigenetic status.

It has been observed that clones show different potential to differentiation (either osteo-chondro, osteo-adipo or osteoprogenitors) [143]. Probably, these variations in clone differentiation reflect biophysical variation among clones. Cellular mechanical properties reflect the underlying structure of the cell, including the cytoskeleton and nucleus. Mechanical differences of similar magnitude have been noted among individual, undifferentiated MSC isolated and passaged together [144]. Several studies suggest that cellular mechanics can be prospectively used to predict differentiation capacity in individual clones to adipogenic, chondrogenic or osteogenic potentials [145, 146].

On the other hand, it has been shown that fast- and slow-growing clonal populations of MSC differ proteomically, with differential expression of proteins including intermediate filaments, calcium-binding proteins and glycolytic proteins [147]. Epigenetic modifications, such as DNA methylation, is associated stem cell differentiation. Investigation of clonal MSC adipogenesis shows that while adipogenesis-associated promoters are hypomethylated in MSC clones, the specific pattern of methylation varies among clonal subpopulations [148].

Manipulation of MSC in the artificial niche

The dose or frequency of MSC therapy cannot be increased infinitely in terms of cost and safety. Thus, there is a need for novel strategies to enhance the capability of survival, homing to the site of damage and improving their therapeutic potency. There are several conditions which may contribute to evolutionary changes and heterogeneity of MSC at the artificial niche, such as culture conditions (O_2 tension, substrate and extracellular cues, inflammatory stimuli or type of culture medium), genetic manipulations or exosome modifications (Fig. 2).

Table 1 Artificial niche factors that influence heterogeneity of MSC

Culture condition	MSC source	Type of study	Effect on MSC	References
Low O_2 tension	hBM-MSC	In vitro	Better retention of their proliferative capacity and differentiation potential. Expression of surface antigen genes Oct4, Sox2 and Nanog	[149, 152]
	hAD-MSC	In vitro	Increased expression of AD-MSC stemness markers Oct3/4 and Nanog, of secreted angiogenesis growth factors and increased proliferation rate. Enhancement of the chondrogenic differentiation ability. Protection against damaging factors, replicative senescence and cryopreservation. Increased immunomodulatory effect by inhibition the proliferation of mitogen-stimulated CD4 and CD8 T lymphocytes	[59, 151, 153, 154, 156, 157, 163]
	hBM-MSC hAD-MSC	In vivo*	Significantly earlier restoration of blood flow. Healing of ischemic lesions. Enhanced of paracrine effect of MSC in diabetes, cancer, liver failure and irradiation-mediated salivary gland damage	[90, 150, 158–162, 164–167]
Three-dimensional (3D) aggregates (spheroids)	Neural Embrionic and hAD-MSC	In vitro	Changes in cell shape and polarity. Improvement of cell–cell interactions. Enhanced differentiation capacity into osteocytes, chondrocytes and non-mesenchymal lineages. Increased production of angiogenic factors and anticancer proteins (TRIL, IL-24 and CD82)	[170, 172–174, 181–183, 256]
		In vitro In vivo*	Increased migration and homing efficiency of MSC to the damaged site with an enhanced engraftment ratio. Improvement of reparative/regenerative and anti-inflammatory properties	[174, 176–180]
Mechanical stimuli	bBM-MSC hMSC	In vitro	Varying stiffness and mechanical loading of MSC result in changes on biochemical signalling, gene expression, cell phenotype and paracrine stimulation Soft environments and low contractility favor adipogenesis. Stiff milieu and high contractility promote osteogenic potential	[185, 187–190, 192]
Inflammatory stimuli	hBM-MSC UC-MSC hAD-MSC	In vitro In vivo*	IFN-γ on MSC culture upregulate the synthesis of indoleamine 2,3-dioxygenase (IDO), cyclooxygenase 2 (COX-2), transforming growth factor beta (TGF-α), and hepatocyte growth factor (HGF) AD-MSC pre-conditioned with IFN-γ, TNF-α and IL-6, show enhanced immunosuppressive properties, anti-inflammatory effects, increased proliferation, mobilization and osteogenic differentiation TLR2 and TLR4 dose-dependent activation enhances AD-MSC osteogenic differentiation, while triggering TLR9 inhibits osteogenesis and proliferation AD-MSC with IFN-γ enhances experimental obliterative bronchiolitis. AD-MSC with TNF-α increases the secretion of interleukin-6 (IL-6) and IL-8, stimulating angiogenesis AD-MSC primed with IFN-γ, TNF-α, and IL-17 enhances immunosuppressive effects	[194–197, 200–202, 204, 206, 207] [198, 199, 203, 205]

* animal model

Culture conditions

O_2 tension The cells are generally cultured in vitro under a O_2 tension such as the one present in our atmosphere (~21%). However, there are models evidencing that MSC in their niches are adapted to lower O2 tensions (from 1 to 7%) [149, 150]. As an example, the adipose niche is known to be hypoxic, usually with around 5% O_2, compared to highly perfused organs [151]. In addition, several studies have shown that MSC cultured under low O2 conditions retain better their proliferative capacity, surface antigen expression profile, expression of "stemness" genes (Oct4, Sox2, and Nanog), and differentiation potential in comparison with those cultured in atmospheric O2 [152–155]. It has been also shown that hypoxic culture conditions could protect AD-MSC against the most common in vitro damaging factors, replicative senescence and cryopreservation [156, 157].

On the other hand, it has also been reported that MSC under low O_2 could drive facilitated release of several trophic factors [59], and especially angiogenesis growth factors such as VEGF and HGF, contributing to an improvement of ischemic lesions [150, 158–162]. In addition, it has been shown that MSC cultured under 1% O_2 display an increased immunomodulatory effect by inhibiting efficiently the proliferation of mitogen-stimulated CD4 and CD8 T lymphocytes [163].

In concordance with all these in vitro data, several in vivo studies showed the advantages of hypoxic pre-conditioning in MSC culture in therapeutic terms. In fact, there are studies reporting that the enhanced paracrine effect of MSC after hypoxic treatment would be beneficial to diabetes [164], liver failure [165, 166], irradiation-mediated salivary gland damage [167] or anticancer effects [90]. Nevertheless, despite all these potential therapeutic advantages, there are issues which should be resolved. For example, hypoxia culture conditions might include a wide range of O_2 tensions from 5% to < 1%, which may have different biological effects on MSC. Therefore, the optimal O_2 concentration for maximizing each therapeutic effect in each MSC type should be determined prior to clinical application.

Substrate and extracellular matrix cues Aspects related with the specific platform on which the cells are grown and different culture conditions are of key importance. Conventional cell culture is generally conducted in a two-dimensional (2D) system (tissue culture flasks—T-flasks) in which cells grow as monolayers. However, under these conditions, the large number of T-flasks needed can lead to flask-to-flask variability, it may increase the chances for contamination, and is certainly very labour intensive [168]. In addition, the 2D system is highly deficient in cell-to-cell or extracellular interactions, which lead to a decrease of stemness of adult stem cells and less therapeutic potential [169]. One alternative is to induce MSC

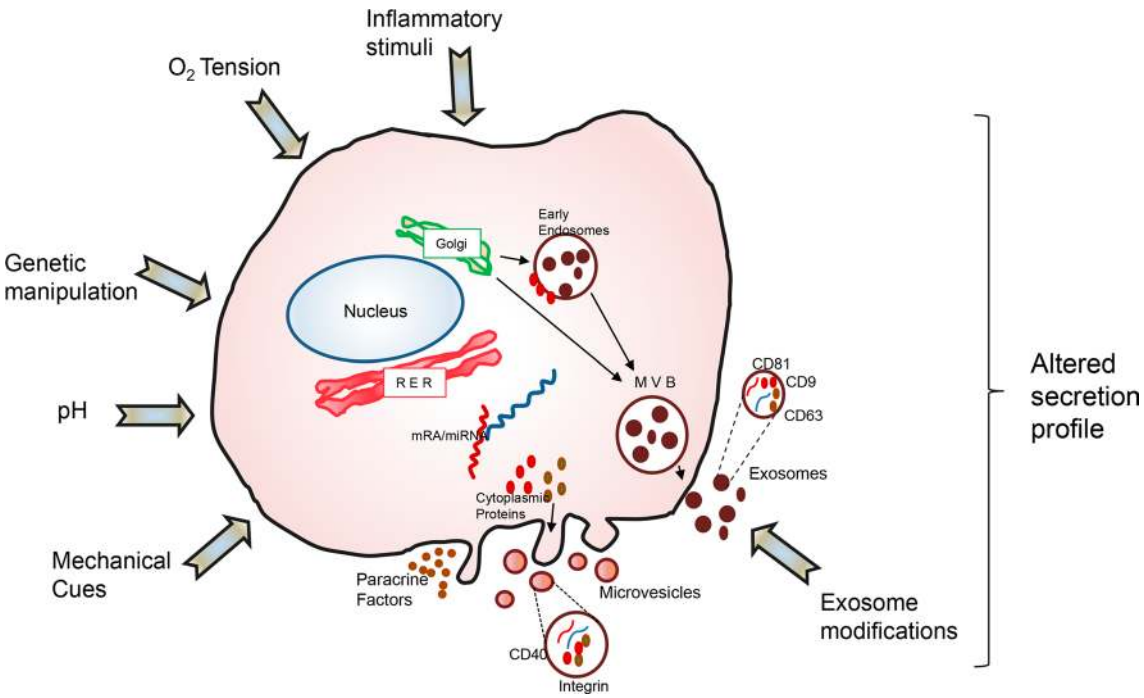

Fig. 2 Factors related to modifiable culture conditions which may influence the quality of MSC to obtain tailor-made secretome-derived products (paracrine factors, microvesicles or exosomes)

to grow as three-dimensional (3D) aggregates (spheroids). Spheroids are multicellular structures in which adherent cells are forced to aggregate with each other using a suspension culture system [170, 171]. Therefore, cell spheroids change the microenvironment to provide cells with 3D context and induce changes in cell shape and polarity whilst encouraging cell–cell interactions, which is considered more physiologically adequate. This method was widely applied to culture neural stem cells (neurospheres), embryonic stem cells (embryonic bodies), cancer cells (tumoroids), and other cells to study developmental and physiopathological cell-to-cell dynamics in vitro [170, 172].

It has been reported that MSC cultured as 3D spheroids have properties far superior to those of 2D MSC cultures, such as enhanced differentiation capacity into osteocytes, chondrocytes [173] and non-mesenchymal lineages (ectodermal or endodermal) [174, 175], increase of the migration and homing efficiency of MSC into the damaged site with an enhanced engraftment ratio after in vivo application [174, 176, 177], increase in reparative/regenerative [177, 178] and anti-inflammatory properties [179, 180], and an increased production of angiogenic factors [181, 182] or anticancer proteins (TRIL, IL-24 and CD82) [183].

Despite all these advantages of 3D cultures, there are several technical questions to clarify in order to improve their therapeutic potential, such as optimal size and total cell number of each spheroid, and culture duration [171, 184].

Mechanical cues The elasticity of the microenvironment and exogenous forces are found have been as determinants of stem cell fate [185]. These cues elicit changes in biochemical signalling, gene expression, cell phenotype and function. Cell response is most uniform in extremely soft or stiff environments, which favor adipogenesis and osteogenesis, respectively [186–188]. In addition, subpopulations of undifferentiated MSC encapsulated in hydrogels respond differentially to compression: calcium signalling is upregulated in one subset of cells, while it is downregulated in another [189].

On the other hand, it seems that the MSC secretome may also vary in response to different mechano-transduction events. In a recent study, conditioned medium from mechanically loaded MSC promoted angiogenesis within human dermal microvascular endothelial cells, and that these findings led to significantly increased levels of MMP2, TGFβ, and FGF [190]. Similarly, when MSC are cultured on polyacrylamide hydrogels of increasing stiffness, VEGF and IGF are upregulated, whilst EGF, IL-6 and IL-8 show biphasic secretory profiles [191]. It has also been shown that after chondrogenic induction through multiaxial mechanical loading, the resulting secretome composition includes soluble factors such as VLC, VEGF, and MMP13 [192].

Inflammatory stimuli Interferon gamma (IFN-γ), a pro-inflammatory cytokine against viral and bacterial infections, is a known source for MSC priming for functional enhancement [193]. Thus, for example, it has been shown that treatment of MSC with IFN-γ upregulates the synthesis of several anti-inflammatory transcription factors, including indoleamine 2,3-dioxygenase (IDO), cyclooxygenase 2 (COX-2), transforming growth factor beta (TGF-α), and hepatocyte growth factor (HGF) [194–197]. It has been shown that pre-treatment course of AD-MSC with IFN-γ improves experimental obliterative bronchiolitis via IDO-dependent suppression of T cell infiltration and induction of regulatory T cells (Tregs) [198], shows pronounced anti-proliferative effects on activated peripheral blood mononuclear cells (PBMC) along with a significant upregulation of PD-L1 expression and COX-2-derived PGE2 secretion [199].

Tumor necrosis factor-alpha (TNF-α) pre-conditioning of MSC exhibit anti-inflammatory effects through upregulation of several immunomodulatory factors [200, 201] can promote bone generation by increasing proliferation, mobilization, and osteogenic differentiation [202], promote endothelial progenitor cell homing and stimulate angiogenesis in a murine ischemic hindlimb model [203].

There are studies conducted to elucidate the optimal combination of cytokines that can maximize the therapeutic effect of MSC. Activation of MSC with TNF-α and IFN-γ has been also shown to increase the production of IL-6, HGF, VEGF, and TGF- and to promote bone formation [204]. Murine AD-MSC primed with IFN-γ, TNF-, and IL-17 attenuates hepatitis through inducible nitric oxide synthase (iNOS)-mediated higher T-cell suppression [205]. Human AD-MSC pre-conditioned with IFN-γ, TNF-α and IL-6, shows enhanced immunosuppressive properties in vitro [206].

Other inflammation inducers used as to maximize their therapeutic capabilities of MSC are toll-like receptors (TLRs). TLRs represent a subgroup of pattern recognition receptors (PRRs) that contribute to the defense mechanism via the innate immune system in response to pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). The expression of TLR1–6, and 9 have been reported in human MSC [207, 208], and these TLR expression patterns may vary depending on the origin of MSC. It has been shown that activation of TLR2 and TLR4 significantly enhances osteogenic differentiation, whereas triggering TLR9 inhibits osteogenesis and ASC proliferation [207].

Culture medium and new technologies Culture media and new technologies may also influence MSC phenotype. Today different media are used, such as fetal bovine serum, xeno-free or chemically defined media. There have already

been significant trends towards chemically defined media that remove the batch-to-batch variability associated with foetal bovine serum [209]. Different defined-media formulations are currently commercially available and have been shown to alter secretion of TGFβ and Ang1 [210], indicating that choice of medium is important.

The 3D self-organized tissue models based on stem cell-derived organoid provide biological models to serve as semiphysiological model. Organoids reproduce biological parameters including the cell–cell and cell–matrix interactions, spatial organization and physiological functions [211].

On the other hand, an alternative for expanding large populations of MSC with higher homogeneity and scalable conditions are suspension bioreactors [212]. These techniques are based on the concept of using dynamic suspension cultures of MSC attached to beads to maximise surface area [213].

Genetic manipulation

AD-MSC transduced with Sox2 and Oct4 show remarkable benefits in their proliferation capability. Nevertheless, this manipulation may inhibit differentiation potential and may have possible adverse effects such as tumor formation during clinical applications [214].

There are data suggesting that the incorporation of anti-inflammatory genes such as IL-10 [215], HGF [216], IDO [217], or Foxp3 [218], could improve the therapeutic potential of MSC. Similarly, MSC transfected with Bcl-2 present better apoptotic tolerance, cell survival and more VEGF secretion [219]. While MSC overexpressing bFGF or platelet-derived growth factor-BB (PDGF-BB) lead to highly proliferating MSC and increased osteogenesis [220]. Other MSC were genetically modified to increase their survival, overexpressing factors such as PI3K [221, 222], SDF1 [223], CXCR4 [220, 224], HGF [225] and IGF [226].

In the central nervous system, BDNF is the predominant neurotrophin complemented by the substantial expression of TrkB [227]. Several studies have shown the involvement of BDNF in the pathogenesis of neurodegenerative diseases and psychiatric disorders, like depression and schizophrenia [228]. Additionally, BDNF acts on cholinergic neurons, which are depleted in Alzheimer's disease [229] and on dopaminergic neurons of the substantia nigra, which are lost in Parkinson's disease [230]. However, recombinant BDNF delivery in clinical trials has not been therapeutically successful [231]. Nevertheless, BDNF overexpressing hMSC protect neurons significantly better from degeneration than native MSC. hMSC were lentivirally modified to overexpress BDNF, and which was more neuroprotective [232].

To enhance anticancer effects, in vitro studies have shown that the expression of interferon-beta (IFN-β) in MSC transfected by adenovirus can effectively kill glioma cells [233]. In a model of lung metastasis of prostate cancer, MSC expressing IFN-β could prolong the survival period, and its possible mechanism is that IFN-β could promote tumor cell apoptosis, inhibit angiogenesis, and increase the activity of natural killer cells [234]. Similarly, adenovirus-transfected MSC expressing interferon-γ (IFN-γ) inhibit proliferation and induce apoptosis in leukemia cells in vitro [235]. On the other hand, it has also been shown that MSC engineered to express IL-12 prevent metastasis and increased tumor cell apoptosis in mice bearing preestablished metastases of melanoma, breast, and hepatoma tumors [236, 237].

Despite all these positive data on genetic manipulation of MSC, several limitations remain still limiting their clinical application. The main concern has to do with the fact that the application of replication-defective viral vectors, such as lenti- and adenoviruses, is closely associated with safety issues including potential tumorigenicity, toxicity, and immunogenicity [238]. A summary of verified MSC genetic modifications and their effects is shown in Table 2.

Several studies demonstrated that the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas system is highlighted as a simple and effective tool for genetic engineering with applicability to the edition of the mammalian cell genome, and which was tested in clinical trials. It has been recently proposed perspectives on how the CRISPR-Cas system may improve the therapeutic potential of MSCs [239, 240]. In this context, there are today the first evidences of successful and effective MSCs secretome managing via CRISPR/Cas9 genome editing technology. Thereby, using CRISPR-Cas9 knockout and transcriptional activation systems, we were able to create both PAI-1 knockout and PAI-1 over-expressing human endometrium-derived mesenchymal stem cells (hMESCs), respectively [241]. It was also reported the use of engineered BM-MSC overexpressing IL-10 using CRISPR activation to treated myocardial infarction in diabetic mice [242].

Exosome modification

MSC are the only human cell type known to have a scalable capacity for the mass production of exosomes for drug delivery [243]. They are smaller, less complex, less immunogenic and their production and have easier storage than their parental cells [244]. In addition, other advantages of exosomes include long circulating half-time [245] or better crossing through the blood–brain barrier [246]. Therefore, exosomes can be easily manipulated and can be modified with certain ligands or proteins on their surface to improve their targeting capability.

Exosomes encapsulated with miR-379 have been administered for breast cancer therapy in vivo and migrated to the tumor site showing antitumor effects [247]. It has also been shown that methotrexate-loaded EVs functionalized with a

Table 2 Influence of genetic manipulation on heterogeneity of MSC

Genetic modification	MSC source	Type of study	Effect on MSC/therapeutic benefits	References
Sox2 Oct4 transduction	hAD-MSC	In vitro	Benefits in their proliferation capability, but may inhibit differentiation potential. Could have adverse effects for clinical applications, such as tumor formation	[214]
IL-10 HGF IDO Foxp3 incorporation	hBM-MSC	In vitro In vivo*	Attenuates the severity of acute GVHD. Enhanced immunosuppressive properties of MSC. Promotes liver allograft tolerance through the generation of regulatory T cells	[215–218]
Bcl-2 engineered	hBM-MSC	In vitro In vivo*	Better apoptotic tolerance, improved cell survival, VEGF secretion and reduced heart infarct size	[219]
bFGF PDGF-BB TGF-β1 overexpressed	hBM-MSC	In vitro	bFGF or PDGF-B lead to highly proliferating MSC and increase osteogenesis. Conversely, adipogenesis is affected. TGF-β1 blocks both osteogenic and adipogenic differentiation, inducing the formation of stress fibers	[220]
PI3K-C2α overexpressed	BM-MSC rat	In vitro In vivo*	The level of apoptotic proteins is downregulated. Increased cell viability of MSC and enhanced myocardial regeneration. Reduction of infarct size and fibrosis area	[221, 222]
SDF-1α overexpressed	BM-MSC rat	In vitro In vivo*	MSC differentiation into endothelial cells. Reduction of infarct size and fibrosis. High vascular density and thicker left ventricular wall. Improvement of left ventricular performance	[223]
CXCR4 overexpressed	hBM-MSC	In vitro In vivo*	Enhanced MSC chemokinesis. Improved cell trafficking and tissue repair. Enhancement of relevant trophic signals. No adverse effects on proliferation and differentiation	[220, 224]
HGF overexpressed	hBM-MSC	In vitro In vivo*	Inhibited collagen deposition and improved cystometric parameters in bladder outlet obstruction	[225]
IGF-I overexpressed	BM-MSC mice	In vitro In vivo*	Paracrine support to EPO-secreting MSC in anemia. Hematocrit elevation. Improvement of Heart function	[226]
BDNF overexpressed	hBM-MSC	In vitro	Lentivirally MSC modification provides significantly neuroprotective effect from degeneration compared to native hMSC	[232]
IFN-β hMSC engineered	hBM-MSC	In vitro In vivo*	In vitro, promotion of tumor cell apoptosis, inhibition of angiogenesis, and increased NK activity In vivo, significantly increased survival in a human U87 intracranial glioma xenograft model. Prolonged survival in a prostate cancer lung metastasis model, compared to controls	[233, 234]
IFN-γ hMSC engineered	hBM-MSC	In vitro	Inhibition of proliferation and induction of apoptosis in leukemia cells	[235]
Ad-FKN engineered	adenoviral vector fractalkine gene	In vitro In vivo*	Ad-fractalkine mediates antitumor effects by induction of both innate and adaptive immunity	[236]
IL-12 expressed	hBM-MSC	In vitro In vivo*	Prevention of breast cancer metastasis into the lymph nodes and internal organs as well as increased tumor cell apoptosis and an antiangiogenic effect on tumor stroma	[237]
(CRISPR)/Cas9	hMESCs BM-MSC	In vitro In vitro In vivo	Obtain PAI-1 knockout and PAI-1 overexpressing hMESCs, provides evidence of successful and effective MSCs secretome managing via CRISPR/Cas9 genome editing technology Overexpression of IL-10 in BM-MSCs. Transplantation of BM-MSCs overexpressing IL-10 inhibited inflammatory cell infiltration and pro-inflammatory cytokines production, improved cardiac functional recovery, alleviated cardiac injury, decreased apoptosis of cardiac cells and increased angiogenesis	[241] [242]

* animal model

synthetic multifunctional peptide facilitated the membrane receptor-mediated internalization procedure both in vitro and in vivo in a glioma model [248].

To enhance vesicle release from cells, other strategies have also been proposed, such as prolonged culture and maintaining cells at low pH [249, 250]. Establishment of immortalized MSC is another strategy to scale up EV production [251]. Overexpression of the *c-myc* oncogene, finally, has been reported to increase EV production in MSC [252].

On the other hand, it has been shown that exosomes derived from MSC cultured under hypoxia provide better

protection in a mouse acute lung injury model than those derived from normoxic MSC [183]. Ischemic preconditioning of MSC has also been to produce EV which reduce cardiac fibrosis and apoptosis compared to control EV [253].

There are recent reports on the use of several human MSC populations treated with sub-lethal concentrations of taxol for 24 h, after which exosomes were isolated and applied to different human cancer populations including A549 lung cancer, SK-OV-3 ovarian cancer, and MDA-hyb1 breast cancer cells. While MSC control exosomes had little or no effect on growth inhibition of the tumor cells, taxol-loaded MSC-derived exosomes were associated with 80–90% cytotoxicity. Highly metastatic MDA-hyb1 breast tumors were induced in NODscid mice, and systemic intravenous application of MSC-derived taxol exosomes revealed a more than 60% reduction of subcutaneous primary tumors. Moreover, the amount of distant organ metastases in lung, liver, spleen, and kidney was reduced by 50% with MSC taxol exosomes, similar to the effects observed with taxol, although the concentration of taxol in the exosomes was about 1000-fold reduced [254].

Conclusions and future perspectives

MSC are widely distributed throughout the human body and an increasing amount of evidence suggests a significant role for MSC in the regulation of tissue homeostasis. In addition, MSC and products derived from their secretomes, such as CM or exosomes, need development because of their broad spectrum of therapeutic potentials, such as regenerative, anti-inflammatory, pro-angiogenic, anti-tumor and antimicrobial activities, already demonstrated in several experimental models in vivo. However, one limiting factor in the clinical translation of the therapeutic potential of MSC is related to their heterogeneity.

MSC demonstrate many dimensions of heterogeneity. They differ with regard to their origin among biological niches, donors, as well as among and within clonal populations. Based on this, we may consider that it will probably be necessary in the future to establish stem cell banks based on the heterogeneity of MSC subpopulations. In addition, to screen for cells prior to their use in the clinic, the properties of the cells being used should be better understood.

It is also relevant to consider that once MSC are isolated, their capabilities can vary widely depending on the culture conditions, including physical and chemical ones. This opens new possibilities to modify the potential of MSC through preconditioning of their cultures or genetic manipulation. In particular, advances in bioengineering and our understanding of how the extracellular environment affects MSC paracrine activity will play a pivotal role in the generation of widespread, successful, clinical MSC therapies. This aspect of the artificial niche represents an opportunity to adapt the possibilities of therapies based on MSC and their derivatives towards personalized medicine tailored to the needs of each patient.

We consider that the most appropriate type of MSC should be chosen for each type of therapeutic application, according to its origin and the result of functional tests after its expansion “in vitro”. Likewise, the use of products derived from MSC, such as the secretome, seems to be the most convenient alternative. In this manner, the disadvantages of administering living cells would be avoided, such as thrombosis and possible microembolism [255], and it implies practical and economic advantages [15]. On the other hand, the production of these biological products should be supported by the development of bioreactor technology, which will allow a strict control and optimization of the culture conditions adapted for each type of MSC, as well as the scalable production of large amounts of secretomes for therapeutic use.

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Compliance with ethical standards

Conflict of interest The authors declare the following competing interests: F.J.V and N.E are co-inventors of a patent (“Human uterine cervical stem cell population and uses thereof”) owned by GiStem Research, of which L.A.C, N.E, L.O.G, J.S, P.P J.S. and F.J.V are shareholders. The funding sponsors had no role in the design of this review, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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