

Review Article

Bioregenerative Applications of the Human Mesenchymal Stem Cell-Derived Secretome: Part-I

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Abstract

Mesenchymal stem cells hold many therapeutic benefits in treating diverse disease states, but autologous requirements, high costs, lack of standardization and other factors limit their widespread application. Additionally, researchers have discovered that many mesenchymal stem cell *in-vivo* benefits originate from their paracrine factors. Therefore, cell-free therapeutics, including mesenchymal stem cell-derived conditioned medium and extracellular vesicles have been suggested as alternative bioregenerative therapies. This literature review summarizes mesenchymal stem cell application, the benefits of cell-free strategies and the components of its secretome. This manuscript is a companion piece to “Part 2: Bioregenerative Applications of the Human Mesenchymal Stem Cell-Derived Secretome,” included in this issue, which contains the results of in-vivo studies applying the conditioned medium and extracellular vesicles to human and animal models, ongoing clinical trials, limitations to cell-free strategies and future directions for the wide-scale adoption of these therapies.

Keywords: Conditioned Medium; Extracellular Vesicles; Mesenchymal Stem Cell-Derived Exosomes; Preconditioning; Bioregenerative Therapies

Abbreviations

ALCAM: Activated Leukocyte Cell Adhesion Molecule; ALS: Amyotrophic Lateral Sclerosis; Apo-EV: Apoptotic Extracellular Vesicle; ARF: ADP-Ribosylation Factor; ASC or AD-MSC: Adipose Mesenchymal Stem Cell; BASP-1: Brain Acid-Soluble Protein 1; BDNF: Brain-Derived Neurotrophic Factor; BM-MSC: Bone Marrow Mesenchymal Stem Cell; CADH2: Cadherin-2;

CCL2: Chemoline-Ligand 2; CD : Cluster of Differentiation; CD40L: Cluster of Differentiation 40 Ligand; CircRNA: Circular RNA; CNTF: Ciliary Neurotrophic Factor; COPD: Chronic Obstructive Pulmonary Disease; CXCL4: CSC Motif Chemokine 5; CYPA: Cyclophilin A; CYPB: Cyclophilin B; Cys C: Cystatin C; DNA: Deoxyribonucleic Acid; DPSC: Dental Pulp Stem Cell; ECM: Extracellular Matrix; ESC: Embryonic Stem Cell; ESCORT3: Endosomal Sorting Complex Required For Transport-3; FGF: Fibroblast Growth Factor; Gal: Galectin; GDN: Glia-Derived Nexin; GvHD: Graft-versus-Host Disease; HSP27: Heat Shock Protein 27; HUCPVC: Human Umbilical Cord Perivascular Cell; IA: Intraarterial; IBS: Inflammatory Bowel Disease;IDO: Indoleamine 2,3-Dioxygenase; IFN- γ : Interferon Gamma; IGF: Insulin-Like Growth Factor-Binding Protein; IL-1Ra: Interleukin 1 Receptor Antagonist; IL: Interleukin; IV: Intravenous; L-EV: Large Extracellular Vesicle; LncRNA: Long Noncoding RNA; LPS: Lipopolysaccharide; MCP: Monocyte Chemoattractant Protein; MFGE8: Milk Fat Globule EGF Factor 8; MHC: Major Histocompatibility Complex; MI: Myocardial Infarction; miRNA:miRNA; MMP: Matrix Metalloprotease; mpCCL2: Metalloproteinase-Processed C-C Motif Chemokine Ligand 2; MS: Multiple Sclerosis; MSC: Mesenchymal Stem Cell; MSC-ex: Mesenchymal Stem Cell Exosomes; MVB: Multivesicular Body; OA: Osteoarthritis; NF- κ B: Nuclear Factor κ B; NGF: Nerve Growth Factor; NO: Nitrous Oxide; NOS: Nitric Oxide Synthase; NT3: Neurotrophin-3; PBSC: Peripheral Blood Mesenchymal Stem Cell; PEDF: Pigment Epithelium-Derived Factor; PGE2: Prostaglandin E2; PLGF: Placental Growth Factor; PL-MSC: Placental Mesenchymal Stem Cell; PRDX1: Peroxiredoxin 1; PTX3: Pentraxin 3; RNA: Ribonucleic Acid; ROS: Reactive Oxidative

Species; SDMSC: Synovial-Derived Mesenchymal Stem Cell; Sem7A: Semaphorin 7A; SVF: Stromal Vascular Fraction; TGF β : Tumor Growth Factor β ; TIMP: Tissue Inhibitors of Metalloproteinase; TNF: Tumor Necrosis Factor; TRX: Thioredoxin; TSG-6: Tumor Necrosis Factor-Inducible Gene 6 Protein; UC-MSC: Umbilical Cord Mesenchymal Stem Cell; UCHL1: Ubiquitin-Carboxy-Terminal Hydrolase 1; VCAM1: Vascular Cell Adhesion Molecule 1; VEGF: Vascular Endothelial Growth Factor; VWF: Von Willebrand Factor; WJ-MSC: Wharton's Jelly Mesenchymal Stem Cell

Introduction

Stem Cells

Stem cells are progenitor cells capable of self-renewal and differentiation into multiple cell types. While there are many ways to classify stem cells based on their differentiation capabilities, one categorization method divides them into embryonic and mature stem cells. Embryonic Stem Cells (ESCs) are pluripotent, meaning they can differentiate to form all embryonic germ layers. Mature stem cells are multipotent, meaning they can differentiate into different cell types within a lineage [1]. For example, mature hematopoietic stem cells can differentiate into formed units in blood, including erythrocytes, thrombocytes and leukocytes [2]. Mature stem cells can be harvested from the umbilical cord or the placenta after birth, as well as autologous and allogeneic body tissues [3]. Stem cells were first isolated and cultured in 1981, but their research was limited due to the moral and religious issues with harvesting stem cells from human embryos [4,5]. However, this field has largely moved beyond embryonic stem cells due to a greater understanding of the capabilities of mature stem cells and the arrival of induced pluripotent stem cells, which are adult stem cells converted to pluripotent stem cells with greater differentiation capabilities [6]. Therefore, there has been an explosion of research interest in the applications of stem cells on a wide variety of medical concerns.

Mesenchymal Stem Cells

Mesenchymal Stem Cells (MSCs) are adult multilineage progenitor stromal cells found in connective tissues. MSCs were initially discovered in bone marrow but have since been found in nearly every organ [7,8]. The MSC populations in different niches are heterogeneous, with various surface markers, proliferation capacities and physiological effects. However, MSCs share many qualities regardless of their harvesting location because they can be developmentally traced back to common embryonic origins [9]. All MSCs are fibroblast-like cells that can differentiate into osteogenic, adipogenic and chondrogenic lineages with appropriate conditioning. Additionally, other sources indicate MSCs can differentiate into myocytes, endothelial cells and neuronal cells [10]. The widely accepted criteria to define human MSCs are (1) plastic adherence in standard cultures, (2) positive expression of the surface antigens CD105, CD73 and CD90 and (3) negative expression of the surface antigens CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLDA-DR [11].

MSCs are capable of limited self-renewal and plasticity *in-vitro* [12]. However, *in-vitro* conditioning techniques like introducing cytokines and growth factors can induce division and prolong the number of passages before the cells become senescent [13]. Changing environmental factors *in-vitro*, like hypoxic conditioning, microgravity, repeated compressions and variable geometry of cultures can also influence MSC division, differentiation and properties when transfected *in-vivo* [14]. For more information, see Secretome Effectors.

MSC Types

MSCs can be gathered from various sources (Fig. 1). This section will highlight some MSCs employed for clinical use. The efficacy of MSCs from various tissues will be discussed in Secretome Effectors.

Bone Marrow Mesenchymal Stem Cells (BM-MSCs) were the first-discovered progenitor cells [16]. BM-MSCs are harvested by a bone marrow biopsy of the iliac crest or sternum [17]. After this process, BM-MSCs are separated from the aspirate or the sample is condensed into Bone Marrow Aspirate Concentrate (BMAC). Literature reviews collecting data on BM-MSC application showed efficacy to advance functional recovery in stroke models [18], frequent benefits in limiting damage from Myocardial Infarction (MI) and hematological malignancies and mixed benefits of improving damaged liver functioning [19-21]. Countless additional studies of their application to various disease models have been done.

Adipose Mesenchymal Stem Cells (ASCs or AD-MSCs) constitute a significant adult stem cell alternative to BM-MSCs. AD-MSC harvesting requires liposuction or dermolipectomy, followed by adipose microfragmentation or digestion via collagenase,

isolation of the Stromal Vascular Fraction (SVF) and potential further purification to derive a pure AD-MSC population [22,23]. A concise review indicated AD-MSCs improve symptoms, remission and recurrence rates of Inflammatory Bowel Disease (IBS); symptoms, functional, radiographic and histological scores in Osteoarthritis (OA); and clinical outcomes in Multiple Sclerosis (MS), Chronic Obstructive Pulmonary Disease (COPD), ischemic stroke, myocardial ischemia, glioblastoma, chronic skin wounds, idiopathic pulmonary fibrosis, chronic liver failure and glioblastoma [24].

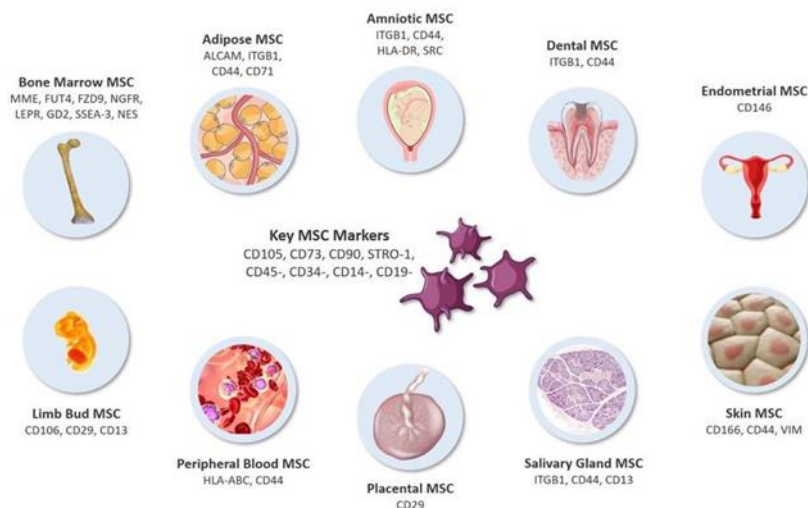


Figure 1: Depiction of some MSCs used in clinical studies. These MSC populations have different key markers for identification, as noted below their bolded locations [15].

While both BM-MSCs and AD-MSCs have been studied in a wide variety of disease models, other adult MSCs have been studied. Peripheral Blood Mesenchymal Stem Cells (PBSCs) are mainly applied to hematological malignancies with limited alternative disease models. Harvesting PBSCs requires an initial priming process, where healthy donors are given hematopoietic growth factors to increase the levels of pluripotent hematopoietic cells in the bloodstream. Because cell counts are closely monitored, this process is relatively safe at short timeframes. Common side effects include bone pain, myalgias, headaches and fevers; these frequently respond to mild analgesics. However, very rare cases of splenic rupture and death have occurred. After priming, peripheral venous apheresis by extracorporeal continuous-flow machines processes 2-3 units of patient blood, segregating stem cells and granulocytes and returning red blood cells and plasma fractions to the patient. Generally, after 1-2 sessions lasting 3-5 hours, sufficient levels of PBSCs are collected [25]. Further isolation of PBSCs is necessary to limit the risk of GvHD due to potential contamination with donor T-cells [26]. Clinical reviews indicate positive outcomes using PBSCs in cartilage injury repair and hematological malignancies [19,27]. Synovial-Derived Mesenchymal Stem Cells (SDMSCs) are often used to treat OA and cartilage injuries, where studies indicate frequent clinical improvements [28]. These cells can be harvested from fibrous or adipose synovium at various locations during synovial joint arthroscopy or arthroplasty [29]. After harvesting, a tissue fragmentation and filtration procedure with or without collagenase digestion can isolate SDMSCs [30].

Dental pulp has gained significant interest as a source of adult MSCs. DPSCs are found in dental crowns with other heterogeneous MSC populations. DPSCs are naturally responsible for repairing tooth damage and regulating the periodontal immune system, explaining why cellular therapy often isolates DPSCs from other native MSCs [31]. DPSCs can be harvested from removed teeth due to caries, periodontic and orthodontic issues [32]. A combination of enzymatic digestion, tissue fragmentation and filtration isolate these cells [33]. DPSC cellular therapy studies were initially focused on addressing oral diseases like caries and periodontal disease. Recently, these have been tested with promising results in ocular, circulatory, neurological, orthopedic and other pathologies [34].

Birth-associated mesenchymal stem cells can be non-invasively harvested from perinatal tissues after birth and include Placental Mesenchymal Stem Cells (PL-MSCs) and Umbilical Cord Mesenchymal Stem Cells (UC-MSCs) [35]. The umbilical cord contains fetal MSC populations found in the cord lining locations like the amnion, subamniotic Wharton's jelly and the perivascular Wharton's jelly areas, which produce WJ-MSCs and HUCPVCs [36]. PL-MSCs are harvested from the fetal placenta. The cord-

placental junction also contains an intermediary population of MSCs [37]. The clinical applications of these different MSC populations have been studied and improvements in recipient health have been demonstrated in GvHD, Ulcerative colitis, Type 1/2 diabetes mellitus, liver disease, spinal cord injury, encephalopathy, cerebral palsy, ALS, heart failure, MI, Lupus and MS [38].

MSC Cellular Benefits

The scientific focus on the applications of MSCs owes to their many properties that hold promise in addressing the cellular origin of medical issues (Fig. 2). MSCs activate extracellular matrix (ECM) production, which enables natural repair cells to migrate to injury sites and aids in tissue repair [40]. When tissues become injured, MSCs naturally mobilize to damaged sites [41]. Chemotactic gradients from signaling molecules secreted at the damaged location mediate this process [42]. Therefore, it was theorized that transfected MSCs aid healing by following chemotactic gradients and physically incorporating into damaged tissues through multipotent action or fusing with native progenitors [43]. MSCs also promote angiogenesis, which is paramount in restoring ischemic tissue and increasing nutrient and healing factor availability [44,45]. The immunomodulatory and anti-inflammatory effects of MSCs have been extensively studied. Through direct and indirect interactions, MSCs can decrease the proliferation and activation of immune cells with inflammatory phenotypes like T cells, macrophages and B lymphocytes [46,47]. At the same time, these MSCs increase the activity of anti-inflammatory phenotype T-cells and B-cells, acting to reduce uncontrolled inflammation [48]. Also, MSCs substantially decrease Reactive Oxidative Species (ROS) levels that damage tissues and hinder healing [49].

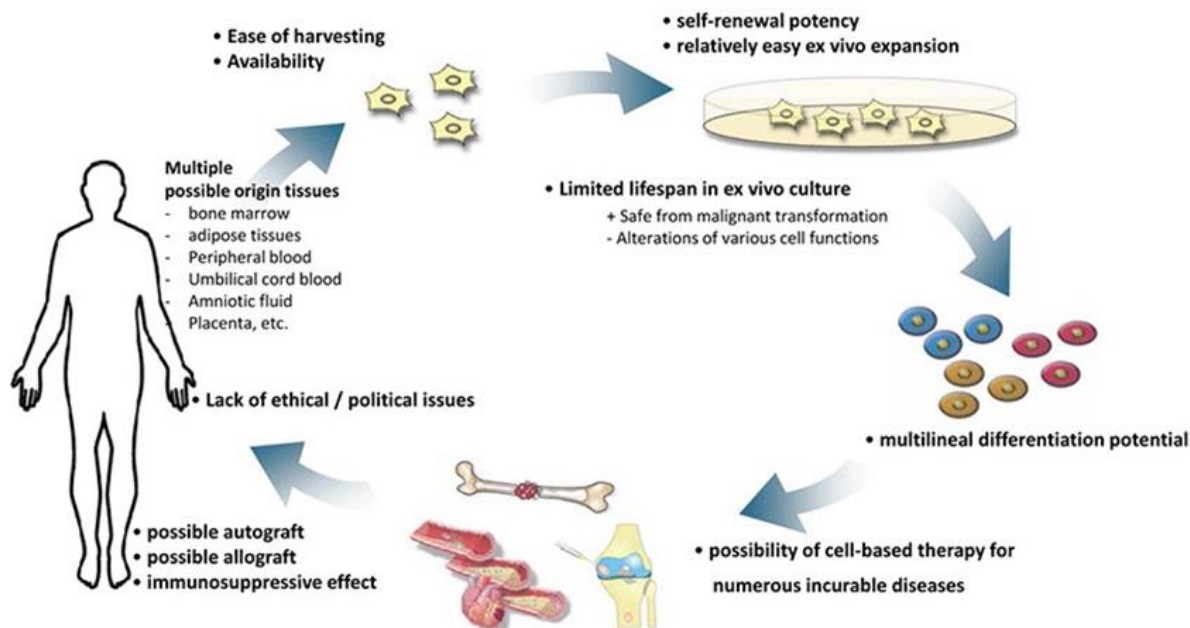


Figure 2: Graphic explaining the advantages of MSC therapy [39].

MSC Issues

The application of different MSC populations presents significant hurdles. Allogeneic MSC applications were initially considered, as it was believed the immunomodulatory properties of MSCs would make them immune privileged. However, studies have illustrated a high risk of immune rejection of allogeneic MSCs and a minor risk of developing GvHD [19,50,51]. While immunological compatibility matches can improve this risk, this process adds major roadblocks to allogeneic MSC therapy. Autologous MSCs largely avoid these immunological issues, but harvesting these cells is often highly invasive and painful [52].

Additionally, studies demonstrate that MSC properties depend on donor characteristics, including age, genetic traits and medical conditions. MSCs from elderly donors are more likely to be senescent with reduced proliferation and differentiation capabilities [53]. Patients with systemic inflammation or obesity produce MSCs with less immunomodulatory effects [54]. Therefore, MSCs from elderly, obese or diseased donors have lower therapeutic potential than those from young and healthy donors [55].

MSCs make up a small percentage of native cells and harvesting yield and quality varies with donor identity [56]. Generally, low MSC yield requires MSC expansion by culturing many passages of cells. This practice can result in senescence, differentiation and mutations [57]. Research indicates senescence increased significantly in later passages, causing changes in cell morphology and reducing cell differentiation and proliferation capabilities [58]. Mutations were also found to occur in later passages due to accumulated replication stressors; while these mutations can decrease transfected cell healing efficacy, they haven't been found to induce tumorigenesis [59,60]. Experimenters must balance the amount of passages to maximize cell growth and minimize cell damage.

MSCs have a risk of unwanted differentiation and tumorigenicity. Studies have shown some transfected MSCs differentiate into undesired cell types that could cause therapeutic issues. There is evidence that MSCs applied to treat ischemic MI in the heart formed calcifications and ossifications and MSCs applied to the liver can form myofibroblasts [61,62]. Unwanted differentiation may be due to long-term culturing or not using correct signaling molecule levels to maintain pluripotency. Two studies indicated long-term cultured MSCs are genetically unstable and may undergo cancerous transformation, but later reports suggested these cultures were contaminated with malignant cells. Two additional studies suggested tumor formation occurred from isolated MSCs, but these studies later reported contamination from tumor cell lines. Technological advances in cell isolation limit the risk of culture contamination [63]. Furthermore, molecular cytogenetic techniques could be applied to a sub-sample of MSCs before transfection to limit the risk of culture-induced mutations [64]. However, these practices are expensive and time-consuming.

MSCs are challenging to isolate due to a lack of one-cell membrane protein that differentiates them from other native cells. Furthermore, there is significant evidence that MSC populations at given locations are heterogeneous and can be divided into different subpopulations, which may have different therapeutic advantages or disadvantages [65]. However, emerging technologies enable greater precision in purifying MSCs and these technologies could conceivably be applied to select specific MSC subpopulations [56]. More research must be done to identify the MSC subpopulations within certain locations, determine if processing can select for or exclude key subpopulations and understand their different therapeutic capabilities [65].

There is major variance in MSC processing and administration techniques. As detailed above, most MSC purification procedures involve a mix of tissue microfragmentation, enzymatic digestion, centrifugation and ultrafiltration to separate the live MSCs from tissue contaminants, non-MSC cells and apoptosed cells. There is no universally accepted framework for location-specific purification procedures, but there is a growing consensus that mechanical separation should be favored over enzymatic digestion to promote genomic stability [30]. Additionally, MSC expansion procedures use various cellular media and chemical additives to extract cells at variable passages. Ideal MSC administration routes are still being determined. MSCs are applied intravenously (IV) or intraarterially (IA) for systemic delivery. For local delivery, MSCs can be applied topically to a wound, injected with media or gel directly into tissue or seeded into a scaffold applied to damaged tissue [66,67]. Further research must determine the most effective, specific procedures to produce and clinically deliver MSCs or MSC byproducts [68].

There is significant variation in the results of MSC clinical trials, likely resulting from divergent locations, sub-populations, purification methods, culture procedures, administration routes and donor characteristics utilized by different groups; for more information, see Secretome Effectors.

MSC Secretome

While researchers initially believed that MSC Cellular Benefits were due to the addition of MSCs to injury sites and in-vitro injury models, this viewpoint is increasingly being reconsidered. When MSCs are applied to living models, their survival time is frequently minuscule compared to their healing effects. While some studies have reported positive integration of transfected MSCs into injury sites many others point to limited engraftment and viability in damaged tissues [69-74]. Furthermore, studies demonstrated less than 1% of systemic MSCs remain in any organ system one week after administration [75]. Therefore, the beneficial effects of MSCs are likely due to factors other than their conversion into regenerated tissue. A growing consensus is being reached that many of the therapeutic effects of MSCs are due to the paracrine factors produced in their secretome, which protect injured tissues and promote endogenous tissue repair [55,76,77].

To understand the effects of the secretome and why these likely explain the cellular benefits of MSCs, one must understand its composition. This secretome can be divided into two groups: the soluble fraction and the vesicular fraction (Fig. 3) [78]. Due to the sheer number of components, it contains, explaining the complete contents of the MSC-derived secretome is beyond the scope of this article. Furthermore, these components are variable even among MSCs with the same immunophenotype, growth characteristics and differentiation abilities, much less in different MSC populations [79]. However, this section will highlight some key components of the secretome that help explain its positive clinical effects.

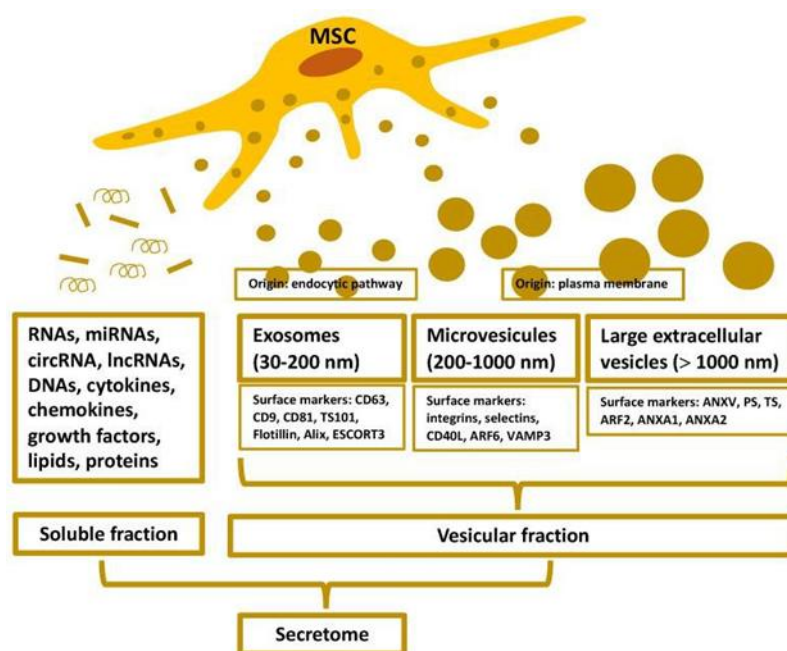


Figure 3: Depiction of the MSC-derived secretome classifying the soluble and vesicular fraction, subclassifying the vesicular fraction and describing the origin, size and characteristic surface markers of secretome components [78].

Soluble Fraction

The soluble fraction includes serum proteins, ECM proteins, ECM proteases, growth factors, angiogenic factors, cytokines and low levels of lipid mediators and genetic material [78-81]. These components can be secreted via classical and non-classical mechanisms [80]. The most extensively studied aspect of the soluble fraction is the proteome. In an extensive study of the ASC proteome, researchers grouped secreted proteins into many therapeutically beneficial groupings, including ECM formation and maintenance, cell motion, blood vessel development, wound healing, immune response, bone development, neuron projection development, regulation of cell development, regeneration and intramolecular oxidoreductase activity [79]. Historical research has also reported the most recognized therapeutic growth factors and cytokines. More research needs to be conducted to determine the effects of lipid mediators and genetic material in-vivo. However, free genetic material is probably largely degraded before impacting native cells, while proteins and signaling molecules are more resistant to degradation [82,83].

Many proteins secreted in the MSC secretome are associated with ECM formation. These include collagens and collagen-maturation enzymes, matricellular proteins and laminins [79]. These proteins can help repair injury sites and improve native cell growth, migration and survival [84]. The MSC secretome has also been shown to be anti-fibrotic, causing decreased ECM overaccumulation and scar formation through proteins like MMPs, TIMP and MFGE8 [85,86]. A complex interplay between these contrasting qualities may explain how the secretome could encourage tissue healing and limit scarring. It supplies ECM proteins to create structures for scaffolding and cell migration while limiting its overaccumulation through proteolytic enzymes and signaling factors promoting ECM degradation and softening [85].

The MSC-derived secretome also promotes cell migration through other pathways. The secretome includes proteins like tropomyosins that aid in physical motion and cytokines that synergistically induce cell migration, angiogenesis and tissue repair [79]. These include VEGF-A, MCP-1/3, MMP2/9 and HGF [87,88]. Cell migration stimulators aid healing by enabling necessary

immune cells, native regenerative cells and neoangiogenic cells to propagate to injury sites. These processes can expedite the removal of damaged tissue and its replacement by vascularized, functional tissue [89].

The angiogenic properties of the secretome are instrumental in its therapeutic benefits. The secretome includes components that encourage vascular regeneration by stimulating arteriogenesis and sprouting angiogenesis, including proteins like N-cadherin, biglycan lysyl oxidase, Cyr61, VEGF, VWF, VCAM1, Angiopoietins, FGF2 and CCL2 [79,90-95]. The secretome contains significantly more factors promoting angiogenesis, as many additional components that regulate ECM, encourage migration and promote cell survival are pro-angiogenic. Angiogenic processes improve blood supply to damaged tissues. This action is vital, as angiogenesis decreases tissue damage and promotes repair by supplying vital nutrients, growth factors and immune cells to injury sites [96].

Signaling molecules involved in regulating cell development and anti-apoptosis are partially responsible for the trophic effects of the secretome. These factors include PEDF, CYPA/CYPB, Cys C, IL-6, Gal-1, IGF-1 and HSP27 [79,114,97]. Trophic effects stimulate cell proliferation and survival, which are important in tissue regeneration and angiogenesis [98].

The MSC secretome is closely associated with immunomodulatory effects (Fig. 4). MSCs have been shown to increase regulator T-cell activation and propagation, cause effector T-cell anergy, modulate the activity of macrophage and dendritic cells and reduce inflammatory B cell activity [99,100]. These immunomodulatory effects are instrumental in reducing inflammation and destructive immune reactions [78]. Factors responsible for immune regulation and the secretome's anti-inflammatory properties include TGF β , HGF, NO, IDO, mpCCL2, IL-1Ra, PGE2 and Gal- 1/9 [101,102].

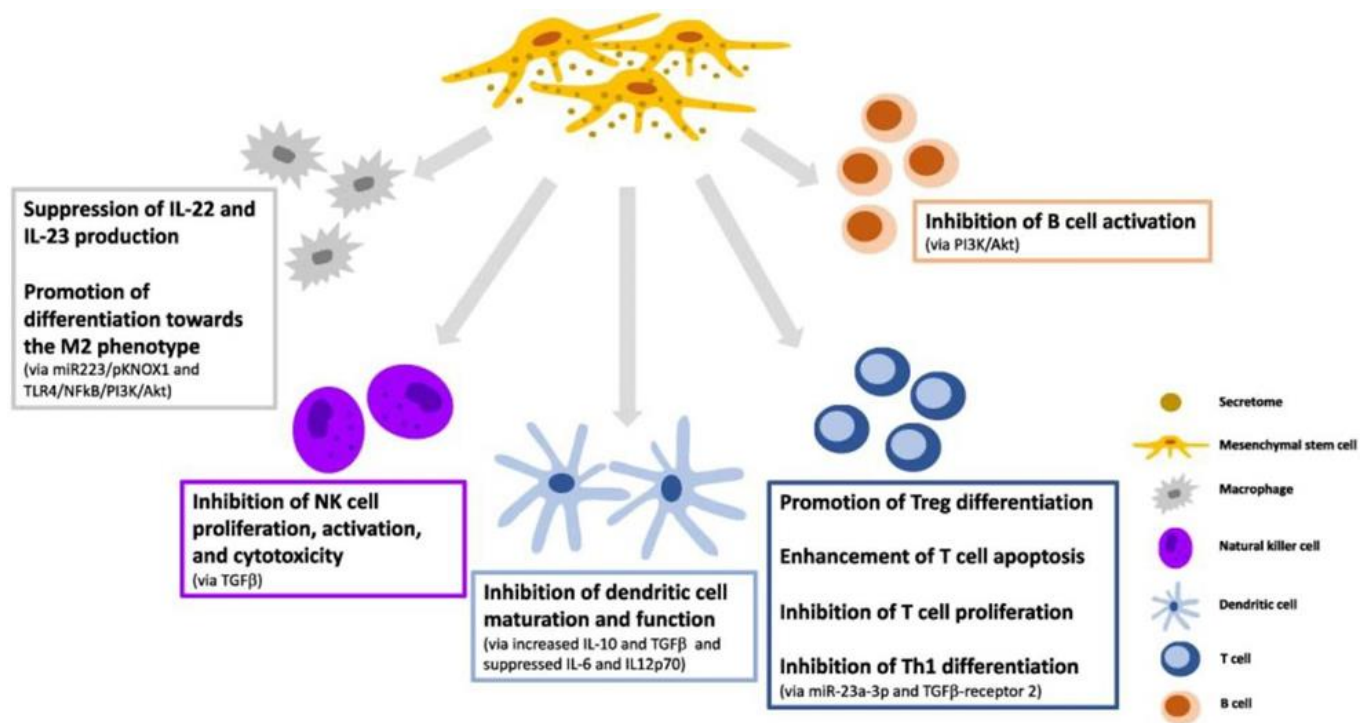


Figure 4: Collection of the immunomodulatory actions of the MSC secretome [78].

It should be noted that the secretome also includes some pro-inflammatory and immunogenic factors, including TGF β , PTX3, TNF α , IL-6, IL-8, IL-1 β and Sem7A [79,102]. These factors enable innate immunity, adaptive immunity and inflammation necessary to stimulate healing processes. However, some are associated with chronic inflammation and autoimmune disease development [103,104]. While the MSC secretome applied in-vivo has recurrently demonstrated balanced immunomodulatory and anti-inflammatory properties which promote healing overly elevated levels of these factors could explain why donor-related characteristics like elevated age, obesity and immune conditions can sometimes cause MSC treatments to be pro-inflammatory and immunogenic [105-107].

The secretome includes components involved in oxygen homeostasis regulation [108]. For example, TRX, Cys C, PRDX1, HSP27 and prolyl 4-hydroxylase help neutralize oxidatively reactive factors [79,109-114]. This process is important in preventing oxidative stress that induces cell death and harms regeneration potential [109].

Many secreted proteins and growth factors have neurotrophic, neurogenic, neurodifferentiative and neuroprotective actions. Growth factors like PEDF, CADH2 and IL-6 increase neuronal outgrowth, survival and differentiation [110,114]. TRX promotes hippocampal neurogenesis [114]. N-cadherin promotes neural interactions with Schwann cells [111]. SEM7A, GDN and BASP-1 promote neurite outgrowth and axon guidance [114]. IGF-1 is a neurotrophic factor that encourages synaptogenesis [112]. BDNF, NGF, NT3, IL-10 and TGF β also act as neurotrophic factors [86,113]. UCHL1 reduces proteasomal degradation, a hallmark of neurodegenerative disease. Cytokines including IL-6 and PEDF are neuroprotective against excitotoxicity, where neurons are overactivated with excitatory neurotransmitters in stroke, traumatic brain injury or neurodegenerative disease [114]. While these properties are essential for regaining broad tissue functionality, they specifically hold promise in nervous system disorders [114].

Vesicular Fraction

The vesicular fraction includes nanovesicles (called exosomes in graphic), microvesicles and Large Extracellular Vesicles (L-EVs) arranged in ascending order of size (Fig. 5). These components are all surrounded by phospholipid bilayers. Nanovesicles and microvesicles generally contain secondary metabolites, proteins, nucleic acids and lipids. These nucleic acids are primarily mRNAs, miRNAs and non-coding RNAs. L-EVs include oncosomes, produced by cancer cells and apoptotic bodies, made by dying cells. L-EVs often enclose organelles, proteins, nucleic acids and lipids. These nucleic acids include DNA, coding RNA and non-coding RNA [78].

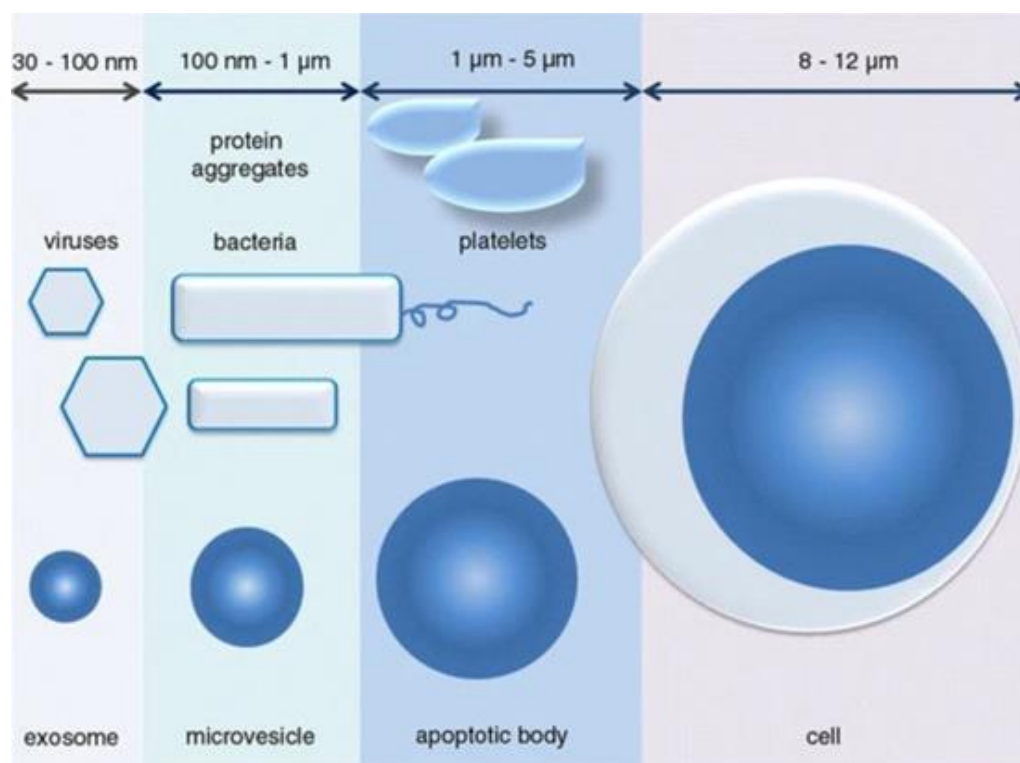


Figure 5: Representation of the major classes of membrane vesicles, including descriptions of their sizes and size comparisons with other viruses, protein aggregates, bacteria, platelets and human cells [115].

Outward budding plasma membranes produce microvesicles and large extracellular vesicles [116]. Nanovesicles are formed through the inward budding of late-stage endosomes, creating Multivesicular Bodies (MVB). These endosomes containing MVBs can then fuse with the plasma membrane to release MVBs, which are called nanovesicles when secreted extracellularly [116,117]. Different sizes and surface markers can differentiate these vesicular fraction sub- categories, although it's challenging to isolate

nanovesicles and microvesicles due to similar characteristics and markers. Therefore, these are grouped as EVs and sometimes separated from L-EVs for therapeutic uses [101]. However, Apo-EVs have been linked to immunosuppressive and anti-inflammatory properties in GvHD and OA, conflicting these protocols [118]. Furthermore, EV isolation is difficult; low recovery rates and reduced functionality are present with ultracentrifugation and low throughput hinders size exclusion and antibody-based capture. Recently, developments in microfluidics, anion-exchange chromatography and ultrafiltration have shown promise in more efficient EV isolation [78].

The therapeutic effects of EVs are due to surface marker interactions or their paracrine delivery of transcriptional factors and functional RNAs to target T-cells through vesicle endocytosis or vesicle-cell membrane fusion [101,115]. EVs express adhesion molecules, which facilitate EV capture by target T-cells [119]. Once connected to target cells, vesicles can undergo endocytosis or vesicle-cell membrane fusion [115]. If undergoing membrane fusion, the EV surface receptors and bioactive lipids are added to the target cell's membrane. This mechanism could induce greater cellular responses to the soluble fraction of the secretome [120,121]. If internalized by endocytosis, EV contents are ferried by endosomes and are often degraded in the lysosome and displayed on cell surfaces [122]. However, some endosomes escape these fates and deliver their contents to the cytosol [123]. Whether through endosomal escape or membrane fusion, EV components in the cytosol can affect host cells. If EV cargoes contain signaling proteins, they can begin signaling cascades. This process has been linked to inducing angiogenesis, immunomodulation and cell division [124]. Especially interesting to scientists is the capability of EV mRNAs to be transferred to and translated in target T-cells [119,120]. The possibility of horizontal transfer of mRNAs enables EVs to change the phenotype of recipient cells. Studies have shown this process can increase native stem cell pluripotency, deliver trophic signals and promote angiogenesis in vivo [121]. In both protein and nucleic acid transfection, EVs can act to reprogram target T-cells to become more like EV source cells. This quality may help explain the long-lasting effects of the secretome.

It should be noted that the MSC-derived secretome can be applied through Conditioned Medium (CM), which contains the soluble and vesicular fraction and MSC-derived EVs, which only include a part of the vesicular fraction [125].

Secretome Effectors

The secretome can be impacted by donor characteristics, tissue source and preconditioning techniques. The age, sex and health status of MSC donors can affect MSC proliferation, differentiation, metabolism and gene expression [107,126]. Some studies suggest these effects also translate to the secretome. Elevated age has been associated with decreased beneficial VEGF and HGF and increased deleterious IL-1 α and TNF- α concentrations in the AD-MSC secretome. However, another proteomic analysis of the secretome found minimal significant differences between MSCs from older versus younger donors and an additional study found the concentration of trophic factors in the secretome isn't affected by donor age [107,126]. MSCs from female donors may have greater anti-inflammatory and proangiogenic factors compared to those from male donors, as they were found to secrete more VEGF and TSG-6 and less TNF- α in primed conditions [107]. Furthermore, this secretome may exhibit greater immunomodulatory effects. However, contrasting studies suggest minimal differences between the soluble fraction contents of female- and male-derived MSC secretomes [127]. MSCs from clinically obese donors produced more inflammatory cytokines and fewer proangiogenic factors [107]. Additionally, these secretomes are less effective in anti-oxidative and immunomodulatory functions [128,129].

As described in MSC Secretome Types, MSCs can be harvested from various tissues and these different populations exhibit different secretome properties. A comprehensive proteome study suggested that BM-MSCs and AD-MSCs secrete more ECM proteins, while UC-MSCs secrete more factors promoting proliferation and differentiation [107]. Another proteomic study comparing BM-MSC, AD-MSC and HUCPVC secretomes suggested all had the potential to confer neuroprotective, neurogenic and neurodifferentiative properties. This study identified BM-MSC secretome as more anti-oxidative, HUCPVC and AD-MSC CM as more neuroprotective and HUCPVC CM as less anti-fibrotic and less conclusive in conferring advantages in neurite outgrowth and neurodifferentiation. While many essential proteins were present in all three secretomes, many additional critical proteins were present in some and absent in others [114]. A study comparing gene expression levels between BM-MSCs, WJ-MSCs and DPSCs determined that all secretomes contained key proangiogenic, trophic and inflammatory factors. However, certain factors were found at higher concentrations in different secretomes [130]. These studies demonstrate that all MSC locations may hold therapeutic potential. However, further studies elucidating the most important soluble factors in treating

specific disease models are needed to select the most appropriate therapy. Furthermore, additional studies on the location's effects on EV populations are required.

Many processing techniques applied to MSC cultures have been tested to influence the secretome's contents (Fig. 6). These methods include physiological preconditioning by inducing hypoxia, near-anoxia or 3D culturing and molecular preconditioning by applying cytokines, chemokines and signaling molecules [80,132].

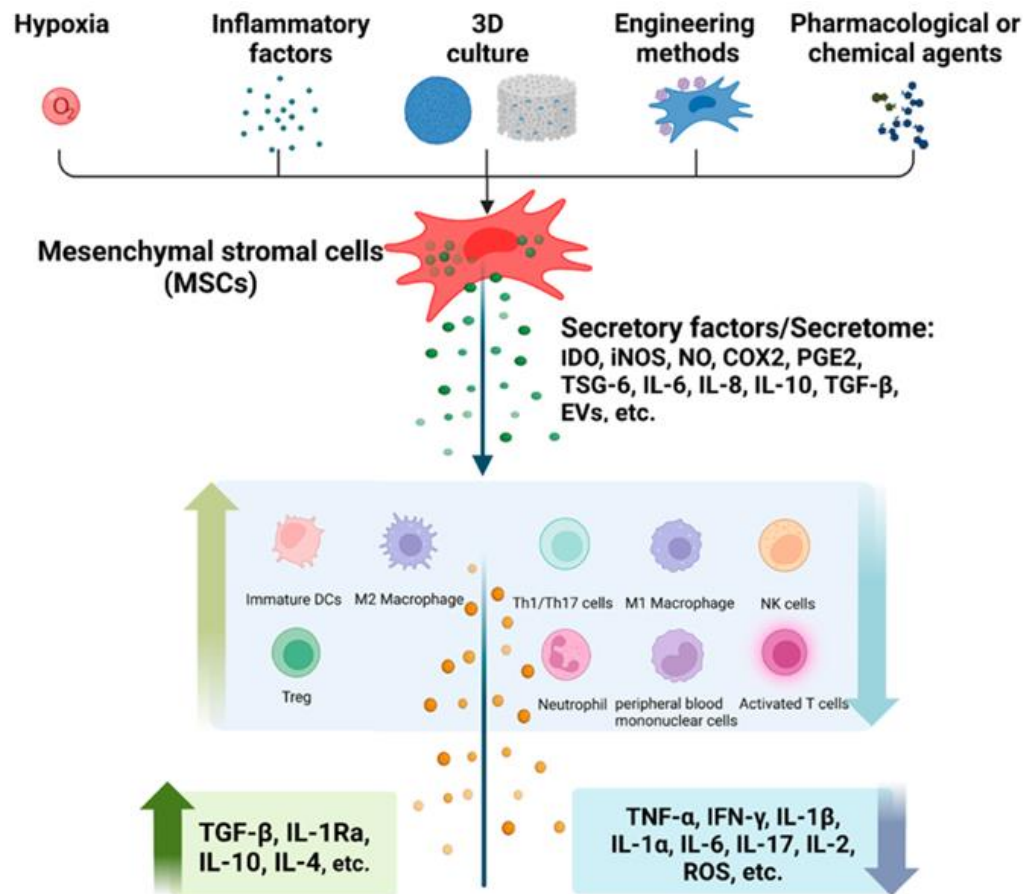


Figure 6: Illustration of some methods used for MSC preconditioning, including critical secreted factors upregulated and downregulated in the secretome and their effects on immunoregulation [131].

Many MSCs are found in hypoxic conditions *in-vivo* and it has been suggested that normoxia may hinder MSC functionality by putting them in non-natural environments and introducing high levels of free radicals into cultures, although this is debated [133,134]. While hypoxic conditions cause some cell death, prolonged hypoxia is associated with increased proliferation and differentiation capabilities [134]. Hypoxic and anoxic conditioning also result in greater expression of many angiogenic and trophic factors in the secretome. These effects are mediated through cellular production of Hypoxia-Inducible Factor (HIF), which changes the expression of proteins involved in cell survival, migration, proliferation, differentiation and angiogenesis [135]. Low oxygen levels are associated with higher expression of crucial paracrine signaling molecules like VEGF, beneficial ILs, PLGF-1 and MCP-1, although the significance of these findings varies with different studies [136-138]. This conditioning also affects EVs, as HIF expression systematically shifts their contents to increase their therapeutic potential. Studies have shown EVs produced by hypoxic MSCs increase angiogenic, anti-apoptotic and anti-oxidative properties *in-vitro* [139].

These effects have translated to some promising *in-vivo* results. CM from hypoxia-treated BM-MSCs was significantly better at restoring rat neurological function after traumatic brain injury compared to normoxia-treated BM-MSCs. A similar study applying BM-MSC CM to rat hepatectomy models found hypoxic preconditioned CM significantly improved survival than

normoxic preconditioned CM. Additionally, hypoxic BM-MSC CM significantly inhibited diabetic cardiomyopathy progression in rat models compared to normoxic CM [140]. However, other studies haven't reported significant differences in the beneficial in-vivo effects of hypoxia- and anoxia-produced CM [141].

MSCs are often cultured in monolayer systems, but 3D culturing can influence the secretome's contents [140]. This model causes MSC aggregation that more closely approximates their natural environment, increasing cell-cell interactions and paracrine signaling in cultures. 3D culturing methods vary widely, as researchers can create spheroids through hanging drops, nonadherent cultures and 3D reactors. They can also use biomaterials in scaffolds and hydrogels to induce 3D configurations [142].

3D cultures confer beneficial immunomodulatory effects on the secretome. Spheroid and biomaterial-cultured MSC secretomes displayed elevated PGE2, HGF and TSG-6 [142]. When applied to LPS-stimulated macrophages, the secretome decreased stimulated macrophage TNF- α and CXCL2 secretion and increased macrophage IL-10 secretion and CD206 expression, which are associated with anti-inflammatory macrophage states [140,143]. This culturing method is also associated with statistically significant increases in VEGF, angiogenin, IL-11 and BMP-2 secretion. Furthermore, spheroid cultures were superior at stimulating native cell proliferation and migration than 2D cultures, suggesting 3D cultures may confer angiogenic and wound healing advantages [138,144]. While 3D culturing is associated with different EV protein contents, some studies show EVs produced by 3D cultured MSCs don't exhibit significantly greater therapeutic effects [145].

Many molecular preconditioning techniques aim to create a pro-inflammatory environment for secretome-producing MSCs, as it is theorized this stressor would increase the immunomodulatory components in the MSC secretome. IFN- γ , IL-1 α and TNF- α are the most frequently used pro-inflammatory cytokines applied to MSC cultures [146,147]. There is evidence that each of these cytokines causes different patterns of secretion and immunomodulation [147]. TNF- β preconditioning is associated with increased IL-6 and IL-8 secretion, which increase proliferation, chemotaxis and angiogenesis linked with wound healing [148]. Additionally, this secretome contains more immunosuppressive molecules like PGE2 and IDO [149]. IL-1 β preconditioning increased the concentration of immunomodulatory factors and decreased the concentration of pro-inflammatory cytokines in intervertebral disk degeneration models [150].

This factor also causes increased IL-6 and IL-10 secretion and decreased T-helper cell activity [149]. IFN- β preconditioning increases immunosuppression by increasing IDO and PGE-2 secretion, decreasing deleterious T-cell activation [140].

Studies have demonstrated conflicting results about the effect of pro-inflammatory preconditioning on the angiogenic potential of the secretome. A study demonstrated this technique increases wound healing and angiogenesis in-vivo [148]. Some studies show increased secretion of pro-angiogenic proteins like IL-6, IL-8, MCP-1 and CXCL1 [146,150]. Others suggest that the pro-inflammatory preconditioned secretome is anti-angiogenic by limiting endothelial cell proliferation, migration, tubulogenesis and sprouting capacity while increasing the secretion of anti-angiogenic signaling factors like TIMP1 [151,152]. These conflicting results may be due to different methodologies in pro-inflammatory preconditioning and further studies are needed to elucidate proper procedures in this technique.

MSC Secretome Commercial Potential

The MSC secretome also has unique characteristics which increase its bioregenerative and commercial potential. Secretome application would bypass difficulties experienced in transfected cell viability and survival in-vivo, potentially allowing for a longer-lasting therapeutic dose of the secretome [81].

Preparation procedures can readily modify the MSC secretome to maximize its therapeutic potential for various conditions. These strategies include altering the physical culture environment, physiologic preconditioning and molecular preconditioning. Cell-free strategies also limit the risk of adverse immunological reactions or immune rejection, as the secretome contains minimal levels of cell proteins like MHCs [153,154]. This property reintroduces the prospects of bioregenerative allogeneic therapies with many potential benefits. Allogeneic MSC secretomes would be readily available for use in time-sensitive or acute clinical pathologies [154,155]. Furthermore, allogeneic MSCs could be administered to patients whose characteristics could

compromise autologous MSC therapeutic potential or limit their ability to donate MSCs. While studies indicate some allogeneic donors should be excluded from MSC donation, the manufacturing process of combining MSCs from various donors could reduce donor-related effects in the aggregate and promote secretome uniformity. Cell-free strategies can be delivered in more ways than MSCs, as there is no risk of damaging cells in application. Therefore, the secretome can be integrated into powders, gels and scaffolds more effectively than MSCs and it can be injected with smaller needles [156-158]. These differences mean the secretome can be better targeted to specific injury models [154].

While these therapies should still test the pluripotency and genomic stability of their MSCs, the risk of unwanted differentiation or malignant cell transfection is nullified with cell-free treatments [64,159,160]. Cell-free products could be produced in dynamically controlled laboratory conditions, increasing production rates and maximizing MSC secretion efficiency. Furthermore, these conditions would improve product homogeneity, enabling the treatment to consistently deliver therapeutic doses at the proper potency of each bioactive component [154]. The MSC secretome is cryostable, meaning it can be frozen for long periods while maintaining its efficacy [161]. Due to all these factors, the MSC-derived secretome is more economically viable than other bioregenerative therapies due to the potential for mass production [162].

Results/Discussion/Conclusion

See “Part 2: Bioregenerative Applications of the Human Mesenchymal Stem Cell-Derived Secretome.”

Conflict of Interest

The authors have no conflict of interest to declare.

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