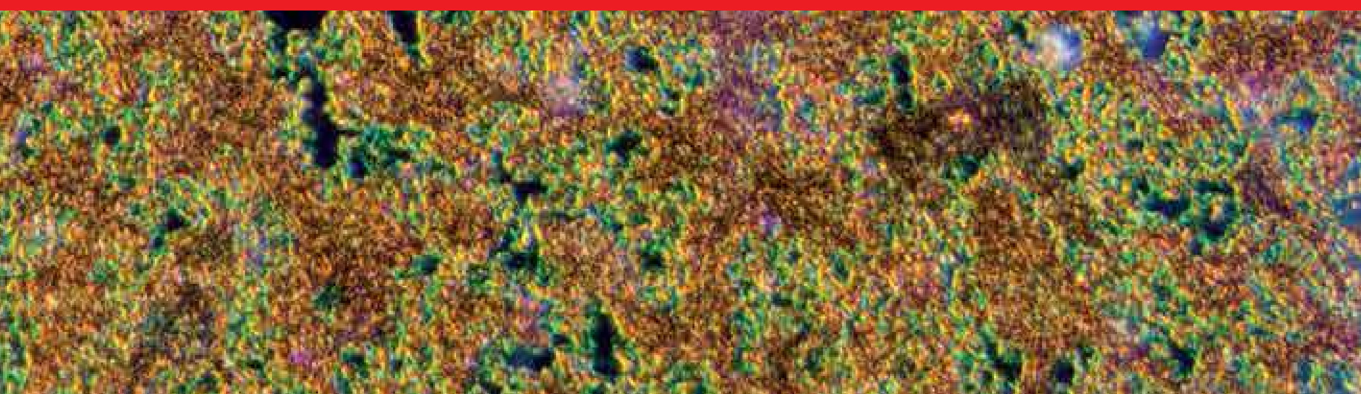
Stromal Cells

Structure, Function, and Therapeutic Implications

Edited by Mani T. Valarmathi



STROMAL CELLS - STRUCTURE, FUNCTION, AND THERAPEUTIC IMPLICATIONS

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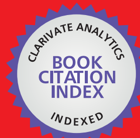

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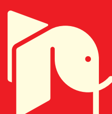

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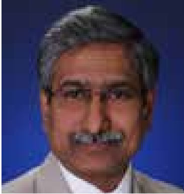

Valarmathi M. Thiruvanamalai is presently an assistant professor at the University of Alabama at Birmingham, USA. He began his scientific career as a cancer geneticist, but soon became captivated with the emerging and translational fields of stem cell biology, tissue engineering, and regenerative medicine. After completing his Bachelor's degree in Chemistry at the University of Madras, he received his MBBS in Medicine and Surgery and MD in Pathology from the University of Madras, as well as his PhD in Medical Biotechnology from the All-India Institute of Medical Sciences, New Delhi, India. For over 15 years, he has had extensive experience in research on various types of stem cells, including adult, embryonic (pluripotent), and induced pluripotent stem cells. Currently, his research work focuses on generating three-dimensional vascularized tissues and/or organs for implantation purposes. He is a member of many prestigious national and international professional societies and scientific organizations, such as ISSCR, TERMIS, AACR, ASIP, ACS, ESC, ISHR, ASGCT, and AHA.

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Preface

Stromal cells are connective tissue cells of any organ, and they support the function of the parenchymal cells of that particular organ. Stromal/stromal stem cells are fundamentally a heterogeneous population of cells with contradictory differentiation potential depending upon their environmental niche. Stromal cell biology is not only intriguing, but equally stromal cell ontogeny *in vivo* remains challenging.

In recent years, there has been substantial advances in our understanding of stromal cell biology, especially stromal cell isolation, characterization, differentiation, and interactions in physiological (epithelial–stromal interactions) as well as pathophysiological (stromal–cancer interactions) contexts. In addition, stromal cells are also utilized more and more as a therapeutic tool not only in the field of gene therapy but also in the translational field of tissue engineering and regenerative medicine. Therefore, the goal of this book is to consolidate the recent advances in the area of stromal/stromal stem cell biology, covering a broad range of interrelated topics in a timely fashion and to disseminate that knowledge in a lucid way to a greater scientific audience.

This book will prove highly useful for students, researchers, and clinicians in stem cell biology, developmental biology, cancer biology, pathology, oncology, as well as tissue engineering and regenerative medicine. This quick reference will benefit anyone desiring a thorough overview of stromal cell structure, function, and its therapeutic implications in human diseases.

The book consists of nine chapters, contributed by leading experts in basic science and clinical care, and is organized into three parts. The first part introduces the structure and function of stromal/stromal stem cells. The second part of the book deals with stromal cell interactions, such as stromal–epithelial interactions and stromal–tumor interactions. Eventually, in contrast, the third part explores the therapeutic potential of stromal/stromal stem cells as a double-edged sword.

I would like to thank the staff of IntechOpen who have produced this book so efficiently, and in particular I am indebted to Danijela Vladika and Nina Kalinic, the publishing process managers, Romina Skomersic, the author service manager, and Anja Filipovic, the commissioning editor, for their valuable source of advice throughout the preparation of this book. Finally, I dedicate this book to my father and the memory of my mother.

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Section 1

Stromal Cell Structure and Function

Chapter 1

Stromal Stem Cells: Nature, Biology and Potential Therapeutic Applications

Amira Hassouna, Marwa M. Abd Elgwad and
Hoda Fahmy

Additional information is available at the end of the chapter

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Abstract

Stromal cells are connective tissue cells of any organ. Mesenchymal stromal cells (MSCs), are multipotent progenitors, which were first described by Caplan and colleagues in 1991. MSCs hold great potential for regenerative medicine because of their ability for self-renewal and differentiation into tissue-specific cells such as osteoblasts, chondrocytes, and adipocytes. Recent studies indicate that MSCs resemble pericytes and emerge from the peripheral stromal region surrounding blood vessels, thus clarifying their broad regenerative potential in adult tissues. The development of uniform protocols for both preparation and characterization of MSCs, including standardized functional assays for evaluation of their biological potential, are critical factors contributing to their clinical utility. Nowadays, due to the capacity of modulating immunological responses, supporting hematopoiesis and repairing tissues, MSCs have been widely used to treat immune-based disorders, such as Crohn's disease, rheumatoid arthritis, diabetes, and multiple sclerosis. Based on animal experiments and clinical studies, the most successful clinical application of MSCs is in the field of hematological disease.

Keywords: mesenchymal stromal cells, stromal stem cells

1. Introduction

Stem cells have the ability of self-renewal, giving rise to a variety of cell lineages. They constitute a significant paradigm of cell-based therapy for various diseases.

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Embryonic and non-embryonic stem cells, are the two principal types of stem cells. Embryonic stem cells (ESCs) originate from the blastocyst's inner cell mass and have the ability for differentiation into cells of all three germ layers. However, teratoma formation and ethical controversy represent an obstacle in their research and clinical application.

On the other side, non-embryonic stem cells, mostly adult stem cells, are fairly specialized and have limited differentiation potential. They can be isolated from various tissues and are currently the most used in regenerative medicine.

Over the last decade, cellular therapy has developed quickly at the level of in vitro and in vivo preclinical research and in clinical trials. Mesenchymal stem cells (MSCs), one type of adult stem cells, have provided a great amount of interest in the field of regenerative medicine due to their unique biological properties [1]. The acronym "MSCs" is restricted to the subset of mesenchymal cells demonstrating stem cell activity by accurate criteria.

1.1. Definition

MSCs are multipotent stromal cells that can differentiate into a variety of cell types, including: osteoblasts (bone cells), chondrocytes (cartilage cells), myocytes (muscle cells) and adipocytes (fat cells which give rise to marrow adipose tissue). MSCs exist in organisms (*in-vivo*) and have been studied as well in tissue culture (*in-vitro*) [2].

1.2. Terminology

Due to the lack of a particularly unique MSCs function, they have been termed 'mesenchymal stem cells' or interchangeably 'mesenchymal stromal cells', 'BM (bone marrow) stromal cells' and 'marrow stromal cells'. MSCs are usually identified by mere plastic adherence and by their morphological appearance, such as the fibroblastoid phenotype. This procedure leads to a diverse population containing both single stem cell-like cells and progenitor cells having various lineage commitment. Compared to hematopoietic stem cells (HSCs), which have been proven to repopulate the bone marrow and give all blood types, and embryonic stem cells (ESCs) which after re-injection into early embryos, were proven to participate in embryonic development of all tissues; MSCs have no established in vivo tests [3].

2. History

In 1970, Friedenstein and colleagues were the first who identified mesenchymal stem cells as colony-forming unit-fibroblasts (CFU-Fs) [4]. Pittenger and colleagues were the first to describe the tri-lineage potential of MSCs [3].

The first clinical trials of MSCs were completed in 1995 when a group of 15 patients were injected with cultured MSCs to test the safety of the treatment [1].

3. Sources

3.1. Bone marrow

BM-MSCs are isolated from bone marrow aspirate. This invasive procedure is painful for the patient with a risk of infection. The commonly used method for the generation of MSCs from bone marrow is density gradient centrifugation [3]. The collected fraction containing mononuclear cells is washed and the cells are seeded on a plastic dish for proliferation.

3.2. Adipose tissue

AT-MSC also termed as adipose-derived stem cells are usually isolated from the biological material generated during liposuction, lipoplasty or lipectomy procedures by enzymatic digestion with collagenase followed by centrifugation and washing [5].

3.3. Peripheral blood

Following a density gradient centrifugation, PB-MSC can be collected from the mononuclear cells' lymphocyte separation fluid fraction. Kassis et al. [6] described another method, which is loading PB-MSC on fibrin microbeads, then separating the cell loaded beads from the mononuclear fraction. This method allows getting enormous amounts of MSCs [6].

Pittenger et al. [3], isolated MSCs from BM by density gradient centrifugation to eliminate unwanted cell types and only 0.001 to 0.01% of the cells isolated from the density interface were identified as mesenchymal stem cells.

3.4. Umbilical cord blood

As umbilical cord blood contains MSCs, it could serve as an alternative source of MSCs to bone marrow. A novel method to obtain single cell-derived and clonally expanded MSCs that have multilineage differentiation potential, is negative immunoselection and limiting dilution. The immunophenotype of these clonally expanded cells is similar with bone marrow mesenchymal stem cells. These cells can differentiate into bone, cartilage, and fat under suitable induction conditions. These cells were also able to differentiate into neuroglial- and hepatocyte-like cells; therefore, these cells may be more than mesenchymal stem cells due to their ability to differentiate into cell types of all 3 germ layers [7].

4. Characteristics

In 2006, the International Society of Cellular Therapy defined characterization of MSCs by the following three criteria [8]:

1. MSCs must be adherent to plastic under standard tissue culture conditions;

2. Certain cell surface markers must be expressed such as CD73, CD90, and CD105, other markers must not be expressed such as CD45, CD34, CD14, or CD11b, CD79 alpha or CD19 and HLA-DR surface molecules;

3. MSCs must have the capacity to differentiate into osteoblasts, adipocytes, and chondroblasts under in vitro conditions.

MSCs generally have low immunogenicity as they do not express MHC class II or costimulatory molecules. Thus, injection of autologous or allogeneic MSCs has been employed in clinical studies. Allogeneic MSC therapy has the potential to expand MSCs therapy to a larger range of patients [9].

The effects of MSCs are generally achieved through two mechanisms:

1. Differentiation of recruited MSCs into functional cells to replace damaged cells, permitting the treatment of organ damage [9].

2. Response of MSCs to inflammatory cytokines, prepares the microenvironment through production of immune regulatory factors that modulate the progression of inflammation by affecting dendritic cells, B cells, T cells, and macrophages.

Furthermore, MSCs also produce a large amount of cytokines, chemokines, and GFs, which stimulate angiogenesis, prevent apoptosis, block oxidation reactions, promote remodeling of extra cellular matrix, and induce the differentiation of tissue stem cells [10].

In addition, under the effect of signals of cellular damage, known as homing signals, MSCs migrate toward areas of injury. This migration property of MSCs is important in regenerative medicine, where various injection routes are utilized depending on the damaged tissue or organ [11].

5. Morphology

MSCs are defined by a small cell body with a few long and thin cell processes. The nucleus is round and large with a prominent nucleolus, in the midst of finely spread chromatin particles, providing the nucleus a clear appearance. A small amount of rough endoplasmic reticulum, polyribosomes, Golgi apparatus and mitochondria are also present. The adjacent extracellular matrix is populated by a few reticular fibrils however other types of collagen fibrils are absent [12].

6. Differentiation capacity

The identification of specific signaling networks and 'master' regulatory genes that control unique MSCs differentiation lineages represents a major challenge. Obtaining a desired differentiation program, or preventing false differentiation of MSCs, needs ability to modulate biological effectors for effective clinical application, as in tissue engineering and regeneration.

6.1. Chondrogenesis

There is similarity between chondrogenic differentiation of MSCs *in vitro* and of cartilage development *in vivo*. In MSC-derived chondrocytes, the following has been positively characterized: expression markers associated with chondrogenesis; including transcription factors (sox-9, scleraxis) and extracellular matrix (ECM) genes (collagen types II and IX, aggrecan, biglycan, decorin, and cartilage oligomeric matrix protein) [13, 14]. Many helpful signaling molecules, involving many transforming growth factor-β (TGF-β), bone morphogenetic protein (BMP), growth and differentiation factor (GDF) and Wnt ligands, have been recognized through naturally occurring human mutations and molecular genetic studies. Chondrogenesis of MSCs from a variety of mesodermal tissue sources is rapidly stimulated by recombinant proteins and/or adenoviral infection of MSCs with TGF-β1 and TGF-β3, BMP-2, BMP-4, BMP-6, BMP-12, BMP-13, and GDF-5 [14, 15]. Through specific intracellular Smad proteins and major mitogen-activated protein kinase (MAPK) cascades, TGF-βs and BMPs signal provide levels of specificity that are widely studied in MSC differentiation contexts, upon receptor binding [16]. Downstream MAPK signaling and Smad effectors crosstalk has declared that MAPK substrates include chromatin histone acetyltransferases (HATs). Smads recruit HATs which enhance Smad transactivation capability [17].

Wnts possess double modulatory function in chondrogenesis. In human MSCs, Wnt7a induces chondrogenesis through various TGF-β1–MAPK signaling pathways when it is transiently upregulated, but in case of sustained expression, Wnt7a turns into chondroinhibitory [18]. Wnt3a controls *bmp2* expression [19], providing a feed forward regulatory loop during chondrogenesis. In ATDC5 cells, chondrogenesis is inhibited by Wnt1 through upregulation of the mesodermal basic helix–loop–helix (bHLH) transcription factor, Twist 1 [20], this effect may be through involving negative sequestration of chondrostimulatory factors or direct repression of target genes.

6.2. Osteogenesis

Two bone morphogenic proteins (BMPs), especially BMP-2 and BMP-6, stimulate osteogenesis in MSCs. BMP-2 acts by induction the p300-mediated acetylation of Runx2, a master osteogenic gene, which leads to enhanced Runx2 transactivating capability. Histone deacetylases 4 and 5 stimulate the degradation of Runx2 by deacetylation, through Smurf1, Smurf2 and E3 ubiquitin ligases [21]. The cytokine TNF-α, involved in inflammation-mediated bone degradation, downregulates Runx2protein levels by increasing degradation by Smurf1 and Smurf2. BMPs, Runx2, and histone deacetyltransferases that are responsible for the therapeutic approaches to MSC-based bone tissue engineering, stimulate existing TNF-α based immunotherapy of bone diseases.

Wnts is another important modulator in osteogenesis. Knockout and dosage compensation in Wnt-pathway-related transgenic animals provide the strongest proof that high levels of endogenous Wnts promote osteogenesis, whereas low levels inhibit osteogenesis [22].

The exciting finding of transcriptional mechanisms, suggesting that a global osteogenic gene, *runx2*, and a specific osteogenic homeobox gene, *tbx5*, are responsible for the balance of bone

formation and loss, show two strong models of transcriptional regulation of osteogenesis, and potentially other MSC lineage differentiation programs.

6.3. Adipogenesis

MSC adipogenesis is stimulated by the nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPARγ) but at the same time it represses osteogenesis. Through binding to various ligands, like long-chain fatty acids and thiazolidinedione compounds, PPARγ induces the transactivation and transrepression of PPARγ. The bipotent coregulators TAZ function as a coactivator of Runx2 and as a corepressor of PPARγ, thus promoting osteogenesis while blocking adipogenesis [23]. In general, osteogenic genes are corepressed by a coactivator of adipogenic genes, but the opposite is also possible. This type of cellular efficiency is very likely, allowing that MSCs may be differentiated to both lineages.

Stretch-related mechanoinduction represents another interesting example of exchange between transcriptional cofactors of adipogenesis. If stretch is induced on mouse embryonic lung mesenchymal cells they form myocytes but they form adipocytes if uninduced. This occurs through activation of specific isoforms of tension-induced/−inhibited proteins (TIPs) [24] chromatin-modifying proteins with intrinsic HAT activity that have other distinctive domains such as nuclear receptor-interacting motifs. TIP-1 which is expressed under non-stretch conditions provides a potential mechanistic endpoint for cytoplasmic RhoA-mediated of adipogenesis; induces RhoA signaling which stimulates adipogenesis [25]. Whereas TIP-3 induces myogenesis. These findings propose a molecular model that connects cell morphology mechanical induction cytoskeletal signaling and transcriptional response during MSC adipogenesis induction.

6.4. Myogenesis

The majority of studies of myogenesis in adult stem cells target skeletal muscle-derived stem cells, or satellite cells. The highly successful stimulation of myogenesis from adult stromal MSCs happened after transfection with activated Notch 1. Other studies, mainly target cardiomyogenesis, represented the importance of cell-cell contact in stimulating cardiomyogenesis through co-cultured MSCs and cardiomyocytes, and the stimulation of MSC cardiomyogenesis in a rat intramyocardial infarct model by Jagged 1, a Notch ligand [26].

In normal conditions, the MSCs are present in low numbers, and on induction of myocardial infarction (MI) these cells proliferate rapidly to participate in wound healing, by generation of fibroblasts and myofibroblasts.

After MI, MSCs penetrate the injured tissue by trafficking through the ECM and repairing the cardiac function. This is through production of *HGF* by apoptotic cardiomyocytes, and not by necrotic cardiomyocytes. MSCs are attracted to the apoptotic cell death site by *HGF* receptor MET, which are responsible for activation of a wide range of signaling pathways. Platelets migrate MSCs to the apoptotic cardiac cells by means of the interaction of a nuclear protein with *TLR-4* expressed on MSCs; high mobility group box-1 (*HMGB1*). On activation of platelet, *HMGB1*/*TLR-4* downregulate MET on MSCs, thus, decreasing the recruitment of the cells.

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Thereby, gene-knockout or blocking of *TLR-4* on MSCs can produce improved infiltration of MSCs to the damaged tissue, thus, raising the efficacy of MSC-based therapy [27].

In myocardium damage, Stromal cell-derived factor-1α (SDF-1) is a chemokine that mediates the homing of the endogenous MSCs. An intracellular storage of the receptor*CXCR4* present in 80–90% of hMSCs but not expressed in large amounts on the surface. When it is expressed by mRNA nucleofection, Ca^{2+} signaling is stimulated through its ligand *SDF-1*α [28]. However, in dilated cardiomyopathy (DCM), another homing factor of MSCs, monocyte-chemotactic protein-1 (*MCP-1*), has been established because of the presence of chemokine receptor type 2 (CCR2), a *MCP-1* receptor, on the cell surface [29]. Many in-vivo and in-vitro studies have been performed to comprehend the mechanism of MSC recruitment to the site of the damaged tissue, starting the process of repair along with its protective role. For the regenerative process to occur, MSCs either differentiate into beating cardiomyocytes or promote a paracrine effect [30].

7. The immunomodulatory effects of mesenchymal stem cells

Beside cell-to-cell contact, the MSCs secret many factors including EVs and soluble factors modulating the inflammatory response. The main paracrine factors are TGF-β, prostaglandin E2 (PGE2), hepatocyte growth factor (HGF), IL-10, IL-6, indoleamine 2,3-dioxygenase (IDO), nitric oxide (NO), and human leukocyte antigen G (HLA-G). Each of these factors plays an important role in regulation of different target immune cells. Other than such soluble factors, MSCs secrete extracellular vehicles (EVs), lipid bilayers that contain and transport the cytoplasmic components of the MSCs. EV is an inclusive term that has recently been suggested to encompass both exosomes and microvesicles. The immunological potential of MSC EVs in vitro, and the ability of these EVs to attenuate an activated immune system in vivo have been reported [31].

7.1. Natural killer (NK) cells

MSCs are capable of inhibiting proliferation and function of NK cells, mediated by IDO, PGE2, and TGF-β1. Many studies have reported that MSCs only partially inhibit the proliferation of activated NK cells and are susceptible to lysis by activated cells. HLA-G5 inhibits NK cell mediated cytolysis and decreases interferon-gamma (IFN-γ) secretion [32].

7.2. Dendritic cells (DCs)

Dendritic cells are antigen presenting cells that arise from monocytes or CD34+ hematopoietic stem cells. After exposure to antigens, they are turned into mature cells. MSCs impair this differentiation process via PGE2 secretion [33].

7.3. Neutrophils

Chemotaxis attracts neutrophils to the wound site, traversing post capillary venules to lyse pathogens with the granules within phagolysosomes, and then undergo apoptosis. MSCs secrete

IL-10 which inhibit neutrophil invasion into the wound. TNF-stimulated gene/protein-6 (TSG-6) is secreted by MSCs, interacts with protein ligands to inhibit rolling and transendothelial migration of neutrophils. Dyer [34] found that TSG-6 interacts with the glycosaminoglycan binding site of CXCL8 (IL-8), a chemokine produced by macrophages and transported to the surface of the endothelium, impairing neutrophil adhesion and migration.

7.4. Macrophages

Macrophages that arrive at the injury site hours later than neutrophils, are phagocytes that cleanse the wound of matrix and cell debris. They typically classified into two main groups: classically activated macrophages (M1) and alternatively activated macrophages (M2). M1 macrophages generally carry antimicrobial characteristics and stimulate a Th1 type response while M2 macrophages stimulate Th2 type responses. In general, M2 macrophages secrete less proinflammatory cytokines, have high production of anti-inflammatory cytokines such as IL-10, and induce resolution of the inflammatory phase. M2b macrophages show the reverse, as they maintain high levels of inflammatory cytokines [35]. Many studies explain the ability of autologous or allogeneic MSCs to polarize macrophages toward an M2 phenotype in vitro mediated by paracrine mechanisms, enhancing expression of M2 associated macrophage genes. Kim and Hematti [36] have suggested a separate definition for MSC-educated macrophages that secrete high IL-10 and IL-6 and low IL-12 and TNF-α, to call them M2 m, differing them from other subcategories. They suggest the possibility of collecting monocytes through leukapheresis and coculturing these mononuclear cells with allogeneic MSCs to provide MSC-educated macrophages prepared for repair of wounds [36].

7.5. B cells

B lymphocytes produce antibodies on exposure for antigens. MSCs may arrest B cell proliferation in the G0/G1 phase of the cell cycle without enhancing apoptosis [37]. IFN-γ inhibits the proliferation, which is probably mediated by MSC production of IDO. IDO is the first and rate-limiting enzyme of the essential amino acid tryptophan catabolism to kynurenine pathway, producing depletion and therefore halting growth. IFN-γ has IDO inducing effects [38].

7.6. T cells

Inhibitory effects of T cell proliferation by MSCs are mediated by both cell-to-cell contact and soluble factors. T cell proliferation was suppressed by TGF-β1 and HGF [39]. MSCs secrete PGE2 which prevents differentiation of CD4+ T cells into Th17 cells. MSCs also release IDO and enhance secretion of IL-10, which also inhibit cell proliferation [40].

8. Isolation and culturing

All MSCs, despite the protocol used for isolation, characterization and expansion, show the minimum criteria suggested by International Society for Cellular Therapy.

hMSCs are isolated based on their adherence ability to plastic surfaces, however, this method leads to the formation of a diversity of cells (stem cells as well as their progenitor cells) [41]. Considered as the best cell source, Bone marrow-derived MSCs (BM-MSCs) are taken as a standard to compare MSCs from other sources.

To ensure the success of the usage of these cells as a dependable source for regenerative medicine, a complete procedure should be established for MSCs isolation, characterization and expansion [42]. Contrary to bone marrow, MSCs from other tissues can be easily collected through non-invasive methods and their proliferation could be sustained up to many passages. Ficoll density gradient method with small modifications is utilized for isolation of MSCs from bone marrow, peripheral blood and synovial fluid [13] and seeded into culture plates. During isolation of MSCs from bone marrow, some hematopoietic cells also adhere to the plastic plate but they are washed away during sub-culturing, leaving only adherent fibroblast like cells [43]. MSCs from various tissue sources (adipose, dental, endometrium, foreskin, placenta, Wharton's Jelly) were isolated after digestion with collagenase and then cultured at varying densities [42]. Novel marrow filter device is recently explored as an efficient method for isolation of BM-MSCs [16], avoiding the risk of external contamination and saving time. Following their isolation from different sources, MSCs were cultured in condition media such as Dulbecco's modified Eagle's media (DMEM), DMEM-F12, DMEM-LG, DMED-HG, αMEM and RPMI (Roswell Park Memorial Institute medium) [44]. The primary culture medium was supplemented with fetal calf serum (FCS), new-born calf serum (NBCS) or10% FBS [45]. Besides the culture media and supplementation, the oxygen concentration is very important in the expansion and proliferation of MSCs [46]. It is also documented when cultured in DMEM culture with low glucose enriched with growth factors like fibroblast growth factor (FGF), epidermal growth factor (EGF) and B27 also leads to MSCs expansion [47]. But most commonly DMEM with 10% FBS is vastly employed in culturing and expanding MSCs *in vitro*, on the other hand, the use of exogenous FBS is highly debated.

8.1. Expression of cell surface markers

One of the essential characteristics of hMSCs is expression of specific set of cell surface markers. According to the International Society for Cellular Therapy standard criteria, MSCs are positive for CD73, D90, CD105 but negative for CD14, CD34, CD45 and HLA-DR [8]. MSCs can be isolated from various human tissues, which express cell surface markers mentioned above along with positive expression of CD29, CD44, CD146, CD140 b specific to tissue origin. The expression of CD34, which is a negative marker, is still controversial [48]. Stage-specific embryonic antigen (SSEA)-4 [49], stromal precursor antigen-1 (Stro-1) and CD146 are reported as are stemness markers for MSCs [50]. MSCs isolated from the human amniotic fluid express HLA-ABC [major histocompatibility complex class I (MHC I)], CD29, CD44, CD90, CD105, as well as SH2 (Src homology 2), SH3 (Src homology 3) and SH4 (Src homology 4). On the other hand, they lack the expression of HLA-DR (MHC II) [51]. Stro-1, a stemness marker for MSCs, is reported positive in dental and bone marrow MSCs, while reported negative in human adipose-derived MSCs (AD-MSCs) [52].

8.2. Msc niche

Schofield 1978 first introduced a stem cell 'niche' term [52]. The niche consists of the elements surrounding the stem cells in their naïve state including the non-stem cells as well as ECM and soluble molecules found in that locale. The above factors act together to maintain the stem cells in their undifferentiated state. Differentiation of the stem cells needs certain signals which must find their way into the niche for the regeneration or repopulation of a tissue.

8.3. Cellular components

The expression of α-smooth muscle actin (α SMA) in MSCs from all tissue types tested, is the basis of a perivascular nature of the MSC niche [53] and the immunohistochemical localization of CD45−/CD31−/Sca-1+/Thy-1+ cells to perivascular sites [54]. These cells also expressed α SMA and some even expressed 3G5, a pericyte-associated cell-surface marker. Doherty et al. [55] suggested that pericytes are in fact MSCs, because their differentiation into osteoblasts, chondrocytes, and adipocytes. MSCs have easy access to all tissues and participate in healing of many different tissues due to their presence in perivascular niches throughout the body.

Cadherins, MSCs transmembrane proteins responsible for cell–cell adhesion, polarity, differentiation, migration [18], interact with Wnts, which implicates in the biology of other stem cell niches [56].

8.4. Soluble components

The nature of bone marrow milieu is hypoxic. Comparison of human MSCs proliferative capacity was better maintained in the former when cultured in hypoxic versus normoxic conditions (2 and 20% oxygen). Additionally, hypoxia has doubled the number of existing CFU-Fs, as well as enhanced the expression of *rex-1and oct-4*; genes which are expressed by embryonic stem cells and are crucial in maintaining 'stemness'. Therefore, through increasing the plasticity and the proliferative capacity of MSCs, hypoxia is considered to have a double effect. However, the mechanism of action of hypoxia on MSCs is still unknown, although there is a possibility through the *oct-4* upregulation by the transcription factor hypoxia-induced factor-2α (HIF-2α) [57].

The effect of proteins secreted in the MSC niche is unexplained. The cell types studied have either induced differentiation or had no effect on MSCs. Finding soluble proteins permitting proliferation while inhibiting MSC differentiation would be ideal for simulating the niche and for MSCs expansion *ex vivo*.

8.5. Extracellular matrix components

However, ECM alone can regulate MSC differentiation, with potential applications for tissue engineering, no specific matrix components have been isolated to maintain MSCs in their naïve state, as a niche matrix would do. For example, osteoblasts on titanium scaffolds leave ECM after decellularization increasing osteogenesis markers, such as alkaline phosphatase and calcium deposition, in MSCs [58]. The ability to design artificial matrices that can resemble

the tissue microenvironment *in vivo* and control the appropriate differentiation of stem cells is a promising approach to therapeutic applications. Molecular information on ECM–MSC interactions, involving integrins, which involved in niche biology in other systems [59], is clearly needed.

9. Applications

9.1. Human mesenchymal stem cells and chronic diseases

MSCs are promising cell source for treatment of autoimmune, degenerative and inflammatory diseases due to the homing ability, multilineage potential, secretion of anti-inflammatory molecules and immunoregulatory effects. MSCs role in treating chronic diseases have been extensively studied in animal disease model.

9.2. Amyotrophic lateral sclerosis

MSCs are capable of differentiating into neurons [60]. An acid sphingomyelinase mouse model was used to conduct the first MSCs transplantation for neurodegenerative disorders. After MSCs injection, an amelioration in the overall survivability of the mouse and a decrease in disease abnormalities were detected [61]. Based on this study, a new study was performed in order to ensure the MSC transplantation efficiency in a neurodegenerative disease that leads to motor neurons degeneration and muscle function distortion, Amyotrophic lateral sclerosis (ALS) [61]. MSCs were isolated from the bone marrow and then reinjected into the spinal cord of the same patients, followed by MRI at 3 and 6 months for MSCs tracking. Results did not reveal any abnormal cells proliferation or structural changes in the spinal cord. However, mild adverse effects occurred which were reversed in few weeks duration e.g. intercostal pain irradiation and leg sensory dysesthesia. In another study, genetically modified AD-MSCs were made to express GDNF to be transplanted in a rat model of ALS, an increased number of neuromuscular connections and an improved pathological phenotype were observed [62].

9.3. Parkinson's disease

Parkinson's disease (PD), a neurodegenerative disorder, characterized by significant loss of dopaminergic neurons. After MSCs transplantation in PD mice model, tyrosine hydroxylase level increased [63]. MSCs participate to neuroprotection by secretion of trophic factors like vascular endothelial growth factor (VEGF), EGF, FGF-2, neurotrophin-3 (NT3), HGF and BDNF without differentiating into neurocytes [64]. Genetically modified hMSCs are used to induce the secretions of specific factors or to increase the dopamine (DA) cell differentiation. BM-MSCs transduction with lentivirus carrying LMX1a gene, resulted in cells which were similar to mesodiencephalic neurons with high DA cell differentiation [65]. Experiments were performed on Parkinson diseased rat, the research group from the university hospital of Tubingen in Germany administered BM-MSCs nasally to treat neurodegenerative patients. MSCs were found in different brain regions after 4.5 months of administration. They have

been found in the cerebral cortex, olfactory lobe, hippocampus and brain stem, suggesting that MSCs could successfully survive and proliferate *in vivo* [66]. Moreover, this type of administration was observed to increase the level of tyrosine hydroxylase and decrease the toxin 6-hydroxydopamine in the ipsilateral striatum and substantia nigra lesions. This novel MSCs administration route could change the face of MSCs transplantation in future.

9.4. Alzheimer disease

Alzheimer disease (AD), one of the commonest neurodegenerative diseases, characterized by symptoms as intellectual disabilities, dementia and memory loss. Till present, no treatment was established to slow down or stop the progression of AD [67]. Researchers use stem cell therapy in AD animal model aiming to decrease the neuropathological deficits. Mostly by activating the alternate microglia, increasing the expression of Aβ-degradation enzymes and decreasing the expression of pro-inflammatory cytokines, that the human AD-MSCs modulate the inflammatory environment [68]. Furthermore, MSCs modulate the inflammatory environment of AD and inadequacy of regulatory T-cells (Tregs) and they could modulate microglia activation [69]. Shin et al. [70] demonstrated that human UCB-MSCs increase the neuronal survival and stimulate Tregs which control microglia activation in AD mice model. Most recently, it was confirmed that MSCs stimulates the cell autophagy pathway, causing increased neuronal survivability and clearing of the amyloid plaque both *in vivo* and *in vitro* [70].

9.5. Autoimmune diseases

MSCs have the ability of regulating immune responses, thus it can treat immune disorders. Other hMSCs can be used for autoimmune diseases treatment, after revealing that human BM-MSCs are able to protect hematopoietic precursors from inflammatory damage [71].

9.6. Rheumatoid arthritis

Rheumatoid arthritis (RA), a joint inflammatory disease resulting from loss of immunological self-tolerance. The use of MSCs in animal models' studies, were successful in slowing disease progression and enhancing the disease recovery. Beside its anti-inflammatory function, IL-10 is an important factor in the activation of Tregs that controls self-reactive T-cells and motivates peripheral tolerance *in vivo* [72]. Similar effects were produced by human BM-MSCs in the collagen-induced arthritis model in DBA/1 mice [73]. These studies suggest that the improvement of the RA pathogenesis in DBA/1 mice model in case of using MSCs, can be caused by activating Treg cells as well as suppressing the production of inflammatory cytokines. However, MSCs were only effective when administered at the onset of disease, in case of adjuvant-induced and spontaneous arthritis model, which suggests that MSCs lost their immunoregulatory properties when exposed to inflammatory microenvironment [74].

9.7. Type 1 diabetes

Type 1 diabetes, an autoimmune disease caused by the destruction of β-cells due the production of auto antibody directed against these cells. As a result, there is decrease in the insulin

production to a level which is failed to control the blood glucose. It has been proved that MSCs can differentiate into insulin producing cells and have the capacity to regulate the immunomodulatory effects [75]. Zulewski et al. [76] isolated Nestin positive cells from rat pancreatic islets which differentiated into pancreatic endocrine cells. Nestin positive cells were isolated from human pancreas and transplanted to diabetic nonobese diabetic/severe combined immunodeficiency (NOD-SCID) mice, which improved hyperglycemic condition [77]. However, these studies were found controversial and it was suggested that besides pancreatic tissues, other tissues can be used as an alternative for MSCs isolation to treat type 1 diabetes. Human BM-MSCs can be differentiated efficiently into pancreatic endocrine cells *in vitro* as well as *in vivo* [78]. There is an option for the use of UCB-MSCs as insulin producing cells. UCB-MSCs were similar to human ESCs, following similar steps producing the differentiated β-cells [79]. Unsal et al. [80] showed that transplantation of MSCs together with islets cells into streptozotocin treated diabetic rat model improve the survival rate of engrafted islets.

9.8. Cardiovascular diseases

Cardiac cells transplantation is a novel strategy for myocardial repair, which is currently applied in animal models. Although MSCs are a good source for cardiomyocytes differentiation, it was found that *in vitro* differentiation is effective only from young cell sources and *in vivo* differentiation of cardiomyocytes is very rare [81]. MSCs, which have differentiated into cardiomyocytes under the effect of cocktail of growth factors [82], were used in treatment of left ventricular heart failure and MI [83]. The systematic injection of BM-MSCs into the infarcted myocardium of rodent models partially produced recompensation [84]. Katritsis et al. [85] reported improvement in myocardial contractibility when autologous MSCs were transplanted with endothelial progenitor cells. Despite the fact that MSCs are proven to be effective in MI and related problems, still the ability of the heart to retain cells is low; only 10% cell retention after 4 h of cells injection and 1% after 24 h [86]. Roura et al. [87] recorded that UCB-MSCs proliferated and then differentiated into endothelial lineage, were retained for several weeks when injected in acute MI mice. Transplantation of UCB-MSCs into myocardial infarction animal model along with fibronectin-immobilized polycaprolactone nanofibers were found very effective [88].

10. Cryopreservation and banking

From all the previous studies, it becomes clear that the use of hMSCs in clinical field will increase in future. For clinical applications, a large number of MSCs in an 'off the shelf' format is required. For this purpose, cryopreservation and banking are necessary to be established. This will allow unique opportunities to improve the potential uses of these cells in research and clinical applications. Keeping in mind its use in future clinical and therapeutic applications, there is a need to ensure the safety and efficacy of these cells while cryopreserving and banking. Cryopreservation media should be optimal so uniform change in temperature during freezing and thawing, long-term storage in liquid nitrogen and employed freezing device are the main factors to consider.

In the cryopreservation media in which cells can maintain their stem cells abilities for long time, the cells require a source of their nutrients as the animal base reagent, like FBS, but previous studies have showed that there is difficulty in removing animal proteins from the hMSCs and that may elicit adverse reactions in the patients who receive these cells for treatment [89]. Therefore, a serum-free media is alternative for the cryopreservation of MSCs and it was successfully used [90]. Lately, instead of using FBS, human albumin and neuropeptide were used. It was observed that MSCs maintained their proliferation potential and cell survival in the culture conditions. Moreover, cryoprotective agents (CPAs) are found to be required for the cryopreservation media to prevent any freezing damage to cells. A large number of CPAs are available [91], DMSO is the commonest CPAs agent used in cryopreservation of MSCs. However, DMSO toxicity to humans and animals hinders its usage in MSCs freezing for clinical applications. Due to these complications, it is necessary to use an alternate CPA. There are many methods along with the introduction of automated cells washing for the removal of DMSO from the frozen thawed cells [92].

The second important factor in cryopreservation of MSCs is the freezing temperature rate. The optimum rate for MSCs preservation is slow freezing at the rate of 1°C/min is [93]. For the purpose of maintaining the rate of temperature during cryopreservation, controlled rate freezers (CRFs) are suitable for regulating the temperature. These CRFs can be programmed to determine the exact temperature the sample is experiencing during freezing [94]. Despite of these advantage, these CRFs do not apply a uniform temperature to all vials during large-scale MSCs banking [95], therefore, the development of advanced CRFs is mandatory for large-scale banking. Lately, Praxair Inc. created advanced CRF, providing unidirectional flow of cryogen to each sample. The safe and efficient cryopreservation as well as the regulatory guidelines are important for large-scale MSCs banking. In the U.S.A., Food and Drug Administration (FDA) is responsible for supervising MSCs based cell therapy products, while in Europe it is the European Medicines Agency that is responsible.

11. Summary and conclusion

Mesenchymal stem cells (MSCs) are plastic-adherent, fibroblast-like, multipotent cells found in the human body having the ability to differentiate into different cell types including osteoblasts, adipocytes and chondrocytes. They are normally present in the umbilical cord, adipose tissue, bone marrow but can also be resident in other tissues and are recruited to sites of wound healing as well as growing tumors.

MSCs are a promising candidate for cell-based tissue regeneration that can potentially revolutionize the current pharmaceutical landscape. The extracellular matrix (ECM), adjacent cells and different types of cytokines and growth factors forming MSC niche microenvironment, are critical for their lineage differentiation. Standardized protocols for cell culture, differentiation, expansion and cryopreservation need to be in place. These factors in combination with safely preconditioned and genetically modified MSCs may pave the way for the development of an effective cellular therapy for countless human immune disorders.

Recently, research and basic knowledge of these cells has fast-tracked, both from fundamental and translational perspectives. There have been important discoveries about the available variety of tissue sources. In addition, novel abilities such as immune-modulation together with improved delivery to the selected optimal tissue site has been discovered. However, the molecular fingerprint of MSCs in these contexts remains imprecise and inadequate. Consequently, without this crucial knowledge the progress is difficult in order to determine with precision the MSCs practical developmental potentials.

Overall, the unavoidable propaganda fluctuation that continued for more than 40 years of work on BMSCs did not reduce the novel biological flavor of these cells. Concurrently functioning as stem cells and as cells providing the microenvironment for other stem cells, BMSCs incorporate properties of the "seed" and "soil." As expectations linked to BMSC plasticity are diminishing, these unique properties of BMSCs challenge both biology and medicine in a quite remarkable fashion.

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Chapter 2

Stromal Cell Ultrastructure

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Additional information is available at the end of the chapter

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Abstract

Regenerative medicine and tissue engineering therapies present an attractive treatment alternative to the current traditional clinical treatments. Stem cells are capable of self-renewal and multilineage differentiation. They also have the ability to create immuno-modulatory microenvironment, and thus help to minimize organ damage caused by the inflammation and cells activated by the immune system. Human bone marrow mesen-chymal stem/stromal cells (MSCs) have great potential for cellular therapy, as they possess the abilities to proliferate as well as to differentiate. MSCs are present in all tissues interacting with tissue cells and easy to isolate and expand in culture. Indeed, histological examination of MSCs is one of the main goals for studying their morphology. Both the light and the electron microscopes are essential tools where the histologist can identify the structure as well as the detailed ultrastructure of these cells. This will guide users to clearly understand their behavior, both *in vivo* and *in vitro*. Thus, the aim of this chapter is to give a spot of light on these cells and their histology.

Keywords: stem cell, stromal cell, biology, histology, ultrastructure

1. Introduction

Stem cells can be defined as undifferentiated cells that have the ability to self-renewal; prolif-erate into undifferentiated cells, and to differentiate into various mature specialized cells [1]. There are different types of stem cells that have been classified according to their potency. Cells are described as pluripotent that is, embryonic cells from the blastocyst (4–14 days after oocyte fertilization), they can differentiate into all cell types of the adult organism. If, in addition, they can form the extraembryonic tissues of the embryo, they are described as totipotent (1–3 days from oocyte fertilization) which can give rise to all the embryonic tissues and placenta. Multipotent stem cells that is, embryonic cells from the 14th day onward, have the ability to form all the differentiated cell types of a given tissue. The stem cells that maintain only one

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lineage are described as unipotent [2]. In the trilaminar embryo, a middle mesodermal layer is formed between the ectodermal and endodermal cell layer. This mesodermal cell layer contains mesenchymal stem cells (MSCs), which develop into connective tissue (mesenchyme) and it maintains the progenitor stem cells that persist after birth [3].

2. Sources of stem cell

2.1. Embryonic stem cells

Embryonic stem cells (ESCs) have the greatest potential to differentiate into all cell types. ESCs are derived from the inner cell mass of the blastocysts. However, the use of ESC is associated with several ethical issues [4]. Also, safety concerns were raised with a high incidence of teratoma formation [5].

2.2. Induced pluripotent stem cells

Induced pluripotent stem cells (iPS) were first achieved by inducing a forced expression of specific genes that can reprogram human and mouse adult somatic cells into the undifferentiated cell [6, 7]. iPS have the same characteristics of ESCs, such as expression of pluripotency markers and differentiation capability [6].

2.3. Fetal stem cells

Fetal stem cells (FSCs) are derived either from a fetus or from extraembryonic structures. Various subtypes of FSCs were described according to their origin (i.e., amniotic fluid, umbilical cord, Wharton's jelly, amniotic membrane, and placenta). FSCs are ideal sources of cells for use in regenerative medicine. They are easily accessible, having a high proliferation rate. In addition, FSCs do not form teratomas [8] and overcome the ethical problem associated with ESCs [9].

2.4. Adult stem cells

In principle, adult stem cells are unspecialized (undifferentiated) cells. They are found in differentiated tissues and considered to be quiescent, but still capable of self-renewal and differentiation. These cells remain in their undifferentiated state until stimulated [10]. Adult MSCs have been isolated from different sites: bone marrow, adult peripheral blood, tooth pulp and liver [11].

3. Mesenchymal stem/stromal cells (MSCs)

3.1. History

The concept of mesenchymal stem/stromal cells (MSCs) was first introduced about half a century ago. In the 1970s, [12] Alexander Friedenstein described a population of bone marrow-derived cells of mesodermal origin. These MSCs were shown to have the ability to self-renew and to

differentiate into a multitude of mesodermal cell types [13–15]. Bone marrow MSCs represent a heterogeneous population derived from the nonblood-forming fraction of bone marrow, but have the ability to regulate hematopoietic cell development. *In vitro*, adult mesenchymal stem cells resident in this bone marrow fraction differentiate into bone, cartilage and fat [16]. Recently, a standardized nomenclature for MSCs has been proposed and the term "multipotent mesenchymal stromal cells" has been introduced [15] to refer to this population of fibroblast-like, plastic-adherent cells [17]. Their asymmetric division produces one identical daughter stem cell and a second progenitor cell that becomes committed to a lineage-specific differentiation program [18].

3.2. Importance and uses

MSCs produce many growth factors and essential cytokines needed for cell proliferation and differentiation [19]. They also support hematopoiesis in bone marrow and play an indirect role in supporting other cell types during tissue repair [20]. Adult stem cells could overcome many of the ethical and technical debate associated with ESC as they are isolated from adult tissues, including bone marrow stromal cells, adipose-derived stem cells and adult skin stromal cells [21]. However, because of their limited differentiation potential (multipotent), they are less likely to form tumors, although some are thought to be related to certain tumors [22].

3.3. Location

The exact location of these cells in vivo is not known, but recent work suggests that MSCs are located in the perivascular spaces as sub-endothelial cells surrounding the vascular sinusoids in the bone marrow [23]. Bone marrow contains three main cell types: endothelial cells, hematopoietic stem cells, and stromal cells. Bone marrow connective tissue network is called the stroma. The stroma consists of a heterogeneous population of cells that provide structural and physiological microenvironment to support hematopoietic cells and forms a complex extracellular matrix, which supports the hematopoietic process [23]. However, the frequency of MSCs in human BM has been estimated to be in the range of 0.001–0.01% of the total nucleated cells. Furthermore, the frequency of MSCs declines with age, from $1/10^4$ nucleated marrow cells in a newborn to about $1/10^6$ nucleated marrow cells in an 80-year-old person [24].

4. Biology of stromal cells/MSCs

4.1. Tissue distribution

Interestingly, MSCs reside in diverse tissues throughout the adult organism [25]. Nowadays, MSC populations have been obtained from many tissues other than the bone marrow, [26] including the adipose tissue [27] and placenta [28].

4.2. Properties

Mesenchymal/stromal cells (MSCs) have the ability to differentiate into a variety of different cells/tissue lineages; osteoblasts, chondroblasts, adipoblasts and reticular stromal cells [29].

MSCs possess potent immunomodulatory and anti-inflammatory effects and have been used as agents in autoimmune diseases [30]. They interfere with pathways of the immune response by means of direct cell-to-cell interactions and soluble factor secretion. In vitro, MSCs inhibit proliferation of T cells, B-cells, natural killer cells and dendritic cells [31].

4.3. Immunobiology

MSCs are believed to have critical roles in repairing damaged tissues. Tissue injury is associated with the activation of immune/inflammatory cells. In addition, inflammatory mediators, chemokines and leukotrienes, are often produced in the microenvironment by phagocytes in response to damaged cells [32]. Nevertheless, the function of the endothelial cells as a barrier is often broken down in damaged tissues. Thus, these inflammatory molecules and immune cells, together with endothelial cells and fibroblasts, result in the mobilization and differentiation of MSCs and replace the damaged tissue cells. The study of endogenous MSC migration is complex. Once MSCs have entered the microenvironment of injured tissues, MSCs start releasing many growth factors, including epidermal growth factor (tissue regeneration), fibroblast growth factor (cell survival and regeneration), platelet-derived growth factor (tissue repair), vascular endothelial growth factor (angiogenesis and wound healing), hepatocyte growth factor (intrinsic neural cell regeneration), angiopoietin-1 (angiogenesis) and stromal cell-derived factor-1 (neuroprotective effect). These growth factors, in turn, promote the development of fibroblasts, endothelial cells and tissue progenitor cells, which carry out tissue regeneration and repair [33].

4.4. Homing

Studies have shown that MSCs have the ability to migrate and to home to a variety of tissues. The migration process is represented by several distinctive steps and starts with the resistance and adhesive interactions between cells flowing through the bloodstream and vascular endothelium. The mechanisms used are assumed to follow the same steps that were described for leukocyte homing.

In the first step, the cells come into contact with the endothelium by tethering and rolling. Different molecules are involved in such process. The selectins on the endothelium are primarily involved and the expression of hematopoietic cell E−/L-selectin ligand which is a specialized form of cluster of differentiations (CD), CD44. This step is mediated by the homing receptors expressed on circulating cells which interact with their corresponding receptors expressed on the layer of endothelial cells. [34]. As regards the second step, the cells are activated by G-protein-coupled receptors, followed by integrin-mediated activation. MSCs express various integrins on their surface, among which integrin $\alpha4/\beta1$, which mediates cell–cell and cell- extracellular matrix interactions by binding to vascular cell adhesion molecule −1 and to the V-region of fibronectin, respectively. In damaged tissues, fibronectin is deposed together with fibrin at the injured site to stop the bleeding. The provisional matrix is then remodeled by macrophages and fibroblasts, determining an increase in V region-exposing fibronectin, which, in turn, allows MSCs to adhere and transmigrate into the extracellular matrix. In the last step, diapedesis or transmigration occur through the endothelium as well as through the underlying basement membrane. In this step one of the

matrix metalloproteinases (MMP) - which are lytic enzymes required to cleave the components of the basement membrane - the gelatinases MMP-2 and MMP-9 are the most important because they specifically degrade collagen and gelatin components of the basement membrane [35].

4.5. Characterization

MSCs isolated directly from bone marrow are positive for CD44. They are also positive for CD29, CD73, CD90, CD105 and CD166. On the other hand, they are negative for the hematopoietic surface markers such as CD11b, CD45, CD31, CD106, CD117 and CD135 [36]. As progress in phenotyping the MSCs and its progeny continues, the use of selective markers has resulted in the enhanced propagation and enrichment of the MSC population, while maintaining them in an undifferentiated state without diminishing the differentiation potential [37].

A part of a work [38] was carried out at Department of Trauma, Hand and Reconstructive Surgery, Johann-Wolfgang-Goethe University Hospital, Frankfurt, Germany. They demonstrated that MSCs expressed typical MSCs specific antigens CD73, CD90 and CD105 (hematopoietic surface marker) and were negative for the hematopoietic marker and lymphocytic markers CD34, CD45, respectively. According to the International Society of Cell Therapy, CD73, CD90 alongside CD105 are positively expressed on MSCs and remain the primary molecules used to identify MSCs [39]. The phenotypic characterization of MSCs from bone marrow has been further realized through the identification of the cytokine expression profile of undifferentiated cells. Constitutive expression of cytokines, such as granulocyte-colony stimulating factor, stem cell factor, leukemia inhibitory factor, macrophage-colony stimulating factor, and IL-6 and IL-11 is consistent with the ability of MSCs to support hematopoiesis [40].

5. Culturing

In order to avoid patient morbidity, the amount of MSCs that could be isolated from BM aspirate should be too small [12]. Therefore, they should be cultured in vitro to enable the expansion of MSCs to generate millions of cells which can be used for further therapeutic applications [39]. It was stated that MSCs retain more potential to differentiate after the third passage (P) [41]. In addition, over 70% of clinical trials used MSCs from 1 to 5 passages [42]. Moreover, a study reported that MSCs from 7 to 9 passages underwent osteogenic differentiation more than cells of later passages. Moreover, recent data indicated that reactive oxygen species-handling mechanisms (i.e., antioxidative activity/reduction potential) become disrupted in later passages, a condition, which was not observed in the lower passage [43].

Although several researchers [41] showed that with the long-term expansion of MSCs and with several sub-culturing, the cells lose their differentiating ability, a study performed [44] reported that no change at the level of genetic expression or differentiation capability of long-term cultured MSCs. Furthermore, MSCs have a stable phenotype over many generations

in vitro [45]. Another study [46] reported that MSCs retained their multilineage differentiation potential till passage 10 (P10) and maintain high levels of telomerase activity and long telomere length up to P10, but steady decline in the efficiency of proliferation in all cell populations after P10. Furthermore, MSCs showed a marked increase in the time required for cell doubling and showed an enlarged, flattened cellular morphology at P15, after which they ceased to undergo cell division but remained viable in culture. Thus, cells from passage 9 were used for differentiation as it was needed to obtain sufficient cell numbers for use through extensive cell quantity amplification and later passages were avoided [47].

6. Histology

Studying the behavior of MSCs in vitro has become an urgent need to give more insights on their behavior in vivo and their mechanisms in initiating osteogenesis. Indeed, histological examination of MSCs is one of the main goals for studying their morphology in vitro by light microscope. Although it is a primary step, yet, it is not sufficient to rely on it alone, to detect their behavior during their differentiation process, and as such it has to be accompanied by ultrastructure examination to correlate between their morphology and behavior.

6.1. Light microscope

MSCs are characterized by being star-shaped cell with thin long processes [48]. Using hematoxylin and eosin stains, MSCs are characterized by pale cytoplasm, large vesicular nucleus and multiple thin processes (**Figure 1**).

6.2. Phase contrast microscope

Regardless of the issue of origin, all MSCs share characteristics by consensus definition: they are spindle-shaped and plastic-adherent. In our study, [38] isolated human bone barrow MSCs

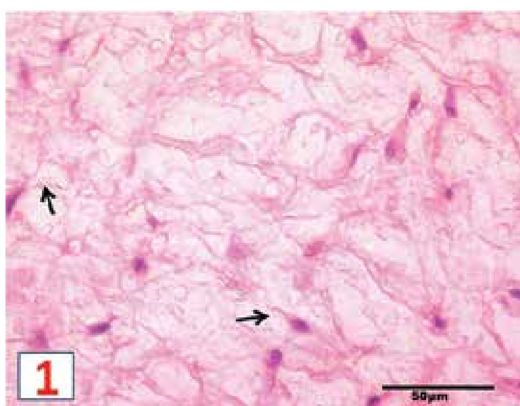

Figure 1. Light microscopic picture of the umbilical cord showing MSCs with many thin processes (arrow). Each cell exhibits a vesicular nucleus. Scale bar 50 μm.

revealed that the cells were adherent to the surface of tissue culture plastic flask. Furthermore, the cells were spindle in shape; which is considered as a second important characteristic of mesenchymal cell morphology. Researchers [38] described a population of adherent cells in culture till P5 (**Figure 2**). Most of the cells exhibited fibroblast-like spindle shape and showed vesicular nuclei with prominent nucleoli. Moreover, in P9, the adherent cells remained attached to the surface with their characteristic spindle shape (**Figure 3**). The cells exhibited vesicular nucleus, prominent nucleolus and multiple processes [38].

6.3. Electron microscope

6.3.1. Scanning electron microscope (SEM)

The two-dimensional morphology of MSCs demonstrated by scanning electron microscope (SEM) [38] showed the spindle-shaped cells with eccentric nuclei and several thin cytoplasmic processes extending from the edge of the cell surface in P5 and P9. In addition, cells in P 9 maintained their spindle shape (**Figure 4**). These SEM results were also reported [49].

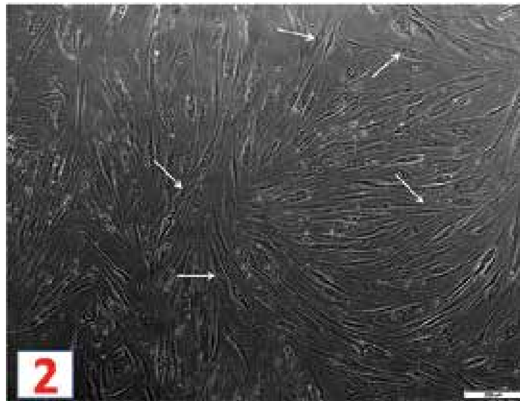

Figure 2. Cultured human bone marrow derived stromal cell from passage 5, showing adherent cells with their characteristic spindle shape (arrow) [38]. Scale bar 200 μm.

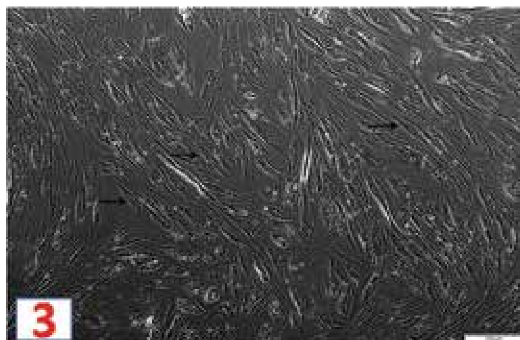

Figure 3. Cultured human bone marrow derived stromal cell from passage 9, showing adherent cells with their characteristic spindle shape (arrow) [38]. Scale bar 200 μm.

6.3.2. Transmission electron microscope (TEM)

Electron microscopic examination of MSCs in culture revealed the presence of euchromatic nucleus associated with abundant cell organelles which are considered as an indicator of an active cell (**Figure 5**). The spindle-shaped cells showed large irregular, euchromatic nucleus and the peripheral heterochromatin was slightly condensed along the inner surface of the nuclear membrane and nuclear pores (**Figure 6**). The cytoplasm showed many elongated profiles of rough endoplasmic reticulum and multiple mitochondria (**Figure 7**). Cytoskeletal structures were seen as fine filaments running parallel to the long axis of the cell near the nuclear membrane as well as beneath the cell membrane (**Figure 6**).

The same features of active MSCs were noticed after 14 days in culture. The cells exhibited a large euchromatic nucleus with numerous profiles of rough endoplasmic reticulum and multiple rounded mitochondria. In addition, the cell surface showed thin pseudopodia (**Figure 7**). Cytoskeletal filaments were irregularly dispersed in the cytoplasm as well as around the nucleus (**Figure 7**). Such observation was explained by the fact that the intracellular organelles architecture is organized by the cytoskeleton [36, 50, 51].

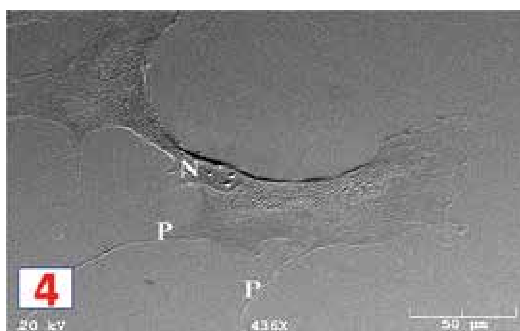

Figure 4. Cultured human bone marrow derived stromal cell, showing spindle shape cell with an eccentric nucleus (N) and multiple processes (P) [38]. Scale bar 50 μm.

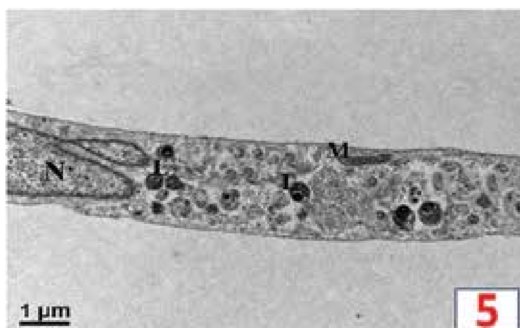

Figure 5. Transmission electron micrograph of cultured human bone marrow derived stromal cell on day 7. The cell is spindle in shape with an euchromatic nucleus (N). The cytoplasm shows mitochondria (M) and multiple lysosomes (L) [38]. Scale bar 1 μm.

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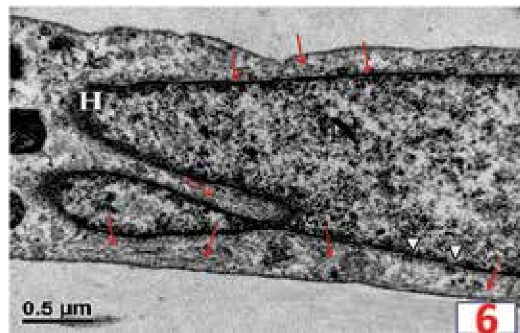

Figure 6. Transmission electron micrograph of cultured human bone marrow derived stromal cell showing part of the same cell exhibiting an euchromatic nucleus (N) with nuclear pores (arrow heads). The peripheral heterochromatin (H) is seen along the inner aspect of the nuclear membrane. Fine cytoskeletal filaments are noticed parallel to the long axis of the cell near the nuclear and cell membranes (arrows) [38]. Scale bar 0.5 μm.

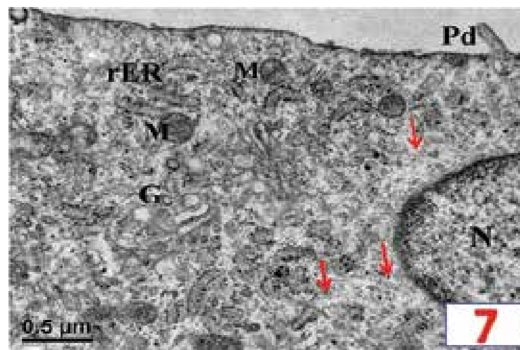

Figure 7. Transmission electron micrograph of cultured human bone marrow derived stromal cell on day 14. The cytoplasm exhibits numerous profiles of rER, mitochondria (M), and well-developed Golgi complex (G). The cell membrane exhibits a pseudopodium (Pd). Cytoskeletal filaments are irregularly dispersed in the cytoplasm (arrows). Part of an euchromatic nucleus is also seen (N) [38]. Scale bar 0.5 μm.

Moreover, after 21 days in culture, the cells showed clearly demarcated nucleolus (**Figure 8**). In addition, numerous large macro vesicles associated with the mature face of the Golgi complex were clearly depicted (**Figure 9**). These cells are now ready for differentiation once in the appropriate media. The structure of these cells would differ during the process of differentiation accordingly.

Another ultrastructure feature of MSCs is the presence of vesicles in the cytoplasm. Intercellular communication can be mediated through direct cell–cell contact or transfer of secreted molecules. Recently, a third mechanism has emerged that involves intercellular transfer of extracellular vesicles. Cells release into the extracellular environment membrane vesicles either of endosomal origin or of plasma membrane origin. They are named exosomes and microvesicles, respectively [52]. In the study [38] carried out on isolated MSCs, showed vesicular trafficking. (**Figure 10**) These vesicles were prominent after the cells were cultured in a

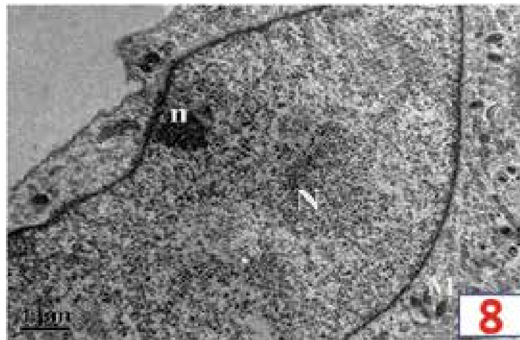

Figure 8. Transmission electron micrograph of cultured human bone marrow derived stromal cells on day 21, showing large euchromatic nucleus (N) with clearly demarcated nucleolus (n). The cytoplasm shows mitochondria (M) [38]. Scale bar 1 μm.

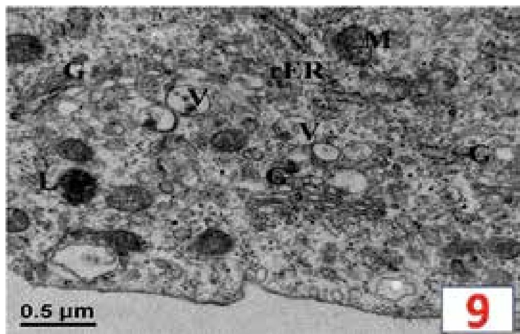

Figure 9. Transmission electron micrograph of cultured human bone marrow derived stromal cells on day 21, showing part of its cytoplasm with multiple well-developed Golgi complexes (G) associated with large secretory vesicles (V), numerous mitochondria (M), and lysosomes (L). The cytoplasm shows profiles of rough endoplasmic reticulum (rER) [38]. Scale bar 0.5 μm.

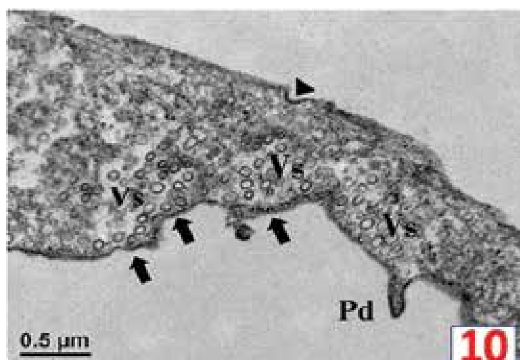

Figure 10. Transmission electron micrograph of cultured human bone marrow derived stromal cells. The cytoplasm shows several cytoplasmic vesicles (Vs) of variable sizes. A coated pit (arrowhead) and numerous subplasmalemmal vesicles are also seen (thick arrows). A surface pseudopodium (Pd) is seen [38]. Scale bar 0.5 μm.

media that stimulated its osteogenic differentiation. Microvesicles vary in size and are formed by the budding of the plasma membrane. Most cell types are known to produce microvesicles either constitutively or when stimulated during apoptosis or activation. The mechanisms involved in the mobilization of secretory microvesicles to the cell periphery, their docking, and fusion with the cell surface require the cytoskeleton (actin and microtubules), associated molecular motor proteins (kinesins and myosins) as well as other factors [53, 54]. The other clearly defined class of secreted membrane vesicles that originate from the endosomes are the exosomes. Exosomes were first discovered by Pan and Johnstone in 1983 [55]. They are formed by the invagination of endolysosomal vesicles to form multi-vesicular bodies. Exosomes are released by exocytosis. First, the cell membrane is internalized to produce endosomes. Subsequently, many small vesicles are formed inside the endosome by invaginating parts of the endosome membranes. Such endosomes are called MVBs. Finally, the MVBs fuse with the cell membrane and release the intraluminal endosomal vesicles into the extracellular space to become exosomes [56].

Exosomes directly interact with the signaling receptors of target cells [57]. After that, the exosomes fuse with the plasma membrane of recipient cells and deliver their content into the cytoplasm [58]. Finally, the exosomes are internalized into the recipient cells. Once in the recipient cell, some of these engulfed exosomes may merge into endosomes and move across the recipient cells to be released into the neighboring cells. In the other case, endosomes fused from engulfed exosomes will mature into lysosomes and undergo degradation [57, 59].

Lipids and proteins are the main components. The protein content of exosomes from different cell types contains different endosome-associated proteins (e.g., RabGTPase, SNAREs, Annexins and flotillin). They are also enriched in proteins that associate with lipid rafts, including glycosylphosphatidylinositol-anchored proteins and flotillin [60]. The other main component of exosomes is the lipid. In comparison to the plasma membrane, exosomes are highly enriched in cholesterol, sphingomyelin and ceramides at the expense of phosphatidylcholine and phosphatidylethanolamine [52]. In addition to the proteins and lipids, various nucleic acids have recently been identified in the exosomal lumen, including mRNAs, microRNAs and other noncoding RNAs [61].

The main functions of exosomes are their capacity to act as antigen-presenting vesicles, to stimulate immune responses [62]. Another main important feature of exosomes is being an ideal drug delivery vehicle. Meanwhile, research has been carried out encapsulating anticancer drugs into exosomes [63].

The function of MSC-derived exosomes has not been well defined. They act as an intercellular communication vehicle for modulating cellular processes. It was recently revealed that exosomes derived from MSCs play important roles in mediating the biological functions of MSCs [64].

A study demonstrated the electron microscopy of exosomes. They were cup-shaped and measured 40–100 nm in diameter. Exosomes are naturally secreted and well tolerated by the body. They are also safely stored and provide many therapeutic applications with avoiding the

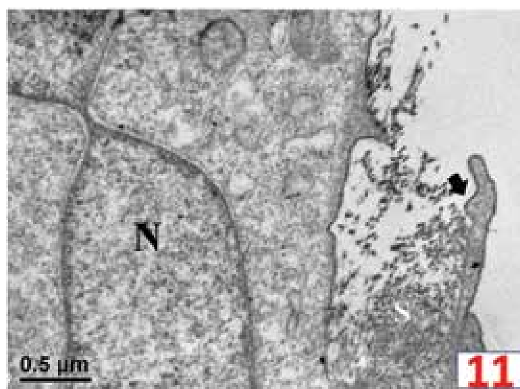

Figure 11. Transmission electron micrograph of cultured human bone marrow derived stromal cells. The cell surface of shows an open fibripositors (short arrow) with large amounts of secretory product (S) is observed. Note the euchromatic nucleus (N) [38]. Scale bar 0.5 μm.

risk of immunological rejection and malignant transformation [65]. Therefore, the use of MSCs to produce exosomes for drug delivery is the subject of the day [66]. Recently, liposomes are preferred drug delivery systems. It is a synthetic vesicle with a phospholipid membrane that has the ability to self-assemble into various sizes and shapes in an aqueous environment [67].

Another morphological feature detected is pseudopodia-like structures extending from the cell membrane (**Figures 7** and **10**). This might explain the capacity of the cells for migration within the receiving tissue. The main role of these structures is to transmit the produced material from one cell into another by extending the pseudopodia and communicating cells with each other as well as in cell signaling [68]. Interestingly, one of the most striking features during differentiation is the observation of finger-like extensions of the plasma membrane known as fibripositors (**Figure 11**). These fibripositors were located at the side of the cell and protrude into the spaces between cells. These fibripositors are the site where collagen fibrils were located. It was reported that the initial stage of extracellular matrix deposition results in arrays of short collagen fibrils completely enclosed within these fibripositors. These fibrils are then subsequently deposited extracellularly [69, 70].

It was reported that fibrils leaving the fibripositors were seen to run along the external surface of the cell. Tracking of fibrils revealed that the collagen fibrils in fibripositors were shorter than those extracellularly. Thus, these data suggested that fibripositors might be a place of fibril assembly. They determined that short fibrils become longer inside closed fibripositors, then protruding fibripositors (open), often project into the matrix, releasing fibrils extracellularly where individual fibrils then coalesce into bundles. Thus, fibripositors are specialized sites not only of fibril assembly, but also share in fibril transport extracellularly [71].

Another study declared that the fibripositors are dynamic structures and their formation and stabilization depend on the actin cytoskeleton [72]. This might explain the existence of the cytoskeletal filaments in the differentiating cells [38]. Accordingly, these cytoskeletal structures might be actin filaments. It is possible that fibripositors have been involved in the alignment of extracellular collagen fibrils in a parallel arrangement [73].

7. Conclusion

The MSCs maintained their undifferentiated histological structure till passage 9 for further tissue engineering. A detailed histological examination using the light and the electron microscopes is essential to understand the function of MSCs. In addition, exosomes represent a promising candidate for drug delivery vehicle.

Abbreviations

ESCs	embryonic stem cells
iPS	induced pluripotent stem cells
FSCs	fetal stem cells
MSCs	mesenchymal/stromal cells
CD	cluster of differentiation
MMP	metalloproteinases
P	passage
SEM	scanning electron microscope
TEM	transmission electron microscope
SNARE	soluble *N*-ethylmaleimide-sensitive factor receptor
Rab	Ras-related proteins in brain
GTP	guanosine triphosphate

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Chapter 3

Adult Stem Cell Membrane Markers: Their Importance and Critical Role in Their Proliferation and Differentiation Potentials

Maria Teresa Gonzalez Garza

Additional information is available at the end of the chapter

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Abstract

The stem cells are part of the cells that belong to the stromal tissue. These cells remain in a quiescent state until they are activated by different factors, usually those generated by an alteration in the parenchymal tissue. These cells have characteristic membrane markers such as CD73, CD90, and CD105. Those are a receptor, which in response to their ligand induces strong changes in different metabolic pathways that lead to these cells, both to generate molecules with different activities and to leave their stationary phase to reproduce and even differentiate. This review describes the metabolic pathways dependent on these membrane markers and how they influence on parenchymal tissue and other stromal cells.

Keywords: stromal cells, stem cells, membrane markers, CD73, CD90, CD

1. Introduction

Stromal cells make up some connective tissues for particular organs and give support by surrounding other tissues and organs. As result, stromal cells provide support, structure, and anchoring for many organs inside the body. The generic term "stromal cells" clearly the phenotypic and functional complexity of these cells. In addition, to their main functions in helping support organs and acting as connective tissues, stromal cells respond with metabolic adaptations to different inductions factors and play an important role in the microenvironment [1]. Stromal cells are able to react to physical and chemical signals of tissue damage. Physical stress such as mechanical stress activates channels (SACs) on the cell membrane [2].

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On cells attached to an extracellular matrix, SACs initiate the remodeling of cell membrane structures called integrins. Membrane receptors rapidly send signals to the nucleus which initiate the synthesis of proteins, which in turn interact with cell metabolism and the surrounding environment to induce the modulation of recovery of parenchymal tissue function. Fibroblasts, pericytes, and stem cells are among the most common types of stromal cells. In this chapter, we analyze the membrane markers of stem cells and assess their capacity to influence surrounding tissues and recover tissue functionality.

2. How adult stem cell markers work?

Stem cells are composed of multiple types of cells, and all of them are characterized as undifferentiated cells able to self-renew and proliferate with high capacity. The international society for cellular therapy minimal criteria to define human MSC: (1) Mesenchymal stem cells (MSC) must be plastic adherent in standard culture conditions. (2) MSC must express CD105, CD73, and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR surface molecules. (3) MSC must differentiate to osteoblasts, adipocytes, and chondroblasts in vitro [3, 4]. Membrane markers are also present on other cells with high proliferation rates such as in the intestinal epithelium, ischemic myocardium, cholinergic synapses and in proliferative lymphocyte, and tumoral cells.

This review aims to analyze why those membrane markers are important to maintain important characteristics of stem cells such as proliferation potential, angiogenic, differentiation, and immunomodulation capacity. We assess how membrane markers promote the growth, proliferation, differentiation, and survival of parenchymal cells where stem cells reside. These cell membrane markers contribute under appropriate stimuli to the capacity of stem cells to differentiate into endoderm, mesoderm, or ectoderm-derived cell tissues.

2.1. CD73 membrane marker

CD73 participates in an autocrine and paracrine manner to the regulation of a variety of physiological processes. The primary structure of CD73 was described by Misumi et al. [5] as a dimer of two identical 70-kD subunits bound by a glycosylphosphatidylinositol linkage to the external face of the plasma membrane. This molecule is an ecto-5′-nucleotidase, which dephosphorylates nucleoside adenosine monophosphate (AMP) into adenosine (ADO). ADO is a potent endogenous physiological and pharmacological regulator of many functions. ADO mediates its effects on tissue regeneration and repair via binding and activation of a family of G protein-coupled receptors (adenosine A1, A2A, A2B, and A3 receptors). Activation of the G protein activates the PKA pathway by activating cyclic AMP. PKA is an enzyme that transfers a phosphate group from ATP to other specific proteins such as the cyclic AMP response element-binding protein (CREB). PKA is a transcriptional coactivator that stimulates the transcription of several genes by a phosphorylation pathway of kinases. Between those kinases, extracellular signal-regulated kinases (ERK) activate many transcription factors such as activating protein 1 (AP1). AP1 controls a number of cellular processes including differentiation, proliferation, and apoptosis [6–8]. Being one of the target genes of Cyclin D,

AP1 transcription factors are also associated with tissue regeneration. Cyclin D is a protein involved in regulating cell cycle progression by regulating the G1–to-S phases [9]. AP1 also induces CREB, another transcription factor responsible for increasing or decreasing the transcription of downstream genes [10]. The presence of CD73 in the cell membrane allows this enzyme to release ADO from extracellular AMP. ADO then binds to a membrane receptor associated with the G protein. Activation of the G protein induces a phosphorylation cascade that allows the activation of transcription factors. The target genes of these transcription factors are those involved with the cell cycle, the synthesis of extracellular matrix, and vascular growth factors (**Figure 1**). Nevertheless, activation of these receptors induces variable responses in different cells.

The pathway generates the liberation of extracellular ADO and could be responsible for the angiogenic effects observed in stem cell transplantation. Because of the dephosphorylate enzymatic activity of CD73 on AMP, the pathway induces the synthesis of VEGF. Indirectly, CD73 is responsible for the angiogenic capacity of stem cells as generating ADO by autocrine signaling will consequently stimulate the production of VEGF, a pro-angiogenic factor. For example, in skeletal muscle cells, activated PKA phosphorylates enzymes involved in glycogen metabolism which simultaneously trigger the breakdown of glycogen to glucose and inhibit glycogen synthesis, thereby increasing the amount of glucose available to muscle cells within seconds. In macrophages, it also induces the synthesis of angiogenic factors, such as VEGF and the proliferation of human retinal endothelial cells [11–13]. The pathway also plays an important role in the proliferation of endothelial cells. Stimulation of A2A receptors could be responsible for wound healing by stimulating both angiogenesis and matrix production [14]. Montesinos et al. [15] proposed that ADOA2A receptor stimulation by ADO promotes the recruitment of circulating bone marrow-derived endothelial precursor cells and differentiation into endothelial cells. CD73 serves as a costimulatory molecule in activating T cells [16].

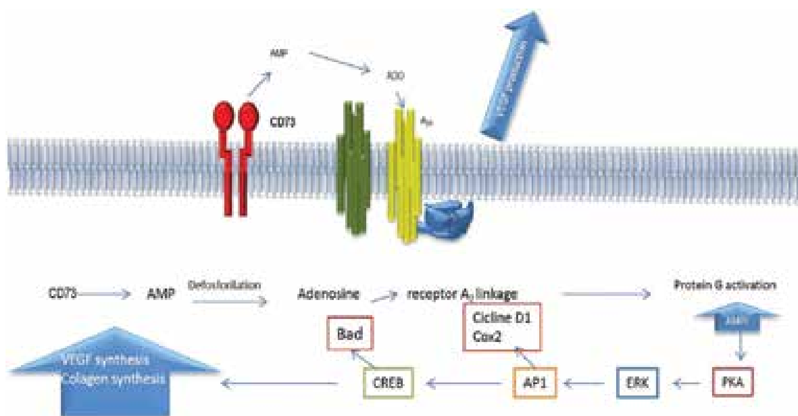

Figure 1. Schematic CD73 signaling pathways. The activity of ectonuclease on extra-cytoplasmic AMP releases (ADO). ADO binds to its receptor which in turn generates the activation of the G protein and triggers the phosphorylation cascade up to transcription factors that will induce the expression of genes responsible for the synthesis of collagen and vascular endothelial growth factors (VEGF).

Several activities for CD73 and its product ADO have been described, including interactions of ADO with its receptor in hematopoietic cells given the activation and angiogenic capabilities of those cells. Probably, in stem cells, the CD73 transmembrane protein is related to the capacity of cells to differentiate into several lineages because the A2A receptor has been inoculated as a possible regulator of osteoblast differentiation in bone tissues [17]. The pathway generated by this membrane marker induces the synthesis of extracellular matrix and promotes collagen production in the skin and in the liver [18–20].

Another activity observed in stem cells is their immunomodulatory potential, which is related to ADO inhibition against inflammatory actions by neutrophils [21]. ADO is also a neuromodulator acting through A1 and A2 receptors. A1Rs are abundantly expressed throughout the brain and control synaptic transmission. Because of its participation in cAMP formation in synaptosomes, CD73 has been proposed as an alternative target in the treatment of some cases of synaptic degeneration and neurodegeneration [22, 23].

CD73 has been related with cardiopathies as ADO produced by the 5′-nucleotidase activity of CD73 could exert control over the mineralization of the aortic valve [24]. Development and maturation of arterial atherosclerotic plaques have been related to the impaired expression of CD73. The production of ADO by CD73 is critical for adaptation to hypoxia in the myocardium, where CD73-catalyzed ADO production acts as a critical control point for the maintenance and regulation of vascular barrier function in multiple tissues under hypoxia [25, 26].

Other stromal cells bearing CD73 are fibroblasts, which are the most common cells in connective tissues. Fibroblasts synthesize the extracellular matrix that includes collagen, glycosaminoglycans, elastic fibers, and glycoproteins, as well as participate in inflammatory responses. Fibroblasts aid to maintain the structural integrity of connective tissues [27–29]. On those activities are involved with the CD73 membrane marker that allows the activation of the G-protein followed by a pathway to induce the activation of the transcription factors responsible for the synthesis of extracellular matrix molecules.

2.2. CD90 membrane marker

Early studies on THY1 and CD90 have suggested their possible relation with cell activation in progenitor's cells with the highest in vitro proliferative potential [30]. THY1 is signaled via integrins, protein tyrosine kinases, cytokines, and growth factors. Several functions have been related to THY1 such as T-cell activation, neurite outgrowth, apoptosis, tumor suppression, wound healing, and fibrosis [31–34]. In order to understand how this membrane receptor induces so many changes in cellular metabolism, numerous studies have been conducted to identify possible activation pathways induced by the activation of THY1. THY1 is a glycophosphatidylinositol (GPI) anchored to conserved cell surface protein with a single V-like immunoglobulin domain. The protein is anchored in the external lipid bilayer of the membrane by a phosphatidylinositol (PI) anchor in membrane microdomains (lipid rafts) [35, 36].

Studies focusing on understanding why this membrane protein induces several changes in cellular pathways have reported that THY1 stimulates neurite outgrowth by activating a second messenger pathway where extracellular signals such as growth factors. Its activation induces a rapid and extensive mobilization of the intracellular second messengers, PI, and

Ca^{2+} [37]. T-cell activation by THY1 causes an immediate phosphatidylinositol (PI) turnover and an influx of extracellular Ca^{2} while releasing very little Ca^{2+} from intracellular stores [38]. Intracellular transduction of the G protein activates phospholipase C that generates inositol phosphate and diacylglycerol (a second messenger) groups from the hydrolysis of plasma membrane phospholipids. Inositol phosphate could be phosphorylated at various positions by enzymes that belong to the family of phosphatidylinositol 5-phosphate 4-kinases. The resulted PI is a second messenger involved in several signaling pathways including signals of cell growth [39–42]. IP3 releases Ca^{2+} from the endoplasmic reticulum by binding to its receptors (IP3R) regulating mitochondrial metabolism, cell cycle entry, and cell survival. Ca^{2+} signals are important for the self-renewal and differentiation of human embryonic stem cells [43–45]. Ca^{2+} forms a complex with the protein calmodulin which regulates the activity of many proteins including various transcription factors [46, 47]. Diacylglycerol is a glyceride of two fatty acid chains covalently bonded to a glycerol molecule through ester linkages and it remains within the plasma membrane where it regulates the protein kinase signaling cascades through protein kinase C (PKC) activation [48].

High capacity for cell proliferation is induced via CDk5 and ERK, generating changes in the cytoskeleton that induce cell proliferation and differentiation, matrix production and immunomodulatory potential. Recently, Chung et al. [49] demonstrated that a subpopulation that is positive for THY1 (CD90) is relatively more capable of forming bone than the CD105 low subset of cells. Considering the possible differentiation and proliferation capacity of cells carrying this membrane protein, stromal cardiac cells with the CD90 antigen were introduced to recover function, and reprogramming capacities in an infarcted heart. Cells obtained from human bone marrow-bearing this membrane marker exhibited robust multi-lineage differentiation and self-renewal potency. In addition, THY1 expression appears to be an indicator of G_0/G_1cell-cycle phase in human stem cells from bone marrow [50–53]. THY1 has possible roles in cell–cell interaction where THY1 mediates adhesion of leukocytes and monocytes to endothelial cells and fibroblasts and performs a signaling event, which results in the activation of cell pathways.

THY1 is a receptor to many molecules such as growth factors, hormones, and the extracellular matrix. Its stimulation induces the synthesis of second messengers that initiate a cascade of reactions that can lead to the cell to proliferation or differentiation (**Figure 2**).

In fibroblasts expressing the endometrial stromal marker CD90 (THY1) [54], CD90 was strongly expressed by functional stroma and perivascular cells and used to isolate pure populations of endometrial stromal stem and progenitor cells [55]. In fibroblasts, these membrane markers are stimulated by peptide growth factors, such as bombesin and PDGF, thereby inducing DNA synthesis and cell division. In addition, since apoptosis is a mechanism during normal wound healing, THY1 has a beneficial effect on lung fibroblast activity where it induces the regulation of apoptosis via Fas-, Bcl-, and caspase-dependent pathways [56].

2.3. CD105 membrane marker

Endoglin, a cell membrane glycoprotein also known as CD105, is over-expressed in proliferating endothelial cells and as consequence is involved in neovascularization. It is a

transmembrane glycoprotein related to the transforming growth factor (TGF)-β receptor. St-Jaques et al. [57] suggested that endoglin on stromal fibroblast-like cells may be regulating the access of TGF-β1 to the signaling receptor complex. It was later confirmed that CD105 is a transmembrane protein that binds to several factors of the TGF-β superfamily, a pleiotropic cytokine that regulates different cellular functions including proliferation, differentiation, and migration [58]. Endoglin binds TGF-β1 and TGF-β3 with high affinity through its association with the TGF-β receptor type II [59]. After TGF-β binding to its receptor via two single pass serine/threonine kinase transmembrane proteins, a phosphorylate kinase activates signaling cascade transduction, which initiates intracellular signaling by phosphorylating members of the Smad family of transcription. The resulting Smad heterocomplex translocates into the nucleus and interacts with numerous transcription factors that in turn regulate the transcription of many TGF-β-responsive genes [60, 61]. Upon ligand stimulation, R-Smads are phosphorylated by receptors and form oligomeric complexes with common-partner Smads (Co-Smads). Oligomeric Smad complexes then translocate into the nucleus where they regulate the transcription of target genes by direct binding to DNA. CD105 co-stimulates the TGF-β receptor to induce CDk5 and other genes by the Smad4 pathway leading to high cell proliferation and collagen production (**Figure 3**).

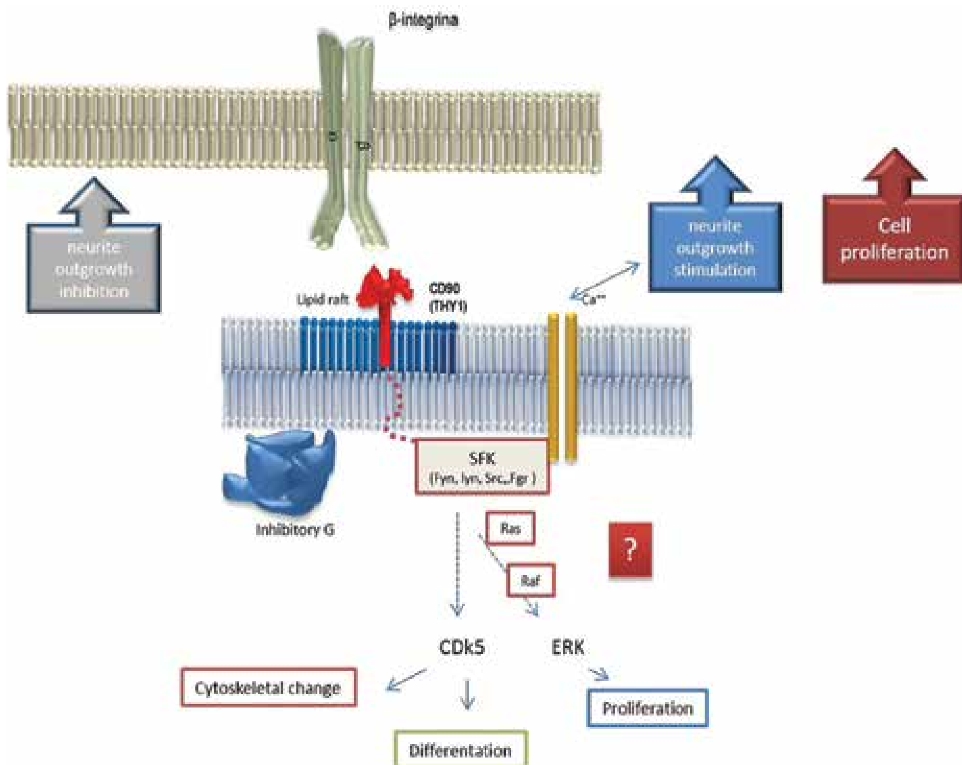

Figure 2. Schematic representation of CD90 pathway induction. GPI is anchored in the cell membrane surface and its activation generates an efflux of calcium and a release of phosphatidylinositol (PI). These second messengers regulate mitochondrial metabolism, cell cycle entry, and cell survival.

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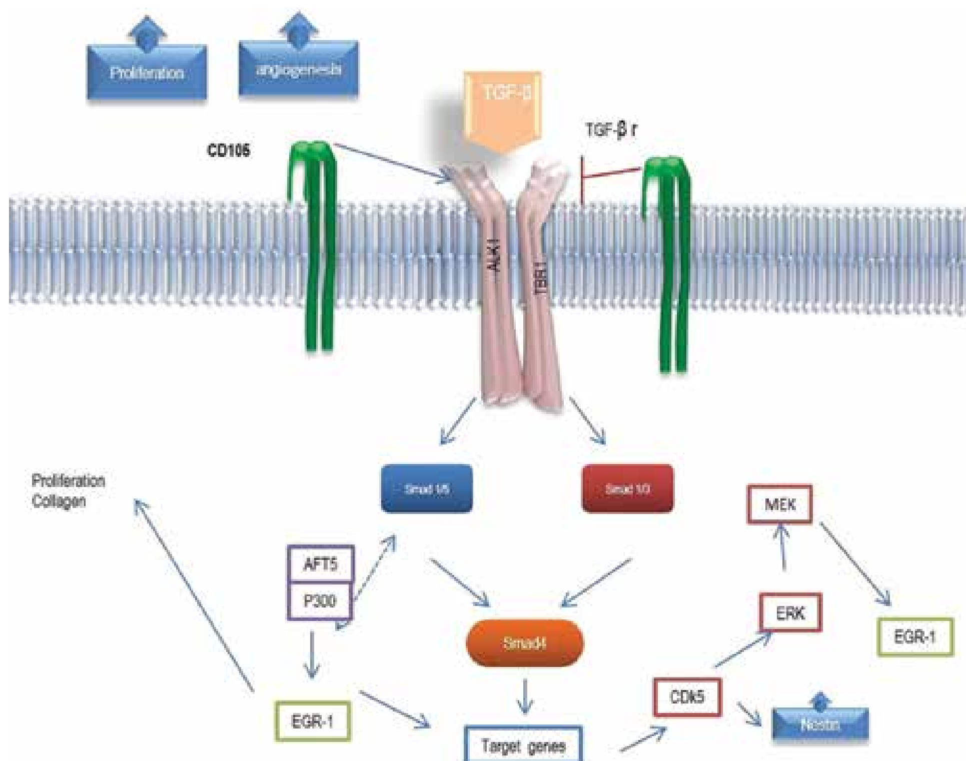

Figure 3. Schematic representation of CD105 pathways. The membrane protein binds to the transforming growth factor receptor (TGFr). Following TGF binding with its receptor, a signaling cascade leads the transcription of different genes related to cell differentiation, chemotaxis, proliferation, and activation across many cells.

The biological functions of TGF-β can only be delivered after ligand activation and they promote or inhibit cell proliferation. The activation of TGF-β is involved in the recruitment of stem and progenitor cell participation in the tissue regeneration and remodeling process [62, 63].

In some cases, the expression of endoglin has been related to its differentiation selectivity. Levi et al. [64] found that a subset of adipose-derived stem cells with low expression of the endoglin cell surface receptor (CD105) had enhanced in vitro and in vivo osteogenic differentiation potential. Nevertheless, more recent research in an osteoarthritis animal model has reported that $CD105^{+}$-MSCs migrated toward the injured knee joint and suggested the use of $CD105^{+}$-MSC as an alternative for cell therapy for these pathologies [65, 66]. Because CD105 is a co-factor component of the TGF-β receptor complex that is expressed in endothelial cells, it has been related to the pathogenesis of vascular diseases and with tumor progression [67, 68]. Nevertheless, TGF-β has been shown to activate two distinct pathways, ALK5-inducing Smad2/3 phosphorylation, and ALK1-promoting Smad1/5 phosphorylation. Those pathways regulate endothelial cell proliferation. Activation of ALK1 stimulates cell proliferation and migration, whereas activation of ALK5 inhibits these responses [69, 70]. Cell therapy may reconstitute the entire hematopoietic system with cells bearing CD105. Since TGF-β1 exerts its action on primitive hematopoiesis by inhibiting cell cycle progression of primitive precursors,

a previous report has shown that the presence of cells bearing CD34 represents an option to recover hematopoietic stem cells. Recently, it has been reported that human stem cells bearing CD34 and CD105 are the best long-term repopulating cells and present high self-renewal capacities [71, 72]. Nevertheless, balance is very important and these cells have been related to pathologies such as fibrosis diseases [73, 74].

3. Conclusions

The membrane markers CD73, CD90, and CD105 allow stem cells and other stromal cells such as fibroblasts to react to stimuli and quickly leave their quiescent state, thereby going into a proliferation state and generating growth factors. Those capacities allow the recovery of parenchymal tissue in which they are found. CD73 is an ectoenzyme that dephosphorylates nucleoside AMP given free ADO. This purine binding to its membrane receptor leads to the activation of the G protein and results in the activation of a pathway that reaches the nucleus. As a consequence, extracellular matrix and growth factors such as VEGF are synthesized. CD90 influences the cell cycle and cell proliferation. CD90 also induce several cytoskeletal changes allowing cell differentiation. CD105 is a co-factor to the TGF-β receptor and following TGF-β union with its receptor a signaling cascade is activated, resulting in the transcription of different effectors including the synthesis of pro-inflammatory cytokines, which have an important role in angiogenesis and proliferation. In conclusion, those membrane markers are related to pathways that regulate the immune response, cell proliferation, and differentiation, thereby allowing lost tissue recovery and the formation of new angiogenic pathways.

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Conflict of interest

The author declares have no competing interests.

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Section 2

Stromal-Tumor Cell Interactions

Chapter 4

Multipotent Stromal Cells in a Tumor Microenvironment

Flavia Alejandra Bruna

Additional information is available at the end of the chapter

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Abstract

Multipotent mesenchymal stromal cells [also referred to as mesenchymal stem cells (MSCs)] as was previously described, are a heterogeneous subset of stromal cells with regenerative potential. Their present tropism for inflamed sites including tumors lesion may be adverse or therapeutic effects arising from MSC administration; in this context, their potential for producing trophic and immunomodulatory factors raises the question as to whether MSCs promote or interact with a tumor microenvironment. Previous studies show a paradoxical effect regarding MSCs, which seems to depend on isolation and expansion, cells source, dose and both route and timing of administration. The occurrence of neoplastic transformation in ex vivo expanded MSCs after a long-term culture has been reported, however, this event has been subsequently described as uncommon, with an estimated frequency of $<10^{-9}$. Furthermore, neither ectopic tissue formation nor MSC-originating tumors have ever been reported so far in hundreds of patients treated with MSC therapy. The biosafety of these cells, both in precancerous and cancerous environments, has been little investigated to date. We found in an animal model of oral cancer that locally or systemically administered allogeneic MSCs do not aggravate the progression of precancerous lesions. Moreover, they preclude cancer progression and tumor growth, particularly at papilloma stage.

Keywords: mesenchymal stem cells, multipotent mesenchymal stromal cells, tumoral microenvironment, cancer

1. Introduction: Properties of the MSC

Mesenchymal stem cells (MSCs) are a promising source for cell therapy in regenerative medicine. The therapeutic properties of MSCs are related to their potentials for transdifferentiation, immunomodulation, and trophic factor secretion. Investigators have isolated MSCs from many

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different tissues, including bone marrow, adipose tissue, umbilical cord blood, peripheral blood, dermis, liver, skin, and skeletal muscle [1–4]. Previously it has been reported that MSCs from different sources (adipose tissue, bone marrow, kidney, muscle, etc.) share characteristics properties (i.e., expression of cell surface antigens, immunomodulatory capability, and tropism toward tumor) [5–8]. On the other hand, it has been reported that MSCs isolated from different sources can be found into tumor microenvironments, and depending on the level of commitment to a certain lineage by MSCs, they can be transdifferentiated faster to certain cell types depending on the source [9]. The MSCs from different source express a distinct set of genes, which is a reflection of its commitment related to their potential of differentiation (including adipocytes, osteocytes, chondrocytes, hepatocytes, fibroblasts, and pericytes) [10, 11]. MSCs can be expanded until five passages preserving their therapeutic potential for use in clinical applications [12, 13]. Additionally, the transdifferentiation of MSCs has rarely been observed in animal models [14]. Regarding the immunomodulator potential, it has been reported that MSCs can secrete various immunomodulators, such as nitric oxide (NO), prostaglandin (PGE2), indoleamine 2,3-dioxygenase (IDO), interleukin (IL)-6, IL-10, and HLA-G [12, 13]. Regarding the immunomodulatory potential of MSCs, there are molecules that can moderate the immune response such as nitric oxide (NO), prostaglandin (PGE2), indoleamine 2,3-dioxygenase (IDO), interleukin (IL)-6, IL-10, and histocompatibility antigen class I, G (HLA-G). These soluble factors modify the function of immune cells and induce T regulatory cells activation ([14]). In addition, MSCs can suppress immune cell activation via cell-to-cell contact. MSCs can also inhibit the proliferation of effector T cells by activating programmed cell death pathways such as apoptosis by the interaction of programmed death signal molecules type 1 (PD-1) with their related ligands PD-L1 and PD-L2. On the other hand, it has been reported that MSCs can induce T cell anergy by inhibiting the expression of CD80 and CD86 in antigen-presenting cells [15–17]. Among the wide range of factors that MSCs secrete, are modulators that can regulate inflammation, apoptosis, angiogenesis, fibrosis, and tissue regeneration [18]. In addition, previous studies reported that MSCs produce trophic factors that promote cell survival (SDF-1, HGF, IGF-1), cell proliferation (EGF, HGF, NGF, TGF-α), and angiogenesis (VEGF) [19, 20]. Faced with the signal of damaged tissue, MSCs can migrate to the site of injury (homing) by sensing chemoattractant gradients of cytokines secreted by the extracellular stromal matrix (MEC) and spreading through the peripheral blood to all the organisms [21–24]. At the site of injury, MSCs are stimulated and activated by local damage and repair factors, such as hypoxia, the cytokine environment, and Toll-like receptor ligands. This cascade of stimuli as a whole promotes the production and the release of abundant growth factors that converge to increase tissue regeneration [28, 29]. In contrast to the use of MSCs in regenerative medicine, recent data suggest that MSCs may increase tumorigenesis or inhibit tumorigenesis [25, 26]. In the tumor microenvironment, the tumor tries to avoid recognition by the immune system while secreting inflammatory mediators to establish and maintain a constant state of inflammation [27]. In addition, the correlation between normal cells, cancer cells, and the matrix within the tumor microenvironments has gained increasing attention, especially because these interactions contribute to certain hallmarks of cancer, such as immunomodulation, angiogenesis, invasion and metastasis, and apoptotic resistance [28–30]. Regarding, if the MSCs promote or suppress tumor development, in several studies shown that MSCs perform homing the tumor microenvironment and then promote the formation of

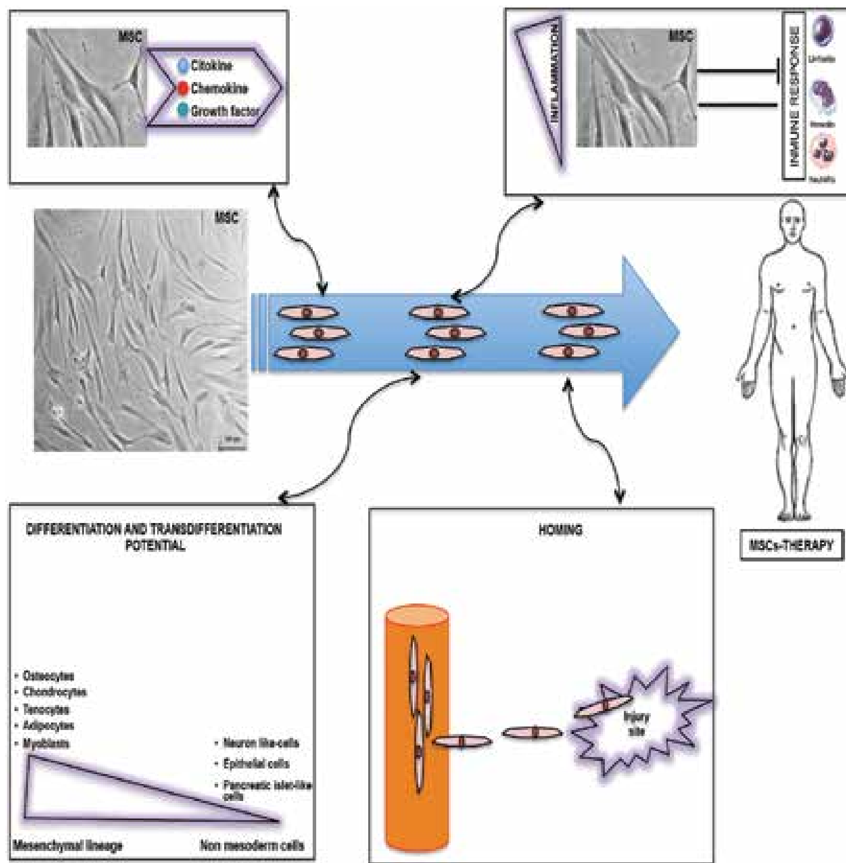

Figure 1. MSC effects in clinical use. The therapeutic potential of MSCs relies on several unique properties as: (i) the capacity to differentiate into various cell lineage, (ii) the ability to secrete paracrine factors initiating healing and regeneration in the surrounding cells, (iii) the ability to reduce inflammation and regulate immune response and to migrate to the exact site of injury.

tumor blood vessels, improving the fibrovascular network and suppressing immune responses, modulating thus the tumor response to antitumor therapy [31–35]. Unlike its tumor-promoting abilities, MSCs can also suppress tumor growth by inhibiting proliferation-related signaling pathways, such as AKT, PI3K, and Wnt, by the secretion of proapoptotic molecules such as Dikkopf type 1 (Dkk1) inhibiting the progression of the cell cycle; in turn, they can negatively regulate the X-linked inhibitor of the apoptosis protein (XIAP) and suppression of angiogenesis [31, 36, 37] (**Figure 1**). In this chapter, we will analyze how MSCs can contribute to tumorigenesis, including (i) transition to tumor-associated fibroblasts; (ii) suppression of the immune response; (iii) promotion of angiogenesis; (iv) stimulation of epithelial-mesenchymal transition (EMT); (v) through contribution to the tumor microenvironment; (vi) inhibition of tumor cell apoptosis; through contribution to the tumor microenvironment; (vi) inhibition of tumor cell apoptosis, and (vii) promotion of tumor metastasis.

2. MSC and cancer: how they relate?

2.1. MSCs can induce epithelial-mesenchymal transition

The epithelial-mesenchymal transition (EMT) is a process characterized by downregulation of proteins associated with cell adhesion present in epithelial cells such as E-cadherin, γ-catenin/plakoglobin, and zonula occludens-1. In turn, it triggers an upregulation of proteins related to the mesenchymal phenotype, such as N-cadherin, vimentin, fibronectin, and alpha smooth muscle actin [38, 39]. The EMT is present during organogenesis and wound healing. EMT has also been described during the development of epithelial tumors, which is associated with a more undifferentiated and metastatic phenotype (poor prognosis) [40]. There are accumulated evidence that suggests that a defective EMT promotes tumor invasion, metastasis, and chemoresistance to medications [41]. In many tumors, the presence of cytokines such as HGF, EGF, PDGF, and TGF-β produced and released by the stroma associated with the tumor, act by inducing EMT and favoring processes such as metastasis [42, 43]. Interestingly, it has been reported that these factors are secreted by MSCs [44] and that they can activate a number of transcription factors of genes that promote EMT, such as Snail, Slug, zinc finger E-box binding homeobox 1 (ZEB1), and Twist related protein-1 (TWIST) to transmit EMT promotion signals [45–47]. A recent study demonstrated the activation of specific genes to induce EMT in breast cancer cell lines when they were co-cultured with MSCs and a decrease in expression of genes related to epithelial differentiation [48]. MSCs also improve the ability to trigger the metastatic cascade in colon cancer cell lines through high expression of EMT-associated genes (ZEB1, ZEB2, Slug, Snail, and Twist-1), in a cell-cell-dependent manner. It should be noted that the decrease in the expression of the E-cadherin gene is related to EMT [48]. In breast cancer cell lines, it has been described that MSCs produce leptin which results in an increase in the expression of EMT genes and associated with metastasis (SERPINE1, MMP-2, and IL-6). On the other hand, in SCID/beige mice co-injected with MCF-7 breast cancer cells and with MSCs containing leptin shRNA, a decrease in the leptin levels produced by the MSCs was observed and consequently a reduction in the tumor volume MCF7 and less metastatic lesions in liver and lung [49]. Other authors have reported that MSCs can fuse with different cancer cells and unleash the classic characteristics of EMT [50–52].

2.2. MSCs can induce transition to tumor-associated fibroblast

MSC to fibroblasts associated with tumors: The tumors consist of cancer cells and different stromal cells that form the tumor cell medium [53]. The tumor stroma consists of an extracellular matrix scaffold (MEC) populated by stromal cells that include fibroblasts, immune cells, and endothelial cells. Fibroblasts can be activated in the tumor stroma and activated fibroblasts (also called myofibroblasts) are called carcinoma-associated fibroblasts (CAF) or tumor-associated fibroblasts (TAF). CAF/TAF are abundant in most invasive tumors and are mainly composed of cells expressing smooth muscle actin α (α-SMA) [54]. These cells can secrete SDF-1 with the consequent promotion of tumor growth and angiogenesis [55], which binds to CXCR4 expressed by tumor cells [55]. Recently, it was reported that MSCs could differentiate into CAFs/TAFs [24, 56, 57]. In fact, MSCs can differentiate into CAF/TAF and increase the production of α-SMA, tenascin-C and fibroblast surface protein (FSP), CCL5/RANTES, and

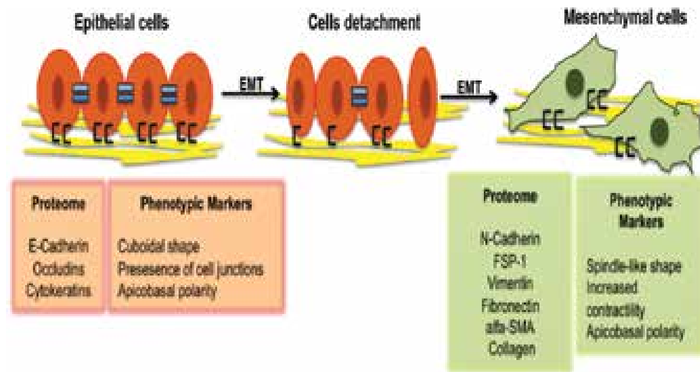

Figure 2. Figure illustrating the epithelial-mesenchymal transition.

SDF-1 by stimulating tumor growth through contribution of angiogenesis and the production of tumor stimulating growth factors [37, 61–63] (**Figure 2**).

2.3. MSCs in tumor microenvironments can modulate the immune response

Immune response in tumor microenvironments: In addition to protecting the host from external invaders, the immune system recognizes tumor antigens and eliminates malignant tumors [58]. Therefore, tumor growth, invasion, and metastasis are important aspects of the tumor's immune escape mechanism [59, 60]. During tumor initiation, TAMs and MSCs migrate to the tumor microenvironments. TAMs act as the main inflammatory component of the tumor microenvironment [61, 62]. In contrast, TAMs can show antitumor activities linked to the M1 phenotype via IFN-γ, TNF-α, TGF-β, PGE2, and IL-10 [72, 77–82]. Also, M1 TAMs secrete free oxygen radicals, nitrogen radicals, and pro-inflammatory interleukins (e.g., IL-1β, IL-6, IL-12, IL-23, and TNF-β) facilitating the killing of tumoral cells. The MSCs can be activated by the pro-inflammatory cytokines IFN-γ, TNF-α, or IL-1βn in tumor microenvironments [30, 52, 69, 83, 84]; additionally, the tumor cells and M2 produce immunomodulatory molecules, such as IDO, PGE2, IL-6, IL-10, HLA-G5, and NO [64, 65]. IDO is the critical rate-limiting enzyme of tryptophan catabolism through the kynurenine pathway, resulting in tryptophan depletion and halting the growth of various cells. In tumor microenvironments, MSCs can be activated by pro-inflammatory cytokines IFN-γ, TNF-α, or IL-1β [66, 67]. Within the immunomodulatory molecules secreted by MSCs, Prostaglandin E2 (PGE2) has a multifunctional role in pathological processes including the regulation of inflammation and cancer. The production of PGE2 by MSCs increases after stimulation with TNF-α or IFN-γ. In addition, PGE2 increases the level of expression of IL-10 and decreases the expression of TNF-α, IFN-γ, and IL-12 in cells of the developing immune system and of macrophages [68, 69]. PGE2 regulates the secretion of IFN-γ and IL-4 in Th1 and Th2 cells, respectively, and promotes proliferation of Treg cells [19]. It has been reported that IL-6 secreted by MSCs inhibits monocyte differentiation toward CD and decreases the activation capacity of CD to T cells [70, 71]. In addition, IL-6 secreted by MSCs resulted in a delay in apoptosis of lymphocytes and neutrophils [72, 73]. Another important molecule in the moderation of the immune response is nitric oxide (NO). NO is produced by inducible NO synthase (iNOS) through stimulation by inflammatory factors such as IL-1, IFN-γ, and TNF- α [72, 74] and also inhibits the functions of T cells [75]. In contrast to

the reported evidence that MSCs can suppress the immune response, Ohlsson et al. reported that administration of tumor cells and MSCs simultaneously caused an increase in the inflammatory component in the stroma, mainly composed of granulocytes and monocytes, whereas when administered separately, this was not observed [75]. In a rat-induced colon cancer model, it was observed that the colon tumor cells inoculated in a gelatin matrix, when implanted subcutaneously, developed larger tumors than animals that surgically received colon cancer cells combined with MSCs. MSCs inhibited rat colon carcinoma by increasing the leukocyte infiltrate [75]. It was observed that the increase in infiltrations of both granulocytes and macrophages was much higher in rats co-injected with tumor cell lines and MSCs than in rats injected with tumors without MSCs. These data suggested that MSCs had pro-inflammatory effects in this model. In this same work, a greater degree of infiltration of granulocytes and macrophages was observed, but to a lesser extent, when only MSCs were added to the gelatin. [75].

2.4. MSCs may promote tumor growth

The tumor microenvironment, is composed of cancer cells, noncancerous cells, and stromal cells, all this as a whole influences the growth of the tumor [28]. The tumor stroma hosts many types of cells, as well as MEC. These cells include different types of immune cells, fibroblasts, endothelial cells, and myofibroblasts [28]. MSCs perform homing at tumor sites and then integrate into the tumor stroma [76, 77]. These cells interact with each other and with cancer cells, resulting in the promotion of tumor growth. The ability of MSCs to promote tumor growth and metastasis was demonstrated in murine models of breast cancer with similar results from cancer cells co-implanted with MSCs [24, 78, 79]. In turn, it was observed that allogenic mice transplanted with B16 melanoma cells did not in the development of tumors when B16 cells were co-injected with MSCs [80]. This finding indicates that MSCs exert essential immunosuppressive and antitumor effects at the onset of the tumor. Human bone marrow-derived MSCs have increased the growth of estrogen receptor-alpha (Erα) positive breast cancer cell lines: T47D, BT474, and ZR-75-1, in an in vitro three-dimensional tumor environment assay, in contrast, have had no effect on the ERα negative cell line MDA-MB-231 [81]. Nonetheless, the growth rate of (another ERα negative cell line) was high in the presence of human MSCs [81]. Another study showed both human fetal MSCs transplanted subcutaneously into BALB/c-nu/nu mice with human adipose-derived MSCs alone or together with cell lines F6 (human mesenchymal stem cells F6) or SW480 (human colon adenocarcinoma cell line) in a ratio 1:1 or 1:10, favoring the growth of these tumor cell lines [79]. Other authors reported that tumor cells procured from primary breast cancer were grown in the presence of human bone marrow-derived MSCs (ratio 1:1). Additionally, this was tested on secondary mice, where a greater tumor-producing ability compared with the cells obtained from primary tumors and grown in the absence of MSCs was observed [82]. In addition, tumor incidence and/or size [83, 84] as well as tumor vascularization [30] increased when breast, lung, colon, or prostate tumor cells were co-injected with MSCs independent of the source of origin from the same. Similar results were observed with MSCs derived from adipose tissue or human bone marrow. The same was demonstrated with tumor cells of osteosarcoma, melanoma, and glioma [85]. Another interesting observation relates to adipose tissue adjacent to the tumor implant (e.g., lung cancer models or to Kaposi's sarcoma xenografts), where a substantial increase in

tumor size was observed along with the appearance of stromal cells of the implant; MSCs derived from adipose tissue may promote tumor growth [86].

The innate tropism of MSCs to injured sites, including established tumors, has been widely reported, although the mechanism behind it has not yet been fully elucidated that the proinflammatory cytokines secreted by the reactive stroma are involved [24]. The most accepted explanation to date is that the tumors behave as unresolved wounds since their stroma closely resemble the healing granulation tissue and produce cytokines, chemokines, and other chemotactic agents [27] and the chemotactic properties of MSC are similar to those of leukocytes [87, 88]. The tropism of MSCs for tumors has been widely studied and exploited with very good results for the supply of antitumor drugs in animal models of lung and breast cancer, melanoma, and glioma [88].

Like any other cell in culture, when long-term MSCs are manipulated in vitro, they can have chromosomal aberrations and produce tumors in healthy animals because they undergo cell crisis [89]; this has been observed mainly in mouse cells, which require extensive cultures to produce a significant number of MSCs free of hematopoiesis [90]. For example, it has been demonstrated that the intravenous administration of MSCs derived from bone marrow in NOC/SCID mice generates cellular aggregates that are retained in the pulmonary capillaries, forming emboli when they are injected in large quantities. Once lodged in the capillaries, they expand and invade the lung parenchyma and form tumor nodules [90]. These lesions rarely contain lung epithelial cells, but have the characteristics of cartilage and immature bone that resembles a well differentiated osteosarcoma. However, until now, no type of transformation has been demonstrated by human MSCs adequately expanded ex vivo for cell therapy (no more than five passages) [90]. The Canadian Trial Critical Care Trials Group recently reported a meta-analysis of randomized, nonrandomized, controlled, and uncontrolled clinical trials, phase I and phase II, where they found no reports associating the administration of autologous or allogeneic MSCs and tumor formation in 36 clinical studies [91]. However, a longer follow-up is necessary to evaluate the tumorigenic potential of human MSCs.

2.5. MSCs might promote metastasis

Along with the increasing number of cancer metastasis mechanisms being discovered, it has been reported that MSCs can induce metastasis in vitro and in vivo [78, 83, 92, 93]. Previous studies showed when human breast cancer cells were co-incubated with MSCs, the gene expression of onco and proto-oncogenes in breast cancer cells was upregulated [48]. These molecular and morphological alterations were accompanied by a metastatic phenotype. Breast cancer cells induce the motility of tumor cells through the secretion of CCL5, increasing invasiveness and metastatic potentials [83]. The invasion mediated by CCL5/RANTES is also closely related to the increased activity of matrix metalloproteinase 9 (MMP-9) [94].

On the contrary, it has been shown that the increase in metastatic capacity when MSCs are co-injected with tumor cells is reversed when the MSCs are injected in a different site from the tumor and this anti-metastatic effect by the MSCs remains independent of tumor distance [83]. Other mechanisms, such as the induction of EMT, the regulation of CSC, and the displacement of mesenchymal niches are also implicated in tumor metastasis [95]. Breast cancer cells co-cultured with MSCs derived from human bone marrow (ratio 1:1) upregulate the expression of

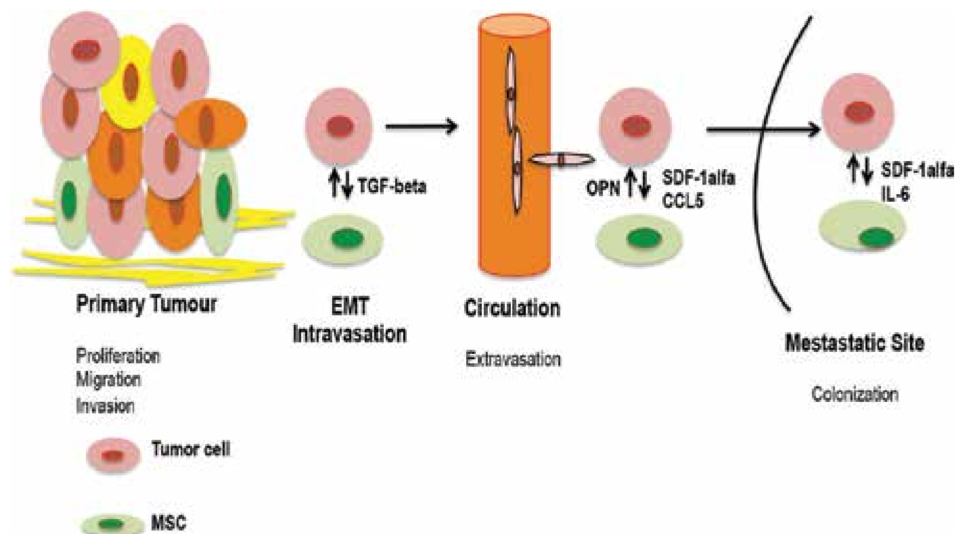

Figure 3. Interaction of tumor cells with MSCs during cancer progression. MSCs can interact with tumor cells at the primary site of the tumor and during metastasis by promoting cancer progression and invasion. One of the mechanisms involved in these processes is that MSCs induce EMT in tumor cells through close cell-cell contact, which could be due in part to the secretion of TGF-β [38, 82]. Studies have shown that secretion of osteopontin (OPN) by tumor cells, induces MSCs to secrete chemokine (motif CC) ligand 5 (CCL5) by stimulating the metastasis of the cancer cell through interaction with its specific chemokine receptor CC type 5 (CCR5) [84]. The migration of tumor cells to and from the metastatic site is mediated by SDF-1, a factor secreted by bone marrow MSCs, which interacts with the CXC receptor chemokine receptor type 4 (CXCR4) expressed in human tumor cell lines of the breast and prostate [33, 101, 102] (adapted from Sarah M. Ridge, Francis J. Sullivan and Sharon A. Glynn. Mesenchymal Stem Cells key players in cancer. Molecular Cancer, Feb. 2017 13:31 1-10. https://doi.org/10.1186/s12943-017-0597-8).

oncogenes and proto-oncogenes associated with tissue invasion, angiogenesis, and apoptosis (i.e., N-cadherin, vimentin, Twist, Snail, and E-cadherin) [48]. These molecular changes have been accompanied by morphological and growth alterations, which are characteristics of a more metastatic phenotype. It has been seen that 0.5×10^5 breast cancer cells co-injected subcutaneously with 1.3×10^6 MSCs derived from human bone marrow have significantly increased the rate of lung metastases in NOD/SCID mice. This effect was lost when the MSCs derived from bone marrow were injected separately from the tumor cells [83]. On the other hand, it has been shown that MSCs derived from bone marrow facilitate cancer cells [MCF-7, T47D low invasive cell lines, and factor 1 derived from stromal cells (SDF-1) null MDA-MB-231 highly aggressive] target to the bone marrow and modify the metastatic niche through the secretion of trophic factor (SDF-1 and CXCR4) and improved neovascularization in a xenogeneic mouse model (**Figure 3**) [96].

2.6. MSCs might inhibit tumor growth

MSCs can not only secrete cell regenerative factors continuously but also secrete factors in response to other various stimuli [97]. Tumor progression is accompanied by hypoxia, starvation, and inflammation. Although many studies have shown that MSCs have tumor promoting properties, many other studies have shown that MSCs have tumor suppressor properties

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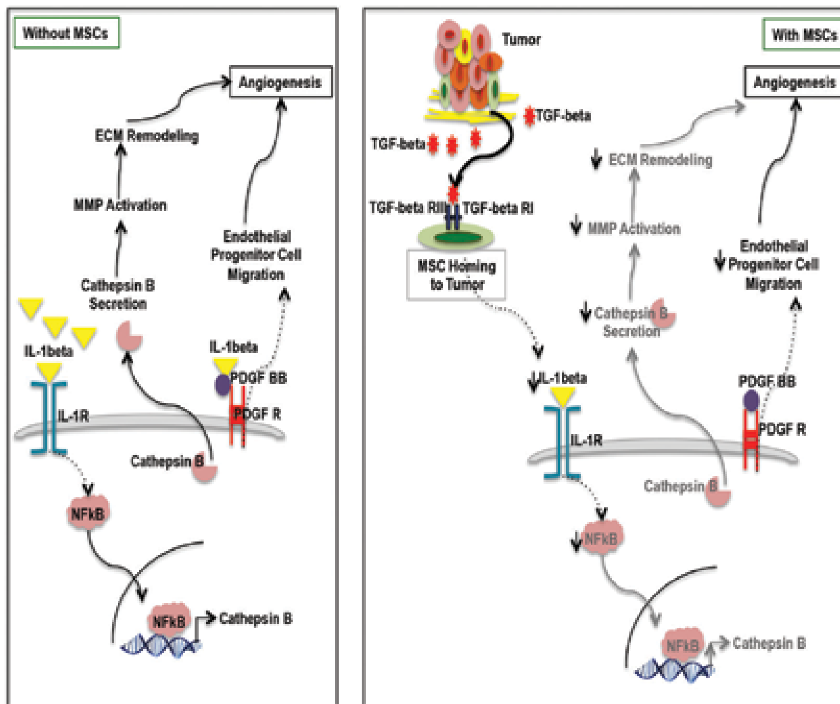

Figure 4. Mesenchymal stem cells can perform homing to the tumor environment. Studies in murine models of gliomas have reported that they can be directed to the tumor site through TGF-beta signaling and, once there, they can suppress angiogenesis within the tumor microenvironment. The proposed mechanisms are the following in sequential order: (1) the glioma microenvironment contains high levels of the proangiogenic cytokine, IL-1 beta. (2) Signaling through the NF-kappa B axis increases the expression of Cathepsin B and activates extracellular matrix remodeling programs that promote angiogenesis. (3) The increase in beta IL-1 potentiates the signaling of PDGF-BB, which promotes the migration of endothelial progenitor cells. (4) Glioma stem cells within a tumor secrete TGF-beta and recruit MSCs through TGF-beta RII and the endoglin/CD105 co-receptor. (5) Within the glioma microenvironment, the presence of MSCs reduces the levels of beta IL-1, negatively regulating Cathepsin B and decreasing PDGF R-beta signaling. It is believed that the downregulation of these signaling cascades in the presence of MSCs inhibits angiogenesis, reduces the density of microvessels and suppresses glioma growth. (Adapted from: https://www.rndsystems.com/resources/articles/mesenchymal-stem-cells-exhibit-tgf-beta-dependent-tropism-gliomas-and-inhibit-angiogenesis).

(**Figure 1**) (reviewed in [30]). In this regard, it is believed that MSCs suppress tumor growth by increasing the infiltration of inflammatory cells [97], inhibit angiogenesis [34], suppress Wnt and AKT signaling, and induce cell cycle arrest and apoptosis [32, 35, 36]. Recently, Ryu et al. reported that when the MSCs derived from adipose tissue were cultured at a high cell density, they synthesized IFN-beta, which then suppressed the growth of MCF-7 cells [98]. In addition, MSCs prepared with IFN-gamma or cultured with three-dimensional systems can express TRAIL, which induces specific apoptosis of tumor cells [97, 99]. In particular, it was demonstrated that in vitro culture of MSCs under hypoxic conditions increased cell proliferation. In addition, the expression of Rex-1 and Oct-4 was increased, leading to the conclusion that MSC scion was increased during hypoxia [100]. In addition, under hypoxic and starvation conditions, MSCs can survive through autophagy and release many antiapoptotic or pro-survival factors such as VEGF, FGF-2, PDGF, HGF, brain-derived neurotropic factor

(BDNF), SDF-1, IGF-1 and IGF-2, transforming growth factor-beta (TGF-b), and IGF-2 binding protein (IGFBP-2) [101, 102]. These factors inhibit the apoptosis of tumor cells and promote tumor proliferation, whereas normal MSCs do not acquire these properties. In addition to the mitogenic properties of growth factors secreted by MSCs, VEGF and FGF-2 can mediate Bcl-2 expression, delaying apoptosis [103], while indirect angiogenic factors can induce VEGF expression and FGF-2 [104]. In addition, SDF-1 was reported to prevent drug-induced apoptosis of chronic lymphocytic leukemia (CLL) cells [105]. In addition, VEGF, FGF-2, HGF, and IGF-1 expressed by MSCs have been reported to stimulate angiogenic and antiapoptotic effects after hypoxic conditioning [101, 106]. Although little is known about how MSCs under hypoxic conditions exert support effects on tumor cells directly, growth factors stimulated by MSCs, stimulated by hypoxia, can provide tumor support effects in the tumor microenvironment through angiogenic and antiapoptotic effects (**Figure 4**).

2.7. MSCs can induce apoptosis of cancer cells and endothelial cells

Depending on the microenvironment, MSCs can exert an antiproliferative effect. Lu et al. demonstrated that MSCs had an inhibitory effect on mouse tumor hepatoma cells in a cell-dependent manner through the activation of intrinsic caspase 3 pathway [107]. Lu et al. reported that MSCs increased p21 gene expression, involved in the arrest of the cell cycle. These data demonstrate that MSCs exerted tumor inhibitory effects in the absence of host immunosuppression, inducing arrest of the G0/G1 phase and apoptosis of cancer cells [107]. The same tumor suppressor activity by MSCs was observed in xenografted SCID mice with disseminated non-Hodgkin lymphoma [108]. A single injection of MSCs which increased the survival of the animals included those who presented more aggressive lymphomas. In turn, significant induction of endothelial cell apoptosis was observed when co-cultured with MSCs, suggesting that MSCs exert anti-angiogenic activity through endothelial cell apoptosis [108]. These findings were consistent with the results reported by Karnoub et al. where they demonstrated that MSCs exhibited potent anti-angiogenic activity in Kaposi's sarcomas with high vascularity and endothelial cell cultures in vitro by inducing apoptosis of tumor epithelial and endothelial cells through the Dkk-1 protein [32, 34]. Additionally, Dasari et al. reported that downregulation of the antiapoptotic inhibitor, inhibitor of the apoptosis protein linked to X (XIAP), in the presence of human umbilical cord blood-derived mesenchymal stem cell (hUCBSC) induced apoptosis of glioma cells and xenograft by the activation of caspase-3 and caspase-9 [109]. Recently, MSCs cultured at high density express IFN type I, which leads to cell death of breast cancer cells, MCF-7 and MDR-MB-231 cells [98]. In addition, MSCs prepared with IFN-gamma or cultured with three-dimensional systems can express TRAIL, which induces specific apoptosis of tumor cells. [97, 98].

2.8. Regulation of cell cycle by MSC

MSCs secrete a variety of cytokines that induce cell cycle arrest of tumor cells, albeit transiently, at the G1 phase through expression of Cyclin A, Cyclin E, Cyclin D2, and p27KIP1 [31, 107, 110]. Human stromal cells of adipose tissue (ADSC) and its conditioned culture medium can suppress tumor growth [107]. In addition, the cell culture medium conditioned by ADSC stimulated the necrosis of the cancer cells after the arrest of the G1 phase in the absence of

apoptosis. Finally, when ADSC was introduced in pancreatic adenocarcinoma, the tumor did not grow [107]. Similarly, tumor cells that were cultured with MSC in vitro were also stopped in the G1 phase [111]. However, when the nonobese diabetic-severe combined immunodeficient mice were injected with MSCs and tumor cells, their growth was more increased compared to the injection of tumor cells alone. Although it has been reported that MSCs can induce arrest of the cell cycle of tumor cells in vitro, little is known about the exact mechanisms. In our experiment, the delay or arrest of the cell cycle can be induced in certain types of tumor cells and under certain co-culture conditions (type of medium, cell concentration, or co-culture time). While we cannot explain the exact mechanism (s), several studies performed by different groups, including hours, have shown that the arrest of the tumor cell cycle occurs. It has been shown that MSCs derived from human bone marrow interfere in vitro with small cell lung cancer (A549), esophageal cancer (Eca-109), Kaposi's sarcoma, and proliferative kinetics of the leukemic cell line [112]. The above was not only observed when 0.5×10^5 tumor cells were cultured together with 0.5×10^5 MSCs derived from human bone marrow but also when exposed to medium conditioned by MSC; the cells were stopped during the G1 phase of the cell cycle in both cases by the negative regulation of Cyclin D2 and the induction of apoptosis [111]. MSCs from other sources, including MSCs derived from human fetal skin and MSCs derived from adipose tissue, have also inhibited the growth of human liver cancer cell lines [32], breast cancer (MCF-7) [111], and primary leukemic cells by reducing their proliferation, colony formation, and oncogene expression [30, 32]. Intravenous injection of 4×10^6 MSCs derived from human bone marrow in nude mice carrying Kaposi's sarcoma has inhibited the growth of tumor cells [32]. A similar effect has been observed in an animal model of hepatocellular carcinoma and pancreatic tumors, since the alteration of cell cycle progression has led to the decrease of cell proliferation [30, 31]; the same has happened with melanoma due to increased apoptosis of capillaries [34], and the growth of colon carcinoma in rats has been inhibited when rat EMFs (cell line MPC1cE) were co-mapped with tumor cells in a relationship 1:1 or 1:10 [33]. MSCs derived from human fetal skin (Z3 cell line) also delayed liver tumor growth and decreased tumor size when injected with the same number of cells from the H7402 cell line in SCID mice [36]. Injection of MSCs derived from human adipose tissue (1×10^3 cells/mm^3) into established pancreatic cancer xenografts has led to apoptosis and the abrogation of tumor growth in nude (nude) Swiss mice [31]. The role of MSCs in cancer remains paradoxical. Evidence to date has suggested that they are pro as well as antitumorigenic [113–115] and such discrepancy seems to depend on the isolation and expansion conditions, the source and dose of the cell, the route of administration, and the model tumor used.

2.9. MSCs and regulation of cellular signaling

The main signaling pathway involved in the control of cell survival is the pathway of phosphoinositide 3-kinase (PI3K)/AKT and WNT/beta-catenin. The activation of this pathway induces proliferation, growth, and migration, and increases cellular metabolism [116–118]. In the biology of a tumor cell, numerous studies have reported the requirement for the activation of the AKT-signaling cascade for the migration, invasion, and survival of tumor cells. Additionally, the WNT pathway has also been associated with the development of various types of carcinomas, including breast, liver, colon, skin, stomach, and ovary [119]. In a murine

model of Kaposi's sarcoma, Kakhoo et al. reported that MSCs injected intravenously were able to migrate to the tumor and inhibit tumor cell proliferation by inhibiting AKT [32]. On the other hand, they observed in glioma cells that PTEN was upregulated in the presence of HUCBSCs, with the consequent downregulation of AKT [109]. In addition to inhibiting the PI3K/AKT pathway, MSCs can also suppress the WNT/beta-catenin pathway through the induced expression of the pro-apoptotic protein DKK-1 [31, 36, 37]. These recent findings demonstrated that beta-catenin can be negatively regulated in different human carcinoma cell lines (hepatocellular, H7402 and HepG2, breast, MCF-7, hematopoietic, K562 and HL60) by the secretion of DKK-1 by the MSCs. On the other hand, when the activity of DKK-1 was inhibited by the use of anti-DKK-1 neutralizing antibodies or interfering RNA, the inhibitory effects of MSCs on tumor progression disappeared [31, 36, 37].

3. Conclusions

Although therapy with MSC in regenerative medicine is considered feasible and safe, the literature reported to date reveals dicrepancies respect to the MSCs impact in the tumor microenvironment. This paradoxical effect could be attributed to the differences in the experimental conditions of isolation and expansion, the source and dose of cells used, the route of administration and its timing, and the host characteristics. This chapter highlights the mechanisms of the effects of tumor support or suppression mediated by the MSCs and analyzes the possible mechanisms involved. MSCs demonstrate a tropism for tumors and once they interact with each other and with cancer cells, they promote tumor growth by: inmunosuppression; promotion of angiogenesis; epithelial-mesenchymal transition; inhibition of apoptosis; and promotion of metastasis. In contrast, many studies have reported that MSCs can prevent tumor growth by increasing leukocyte infiltration, inhibiting angiogenesis, suppressing Wnt and AKT signaling. Further investigations are necessary to establish the biosecurity of cell therapy in the presence of precancerous lesions.

Conflict of interest

The author discloses no potential conflicts of interest.

Notes/Thanks/Other declarations

Not applicable.

Appendices and nomenclature

NO	nitric oxide
PGE2	prostaglandin

IDO	indoleamine 2,3-dioxygenase
IL-6	interleukin-6
IL-10	interleukin-6
HLA-G	histocompatibility antigen, class I, G
PD-1/2	programmed death-1/2
PD-L1/2	programmed death-1/2 ligand
CD80	T-lymphocyte activation antigen CD80
CD86	T-lymphocyte activation antigen CD86
SDF-1	stromal derived factor-1
HGF	Hepatocyte Growth Factor
IGF-1	insulin dependent growth factor-1
EGF	epithelial growth factor
NGF	neurotrophic growth factor
TGF-α	transforming growth factor-alpha
VEGF	vascular endothelial growth factor
ECM	stromal extracellular matrix
MSCs	multipotent stromal mesenchymal stem cells
AKT	Serine-threonine kinase
PI3K	Phosphoinositide 3-kinase
Wnt	Wingless-Type MMTV Integration Site Family, Member 1
XIAP	X-linked inhibitor of apoptosis protein
EMT	epithelial–mesenchymal transition
PDGF	platelet derived growth factor
TGF-β	transforming growth factor-beta
ZEB1/2	zinc finger E-box binding homeobox 1/2
TWIST	twist related protein-1
SERPINE1	serpin family E member 1
MMP-2	metalloproteinase-2
SCID	severe combined immunodeficiency
CAFs	carcinoma-associated fibroblasts

TAFs	tumor-associated fibroblasts
α-SMA	α-smooth muscle actin
FSP	fibroblast surface protein
CCL5/RANTES	chemokine (C-C motif) ligand 5
DCs	dendritic cells
IgG	immunoglobulin G
NK	natural killer cells
TNF-α	tumor necrosis factor-alpha
IFN-γ	interferon-gamma
MHC-class I/II	major histocompatibility complex
Erα	estrogen receptor-alpha

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Chapter 5

Stromal-Epithelial Interactions during Mammary Gland Development

Żaneta Dzięgelewska and Małgorzata Gajewska

Additional information is available at the end of the chapter

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Abstract

Mammary gland is an organ, which undergoes the majority of its development in the postnatal life of mammals. The complex structure of the mammary gland comprises epithelial and myoepithelial cells forming the parenchymal tissue and adipocytes, fibroblasts, vascular endothelial cells, and infiltrating immune cell composing the stromal compartment. During puberty and in adulthood, circulating hormones released from the pituitary and ovaries regulate the rate of development and functional differentiation of the mammary epithelium. In addition, growing body of evidence shows that interactions between the stromal and parenchymal compartments of the mammary gland play a crucial role in mammogenesis. This regulation takes place on a paracrine level, by locally synthesized growth factors, adipokines, and cytokines, as well as via direct cell-cell interactions. This chapter summarizes the current knowledge about the complex nature of interactions between the mammary epithelium and stroma during mammary gland development in different mammalian species.

Keywords: mammary epithelial cells, mammogenesis, adipocytes, fibroblasts, immune cells, endothelial cells

1. Introduction

The origin of the mammary gland in the fossil record appeared about 220–300 million years ago in the Carboniferous geological period and was evolving for 130 million years to its current mammalian form [1]. In its earliest evolutionary form, the glandular structure ancestral to the mammary gland had functioned as a source of secretion that helped eggs withstand desiccation associated with incubation on land and appeared among tetrapods or among the

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basal amniotes-vertebrates. Comparison of mammary-expressed genes between mammalian taxa revealed the sheared presence and high degree of conservation of the genes. Mammary gland fully developed prior to emergence of diverse groups of mammals, and the milk compounds (fat globules, whey proteins, casein micelles, and sugars) are structurally similar across all mammalian species [2].

In contrast to most organs that achieve morphological maturity during prenatal development in the process defined as morphogenesis, the majority of mammary gland development leading to its complex morphological maturity occurs mostly during postnatal life of mammals [3]. During embryogenesis, the mammary gland development is driven mostly by mesenchymal cells. In postnatal life, subsequent stages of glandular development: mammogenesis (development of mammary epithelial tissue), lactogenesis (functional differentiation of the mammary epithelium leading to initiation of milk secretion), galactopoiesis (maintenance of milk secretion), and involution (regression of the glandular epithelium), take place under significant regulation of hormones. In parallel, the intraglandular milieu plays also an important role in controlling the progress of events related to mammary gland morphogenesis.

2. Stages of mammary gland development

At the embryonic period, the mammary gland is derived from ectoderm cell migration, followed by the formation of disk-shaped placodes. The mammary buds arise as a result of proliferation of the basal cells of the ventral epidermis due to factors secreted by mesenchymal cells present in the mammary bud in a process referred to as branching morphogenesis [4–6]. The mesenchyme is instructive and provides critical information to drive mammary gland development. Two different mesenchymal tissues with different properties are involved in this and, with other cells, become a part of stroma compartment. First type of mammary mesenchyme, termed the fibroblastic mesenchyme, is composed of fibroblastic cells surrounding the epithelial rudiment and the second comprise the fat pad cells, thus is known as the fat pad mesenchyme. A solid cord of epithelial cells extends from the mammary bud and grows through the fibroblastic mesenchymal tissue into the fat pad precursor mesenchyme, which at this stage is a small collection of preadipocytes. In rodents, a single epithelial sprout reaches the fat pad and begins to branch by equal division of the terminal bud. The terminal end buds (TEBs) are created as an outer layer of cap epithelial cells surrounding multilayered body epithelial cells located at the front of the branch that invades into the mammary mesenchyme. The body epithelial cells give rise to mammary epithelial cells and the cap cells are myoepithelial precursors. TEBs move forward through mesenchymal cells leading to formation of a rudimentary ductal system. In rodents, it is composed of 10–15 branches that are generated without hormonal input, and the rudimentary ducts remain largely quiescent until puberty [6, 7]. In humans, several sprouts form, creating multiple mammary trees that unite at the nipple, whereas in ruminants the rudimentary ductal network is connected to a small cisternal cavity that connects to the teat cistern and ultimately communicates with the teat meatus [6–8].

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After birth, in the postnatal life until puberty, the gland remains quiescent and exhibits only minimal ductal growth. Interspecies differences occur in the extent of mammary gland development that occurs in neonates. In mice, the mammary tree consists of long, infrequently branching ducts and TEBs. Human mammary gland has a more complex structure composed of approximately 15–20 lobes of glandular tissue, each containing a lactiferous duct that opens onto the breast surface through the mammary pit [9]. In the case of ruminants, the mammary gland consists of terminal ductal units (TDU), which are formed during prenatal development accomplished through the coordinated growth, branching and extension of TDU, as well as growth of the loose connective tissue that surrounds the TDU as it invades the mammary fat pad [8].

With the onset of puberty, a combination of systemic and paracrine hormones induces TEBs to reappear at the ductal tips accompanied by a significant increase in the growth rate. Elongation and branching of the ducts, regulated by proliferation and migration of TEBs cells, rely on both endocrine and local growth regulatory signals, extracellular matrix (ECM) remodeling, and stromal influence. With the beginning of puberty, the epithelium bifurcates and invades into the surrounding stroma creating a tree-like structure of mammary ducts. The majority of mammary ductal morphogenesis occurs with onset of ovarian function because of the cyclic influence of reproductive hormones. Further, with each estrus cycle, the alveoli and ducts undergo cyclic expansion and maturation, followed by a modest regression phase as ovarian hormone levels rise and fall, respectively. These events are under the control of a complex interplay of circulating essential steroids (estrogen and progesterone), polypeptide systemic hormones (e.g., prolactin), metabolic hormones that are responsible for coordinating the body's response to metabolic homeostasis (e.g., growth hormone—GH, glucocorticoids, insulin, leptin), as well as locally acting paracrine hormones and growth factors (e.g., insulin-like growth factor I—IGF-I, hepatocyte growth hormone—HGF, transforming growth factor-β—TGF-β, epidermal growth factor—EGF) [10]. It is worth noting that the hormone acting network regulating the development of the mammary epithelium varies between different species.

The mammary gland is able to undergo its terminal differentiation only in female mammals during pregnancy and lactation. With the onset of gestation period and increased levels of progesterone, alveolar structures give rise to lobuloalveolar structures capable of milk production during lactation. After weaning of the offspring (or termination of milking), the gland undergoes post-lactating regression referred as involution, with loss of most of epithelial components gained during the preceding event. Early involution is evidenced by apoptotic death of alveolar secretory epithelial cells which subsequently are removed by efferocytosis (the process of engulfing and destroying apoptotic cells) [11]. Second phase of the mammary gland involution is defined by degradation of basement membrane and ECM proteins and reduction of lobuloalveolar structures.

3. Structure of fully developed mammary gland

Fully developed mammary gland is created by two compartments: epithelial and stromal. The epithelial compartment, termed parenchyma, is composed of the branching network of ducts

and lobuloalveolar structures comprised of mammary epithelial cells of two primary lineages: myoepithelial (basal) cells and epithelial (luminal) cells, forming a bilayered structure, which is embedded in the stroma [12]. Mammary ducts consist of apically orientated luminal epithelial cells that line ducts with alveolar structures at the ends and of basally orientated myoepithelial cells surrounded by a laminin and collagen-rich basement membrane (BM). Luminal epithelial cells are separated from all kinds of stromal cells, laying on top of myoepithelial cells. The functionally distinct basal layer contains myoepithelial cells with contractile properties and cells with demonstrated stem cell activity, referred as mammary repopulating units (MRUs). These cells have an ability to regenerate the bilayered glandular structure of inner luminal and basal outer epithelial cells [12]. The myoepithelial and stromal cells produce the basement membrane, which is a thin sheet composed of collagen IV, laminins, entactin, and proteoglycans, and forms physical barrier separating the epithelial and stromal compartments [3]. The stromal compartment is composed of two mesenchymal lineages: adipocytes and fibroblasts, as well as infiltrating immune and vascular endothelial cells [5]. These cells synthesize extracellular matrix (ECM) components essential for three-dimensional microstructure of the stroma. Stromal ECM components include collagens, which are the major structural proteins, as well as proteoglycans, hyaluronic acid, fibronectins, and tenascins [13, 14] (**Figure 1**).

Stromal-epithelial interactions regulate mammary epithelial growth and differentiation during embryonic and postnatal development through soluble factors that are released into the environment, as well as through insoluble factors that are present in the stroma itself, referred as matrikines and matricryptins [14]. The stroma accounts for roughly 60% of the total tissue

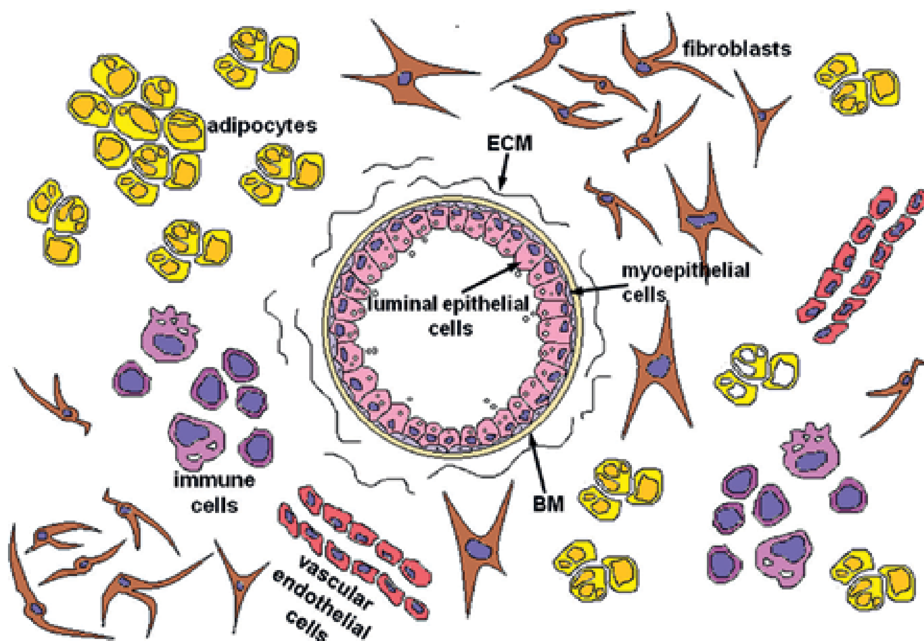

Figure 1. Schematic representation of cells found within the structure of fully developed mammary gland. Scheme presents cross section of mammary alveolus surrounded by stromal components (cells and extracellular matrix).

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volume and exerts a dominant effect on tissue morphogenesis. Ratio between stromal and epithelial compartment changes at all stages of mammary gland development, still staying in its own harmony milieu. Stromal cells architecturally support the epithelium, providing structure, nutrients, blood, and immune defense. Large amount of data suggest that the mammary stroma not only provides a scaffold but also regulates mammary epithelial cells (MECs) function via paracrine, physical, and reciprocal signaling between MECs and underlying stromal cells, modifying proliferation, survival, polarity, differentiation, and invasive capacity of the mammary epithelium [4, 15]. The importance of stromal cells is reflected by the fact that signals emitted by embryonic mesenchyme dictate the differentiation of epithelial cells, and mammary epithelial cells form salivary gland-like structures when placed on top of salivary gland mesenchyme [16]. On the other hand, outgrowth of salivary epithelium in contact with mammary mesenchyme resembles a mammary gland ductal tree and responds to hormonal stimuli [16]. The following paragraphs of this chapter present the complex interactions between the mammary epithelium and different stromal cells that direct the progression of normal mammary gland morphogenesis.

4. Role of stromal cells in regulation of mammary gland development

4.1. Adipocytes

Adipocytes constitute the most abundant type of cells within the stroma of the mammary gland. Fat cells predominate in the stromal compartment of the mammary glands of rodents (mice and rats), whereas in the mammary glands of humans and ruminants adipocytes of white adipose tissue form the structure of a fibrous-adipose stroma along with fibroblasts. Adipocytes create a specific microenvironmental niche for MECs as the source of triglycerides and thus a source of energy, as well as a scaffold liable to invade, and a supply of various biologically active compounds.

Adipose tissue modulates epithelial development, remodeling, and function in a state-dependent manner. During embryonic morphogenesis, the fat pad together with the fibroblastic mesenchyme appears before ectoderm cell migration, creating environment and scaffold for mammary buds development. At this stage, each type of mesenchymal cells has different properties. It has been shown that fat pad mesenchyme induces elongation and branching of the mammary epithelium [5]. Lack of white adipose tissue in transgenic Z-ZIP/F1 female mice leads to compromised ductal growth during prenatal development, manifested by formation of only few underdeveloped ductal structures showing severe, abnormal distension [17]. Interestingly, these transgenic Z-ZIP/F1 mice produce a mass of lobuloalveolar structures in the mammary gland during pregnancy, which suggests that interactions between MECs and adipocytes are not essential for the functional differentiation of the mammary epithelium [17]. An alternative in vivo model of adipocytes depletion (FAT-ATTAC mice) allowed scientists to explore further the role of mammary-associated adipocytes. In FAT-ATTAC mice, elimination of adipocytes can be induced at any developmental

stage through induction of apoptotic cell death by administration of a FK1012 analog, which leads to the forced dimerization of a caspase-8 fusion protein uniquely expressed in adipose tissue [18]. This model allows for selective ablation of mammary adipocytes in female mice without affecting other fat pads. Under these conditions, Landskroner-Eiger and co-workers [18] demonstrated that the presence of adipocytes is necessary for proper formation of the extended ductal network in the mammary gland during puberty as well as for the maintenance of the normal alveolar structures that develop during adulthood. Ablation of adipocytes in mice starting from 2 weeks of age resulted in reduced ductal growth. Alterations in ductal features were caused by the loss of mechanical and physical support provided by adipocytes. However, when the loss of local adipocytes was initiated at 7 weeks of age in FAT-ATTAC mice model, an excessive lobulation was observed in the mammary gland. These observations indicate that adipocytes are critically involved in maintaining proper architecture and functionality of the mammary epithelium [18]. The important role of adipocytes in normal morphogenesis of the mammary epithelium was further confirmed in in vitro studies. MCF-10A human mammary epithelial cells co-cultured with human adipose-derived stem cells (hASCs) in Matrigel/collagen gels spread on silk scaffolds were able to create both alveolar- and duct-like structures. In contrast, monoculture of MCF-10A resulted in formation of only alveolar structures [19]. Consistently, EpH4 murine mammary epithelial cells cultured within adipose-rich collagen I formed branched mammary epithelial tubules within 24 h of culture [20]. It should be noted that the mammary-associated adipocytes also undergo massive morphological changes between the periods of lactation and involution. During lactation, adipose tissue serves as a major lipid store utilized as a source of energy for milk production. That is why in lactating mammary gland fat cells undergo lipid depletion and appear as long projections. At the time of involution, when milk synthesis ceases and mammary epithelium regresses, adipocytes regain their lipid stores, but some adipocytes undergo dedifferentiation into preadipocytes or are eliminated via apoptotic cell death [21].

4.1.1. Adipokines

Beyond the function of adipocytes as the energy storage depot, currently it is well accepted that these cells are actively producing and secreting a wide range of endocrine factors referred to as adipokines. Adipokines are signaling molecules that regulate various physiological processes in the body. In the context of the mammary gland, adipokines are thought to regulate normal development of this organ [22]. This group of compounds is also locally synthesized by adipocytes of the mammary stroma and act through juxtacrine or paracrine signals modulating epithelial cells proliferation. In vitro studies on normal human MECs (NMuMG cell line) elegantly demonstrated the effect of signaling molecules secreted by adipocytes. NMuMG cells were incubated for 24 or 48 h in the presence of conditioned medium derived from adipocytes (3T3-L1 cell line) at various degrees of differentiation: preadipocytes (preA), poorly differentiated adipocytes (pDA), and mature adipocytes (MA) [23]. After 24 h treatment human MECs showed significantly increased proliferative activity when cultured in conditioned media from pDA and MA, whereas after 48 h incubation the effect of increase proliferation was observed in the case of all conditioned media (preA, pDA, and MA) [23]. Another study revealed that 24 h treatment with conditioned medium from mature adipocytes

induced branching morphogenesis of mammary tubules, with sites localized to the ends of the tubules, without appreciable lumen formation, which indicates that the biologically active molecules produced by adipocytes influence mostly the ductal growth [20].

Adipokines detected in the mammary gland include hormones (leptin and adiponectin), growth factors (HGF, IGF), cytokines (interleukin 6—IL 6, tumor necrosis factor alpha—TNFα), as well as ECM components (collagen VI). It has been proven that HGF is especially important stimulator of branching morphogenesis [20]. HGF secreted by human pre-differentiated hASCs affected the duct-like structure formation by mammary epithelial cells (MCF10A) in co-culture [19]. Moreover, systemic hormones (prolactin, GH) not only exert their action directly in epithelial cells but also can act indirectly via the stromal compartment of the mammary gland. Studies have shown that GH stimulates the mammary gland adipocytes to produce IGF-I [17]. It is also evident that pubertal branching morphogenesis in vivo is stimulated by steroid hormones, including estrogen, which act on receptors located in the stroma to induce production of mitogens including HGF [20].

Leptin and adiponectin are the most extensively studied hormones synthesized by adipose tissue. They are found in higher concentrations in the mammary tissue than in blood and thus may be a part of an important paracrine or juxtacrine signaling system between adipose-rich stroma and epithelial cells [24]. Leptin, which was the first known adipokine discovered by Friedman and Coleman in 1994, is a 16 kDa nonglycosylated protein encoded by the *Ob* gene. This protein hormone is secreted mainly by adipose tissue to regulate body energy balance, suppressing food intake and thereby inducing weight loss. In the context of mammary gland physiology leptin actions are associated with regulation of the metabolic changes occurring during pregnancy and lactation, due to the fact that it is the key hormone regulating the metabolic adaptation of nutrient partitioning during the energy consuming processes [25]. MECs express leptin receptors (OB-Rb) and therefore may undergo direct regulation by leptin, whereas local production of leptin by mammary adipose tissue is under control of several hormones: insulin, glucocorticoids, and prolactin. Prolactin, the main lactogenic factor, was shown to regulate leptin and leptin receptor gene expression in the bovine mammary gland [26]. It is believed that prolactin may be the key signaling factor stimulating the mammary gland to interact with leptin in the regulation of milk synthesis during lactation [27]. In the presence of prolactin, leptin was shown to enhance the expression of α-casein gene (milk protein gene) in bovine mammary gland, indicating that leptin and prolactin interact to alter milk synthesis during lactation [27]. Estradiol, which is known to regulate ductal morphogenesis in the mammary gland, also plays an important role in the regulation of the extracellular levels of leptin, as well as adiponectin in normal human mammary gland [28].

In contrast to leptin, circulating levels of adiponectin are inversely correlated with the body mass index (BMI). Adiponectin is a 240 amino acid protein of approximately 28–30 kDa existing as a monomer, although it forms dimmers and multimers, circulating as low, medium, and high molecular weight isoforms. Two types of receptors, adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2), have distinct distribution patterns in different tissues. Both receptors were shown to be expressed in normal mammary epithelial cells [29, 30]. Binding of adiponectin to its receptor activates adenosine monophosphate-activated protein

kinase (AMPK), a nutrient-sensing enzyme, which regulates several key pathways involved in protein synthesis and cellular energy metabolism. One of the few researches on bovine mammary gland disclosed that adiponectin expression in the mammary gland decreases in the peak and late-lactation period, although adiponectin receptor 1 (AdipoR1) expression increases in the same period [30]. Moreover, leptin/adiponectin ratio is directly proportional to the size of stem cell population in vivo. It was evidenced that leptin alone is sufficient to stimulate mammary stem cell self-renewal, leading to significant increase in the stem cell population. In contrast, unopposed adiponectin decreases the size of the mammary stem cell pool in vitro. It is believed that leptin and adiponectin may function as both endocrine and paracrine/juxtacrine factors to modulate the size of the normal stem cell pool [24].

Recent studies have shown chemerin as a novel adipokine, which may actively take part in regulation of the mammary gland lactogenesis. Chemerin, also called retinoic acid receptor responder protein 2 (RARRES2), is a 16 kDa chemoattractant cytokine (chemokine) mainly expressed in and secreted from white adipose tissue. Chemerin is secreted as a 143-amino acid inactive precursor, pro-chemerin, and is activated by proteolytic removal of six to seven amino acids from its C-terminus by proteases such as elastase or cathepsin G. Three G protein-coupled receptors are able to bind chemerin with high affinity, namely chemokine receptor-like 1 (CMKLR1), G protein-coupled receptor 1 (GPR1), and C-C chemokine receptor-like 2 (CCRL2). Chemerin inhibits cAMP production and promotes phospholipase C activation, IP3 release, calcium mobilization as well as activation of PI3K and MAPK pathways [31]. In bovine mammary gland, the expression of chemerin was greater in adipose tissue of postpartum dairy cows versus pregnant cows, and two out of three chemerin receptors (CMKLR1 and CCRL2) were expressed in bovine MECs [31]. Studies with immortalized bovine MECs treated with chemerin revealed upregulated expression of genes associated with fatty acid synthesis, glucose uptake, and casein synthesis; thus, it is postulated that chemerin may play a role of lactogenesis regulator in bovine mammary epithelium. Surprisingly, adiponectin reduced the expression of CMKLR1 receptor, without altering CCRL2 expression [30]. These results imply that adiponectin is not only able to counteract the effects of leptin but also able to regulate the influence of chemerin on mammary epithelial cells.

4.1.2. Other adipocyte-related molecular regulators of mammogenesis

Adipocytes of the mammary stroma also express retinoids (RARs), which are potent transcription regulators [32]. Co-cultures of primary adipocytes, or in vitro differentiated adipocyte cell line, with mammary epithelium showed that when activated, adipocyte-RARs contribute to generation of secreted proliferative and pro-migratory factors affecting branching morphogenesis [33]. RARs expressed by adipocytes were shown to be important regulators of secreted growth factor—pleiotrophin (PTN), involved in paracrine regulation of epithelial ductal tree development [33]. Adipocyte-RARs induced parathyroid hormone receptor (PTHR) expression leading to increased expression of PTN, which in turn regulated mammary epithelial migration.

Adipocytes also express vitamin D receptor (VDR), which is expressed in both epithelial and stromal compartment of the mammary gland and is known to participate in regulation of hormone-induced growth and differentiation throughout development [34]. VDR complexes with the active ligand, 1a,25-dihydroxyvitamin D3 (1,25D3), to induce cell cycle arrest,

differentiation, and apoptosis in human MECs, regulating growth in normal and transformed cells [34–36]. The ability of human MECs to synthesize 1,25D3 locally within the mammary epithelium to regulate cellular growth and differentiation may constitute a potential mechanism by which elevated serum 25D3 is associated with a decreased risk of developing breast cancer or metastatic progression [37]. Ching and co-workers [38] investigated the hypothesis that adipocytes from the mammary stroma express the signaling components necessary to participate in vitamin D3 synthesis and act via VDR, potentially modulating ductal epithelial cell growth and differentiation. Mammary adipocytes expressing VDR were shown to participate in bioactivating 25-hydroxyvitamin D3 (25D3) to the active ligand, 1,25D3, and secrete it to the surrounding microenvironment. Active vitamin D3 in turn was able to inhibit the ductal epithelial cell growth [38]. Similar results were obtained by Matthews and co-workers, who used a different animal model in their studies [39]. This group generated CVF transgenic mice with adipose-specific *Vdr* gene deletion and noted that adipose deletion of *Vdr* significantly enhanced mammary epithelial density and branching, supporting the hypothesis that vitamin D receptor in mature adipocytes exerts anti-proliferative effects on the mammary epithelium [39].

4.1.3. Influence of mammary epithelium on stromal adipocytes

In terms of investigating interactions between epithelial and stromal compartments of the mammary gland, it is important to expand our knowledge about reciprocal cell-cell interactions within the gland. There are still relatively few studies focused on the influence of MECs on the adipocytes population. A vivid example is an in vitro model of three-dimensional (3D) collagen gels containing differentiated adipocytes, which were used to investigate the mutual interactions between adipocytes and MECs during branching morphogenesis [20]. In this research, 3T3-L1 mouse preadipocytes were embedded in collagen, differentiated, and then treated with MECs-derived conditioned medium. Samples treated with conditioned media formed fewer and smaller fatty clusters and showed lower expression of lipoprotein lipase (LPL) and adipogenic transcription factor PPARγ2. These data suggest that MECs either inhibited or delayed differentiation of the preadipocytes [20]. In vivo, during embryonic mammary gland development, the fat pad is present before the epithelium invades, and epithelial compartment invades the stroma causing its reduction [20]. Similar conclusion was made by investigators who demonstrated that MECs produce the enzyme galactose 3-*O*-sulfotransferase 2 (GAL3STS2), which was able to inhibit the expression of adipogenic transcription factor C/EBPb and fatty acid-binding protein 4 (FABP4)—a marker of adipocytes differentiation [40]. In addition, accumulation of triglycerides was also inhibited under the influence of GAL3STS2. The authors postulate that GAL3ST2 may generate multiple signals related to integrin activation, including its effect on preadipocyte differentiation [40]. Taken together, it seems that epithelial compartment reduces the adipose tissue during mammary gland morphogenesis and works as negative feedback creating an appropriate/ favorable microenvironment for itself.

4.1.4. Summary

Stromal adipocytes play a profound role in regulation of mammogenesis during both embryonic and postnatal development of the mammary gland. These cells are necessary for proper ductal elongation and branching and are critically involved in maintaining proper architecture and

function of the mammary epithelium. This effect is exerted through direct cell-cell contact with the mammary epithelial cells as well as through paracrine signals induced by secreted adipokines. This group of biologically active molecules includes HGF supporting ductal morphogenesis, leptin and adiponectin that may modulate the size of the mammary stem cell pool within the glandular tissue, as well as chemerin, which may be a novel, local regulator of lactogenesis, as it is involved in regulation of fatty acids and milk protein synthesis and glucose uptake (**Figure 2**).

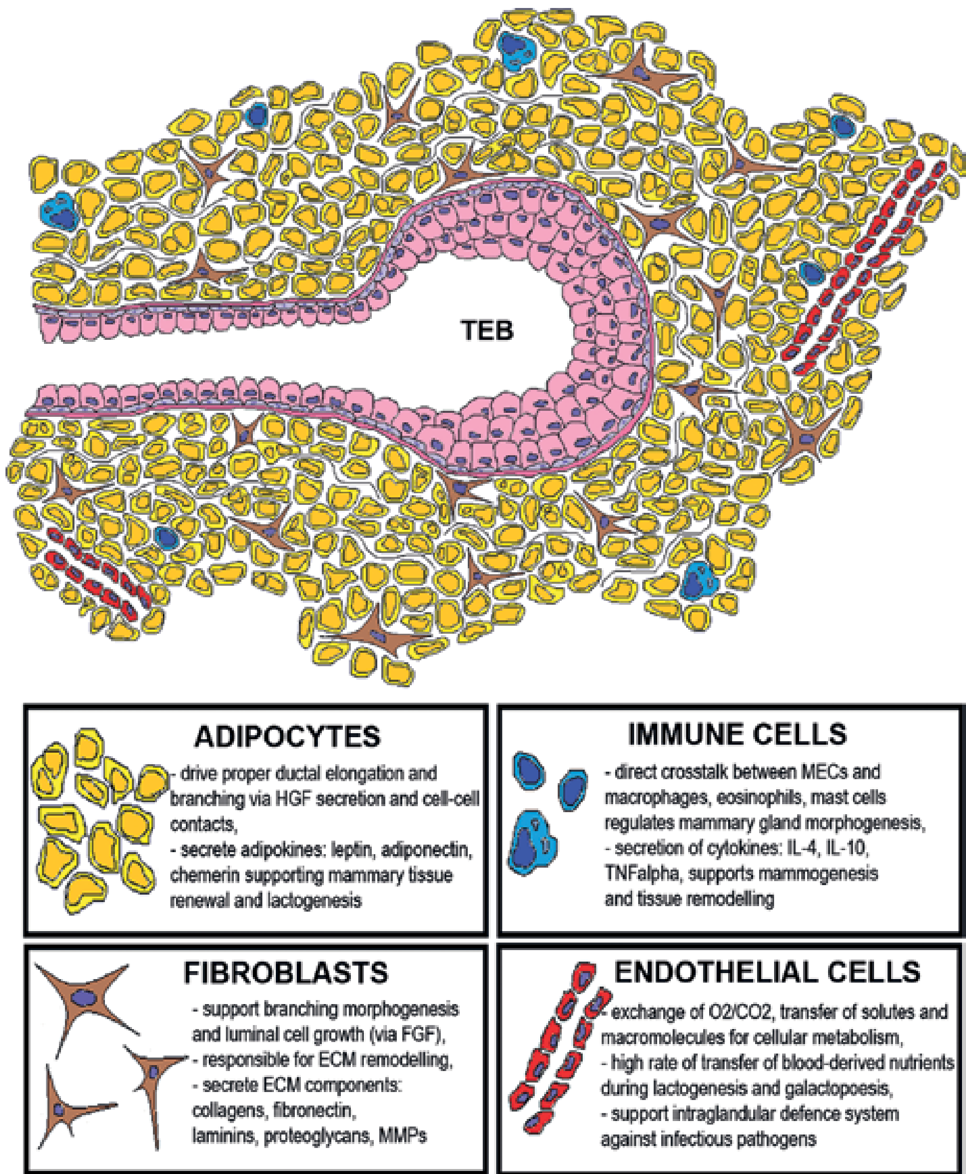

Figure 2. Schematic representation of different types of interactions between mammary epithelial cells [forming terminal end bud (TEB)] and stromal cells during mammogenesis.

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4.2. Fibroblasts

Fibroblasts, together with adipocytes, are the major cellular components of the mammary stroma and play an integral role in regulating mammary gland development. As mentioned previously, during prenatal period of mammogenesis, the fat pad and fibroblastic mesenchyme appear before ectoderm cell migration, creating the environment and scaffold for emerging mammary buds [4]. Fibroblastic cells of the mesenchyme are in direct contact with the developing epithelial rudiment, and their signals first determine the identity of MECs [41]. In parallel, the epithelium also influences mesenchymal maturation. Research done on murine model of mammary gland morphogenesis revealed that by day 14 of mouse embryonic development the mammary mesenchyme condenses to form a few layers of fibroblast-rich cells closely surrounding the epithelial rudiment, and it is distinct from the fat pad precorsor tissue, which develops from more deeply located subcutaneous mesenchymal cells [41].

Moreover, it has been shown that more than one phenotype of normal fibroblasts can be distinguished within the stromal compartment of the mammary gland, and each has the potential for various epigenetic effects on normal epithelial cells depending on their proximity to the parenchyma [42]. Intralobular fibroblasts can be distinguished from interlobular fibroblast as they differ in the expression patterns of several proteins such as collagen type XIV and CD13 [43]. Morsing and co-workers conducted a study using fluorescence-activated cell sorting analysis, by which they were able to isolate and characterize two lineages of stromal fibroblasts from human mammary gland, and showed their different impact on the mammary epithelium [44]. Lobular fibroblasts were characterized by high expression of a surface glycoprotein CD105 (which is a part of the TGF beta receptor complex) and low expression of CD26 surface marker, also known as dipeptidyl peptidase-4. In terms of biological properties, $CD105^{high}/CD26^{low}$ lobular fibroblasts resembled mesenchymal stem cells and supported luminal epithelial growth and branching morphogenesis. On the other hand, the second identified fibroblastic cells subpopulation, termed interlobular fibroblasts, showed low expression of CD105 and high expression of CD26 and did not exert such impact on the branching morphogenesis of epithelial progenitors [44]. It has been suggested that the interstitial stroma serves mainly to form a barrier between capillaries and epithelium, across which epitheliotropic stimuli from the blood supply must pass [44].

It is worth noting that contrary to the overall structure of the mammary parenchyma, which is similar among mammalian species being composed of bilayered luminal and basal epithelial cells, the relative abundance of connective tissue is more species-specific. Stroma surrounding the lobules and ducts (intra and interlobular stroma) in mice is sparse, and there is little non-cellular fibrous connective tissue between ducts, whereas the white adipose tissue is abundant. In humans, the ratio of fibrous connective tissue to adipose is opposite, with an abundance of stroma surrounding the alveoli and ducts, predominance of fibrous connective tissue between ducts, and reduced adipose content [14]. Interestingly, in the case of outbred Sprague Dawley female rats, the organization of the mammary stroma is intermediate between mice and humans, and it is thought that its histological pattern is more similar to the one observed in humans than mice. The mammary stroma in cattle is also more fibrous

and contains less adipose tissue than the fatty mouse mammary stroma. The morphology of the bovine mammary gland resembles that of the human breast, because the mammary epithelium is generally closely associated with fibrous connective tissue, which in this case is extensively developed [45].

4.2.1. Fibroblast-mammary epithelial cell interactions during mammogenesis

The composition of the mammary stroma largely determines the progression of glandular epithelium development. Attempts to recapitulate human breast epithelial morphogenesis by introducing human MECs into the cleared mammary fat pads of mice were unsuccessful for a long time, due to improper composition of murine stroma comprising mainly adipocytes. Kuperwasser and co-workers used a different approach, creating a model of humanized mouse mammary gland by injecting immortalized human mammary stromal fibroblasts labeled with green fluorescence protein (GFP) into the cleared mice mammary fat pad prior to injection of human breast organoids. Addition of human fibroblasts to the murine fat pad effectively stimulated human MECs proliferation and promoted organization of differentiated acini structures [46]. This experiment pointed to tight stromal-epithelial species affinity [46]. A follow-up study was made, in which human macrophages were also injected. This procedure intensified humanization of the murine fat pad by enhancing fibroblast proliferation and engraftment of the mammary fat pad, thereby providing a larger stromal scaffold for breast epithelial growth and acini formation [47].

4.2.2. Paracrine factors produced by stromal fibroblasts

Stromal fibroblasts play a significant role in the development of the mammary gland, not only by creating a complex scaffolding network but also being a source of bioactive compounds. Fibroblasts may also take part in transmission and modulation of signals from superior hypothalamic-pituitary-gonadal axis (HPG). During puberty, mammary fibroblasts surrounding the branching TEBs become activated in response to estrogen and GH released by the ovaries and pituitary gland, respectively [48]. Stromal fibroblasts express growth hormone receptor (GHR) and through secretion of IGF-I may modulate epithelial compartment growth especially in pubertal state [6].

In general, fibroblasts exist in a relatively quiescent state, proliferating slowly and synthesizing only low levels of ECM proteins and matrix metalloproteinases (MMPs) to maintain ECM integrity [48]. During branching morphogenesis, fibroblasts actively synthesize growth factors and proteases. For example, signaling pathways induced by fibroblast growth factors (FGFs) play a major role in the process of mammary placode development [49]. FGFs family contains 18 secreted proteins that can interact with four FGF receptors (FGFRs) having tyrosine kinase activity. These secreted FGFs function as auto- or paracrine factors, but some also show an endocrine function. In addition, there are intracellular FGFs (iFGFs), which are non-signaling proteins serving as cofactors for voltage-gated sodium channels and other molecules [50]. Interaction of FGF ligands with their receptors is regulated by protein or proteoglycan cofactors and by extracellular-binding proteins. The first line of evidence confirming the role of FGF signaling in embryonic stage of mammogenesis came after it was demonstrated that mice

lacking either FGF10 or FGFR2b fail to form mammary placodes 1, 2, 3, and 5 [51]. In mouse embryos lacking *Fgf10* gene, an epithelial sprout derived from placode 4 failed to branch, which completely inhibited the formation of a primitive epithelial network in the neonatal mice after birth [51]. In humans, a birth defect known as Poland syndrome, which is characterized by the underdevelopment of the somite-derived pectoral muscle on one side of the body and a corresponding hypoplasia of the overlying breast on the same side, arises from disruption in FGF10 signaling, because *Fgf10*+/− glands show reduced thickening of the ectoderm along the mammary line and subsequent loss of buds 3 and 5 [6]. Furthermore, secreted FGFs are known to stimulate TEBs promoting luminal epithelial cell expansion, ductal branching, and their differentiation into myoepithelial cells. The majority of FGFs is involved in branching process and involution, both of which require ECM rearrangement. In the case of pregnancy, signals through FGFR2-IIIb are essential to stimulate normal lobuloalveolar development [48]. Recent studies revealed that *Spry2* gene, which encodes an inhibitor of signaling via receptor tyrosine kinases, is essential for regulation of both FGF2-based ductal elongation and FGF10-mediated epithelial invasion during normal mammary gland development. For example, loss of *Spry*2 expression results in increased FGF signaling activities, causing more rapid ductal elongation and epithelial invasion, which leads to accelerated epithelial invasion during pubertal branching. Conversely, a decrease of FGF signaling leads to slower than normal ductal elongation and invasion, resulting in stunted epithelial invasion during postnatal branching of the mammary gland [52]. It was also revealed that basal epithelial cells lacking *Fgfr2* gene did not generate an epithelial network due to failure in luminal differentiation, and *Fgfr2*−/− epithelium was unable to undergo ductal branching initiation, which depends on directional epithelial migration [53]. The results of the abovementioned studies demonstrated that distinct types of FGFs stimulate epithelial cells on different levels. FGF2 controls the ductal elongation process, which depends on cell proliferation and expansion, while FGF10 regulates the branch initiation process depended on directional epithelial migration.

Other fibroblast-derived bioactive compounds like TGF-β1, HGF, or stroma cell-derived factor-1 (SDF-1) also known as CXCL12, were shown to influence mammary parenchyma development in a paracrine manner [54, 55]. HGF is a multi-functional cytokine stimulating invasion, motility, and morphogenesis. Its presence was found in conditioned media from human mammary fibroblasts [56, 57]. Fibroblast-derived conditioned media containing HGF were shown to induce tubulogenesis and branching morphogenesis of TAC-2 mouse mammary epithelial cell line [20]. In addition, it is well documented that fibroblastic HGF mediates the proliferation of estrogen receptor positive (ER+) mammary epithelial cells [43]. HGF was identified as one of the major mediators of this effect, because in in vitro experiments the proliferative activity of MECs cultured in fibroblast-derived conditioned medium was completely abolished by a neutralizing antibody against HGF [41].

Another important growth factor—TGF-β1, secreted by the mammary stroma, acts in an auto/paracrine manner to regulate glandular morphogenesis and remodeling by preventing inappropriate side branching. The presence of TGF-β1 was detected in mature periductal ECM in mice, and it was specifically downregulated at sites where side branches were being initiated [58]. Furthermore, TGF-β1 plays an important role in regulation of growth and activity of fibroblasts. This growth factor functions by signaling to cell surface type II receptors, which recruit

type I receptors, resulting in activation of downstream signaling cascades, including canonical Smad pathways that modulate gene transcription [59]. TGF-β signaling in fibroblasts functions to modulate expression of tissue remodeling factors, including ECM proteins, proteases, and angiogenic factors. During lactation, the expression of TGF-β1 is significantly downregulated, which may prevent TGF-β1 from negatively regulating the expression of milk proteins. Upon the onset of involution, when the gland remodels toward its pre-pregnant state, there is an upregulation of TGF-β1 transcripts. TGF-β1 signaling may further contribute to the remodeling of the involuting gland by inducing ECM production, upregulating MMPs expression, and by recruiting immune cells [14, 19]. Recent studies revealed that TGF-β1 promotes mammary fibroblast proliferation and may cause severe side effect in mammary gland structure and function in dairy cows [60]. TGF-β1 not only affects the development of the epithelial compartment by inhibiting formation and differentiation of mammary ducts and induction of apoptosis. Treatment of bovine mammary fibroblasts with TGF-β1 significantly promoted their proliferation and accelerated the cell cycle. Further research using a mouse model showed that TGF-β1 significantly increased the proportion of fibroblasts and accelerated the cell transition from the G1 to G2/M phases. Thus, TGF-β1 is a cytokine which may also cause negative effect in the mammary gland by contributing to the development of mammary gland fibrosis [60].

4.2.3. Fibroblast-derived extracellular matrix components

As mentioned earlier, fibroblasts together with other stromal cells synthesize the main amount of ECM components, such as collagens (collagen I, III, and V), proteoglycans, elastin, integrins, and fibronectin; thus, these stromal cells are responsible for mammary tissue architecture and stiffness [5, 48]. ECM can be described as an interconnected meshwork of secreted proteins interacting with cells to form a functional unit [14]. Additionally, mammary gland fibroblasts synthesize many matrix metalloproteinases (MMPs), like MMP2, MMP3, MMP14, that are able to remodel the ECM and release growth factors and cytokines harbored or embedded within the ECM [19]. MMPs consist of a family of over 20 zinc-dependent proteinases synthesized as latent enzymes, in a zymogen form, activated post-translationally and regulated by endogenous inhibitors referred to as tissue inhibitors of metalloproteinases (TIMPs) [5, 56, 57]. MMPs are secreted by stromal cells, but MMP2 and MPP3 exclusively by fibroblasts [61]. MMPs are important for ECM remodeling as well as for the microenvironmental signaling necessary to carry out morphogenic programs within the mammary gland [5]. Increased level of the active MMP3 leads to excessive side branching, and advanced alveolar morphogenesis but as a side effect is responsible for causing production of reactive oxygen species (ROS) leading to genomic instability [5]. MMP3, described also as stromelysin 1 (Str1), is expressed by mammary fibroblasts in vivo at elevated levels in the glands of virgin animals during ductal elongation. The highest level of MMP3 is found around the end buds and rear branch points, where mammary epithelial cells display the highest mitotic activity [57]. Overexpression of another matrix metalloproteinase—MMP14 in the mammary gland was demonstrated to cause excessive side branching and advanced alveolar morphogenesis [56]. The hemopexin domain of MMP14 is important for sorting mammary epithelial cells to points of branching. It has also been shown that only the short intracellular domain of MMP14, which does not contain kinase activity, is needed to resource branching morphogenesis in MMP14-deficient

cells [5]. MMP14 intracellular domain interacts with β1-integrin on the basal surface of cells, and this interaction is required for transducing the extracellular signals needed for epithelial cells to invade [5].

The role of fibroblasts should also be described in the context of the mammary gland remodeling observed extensively during post-lactating involution. Mammary involution is analogous to a wound healing response, involving complex epithelial-stromal cell interactions, degradation of basement membrane driven by protease production originating from fibroblasts. Stromal fibroblasts contain elevated fibronectin, laminins, and higher level of fibrillar collagens to remodel mammary tissue during involution [48]. Fibrillar collagen-epithelial interactions, especially collagen I, III, and V, are crucial during this process [14]. Studies revealed that the epithelial compartment is highly malleable and that cell fate and tissue function are strongly influenced by the stromal compartment of the gland [48].

4.2.4. Different properties of fibroblasts derived from normal and cancerous stroma

When discussing the role of stromal fibroblasts in mammary gland biology, one needs to mention about epithelial-stromal interactions in the context of breast cancer development. Fibroblasts arising from tumor stroma, described as cancer-associated fibroblasts (CAFs), compared to normal fibroblasts, have acquired distinct properties mainly leading to the promotion of cancer cell proliferation and invasion. CAFs, which are characterized by their high expression of alpha smooth muscle actin, are detected in large numbers in malignant breast cancers and their presence is correlated with poor clinical outcome [62]. Particularly in breast cancer, the progression from ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC) is believed to be actively driven by complex interactions with the surrounding microenvironment including interactions with various activated stromal fibroblasts [63]. It is believed that CAFs contribute to cancer cell survival and progression not only through enhanced secretion of cytokines, growth factors, and proteases such as TGFβ1, HGF, SDF-1, and MMP2, respectively, but also by secreting high levels of nutrient-rich ECM, promoting persistent chronic inflammation within the tumor microenvironment and inducing epithelial-to-mesenchymal transition (EMT) of tumor cells [48]. During EMT, downregulation or loss of the epithelial adhesion molecule E-cadherin and upregulation of N-cadherin represent a key step in the acquisition of the phenotype for many tumors. Interestingly, normal fibroblasts induce a strong E-cadherin enhancement even in cancer mammary epithelial cells; thus, these fibroblasts appear to favor the maintenance of the normal tissue architecture [64]. In vitro studies investigating the relationship between mammary carcinoma cells and stromal cells revealed that normal mammary fibroblasts function to suppress tumor progression by negatively regulating expression of oncogenic signaling factors [65]. Furthermore, co-culture of cancerous cells with stromal fibroblasts has been shown to induce significant changes in tumor development and progression [56].

4.2.5. Summary

Fibroblasts are the principal component of the stromal connective tissue. These cells are responsible for ECM remodeling and secrete FGFs and ECM components, such as collagens,

fibronectin, laminins, elastin, proteoglycans, and MMPs. Due to their properties, fibroblasts support the luminal epithelial growth and branching morphogenesis as well as participate in the mammary gland tissue remodeling during involution (**Figure 2**).

4.3. Ivmmune cells

Regulation of the mammary gland morphogenesis also pertains to the involvement of immune cells and the utilization of immune-related signaling molecules [67]. The immune system may contribute to mammary development at each stage via cytokine secretion and recruitment of macrophages, eosinophils, neutrophils, mast cells, and lymphocytes (T and B cells) [48, 66]. The gland is intercalated with extensive vascular and lymphatic networks present throughout the fat pad. During pubertal mammary gland development, the lymphatic network develops in close association with the mammary epithelial tree and blood vasculature. The presence of immune cells within the surrounding stroma was shown to be important for ductal branching as these cells are recruited to the branching tips of the epithelium to mediate invasion into the fat pad [67].

4.3.1. Regulation of immune cells present within the mammary stroma

Immune microenvironment of the mammary gland is also driven by the hypothalamic-pituitary-gonadal axis. Hormones act directly on epithelial cells and may modulate immune impact on tissue remodeling. Estrogen, progesterone, and prolactin each regulate immune cell functions, which in turn support the morphogenic processes occurring in the pubertal and adult mammary gland [68]. The effects of these hormones on immune cells can be either direct or indirect. The direct effects are mediated when the immune cells express receptors for estrogen, progesterone, and prolactin, which are activated by their respective ligands. The indirect effects of these hormones on immune cells are mediated by paracrine signals derived from MECs and the surrounding stroma [66]. It has been shown that mice lacking the expression of estrogen receptor alpha (ERα) and amphiregulin (a member of EGF family) and showing deficient signaling driven by EGF receptor (ERBB1) fail to develop mature ductal trees and have inhibited recruitment of macrophages and eosinophils to the site of tissue remodeling [66]. In vitro experiments demonstrated that estrogen-stimulated macrophages significantly enhanced fibroblast proliferation and invasion by tumor necrosis factor (TNFα) and MMP9 secretion, thus modifying stromal tissue compartment for epithelial expansion [47].

The profile of immune cells within the microenvironment of the mammary gland varies depending on the changes in hormonal stimuli occurring during the estrus/menstrual cycle. In humans, during the luteal phase of the menstrual cycle, the dominant subpopulation of Th lymphocytes is the Th2 cells secreting IL-4 and IL-10. Increasing concentrations of estrogen increase the abundance of regulatory T cells (Treg) in blood and enhance their immunosuppressive functions [66]. It is speculated that since estrogen and progesterone regulate the number of Treg cells in blood, the abundance of these cells in the mammary gland may also be hormone-dependent and fluctuate over the menstrual cycle. In addition, progesterone during pregnancy and prolactin during lactation were shown to stimulate the recruitment of Th2 cells. These hormones induce MECs to produce Th2-like cytokines, such as IL4, IL5, IL10, and IL13 [69, 70].

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4.3.2. Role of eosinophils in mammary gland morphogenesis

Eosinophils belong to immune cells found around the growing TEBs. These cells are attracted by eotaxin, another chemokine produced by mammary gland [43]. Eosinophil knockdown mice show altered elongation and branching during mammary gland development as well as insufficient milk productions at the time of lactation [48]. Similar abnormalities can be noted in knockout mice with deficiency of interleukin 5 (IL-5), a cytokine to which eosinophils are particularly responsive [71]. Mammary tissues from IL-5-deficient females had fewer TEBs, less well-branched mammary ducts, and lower overall density of the mammary gland structures. Furthermore, IL-5-deficient pups nursed by IL-5-deficient mothers were notably underweight, with a high percentage of pre-weaning mortality, in contrast to well-developed IL-5-deficient mice which were nursed by IL-5-sufficient foster mothers [71]. Interestingly, overabundance of eosinophils during puberty results in retarded morphogenesis of the mammary epithelium, suggesting the existence of mechanisms controlling the number of these cells that reside in the gland and are involved in MECs expansion during morphogenesis [66]. In addition to eosinophils, mast cells were also shown to be important for normal mammary gland development. Mice deficient in mast cells have defective mammary branching during puberty. It may be associated with the lack of vascular endothelial growth factor (VEGF) released by these cells that assist in mast cell degranulation [48]. Through activation of their serine proteases and degranulation, mast cells are involved in normal branching during puberty, and they accumulate and possibly activate plasma kallikrein, thus activating the plasminogen [5]. Furthermore, it was demonstrated that inhibition of this mast cell-associated protease during involution caused an accumulation of fibrillar collagen and delayed repopulation of adipocytes, thus preventing the gland from regaining the pre-pregnant state [14].

4.3.3. Role of macrophages in mammary gland development and remodeling

The role of macrophages at different stages of glandular morphogenesis as well as remodeling are better recognized. In the pubertal mammary gland, macrophages are recruited to the highly mitotic terminal end buds from which ducts elongate and branch to give rise to a mature ductal tree [48]. Macrophage colony stimulating factor-1 (CSF1) secreted by myoepithelial cells is a key cytokine that regulates the recruitment, proliferation, and survival of macrophages [48, 72]. Estrogen-regulated CSF1synthesis is essential for expanding of epithelial ducts and buds and alters structural alignment of collagen fibers around the expanding TEBs [70]. Macrophage abundance changes over the estrous cycle, peaking at metestrus and diestrus phases, and being the lowest at proestrus and estrus [66]. Studies on Csf1$^{op/op}$ mice, which are homozygous for a null mutation in *Csf1* gene, revealed that these animals exhibited multiple defects and had reduced macrophage numbers in most tissues including the mammary gland [72]. Depletion of mammary gland macrophages observed in Csf1$^{op/op}$ mice altered the mammary stem/progenitor cell activity, which was reflected in a substantially reduced outgrowth potential of the mammary epithelium. The mammary glands of Csf1$^{op/op}$ mice displayed lower number of TEBs as well as reduced ductal branching and elongation. During pregnancy, Csf1$^{op/op}$ glands developed precocious alveolar units but failed to switch to the lactational state resulting in impaired lactation [72]. These observations prove a continued requirement for normal macrophages during ductal morphogenesis and their stimulatory role on the putative basal progenitor cells. Macrophages also mediate the switch from

pregnancy to lactation through regulation of tight junction permeability. In mice, activation of NF-κB by toll-like receptor 4 (TLR4) signaling pathway increases permeability of the milk-blood barrier [66].

Post-lactating involution, which is analogous to a wound healing response, involves complex stromal-epithelial interactions, activation of elements of both innate and adaptive immune system, as well as stimulation of inflammatory cytokines and proteinases expression. This process is mediated in part through Jak/Stat signaling pathway and is characterized by the apoptotic death of MECs and their removal and engulfing by phagocytic cells: macrophages and epithelial cells by process of efferocytosis [11]. Tissue resident and infiltrating macrophages have special role in that process. Specific depletion of these cells in the involuting mammary gland leads to a reduction in both lysosomal-mediated and apoptotic cell death [73]. Involution is associated with the polarization of macrophages away from proinflammatory (M1) phenotype to an alternatively activated state (M2) [74]. This phenotypic switch is STAT3-dependent and occurs within an infiltrating macrophage population from day 3 of involution [75]. Re-emergence of adipose tissue is an important feature of involution associated with infiltration of macrophages into the gland form [14]. In the mouse mammary gland, gene expression profiling during postlactational tissue regression showed an increase in genes linked to the immune system, which coincides with increasing levels of interleukins: IL-4 and IL-13 acting as macrophage chemoattractants [76]. Furthermore, ECM can fragment into matrikines and matricryptins that also serve as attractants for the peripheral immune cells [14]. Fragments of collagen I, collagen IV, laminins, and nidogen-1 have all been shown to promote chemotaxis of monocytes and neutrophils within the interstitial tissue. Once in the mammary gland, macrophages and neutrophils secrete proteases such as MMP9 and elastase that are involved in further ECM breakdown [73]. Thus, without the influx of macrophages or neutrophils, the remodeling of the mammary tissue during involution, that serves to return the gland to the non-secretory postpartum state, could be delayed or incomplete [14].

ECM fragments not only aid the immune cells infiltration into the mammary gland but also may act as ligands to receptors present on leukocytes residing in the mammary gland. Fragments of biglycan, heparan sulfate, and hyaluronan have been shown to act as ligands for toll-like receptor 4 (TLR4) [14]. Toll-like receptors (TLRs) are part of the pattern recognition receptor family expressed on the cell surface of innate immune cells and dendritic cells. Binding the ligand to its TLR activates the immune cell or induces secretion of cytokines by these cells, resulting in further activation of cells of the adaptive immune system. For example, binding of soluble biglycan TLR 2/4 on macrophages stimulates them to synthesize and release a proinflammatory cytokine interleukin-1β [77]. Other ECM components, such as heparan sulfate and hyaluronan, have been shown to bind to the TLR4 on dendritic cells, causing their maturation [78, 79]. In turn, mature dendritic cells are able to activate cells of the adaptive immune system, which migrate to the site of ECM remodeling [14]. Also the presence of B lymphocytes in involuting mammary gland may be connected with the chemoattractive properties of ECM fragments. In vitro studies revealed that interleukin-4 and fibronectin stimulated B cells motility, and both compounds are known to be upregulated during involution. In fact, the presence of B cells during early to mid involution has been confirmed, prior to the peak in macrophage recruitment [35].

4.3.4. Summary

The intricate interactions between immune and epithelial cells are an inherent part of the mammary gland physiology. Paracrine factors secreted by Th2 lymphocytes and macrophages (IL-4, IL-10, and TNFα) as well as direct crosstalk between MECs and macrophages, eosinophils, mast cells are involved in regulation of all stages of mammary gland morphogenesis, from early embryogenesis, puberty, through pregnancy, lactation and involution (**Figure 2**).

4.4. Vascular endothelial cells

Mammary gland development, occurring during pre- and postnatal life of female mammals, serves to create a highly branched network of ducts and alveoli made of secretory epithelium that actively synthesizes and secretes milk at the time of lactation. To fulfill its function, the mammary gland also requires an expanded network of vascular endothelium. Currently, it is thought that the vasculature not only provides nutrients to the developing and functionally active mammary parenchyma, but also it is important for maintaining homoeostasis of the mammary epithelium.

4.4.1. Development of mammary blood vasculature

Vasculature in the mammary gland undergoes repeated cycles of expansion and regression concomitantly with the cycles of growth, differentiation, and regression of the mammary epithelium [80]. The development of blood vessels occurs in parallel with mammogenesis. In the course of vascularization, first the process of de novo blood vessel formation takes place in the embryonic life, followed by angiogenesis which serves to form new vessels from pre-existing ones [80]. Angiogenesis is driven in main part by epithelial and stromal cells through secretion of the vascular endothelial growth factor (VEGF) and matrix metalloproteinases, especially MMP-9. Furthermore, studies have shown that development of the vessels in the mammary gland is driven by the same hormones that stimulate growth of the glandular parenchyma, that is the metabolic and sex hormones and the growth factors [81].

Before pregnancy, the mammary vasculature is composed of a thin layer of simple squamous endothelial cells forming a complex vascular network along with myoepithelial cells and connective tissue [82]. The structure of the glandular vasculature has been the best characterized in the mouse mammary gland. It is described as the basket-like capillary beds surrounding the alveoli clusters [83]. The capillary vessels run in parallel or encircle the mammary parenchyma and branch throughout the adipose tissue [82]. In humans, a high number of small capillaries are surrounding the ductal structures, whereas the acini of the lobular structures are interspersed by fewer, but significantly larger capillaries, which are sinusoidal in shape [80]. Such morphology provides a slower blood flow, thus a prolonged contact of the lobuloalveolar epithelium with circulating hormones and nutrients. During pregnancy, the growth of the mammary vessels intensifies along with expanded development of the parenchyma in order to increase the cell number and surface area to provide a maximal interface for nutrient transfer and milk secretion after parturition. Furthermore, increased surface area of the luminal endothelium is also accomplished by formation of microvilli and marginal folds

on individual endothelial cells [82]. Studies on bovine model of mammogenesis showed that the blood volume expands in the pregnant animal, and about 15% of the cardiac output is directed to the fetoplacental unit toward the end of pregnancy, but at parturition most of the blood flow is redirected from the uterus to the mammary glands [81].

Functional differentiation of the mammary gland during lactogenesis is also tightly connected with further changes in morphology and properties of the endothelial cells, which occur in order to support the efficient milk synthesis and secretion. The vasculature of the lactating gland is composed of a well-developed capillary meshwork enveloping the secretory alveoli with basket-like honeycomb structures [84]. The mammary endothelial cells show elevated number of mitochondria supporting their increasing demands for energy during milk production period. A higher number of pinocytotic vesicles is also observed in the endothelial cells, providing efficient transportation of plasma solutes and molecules, such as glucose [85]. In addition, increased capillary permeability occurs during early lactation. Capillaries have thinner walls and are in closer contact with the mammary alveoli, which also aids the enhanced transfer of nutrients and fluids in the functionally active gland [80, 82]. Studies done on rodents have shown that the development of the mammary vasculature, measured as the number of capillaries per individual lobular ductile, surpasses the development of the parenchymal network during lactation [82, 86]. This underlines the important role of the glandular vascular system supporting the optimal function of the mammary gland during the milk production period.

After weaning or termination of milking, when mammary gland involution takes place, the endothelium undergoes regression similarly to the mammary epithelium. Although the mechanisms controlling endothelial regression have not been well recognized so far, it seems that apoptotic cell death at least partially accounts for the remodeling of the vasculature [84]. It is worth noting that the timing of endothelial and epithelial regression is not equal, and MECs apoptosis precedes the death of the endothelial cells [84]. This indicates that the changes in the structure of the mammary gland are initiated in the parenchymal compartment and the altered microenvironment of the gland induces the changes in the vasculature. It is possible that the vascular regression is induced mechanically by disruption of the contact and anchoring between the endothelium and the collapsing mammary epithelial cells. The signals could be mediated by integrins and their cognate intracellular signal transducers, such as members of the Src family and the focal adhesion kinase (FAK); however, further studies are needed to confirm this hypothesis. Djonov and co-workers [84] also suggested that the massive endothelial regression cannot be exclusively due to apoptotic cell death since apoptotic endothelial cells were observed only occasionally in the involuting gland [87]. The authors proposed another mechanism involving regressive remodeling of the endothelium, which they termed angiomeiosis, taken from the Greek words angio (vessel) and meiosis (dwindling, retraction).

4.4.2. Function of endothelial cells in immune response to infections in the mammary gland

One of the most important functions of the endothelial cells is the ability of these cells to regulate the immune response of the host to protect the mammary gland during pathogen

exposure. This function is especially relevant in regard to bovine mammary gland which is highly prone to infections due to extended period of lactation connected with intensive milk production. Exposure to pathogens initially triggers a response from MECs and resident immune cells which produce and secrete a variety of inflammatory mediators, such as cytokines. These inflammatory mediators also activate the endothelial cells, increasing vascular permeability which is necessary for the influx of neutrophils to ingest pathogens and limit extravascular tissue damage [82]. Endothelial cells produce a variety of vasoactive mediators, such as nitric oxide (NO), prostacyclin (PGI_2), endothelin-1, and histamine. At the onset of inflammation, endothelial nitric oxide synthase (eNOS) becomes activated by increased intracellular calcium levels, leading to conversion of arginine to citrulline and NO. Subsequently, NO activates cellular pathways that result in inhibition of calcium influx into the endothelial cells, thus relaxation of the actin cytoskeleton. In addition to NO biosynthesis, constitutive cyclooxygenase-1 (COX-1) is activated by increased intracellular calcium and facilitates the synthesis of PGI_2 and oxylipid. By releasing the vasoactive mediators, endothelial cells modulate the vascular tone in order to provide an optimal endothelial surface to facilitate rolling, attachment, and migration of leukocytes that serve to regulate an appropriate immune response to infection [82]. However, during very early stages of infection and inflammation, an opposing process of vasoconstriction is also very important to protect the host's organism in the event of mechanical injury and bleeding. Interestingly, production of vasoconstrictors, such as platelet-activating factor (PAF), by endothelial cells may in turn induce increased production of NO, to prevent sustained vasoconstriction [88]. This suggests that modulation of vascular tone during the initial inflammatory response is tightly regulated to prevent unnecessary damage to blood vessels and interstitial tissue [82].

Endothelial cells, lining the extensive vascular network of the mammary gland, may also contribute to the production of inflammatory mediators, especially IL-1, IL-6, IL-8, and granulocyte colony-stimulating factor (GM-CSF), during inflammation of the mammary gland (mastitis). IL-8 directly stimulates bovine neutrophil migration, phagocytosis, priming, and enzyme degranulation. Both epithelial and endothelial cells contribute to the production of IL-8 during *Escherichia coli* infection. In cows experimentally infected with *E. coli* via injection in the teat canal, MECs showed increased levels of IL-8 mRNA until 24 h post infection, whereas endothelial cells showed increased levels of IL-8 mRNA 24 h after infection, resulting in sustained IL-8 level in tissue [89]. Studies on bovine mammary endothelial cells demonstrated that in early reaction to *E. coli* infection vascular-derived PAF seems to play a prominent role [90]. PAF is a potent phospholipid mediator and endothelial cells work as a target and a source of this molecule. In bovine mammary endothelial cells stimulated in vitro with endotoxin obtained from *E. coli*, PAF biosynthesis began as early as 30 min after the endotoxin challenge and peaked at 1 h following the challenge. The biosynthesis of PAF preceded the endotoxin-induced IL-1β and IL-8 mRNA expression that reached peak expression between 4 and 12 h following stimulation. These results suggest that vascular-derived PAF is an early proinflammatory mediator during pathogen invasion in bovine mammary gland [90]. Therefore, the endothelium enables the progression of a self-limiting inflammatory response to milk-producing tissue through modulation of vascular tone and blood fluidity, vascular permeability, endothelial adhesiveness, and production of inflammatory mediators.

4.4.3. Lymphatic vasculature in the mammary gland

When describing the vasculature present within the structure of the mammary gland, one needs to mention also the lymphatic vasculature, which plays a distinct role in the gland's function. Lymphatic vessels serve to return the interstitial protein-rich fluid to the bloodstream, absorb dietary fats and fat-soluble vitamins from the digestive tract, and traffic the immune cells to the site of their physiological destination, as well as at the time of infection [91]. Very little is known about the course of lymphatic vessel formation during mammogenesis. Betterman and co-workers described the process of lymphangiogenesis during the postnatal development of the mouse mammary gland [91]. The authors showed that lymphatic vessels share an intimate spatial association with epithelial ducts and large blood vessels. Lymphatic vessels were observed to encircle epithelial ducts in the mammary glands of virgin and pregnant mice; however, these vessels were not dispersed throughout the stroma and were excluded from alveoli during pregnancy [91]. In contrast, lymphatic vessels in the rat mammary gland were found throughout the interlobular connective tissue and in close association with the alveoli during pregnancy, pointing at substantial interspecies differences [92]. The results of the study performed by Betterman and co-workers [91] have indicated that myoepithelial cells are the source of prolymphangiogenic growth factors, such as VEGF-C and VEGF-D, that drive the expansion of lymphatic vasculature. Interestingly, the lymphatic vessels were not observed in close proximity to alveoli in the pregnant and lactating murine mammary glands. This phenomenon could be caused by insufficient prolymphangiogenic stimuli originating from myoepithelial cells which form a discontinuous sheath around the secretory MECs of the alveoli. Alternatively, the absence of lymphatic vessels could result from repulsive bioactive compounds secreted by the alveolar epithelium [91]. Among the considered molecules showing possible properties of repelling the lymphatic vascular growth is soluble VEGF receptor 2 (sVEGFR-2), which was shown to maintain the lymphatic state of cornea by sequestering endogenous VEGF-C [93].

4.4.4. Summary

Mammary vasculature supports three aspects of mammary gland physiology: (1) capillary endothelial cells form a semipermeable barrier that facilitates the exchange of serum compounds to provide oxygen, remove CO_2, and transfer solutes and macromolecules for cellular energy metabolism; (2) vascular endothelium provides a high rate of transfer of blood-derived components, such as glucose and amino acids for efficient synthesis of milk; (3) it also plays a significant role in orchestrating host defense to infectious pathogens, which is especially important in extensively active bovine mammary gland producing milk volumes that exceed the nutritional requirements of the offspring. Still, the intricacy of the epithelial-endothelial interactions and their impact on mammary gland development remain largely undiscovered. Further research is needed to gain more knowledge about the role of endothelial cells in the complex interactions between the stromal and epithelial compartments of the mammary gland (**Figure 2**).

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Conflict of interest

The authors declare no conflict of interest.

Nomenclature

BM	basement membrane
CAFs	cancer-associated fibroblasts
CSF1	colony stimulating factor-1
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	epithelial-to-mesenchymal transition
ERBB1	epidermal growth factor receptor
ERα	estrogen receptor alpha
FGF	fibroblast growth factor
FGFRs	fibroblast growth factor receptors
GH	growth hormone
GHR	growth hormone receptor
HGF	hepatocytes growth factor
IGF-I	insulin-like growth factor-I
IL	interleukins
MECs	mammary epithelial cells

MMPs	matrix metalloproteinases
MRUs	mammary repopulating units
TDU	terminal ductal units
TEBs	terminal end buds
TGF-β	transforming growth factor-beta
TIMPs	tissue inhibitors of metalloproteinases
TLRs	toll-like receptors
TNFα	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor
VEGFR-2	vascular endothelial growth factor receptor-2

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Chapter 6

Molecular Aberrations in Bone Marrow Stromal Cells in Multiple Myeloma

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Additional information is available at the end of the chapter

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Abstract

Multiple myeloma (MM) is a B-cell malignancy characterized by an accumulation of malignant plasma cells within the bone marrow. Bone marrow mesenchymal stromal cells (BMMSCs) represent a crucial component of MM microenvironment supporting its progression and proliferation. Alterations in BMMSC of MM (MM-BMMSC) have become an important research focus. In this study, we analyzed MM-BMMSC and their modification through interaction with plasma cells in 128 MM patients. MM-BMMSC displayed a senescence-like state that was accompanied by an increase in senescence-associated β-galactosidase activity, a reduced number of colony-forming units, an accumulation of cells in S phase of the cell cycle, and the overexpression of microRNAs (miR-16, miR-223, miR-485-5p, and miR-519d) and p21. MM-BMMSC showed a reduced expression of mitochondrial stress response protein SIRT3 and an increased mitochondrial DNA mass that led to a higher amount of reactive oxygen species compared to healthy donor BMMSC. The interaction between MM cells and MM-BMMSC is a complex mechanism that relies on multiple interacting signaling pathways. Observed aberrations in MM-BMMSC should be confirmed in an *in vivo* model in order to clarify the importance for the pathogenesis of MM. Eventually, the result of MM therapy could be improved by understanding the interaction between MM cells and MM-BMSCs.

Keywords: multiple myeloma, bone marrow stromal cells, molecular aberrations

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1. Introduction

Multiple myeloma (MM) is a B-cell malignancy characterized by an accumulation of malignant plasma cells within the bone marrow (BM) [1]. Contact between MM cells and their microenvironment plays a crucial role in MM survival and proliferation and is able to promote tumor progression and drug resistance. Bone marrow mesenchymal stromal cells (BMMSCs) represent a central component of MM microenvironment supporting its progression and proliferation [2–4]. Alterations in BMSC from MM patients (MM-BMMSC) have become an important research focus. Several studies and our previous data have suggested the genesis of constitutive abnormalities within the BMMSC population through direct and indirect interactions with MM cells [5–8]. The development of a senescence-like state in BMMSC and thereby a modulated secretory profile, worsened osteogenic differentiation potential and inhibition of the T-cell proliferation, was reported [6, 8, 9]. Senescent BMMSCs display an increased senescence-associated β-galactosidase activity (SAβGalA) and irregular cell morphology. Usually, the cell cycle of senescent cells is arrested at the G_1/S-transition point in combination with the overexpression of different cell cycle inhibitors as p21 and p16. In spite of the aberrant growth characteristics, senescent cells remain metabolically active, and therefore, the secretion of pro-inflammatory mediators could promote tumorigenesis in neighboring premalignant cells [10–12]. The secretion of pro-inflammatory mediators by senescent BMMSC could therefore promote tumorigenesis in neighboring premalignant cells [13].

Two imprinted clusters in the human genome might contribute to the generation of senescence and the induction of cellular changes in MM-BMMSC [14–17]. The DLK1-DIO3 imprinted domain is located on chromosome 14q32.2, and cluster C19MC is located on chromosome 19q13. The DLK1-DIO3 expresses the non-coding transcripts MEG3, anti-RTL1, 53 microRNAs (miRNA), and 2 snoRNA clusters on the maternal chromosome. The paternal chromosome is responsible for the transcription of the protein-coding genes DLK1, RTL1, and DIO3 [18, 19]. Allelic expression of these genes is controlled through methylation of a regulatory region (IG-DMR) located upstream of the cluster [20]. The C19MC codes for 59 miRNAs are processed into one primary transcript from the paternal chromosome. Its expression strongly correlates with the epigenetic modulation of a CpG site located upstream [21].

In addition, there are evidence that the presence of cancer-associated fibroblasts (CAFs), characterized by high α-SMA, FAP, and FSP-1 expression, in the BM samples of MM patients, contributes to altered, tumor favorable, cell-cell interactions and cytokine secretions [22–24].

BMMSC represents an essential part for assistance of MM partly by the secretion of tumor supportive cytokines as interleukin 6 (IL-6) and vascular endothelial growth factor (VEGF) [2]. Both of them play a major role in the aberration of multiple signaling pathways such as PI3K, JAK/STAT3, Raf, and NF-κB [25]. In addition, MM cells produce inflammatory molecules, such as TGFβ and TNFα, which lead to NF-κB activation tumor-promoting effects. The activation of the NF-κB pathway in both MM cells and BMMSC results in the downstream upregulation of adhesion molecules and a consequent increase in cell-cell interactions [26, 27].

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It is known that the increased activation of NF-κB together with the overexpression of adhesion molecules can induce a therapy resistance [4, 26, 28, 29]. It can increase the secretion of tumor supportive soluble factors by BMMSC and may possibly lead to the generation of cell adhesion-mediated drug resistance [30–32].

Furthermore, additional interaction pathway, such as Notch signaling, is a factor between BMMSC and myeloma cells [33]. For example, it has been shown that malignant plasma cells overexpress the Notch ligand Jagged-2. An inhibition of Notch interaction induces myeloma cell apoptosis [34]. Notch signaling is important not only for the interaction of the myeloma cell with its surrounding cells but also for intercellular signaling between the malignant plasma cells. Downstream leads the Notch receptor-ligand interaction to an increased release of angiogenic and tumor-stimulating factors, such as VEGF, IL-6, and IGF-1 [35]. In addition, direct interaction between adhesion molecules (e.g., VCAM-1-VLA-4 interactions) and cell surface receptors such as Notch mediates therapy resistance and disease progression [36]. Furthermore, the formation of exosomes by BMSC, which actively transports modulatory substances, such as chemokines and miRNAs to the malignant MM cell, promotes survival and growth of MM cells. However, further investigations are needed to identify the exact mechanisms of exosome-mediated tumor promotion [37, 38].

It is known that cancer cells undergo dramatic alteration of metabolic pathways. Cancer cell survival and proliferation depend on metabolic processes, like glucose-uptake via altered glycolysis, also known as the Warburg effect. Sirtuins (SIRTs) are a family of deacylases and ADP-ribosyltransferases with clear links to regulation of cancer metabolism. Through their unique ability to integrate cellular stress and nutrient status in coordination with metabolic outputs, SIRTs are well poised to play pivotal roles in tumor progression and survival [39]. SIRT3 is the main mitochondrial deacetylase, which controls the activity of many metabolic enzymes in the mitochondria. SIRT3 deacetylates mitochondrial proteins that act in mitochondrial metabolism, including the oxidation of fatty acids, glutamine metabolism, and the production of mitochondrial reactive oxygen species (ROS) [40]. It was found that the increased level of cellular ROS observed with the loss of SIRT3 leads to a change in the cellular metabolism with respect to glycolysis. It is possible that the SIRT3 deficiency leads to a cancer resolution, coordinating the metabolic shift in the Warburg phenotype [41].

Despite some knowledge of the constitutive changes in the BMMSC of MM patients, the molecular mechanisms and pathways that induce abnormalities are largely unknown.

2. Patients and methods

2.1. Patients and donor characteristics

BM samples from 116 MM patients were studied: 69 patients with MM at the time of diagnosis and 47 at relapse. All patients had indications for treatment. The main clinical characteristics

of patients are shown in **Table 1**. Twelve bone marrow aspirates were received from healthy donors (HDs) as control. Written informed consent was obtained from all patients and donors in accordance with the Declaration of Helsinki and the ethical guidelines of the Charité University School of Medicine, which approved this study (Votum No.: EA4/131/13).

2.2. Isolation of BMSC and CD138+ plasma cells

BMMSCs from patients and donors (HD-BMMSC) were isolated using adhesion method and cultivated as previously described [42–44]. The colony-forming unit fibroblast (CFU-F) assay was used to study the self-renewal capacity of BMMSC. The staining was carried out with the Hemacolor Rapid Staining Kit from Merck according to the manufacturer's instructions. The evaluation was done by counting blue colonies.

Non-hematopoietic cell characteristics were identified by flow cytometry by the absence of CD105-FITC, CD90-FITC, CD45-PE, and CD34-PE (Miltenyi). Data were acquired and analyzed with a FACS Calibur Flow Cytometer (BD Biosciences). Control CD138^{+} plasma cells were isolated from HD mononuclear cells using magnetic-activated cell sorting (MACS) with a CD138 antibody (Miltenyi) as recommended by the manufacturer's protocol and seeded in culture flask with RPMI media with 20% of fetal calf serum and antibiotic/antimycotic.

Characteristics	**All patients (*n* = 116)**	**Patients at diagnosis (*n* = 69)**	**Patients at relapse (*n* = 47)**	**Donors (*n* = 16)**
Age, median (range)	63 (33–87)	64 (33–87)	62 (57–84)	69 (38–81)
Gender (M/F, %)	66/34	67/33	66/34	62/38
Ig expression (%)				
IgG	58	60	53	
IgA	13	14	13	
IgD	1	0	2	
Light chain	27	25	32	
Non-secretary	1	1	0	
Stage on Durie-Salmon (%)				
I A	10	13	7	
I B	6	8	2	
II A	8	6	11	
II B	5	3	7	
III A	55	54	57	
III B	16	16	16	
Bone marrow infiltration % median (range):	50 (10–100)	60 (10–100)	40 (5–90)	

Table 1. Patients and donor characteristics.

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2.3. Co-culture and transwell culture experiments

KMS12-PE cells received from DSMZ (ACC606) were cultured in enriched RPMI media. For co-cultures, MM-BMMSCs were seeded in a six-well plate and incubated for 4 h. Then, KMS12-PE myeloma cells were added followed by incubation for 72 h. After incubation, KMS12-PE cells were removed. The absence of CD138+ cells was confirmed using microscopy and checked with FACS analysis. MM-BMMSCs were washed twice with PBS and applied for future analysis. Co-cultured KMS12-PE myeloma cells were suspended in TRIzol for future analysis.

For transwell cultures (0.4 μM pore size, Corning), 2×10^4 MM-BMMSCs were seeded in the lower chamber of a 12-well plate and incubated for 4 h. Then, 2×10^4 KMS12-PE myeloma cells were added to the upper chamber. Incubation was performed for 72 h. Cultures without KMS12-PE cells served as negative control for transwell cultures and co-cultures.

2.4. Detection of SAβGalA and cell cycle analysis

SAβGalA was measured using the median fluorescence intensity (MFI) as previously reported [45]. Co-cultures of HD-BMMSC and HS-5 stromal cells (CRL-11882) were used as controls. In addition, β-galactosidase activity was analyzed using the "Senescence Cells Histochemical Staining Kit" (Sigma-Aldrich) as recommended by the manufacturer. Cell cycle analysis was performed using the "Cell Cycle Assay Kit" (Abcam) as recommended in the prescription. Data were studied using a logarithmic scale.

2.5. Quantitative real-time PCR (qPCR)

Total RNA was extracted using TRIzol as described previously [46]. RNA was treated with DNase (Ambion) and poly(A)-polymerase (NEB) according to the manufacturer's instructions. About 800 ng of RNA was used for cDNA synthesis with a Transcriptor First Strand cDNA Synthesis Kit (Roche) and 2.5 μl of poly(T)VN adaptor primer (10 pmol) in a 20 μl reaction.

qPCR was performed with the FastStart Universal SYBR Green Master Mix (Roche). Primers were designed for each mRNA target using Primer3, OligoCalc, and OligoIDT. MiRNA detection was conducted using a specific miRNA primer and a universal reverse primer complementary to the adaptor sequence [47]. GAP-DH (for mRNA) and 5.8S rRNA (for miRNA) were chosen as housekeeping genes. QPCR was carried out with the Rotor Gene 6000 Real-Time PCR cycler. Cycling condition comprised 10 min at 95°C, 45 cycles of 15 s at 95°C and 60 s at 59°C, followed by a melting curve analysis from 60 to 98°C, rising by 1°/s. Efficiencies of qPCR were determined using linear regression analysis [48, 49] using LinRegPCR software, and relative quantifications were estimated with the Pfaffl method [50]. Received data were analyzed with the Rotor Gene 6000 software.

2.6. Quantitative methylation-specific PCR (qMSP)

DNA isolation was performed using Puregene reagents (Qiagen) according to the manufacturer's instructions. Genomic DNA was subjected to bisulfite treatment with the EpiTect Fast Bisulfite Conversion Kit (Qiagen) as recommended in the manual. Primers were used as

described by Murphy et al. [51] for DLK1-DIO3 and Fornari et al. [52] for C19MC. Reactions were performed with 30 ng treated DNA using SYBR Green Master Mix (Roche). Quantification was carried out using a standard curve generated using a dilution series of fully methylated with unmethylated DNA (Applied Biosystems). Each sample was analyzed in duplicates, and Ct values above 32 were excluded.

2.7. Copy number (CN) variation analysis

Three genomic regions located along each of the clusters were chosen for CN estimations of DLK1-DIO3 and C19MC. Assay qBiomarker Copy Number (Qiagen) was used. Genomic DNA from the stromal cell line HS-5 (CRL-11882) was applied as a calibrator. Analysis was performed with 5 μl of SYBR Green Master Mix, 0.5 μl of respective copy number assay, and 2 ng of genomic DNA in a total volume of 10 μl. Relative quantification was achieved by the $\Delta\Delta C_t$ method.

2.8. Transfection of SIRT3 siRNA

The transient knockdown of SIRT3 was performed in HD-BMMSC using siRNA (Qiagen). The transfections were carried out in 6-well and 24-well plates. For a 24-well plate, 33 nM siRNA was mixed with 6 μl HiPerFect Transfection Reagent in 100 μl serum-free medium and incubated for 10 min at room temperature. The cells were incubated for 48 h and then used for future analyses. For a six-well plate, the cell number was constant, and the reagent volumes were scaled up accordingly.

2.9. Determination of mitochondrial membrane potential and reactive oxygen species

Investigation of ROS amount was carried out using the DCFDA—cellular Reactive Oxygen Species Detection Assay Kit (Abcam) as recommended in the instruction. Analysis was conducted using the median fluorescence intensity.

Analysis of mitochondrial membrane potential ($\Delta\Psi m$) was performed with the Mitochondria Staining Kit (Sigma) using JC-1 dye. Results were analyzed using the ratio of JC-1 aggregates (median value of FL2 channel) to JC-1 monomers (median value of FL21 channel).

2.10. Indirect enzyme-linked immunosorbent assay (ELISA)

Proteins from complete cell lysates of BMMSC were detected with a Coomassie (Bradford) Protein Assay Kit (Pierce) and were adjusted with BupH Coating Buffer (Pierce). Analyses were performed according to the commercially available indirect ELISA protocol from Abcam. Detection was performed with 1-Step pNpp-Substrate (Pierce). Absorption was measured at 405 nm. All measurements were performed with three technical replicates. A dilution series of complete cell lysates of the HS-5 cell line was used for standard curve generation.

2.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software (La Jolla, CA, USA). The data shown represent the mean ± standard error of the mean (SEM). Comparisons of

HD-BMMSC with MM-BMMSC were performed using the Mann-Whitney *U* test. The Wilcoxon signed-rank test was used for the analysis of co-cultures. Results were considered statistically significant when $p \leq 0.05$.

3. Results

3.1. MM-BMMSCs are characterized by high senescence state and cell cycle abnormalities

Analysis of β-galactosidase activity revealed a significantly higher SAβGalA in MM-BMSC when compared with HD-BMDSC (**Figure 1A**). Since no significant differences in senescent cells between passages 1 and 4 were observed in both MM-BMMSC and HD-BMMSC, we can exclude the effect of cultivation on SAβGalA. These results were confirmed by a histological β-galactosidase staining of HD-BMMSC and MM-BMMSC in passage 4.

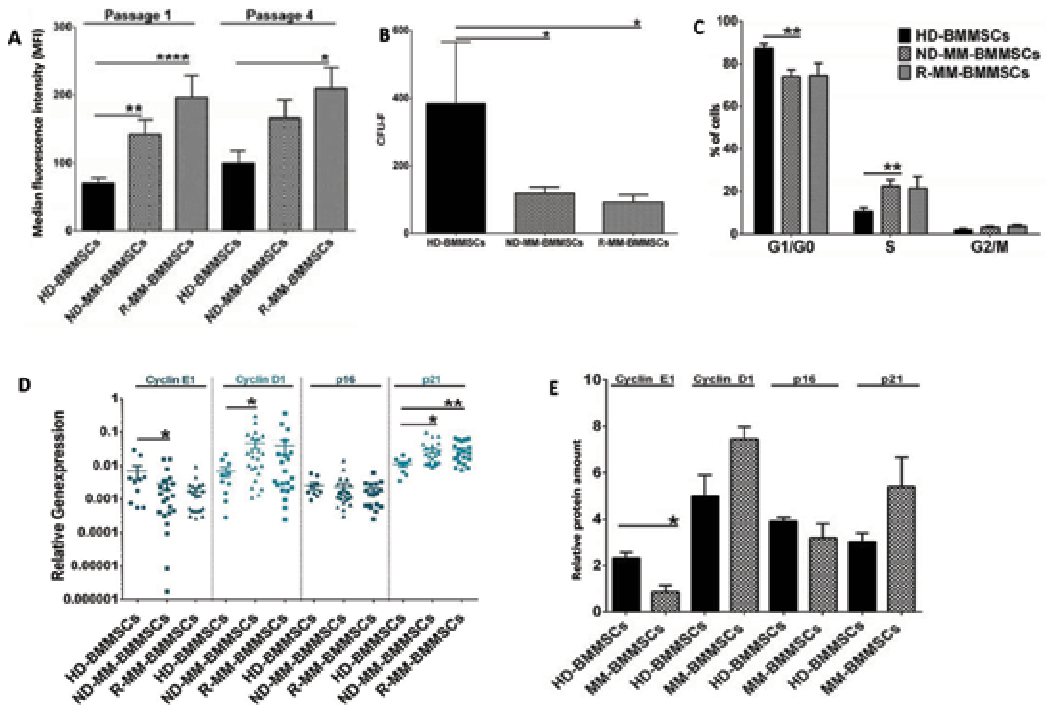

Figure 1. MM-BMMSC exhibits a higher senescence state and a lower self-renewal capacity than HD-BMMSC. *P* values: * <0.05; ** <0.01; *** <0.001; and **** <0.0001. All data were analyzed using the Mann-Whitney *U* test and unpaired *t*-test (ELISA analysis). (A) Flow cytometric analysis of SAβGalA. ND-MM-BMMSCs and R-MM-BMMSCs displayed higher activity of SAβGalA in passages 1 and 4 of cell cultures compared to HD-BMMSCs. (B) The colony-forming unit fibroblast (CFU-F) assay was used to study the self-renewal capacity of BMMSC. MM-BMMSC showed a lower self-renewal capacity compared to HD-BMMSC. (C) Cell cycle analysis showed a higher amount of cell in S phase and amount in G_1/G_0 phase in MM-BMMSC compared to HD-BMMSC. (D) QPCR analysis displayed decreased cyclin E1, increased cyclin D1 and p21 expression in MM-BMMSC compared to HD-BMMSC. (E) Measurement of the protein level in HD-BMMSC and MM-BMMSC. Cyclin E1 was significantly decreased in MM-BMMSC compared to HD-BMMSC, whereas cyclin D1 and p21 were increased. The protein amount of p16 was slightly reduced in MM-BMMSC compared to HD-BMMSCs. ND-MM-BMMSCs, new diagnosed MM patients; R-MM-BMMSCs, MM patients in relapse; HD-BMMSCs, healthy donor control.

The colony-forming unit fibroblast (CFU-F) assay was used to study the self-renewal capacity of BMMSC. MM-BMMSC showed a lower self-renewal capacity compared to HD-BMMSC (**Figure 1B**). Similar to the senescence study, MM-BMMSC obtained from relapsed patients showed a significantly lower self-renewal capacity than MM-BMMSC, which forms newly diagnosed patients.

MM-BMMSCs are characterized by a lower expression of cyclin E1 and an overexpression of cyclin D1 when compared with HD-BMMSC (**Figure 1D**). In addition, the cell cycle inhibitor p21 was upregulated in MM-BMMSC compared to HD-BMMSC ($p < 0.05$). No changes were observed in the mRNA level of p16. Changes in the mRNA levels were also confirmed using protein analysis ($p < 0.03$; **Figure 1E**). Cyclin E1 was decreased in MM-BMMSC compared to HD-BMMSC ($p = 0.0416$). Cyclin D1 and p21 protein levels were 1.5- to 1.8-fold increased. Protein measurement also showed a slightly reduced level of p16 in MM-BMMSCs, but this change was below 1.5-fold. These results correlated with a higher number of cells in S phase and a reduced number of cells in G_1/G_0 phase compared to HD-BMMSCs ($p < 0.008$; **Figure 1C**).

3.2. Co-culturing of KMS12-PE cell line represses the senescence entry of MM-BMMSCs

Co-cultures of the KMS12-PE cell line with MM-BMMSC and HD-BMMSC were carried out to analyze whether MM cells can exert an influence on the senescence characteristics of BMMSC. Experiments were performed with MM-BMMSC ($n = 20$) and HD-BMMSC ($n = 3$). After co-culturing BMSC with MM cells, an inhibition of senescence entry in MM-BMMSC was observed. SAβGalA activity was significantly reduced (**Figure 2A**). A similar effect was detected using transwell cultures to prevent cell-cell contact between MM-BMMSC and KMS12-PE cells ($p < 0.0313$; **Figure 2A**). No effect on the activity of SA-βGal was observed in HD-BMMSC and the HS-5 cell line co-cultured with KMS12-PE myeloma cells. Interestingly, $CD138^+$ plasma cells from healthy donors induced a downregulation of SAβGalA activity in MM-BMMSC. However, this influence was three- to sixfold lower than that of observed in co-cultures with KMS12-PE cells. These results indicate that MM cells have a higher and more specificity proliferation stimulation effect on BMMSC compared to CD138+ plasma cells.

Also, mRNA expression of co-cultured and transwell cultured MM-BMMSCs was measured (**Figure 2B**). No changes were found for cyclin D1 and p16, whereas cyclin E1 was upregulated in both co-cultured and transwell cultured MM-BMMSC ($p < 0.05$). BMSC interaction with MM cells has induced an upregulation of p21. This effect was lower in transwell cultured MM-BMMSC compared to co-cultured MM-BMMSC ($p < 0.008$).

However, some contrary results were detected at the protein level. We have found a reduction in p21 in co-cultured MM-BMMSC (**Figure 2C**). In addition, cyclin D1 protein expression was 1.8-fold reduced upon co-cultivation with KMS12-PE myeloma cells, whereas no change was seen on mRNA level ($p = 0.0033$). The mRNA and protein analysis of cyclin E1 and p16 were concordant.

Next, we analyzed cell cycle distribution of co-cultured and transwell cultured MM-BMMSC (**Figure 2D**). Both cell culture systems led to a slight reduction in cells in S phase compared to MM-BMMSC cultured alone ($p = 0.008$) and an increase in the percentage of cells in G1/G0 phase

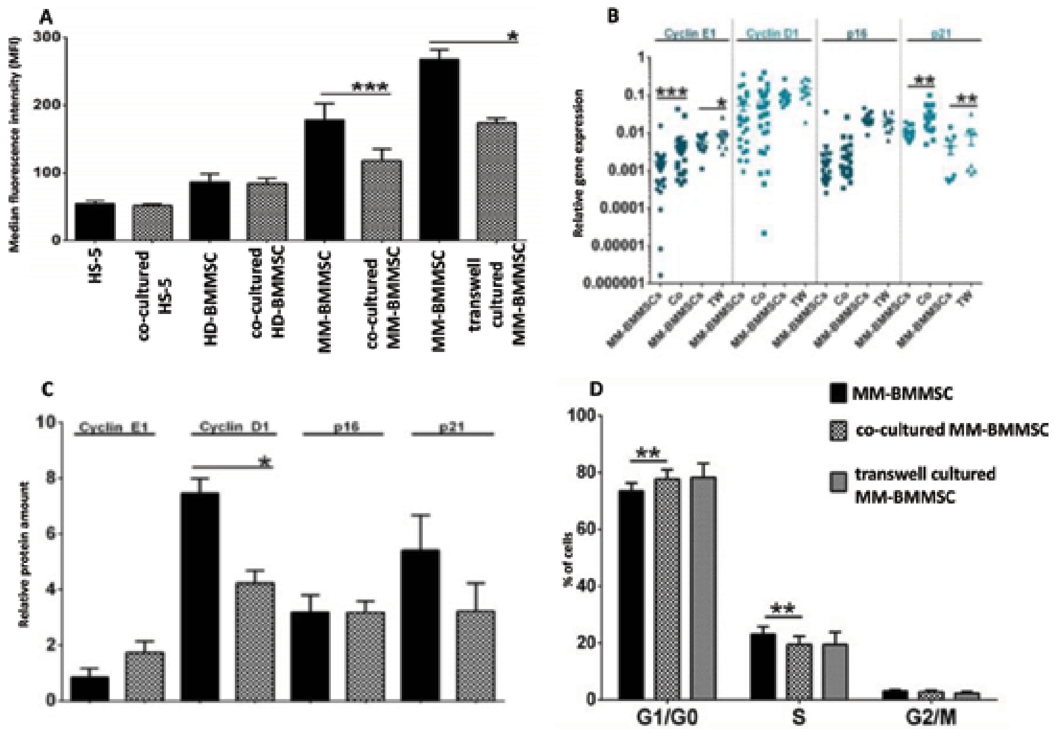

Figure 2. KMS12-PE myeloma cells reduce SAβGalA and modify cell cycle characteristics of MM-BMMSC. *P* values: * <0.05; ** <0.01; *** <0.001; and **** <0.0001. All data were analyzed using the Wilcoxon signed-rank test and paired *t*-test (ELISA analysis). HS-5 (CRL-11882)—BMSC line. (A) KMS12-PE myeloma cells reduce SAβGalA in MM-BMMSC upon co-cultivation and cultivation in transwell. The MFI in MM-BMMSC was significantly reduced in both culture systems. No changes were observed for co-cultured HD-BMMSC and HS-5 cells indicating specificity of the measured effect for MM-BMMSC. (B) Cell interaction with KMS12-PE myeloma cells induced increased cyclin E1 and p21 expression in MM-BMMSC compared to MM-BMMSC cultured alone. (C) Protein expression analysis of co-cultured MM-BMMSC ($n = 3$) compared to mono-cultured MM-BMMSC. Cyclin E1 was increased, whereas cyclin D1 and p21 were reduced in co-cultured cells compared to mono-cultures. No change was seen for p16. (D) Cell interaction with KMS12-PE myeloma cells induced an increase in cells in G_1/G_0 phase and reduced the amount of cells in S phase in co-cultured and transwell cultured MM-BMMSCs ($n = 8$) compared to the same MM-BMMSC cultured alone.

when compared with mono-cultured MM-BMMSC ($p = 0.008$). Transwell cultured MM-BMMSCs showed the same tendency, but significant changes were not detectable.

3.3. Deregulation of microRNA expression in MM-BMSC

We chose six microRNAs, which were previously reported to be deregulated in MM cells and to play a possible role in the generation of senescence or cell cycle arrest (miR-16, miR-485-5p, miR-519d, miR-221, miR-126, and miR-223). Analysis revealed an overexpression of miR-16, miR-223, miR-485-5p, and miR-519d (all with $p < 0.025$) in MM-BMMSCs compared to HD-BMMSCs. No expression differences were detected for miR-221 and miR-126 (**Figure 3A**).

We revealed the overexpression of miR-485-5p and miR-519d in MM-BMMSCs. These microRNAs are located on two imprinted clusters on chromosomes 14 (DLK1-DIO3) and 19 (C19MC), respectively, and are reported to play a role in senescence generation [21, 31, 32].

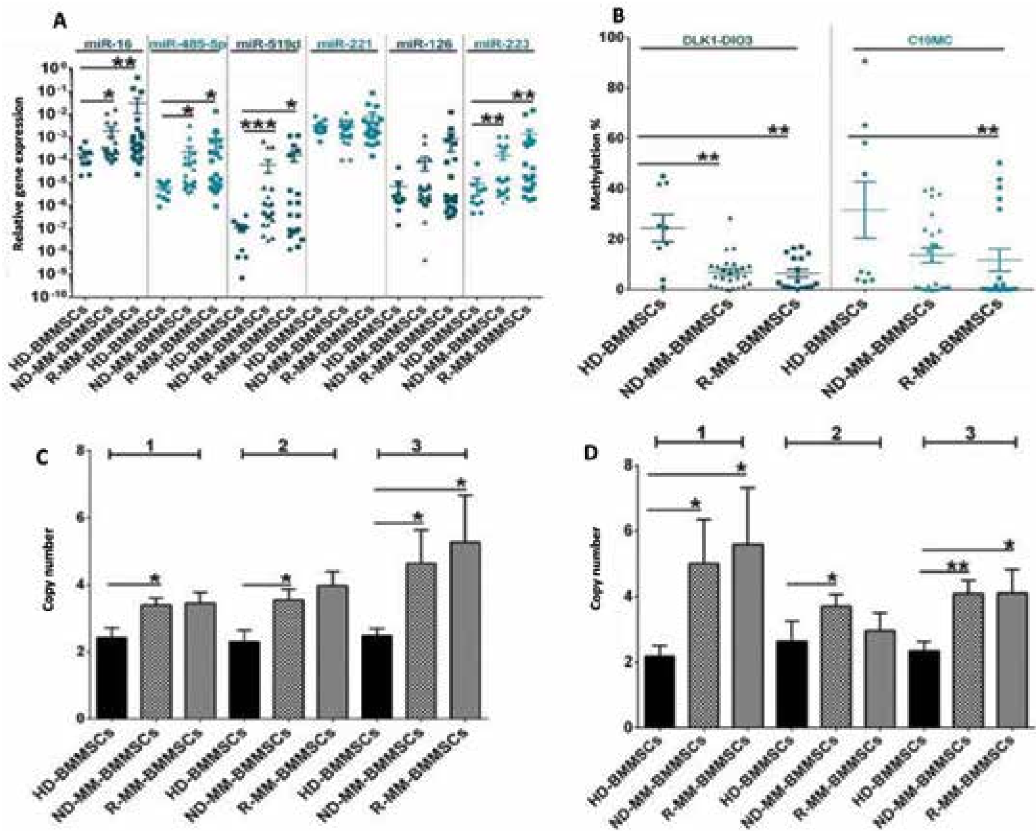

Figure 3. Overexpressed microRNAs in MM-BMMSC are associated with hypomethylation and CN accumulation of DLK1-DIO3 and C19MC. *P* values: * <0.05; ** <0.01; *** <0.001; and **** <0.0001. All data were analyzed using the Mann-Whitney *U* test. (A) ND-MM-BMMSC and R-MM-BMMSC showed high overexpression of miR-16, miR-485-5p, miR-519d, and miR-223 compared to HD-BMMSCs. (B) The regulatory regions of DLK1-DIO3 and C19MC were hypomethylated in ND-MM-BMMSC and R-MM-BMMSC compared to HD-BMMSC. (C) CN analysis of C19MC displayed CN accumulation in all three regions in MM-BMMSC compared to HD-BMMSC. (D) CN analysis of DLK1-DIO3 displayed CN accumulation in all three measured positions in MM-BMMSCs compared to HD-BMMSC.

Given that the expression of both clusters is controlled by methylation of their regulatory regions, we analyzed their methylation status using qMSP (**Figure 3B**). Hypomethylation of both clusters in MM-BMMSCs compared to HD-BMMSCs was observed. For DLK1-DIO3, MM-BMMSC exhibited an approximate fivefold lower methylation level of the IG-DMR. The C19MC exhibited a 2.5-fold lower methylation level in MM-BMSC compared to HD-BMMSC ($p = 0.0062$). CN analysis of both clusters displayed CN accumulation in all three regions in MM-BMMSC ($n = 38$) compared to HD-BMMSC ($n = 8$; **Figure 3C** and **D**).

3.4. Co-culturing of MM-BMSC with the KMS12-PE cell line induces the changes of microRNA expression in both cell types

The expression of four miRNA (miR-16, miR-223, miR-485-5p, and miR-519d) after co-culturing and transwell cultured MM-BMMSC was measured using qPCR (**Figure 4A**). MiR-223 was down-regulated in co-cultured MM-BMMSC ($p < 0.007$), whereas no effect was detected in transwell

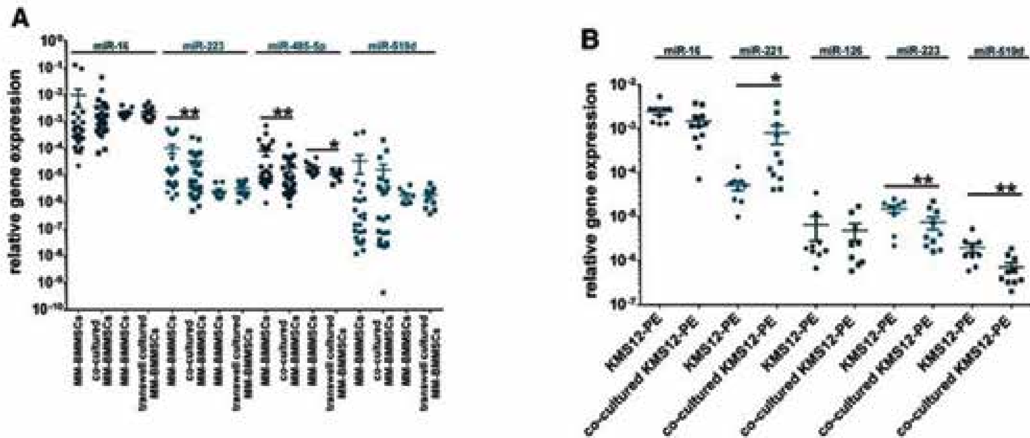

Figure 4. KMS12-PE myeloma cells downregulate miR-223 and miR-485-5p in MM-BMMSC. *P* values: * <0.05; ** <0.01; *** <0.001; and **** <0.0001. All data were analyzed using the Wilcoxon signed-rank test. (A) Co-cultured MM-BMMSC ($n = 25$) displayed reduced expression of miR-223 and miR-485-5p. Transwell-cultured ($n = 10$) MM-BMMSC showed no changes in miR-223 expression but also decreased miR-485-5p levels. Intensity of changes in miR-485-5p decreased when cell-cell contact was prevented by transwell cultivation. (B) Cell interaction with MM-BMMSC induced changes in the microRNA expression of KMS12-PE myeloma cells ($n = 10$). MiR-221 was upregulated, whereas miR-223 and miR-519d decreased in co-cultured KMS12-PE myeloma cells.

cultured MM-BMMSC. In contrast, downregulation of miR-485-5p was detected in both cell culture systems ($p < 0.03$). Interestingly, cell-cell interaction also altered miRNA expression of KMS12-PE myeloma cells. We found upregulation of miR-221 and significantly downregulation of miR-223 and miR-519d ($p < 0.02$; **Figure 4B**). Expression of miR-485-5p was not detectable in KMS12-PE myeloma cells.

3.5. KMS12-PE cells modulate the gene expression of MM-BMMSC

To explore the influence of KMS12-PE cells on gene expression of adhesion molecules, qPCR analysis of MM-BMMSC, co-cultured for 72 h with KMS12-PE cells in passage 4, was performed ($n = 25$). In mono-cultured BMSC, an upregulation of VCAM-1 ($p = 0.33$), ICAM-1 ($p = 0.33$), and IKK-α ($p = 0.05$) was demonstrated. Furthermore, the expression profile of miRNAs, targeting the analyzed genes or correlating with senescence, was studied (miR-16, miR-221, miR-126, miR-223, miR-485-5p, and miR-519d). MiR-16, miR-223, miR-485-5p, and miR-519d were significantly upregulated ($p = 0.02$; $p = 0.004$; $p = 0.02$; and $p = 0.002$, respectively), whereas miR-221 and miR-126 showed no considerable differences to BMSC obtained from healthy donors. After co-culturing of MM-BMSC with KMS12-PE cells, an enhanced expression of adhesion molecules was apparent. This includes the upregulation of VCAM-1 ($p = 0.0078$), ICAM-1 ($p = 0.2425$), and NF-κB activator IKK-α ($p = 0.0573$), though the values for ICAM-1 and IKK-α were not significant. Hence, MM cells seem to further boost the aberrant expression of adhesion molecules in MM-BMMSCs. Regarding microRNAs, a significant downregulation of miR-223 and miR-485-5p ($p < 0.009$) was detected. In addition, miR-16 and miR-519d showed a trend toward downregulation, though the changes were not significant. No expression alterations to miR-221 or miR-126 were detected (data not shown).

3.6. Expression of metabolic regulators in MM-BMSC

We investigated whether metabolic changes in MM-BMMSC could be responsible for the early aging status of the cells. For this purpose, we analyzed the expression of the gene and protein of the metabolic molecules SIRT3 and UCP2 and the lactate transporter MCT1 and MCT4.

There were no significant differences in the gene expression of MCT1, MCT4, and UCP2 in MM-BMMSC compared to HD-BMMSC (data not shown). In contrast, a significant lower expression of SIRT3 was detected in MM-BMMSC (p < 0.001; **Figure 5A**). All data were reproduced at the protein level. In addition, it was investigated whether MM-BMMSCs have an increased mitochondrial mass in comparison with HD-BMMSC. For this purpose, mtDNA was quantified and was normalized to the content of nuDNA. It was shown that MM-BMMSCs show a significant increase in mitochondrial mass compared to HD-BMMSC (p = 0.0149; **Figure 5B**). These changes were not detected in MGUS-BMMSC (n = 4), suggesting an association with disease progression.

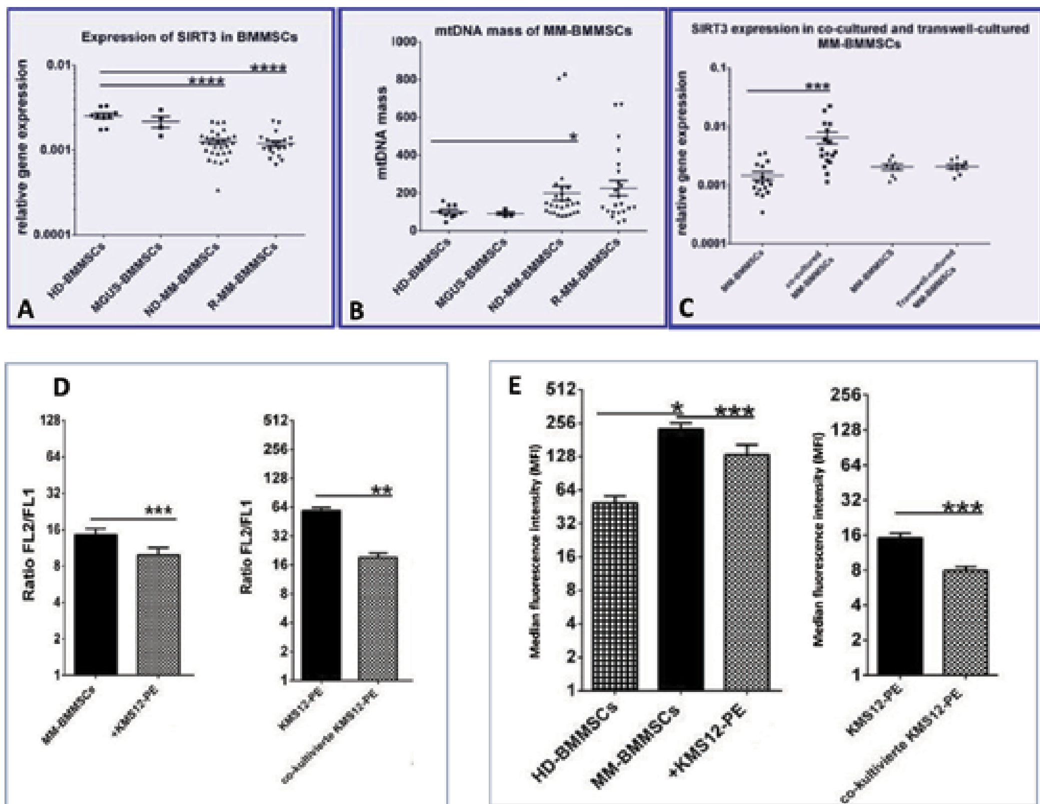

Figure 5. SIRT3 expression and mtDNA mass in MM-BMMSC. P values: * <0.05; ** <0.01; *** <0.001; and **** <0.0001. All data were analyzed using the Wilcoxon signed-rank test. (A) MM-BMSC displayed a twofold decrease in the expression of SIRT3 compared to HD-BMSC. MGUS-BMSC showed no changes. (B) MM-BMSC showed a twofold increase in mtDNA mass compared to HD-BMSC. (C) Co-cultured MM-BMSC displayed a fourfold increase in SRT3 mRNA level. No changes were seen in transwell cultures. (D) Co-cultivation KMS12-PE and MM-BMSC induced depolarization of $\Delta\Psi$m. (E) Co-cultivation KMS12-PE and MM-BMSC reduced the amount of ROS in both cell systems.

To explore the influence of MM cells on SIRT3 expression in BMSC, co-culturing for 72 h with KMS12-PE cells (n = 20) and transwell experiments (n = 10) was performed. Interestingly, we found a fourfold upregulation of SIRT3 expression in MM-BMMSC when co-cultured with KMS12-PE myeloma cells (**Figure 5C**). No changes were seen in transwell cultures.

Moreover, co-cultivation induced depolarization of ΔΨm leading to an approximately two-fold JC1 monomers increasing in MM-BMSC and MM cells (**Figure 5D**). Co-cultivation of KMS12-PE and MM-BMSC reduced the amount of ROS in both cell systems (**Figure 5E**).

To further elucidate the involvement of SIRT3 in metabolic and senescence-like alterations of MM-BMMSCs, siRNA was used to transiently “knockdown” this gene in HD-BMMSC.

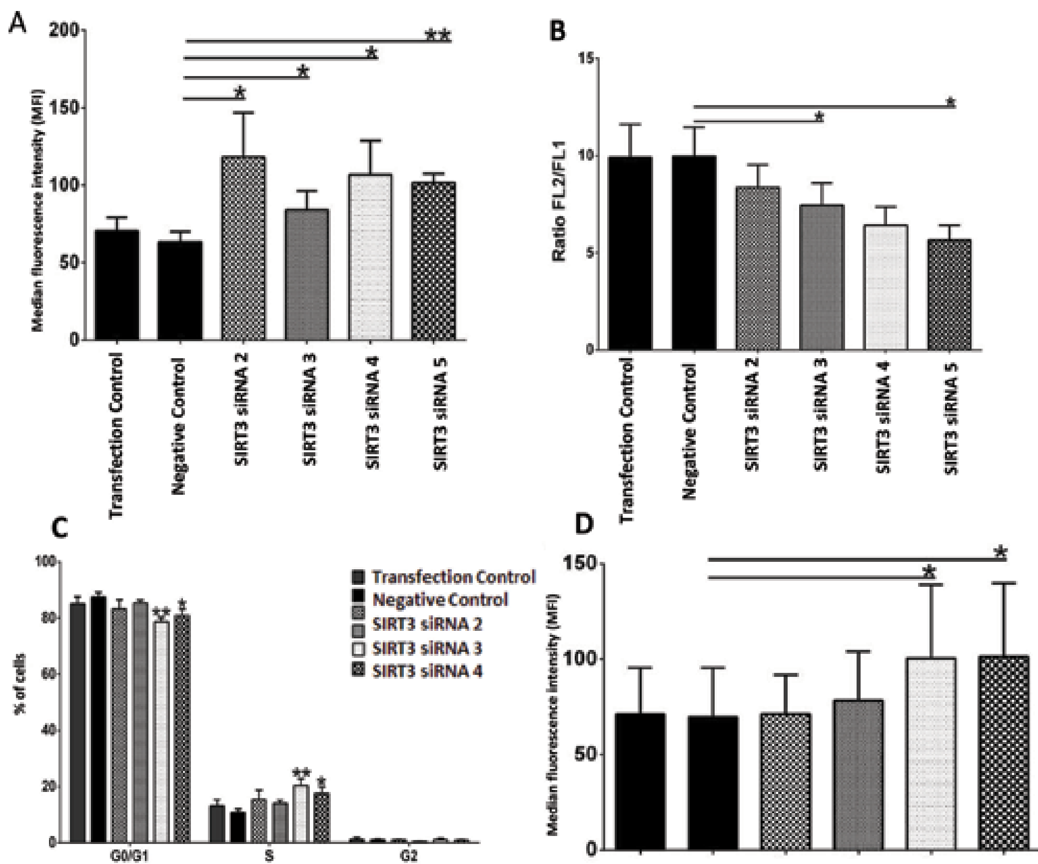

Figure 6. Influence of SIRT3 on ROS in HD-BMMSC. P values: * <0.05; ** <0.01; *** <0.001; and **** <0.0001. (A) The knockdown of SIRT3 in HD-BMMSC caused an increase in the ROS content of all four siRNAs tested compared to the negative and transfection control. (B) Influence of SIRT3 on ΔΨm in HD-BMMSC. The “knockdown” of SIRT3 in HD-BMMSC caused a reduction in the FL-2/FL-1 ratio. For siRNAs 2 and 3, only the proportion of FL-1 negative cells was reduced (R-4), whereas siRNAs 4 and 5 also caused an increase in FL-2 negative cells (R-3). (C) Influence of SIRT3 on cell cycle in HD-BMMSC. The “knockdown” of SIRT3 in HD-BMMSCs led to an accumulation of cells in S phase of the cell cycle (siRNAs 4 and 5). siRNAs 2 and 3 produced effects of the same tendency, but these were very low (<5%). (D) Influence of SIRT3 on senescence-associated β-galactosidase activity HD-BMMSC. Transfection of HD-BMMSCs with siRNAs 4 and 5 produced an increase in SAβGalA. In contrast, no significant effects were observed for siRNA 2 and siRNA.

Subsequently, the ROS amount, mitochondrial membrane potential, cell cycle, and SAβGalA of the cells were investigated. Two different HD-BMMSCs were used for these analyses, and from each study, 2–3 replicates were performed. The donors were 73 and 74 years old. Furthermore, four different siRNAs against SIRT3 were used. SIRT3 knockdown in HD-BMMSC induced 1.4- to 1.9-fold increase in ROS levels ($p < 0.05$; **Figure 6A**). This was associated with dissipation of ΔΨM between 1.4- and 1.8-fold depending on the siRNA that was used for transient knockdown of SIRT3 ($p < 0.04$; **Figure 6B**). Furthermore, the inhibition of SIRT3 mimicked cell cycle arrest in S phase previously reported in BMMSC of myeloma patients. The percentage of BMMSC in S phase increased upon SIRT3 knockdown between 6.7 and 9.6% ($p < 0.039$; **Figure 6C**). In addition, it was investigated whether the depletion of SIRT3 increases senescence-associated β-galactosidase activity. It was found that transfection of HD-BMMSC with SIRT3 siRNAs 4 and 5 resulted in an approximately 1.5-fold increase in SAβGalA ($p < 0.03$). In contrast, HD-BMMSCs transfected with siRNA 2 did not show any changes. Similarly, transfections with siRNA 3 caused only minimal changes in HD-BMMSCs (**Figure 6D**).

4. Discussion

MM-BMMSCs play a critical role in MM tumor growth and survival. Several studies suggest the existence of constitutive abnormalities in MM-BMMSC, and these lead to abnormal cell characteristics and increased tumor support [5, 6, 9, 23, 53, 54]. In this study, we explored the cellular and genetic aberrations of MM-BMMSCs in order to further identify the molecular mechanisms for these changes.

The enhanced and early senescence of BMMSC has been previously reported for different hematological disorders, including MM [9, 55]. Here, a significant higher senescence level of MM-BMMSC compared to HD-BMMSC was identified. When combined with our qPCR results that revealed an enrichment of cyclin D1 mRNA and the reduced expression of cyclin E1, an arrest of the cell cycle in G_1 phase can be assumed. In contrast, André et al. related senescence to an accumulation of MM-BMMSCs in S phase [9]. These contrasting results could be due to diverse patient samples as well as different cell isolation and culture treatment methods. However, early senescence indicates the impairment of MM-BMMSCs. With regard to the relapsed analysis group, therapy might lead to increased cellular stress for MM-BMMSCs resulting in higher senescence levels.

Distinct changes to gene expression profiles were also reported [24, 56–59]. In addition to the abovementioned changes in cyclin expression, an upregulation of the cell adhesion molecules VCAM-1 and ICAM-1, as well as the NF-κB member IKK-α, was found, consistent with previous studies [6, 9, 53]. Overexpression of the cell adhesion molecules and the NF-κB pathway without MM-BMMSC in contact with MM cells suggests the generation of a constitutive myeloma favorable microenvironment.

In contrast to the above studies, data relating to microRNA expression in MM-BMMSCs are limited. Here, overexpression of miR-16, miR-223, miR-485-5p, and miR-519d was identified. These microRNAs possibly influence cell cycle regulation, cell differentiation, and cell migration.

Alterations to MM-BMMSCs could therefore result from the specific deregulation of microRNA expression and their corresponding downstream targets [15, 52, 60–66]. The relapsed analysis group displayed a higher senescence level and a strongly increased microRNA expression (mean fold change > 100), supporting their possible function as cell cycle modifiers. Therapy seems to enforce senescence in MM-BMMSCs due to higher cellular stress and could lead to an even more altered cellular phenotype at relapse.

Overexpressed miR-485-5p and miR-519d are associated with two imprinted clusters on chromosomes 14 (DLK1-DIO3) and 19 (C19MC), respectively. Since both clusters exhibit a complex composition, including tumor-suppressive as well as tumor-promoting microRNAs, changes to their epigenetic regulation could account for important changes to the cellular characteristics of MM-BMMSCs [21, 66]. Here, analysis revealed hypomethylation and amplification of both clusters, possibly resulting in a higher transcriptional rate of cluster-associated genes. Several studies have reported the accumulation of genomic and global methylation changes due to in vitro cultivation of BMMSCs [67–72]. Indeed, minimal changes in the HD-BMMSC population, for example, hypo- and hypermethylation, as well as CN values between 2.2 and 2.8, were found. However, these alterations were less than those found in MM-BMMSCs, with distinct clustering of MM-BMMSC values below 20% methylation level and a mean value of more than 3.5 copies of the DLK1-DIO3 and C19MC genomic regions. The detected aberrations could be due to the existence of a CAF population in the MM-BMMSCs because some data highlight the presence of DNA hypomethylation and genetic instability in CAFs [24, 56, 73]. However, genetic instability in CAFs is controversial [74]. Hence, it cannot be excluded that CN variations of DLK1-DIO3 and C19MC result from hypomethylation or vice versa.

Moreover, the effect of MM cells on previously identified gene expression variations was investigated. In this context, a proliferation stimulating influence of KMS12-PE myeloma cells on MM-BMMSCs was apparent. Thus, KMS12-PE cells appear to repress MM-BMMSC senescence entry and increase the cell vitality. This modification could be associated with an increase in cyclin E1 mRNA levels.

Lastly, we investigated whether metabolic changes in MM-BMMSC could be responsible for the early aging status of the cells. For this purpose, we analyzed the expression of the gene and protein of the metabolic molecules SIRT3 and UCP2 and the lactate transporter MCT1 and MCT4. There were no significant differences in the gene expression of MCT1, MCT4, and UCP2 in MM-BMMSC compared to HD-BMMSC. In contrast, a significant lower expression of SIRT3 and a significant increase in mitochondrial mass compared were detected in MM-BMMSC. Interesting, no changes were detected in MGUS-BMMSC, suggesting an association with disease progression.

Our results suggested that MM cells influence the mitochondrial function of MM-BMMSC. This interaction leads to decrease the ROS levels in both cell types and could support their survival and growth. Moreover, the sustained induction of mitochondrial stress response could be the reason for premature senescence in MM-BMMSC. Therefore, the result of MM therapy could be improved through the disabling of metabolic interactions between MM cells and MM-BMMSC.

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Section 3

Stromal Cell Therapies

Chapter 7

Mesenchymal Stromal Cells as a Therapeutic Intervention

Ivonne Hernandez Schulman and Joshua M. Hare

Additional information is available at the end of the chapter

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Abstract

Mesenchymal stem cells, also known as mesenchymal stromal cells (MSCs), are a safe and promising biologic therapeutic for inducing tissue repair and regeneration in a broad array of chronic diseases. The mechanisms underlying the beneficial effects of MSCs include immunomodulation, reduction in inflammation and fibrosis, and stimulation of neovascularization and endogenous regeneration. Accumulating evidence from a multitude of clinical trials support the notion that both autologous and allogeneic MSCs are not only safe but also possess the capacity for repair of diverse organ systems and amelioration of multiple chronic disease processes. However, there are many questions regarding the underlying mechanisms of action, the most efficacious cell characteristics, tissue source, dose/concentration, route of delivery, and timing of administration, interactions with concurrent therapies, sustainability of effect, donor and patient characteristics, and adverse effects, including infections and malignancy, that remain to be resolved. Answering these questions will require well-designed and rigorously conducted multicenter clinical trials with well-established and defined clinical endpoints and appropriately defined patient populations, number of patients, and duration of follow-up. This chapter will review the current state of knowledge in the use of MSCs as a therapeutic strategy for organ structural and functional repair in chronic diseases.

Keywords: cell transplantation, mesenchymal stem cells, regenerative medicine

1. Introduction

Mesenchymal stem cells (a.k.a. mesenchymal stromal cells, MSCs) hold enormous promise as a durable, sustainable, and novel cell-based biologic therapeutic for a diverse range of clinical applications aimed at preventing or reversing organ injury and promoting tissue regeneration.

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Substantial data have accumulated regarding the safety of administering both autologous and allogeneic MSCs to patients with a broad array of diseases. In addition, it is increasingly clear that MSCs exert anti-fibrotic, pro-angiogenic, regenerative, and immunomodulatory effects, and therefore, offering therapeutic potential in a wide range of presently untreatable conditions. The growing evidence supporting the use of MSCs as therapeutic strategy includes their relative ease of isolation and expansion in culture, multilineage differentiation capacity, immunomodulatory, anti-inflammatory, anti-microbial, and regenerative effects, homing and migratory capacity to injury sites, safety profile in allogeneic transplantation, and few ethical considerations [1, 2]. The use of large animal models in preclinical studies has been instrumental in deciphering the underlying mechanisms of action of MSC therapy [3]. Moreover, substantial human phenotypic data has demonstrated that MSC therapy is safe [4–10] and holds the potential for repair and regeneration of diverse organ systems and amelioration of multiple chronic illnesses for which there is currently no cure [4, 6, 7, 9–24]. There are currently various MSC sources under investigation in preclinical and clinical studies, namely bone marrow, adipose tissue, umbilical cord blood, umbilical cord, and amniotic membranes/placenta (**Figure 1**). Multiple mechanisms of action underlie successful MSC therapy, including MSC engraftment and differentiation, and more importantly, the secretion of bioactive paracrine molecules that inhibit apoptosis, fibrosis, and inflammation and promote neovascularization/neo-angiogenesis and endogenous stem cell recruitment, proliferation, and differentiation [25–27] (**Figure 2**). In particular, cell-cell interactions between MSCs

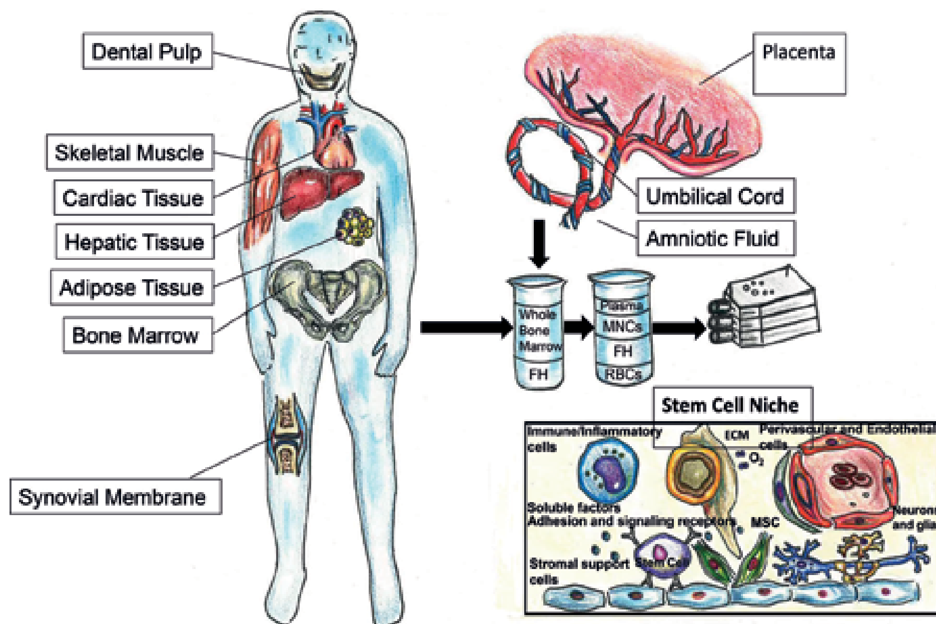

Figure 1. Mesenchymal stem cell tissue sources, ex vivo expansion, and role in stem cell niche. Initially identified in bone marrow, MSCs can be isolated from various tissues in the body. To isolate MSCs from a bone marrow biopsy, first the mononuclear cells are isolated from red blood cells by Ficoll density centrifugation, and subsequently, the MSCs are separated from the mononuclear cells by plastic adherence in culture. Inset: the constituents of a stem cell niche are depicted in this schematic. ECM extracellular matrix. *Adapted from Wagers AJ et al., Cell Stem Cell, 2012.*

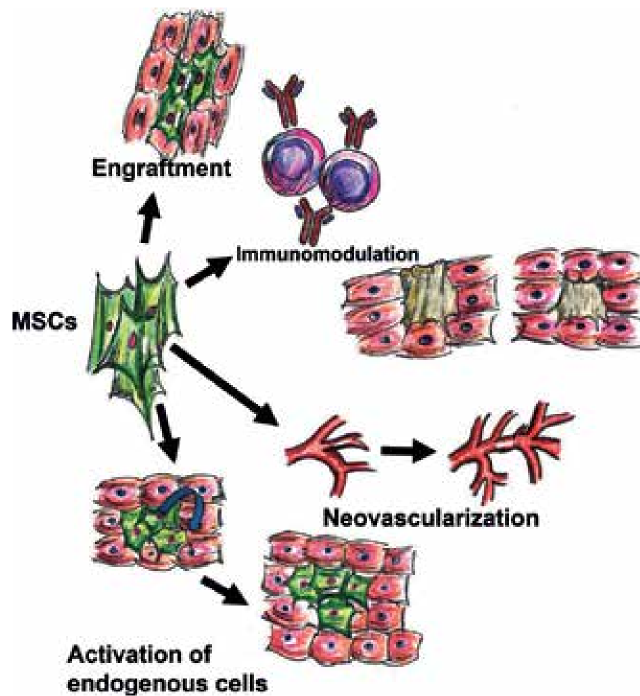

Figure 2. Mechanism of action of mesenchymal stem cell therapy.

and endogenous host cells within stem cell niches provide structural support and produce the soluble signals that regulate stem cell function in tissues[1, 28–30] (**Figure 1** inset). An in-depth molecular understanding of how MSCs produce the therapeutic benefits demonstrated in numerous clinical trials is critical for the development and design of new clinical trials as well as for the development of newer generations of MSC products that have greater efficacy and sustainability. This chapter will review the current state of knowledge in the use of MSCs as a therapeutic strategy for organ structural and functional repair.

2. Biology of mesenchymal stem cells

MSCs are non-hematopoietic stem cells with multilineage potential that originate from the mesodermal germ layer. The pioneering studies conducted by Friedenstein et al. provided the first evidence that these fibroblast-like cells, described as spindle-shaped and clonogenic in culture conditions could be isolated from bone marrow via their inherent adherence to plastic in culture [31, 32]. MSCs are an integral part of the stromal microenvironment and support hematopoietic stem cells and regulate hematopoiesis, although they comprise only ~0.01–0.001% of the total nucleated cells in the bone marrow [33, 34]. Moreover, MSCs have been isolated from virtually every tissue type, including adipose tissue, liver, lung, skeletal and heart muscle, synovial membrane, amniotic fluid, placenta, umbilical cord blood, and dental pulp, suggesting that they reside in all organs [35–37].

MSCs are readily expanded *in vitro* and have the capacity, as classically defined, to differentiate into osteoblasts, chondrocytes, and adipocytes [38, 39]. Studies also strongly support a role for MSCs in neovascularization, with the capacity for differentiation into both endothelial [40, 41] and vascular smooth muscle cells [40]. Finally, MSCs can differentiate into myocytes: skeletal myocyte differentiation is widely accepted, whereas there is ongoing controversy as to whether MSCs have a robust ability to form cardiomyocytes [40, 42–45].

No single cell surface marker specifically identifies MSCs. The International Society for Cellular Therapy has provided minimum criteria for defining multipotent human MSCs including (1) plastic-adherence under standard culture conditions; (2) expression of CD105, CD73, and CD90 and absence of hematopoietic cell surface markers, CD34, CD45, CD11a, CD19, and HLA-DR; and (3) *in vitro* differentiation into osteocytes, adipocytes, and chondrocytes under specific culture conditions [46]. However, MSCs can lose/acquire surface markers as they are isolated and expanded [47]. Furthermore, MSCs isolated from different tissues may exhibit a molecular fingerprint specific for their tissue of origin and thus vary in their differentiation capacity [48–50].

Bone marrow-derived MSC precursors (MPCs) have also been identified based upon specific cell surface marker expression, the most important being stromal precursor antigens (STRO-1, STRO-3) and CD271 [51–56]. *In vitro* studies suggest that the STRO-1 and STRO-3-enriched MPC populations have superior proliferative ability, multilineage regenerative capacity, and paracrine activity compared to MSCs [51, 54, 55], whereas CD271^{+} selection significantly increases clonogenic outgrowth of MPCs [52]. Preclinical studies using large animals have shown the efficacy of MPCs in acute MI and chronic ischemic and non-ischemic models of cardiomyopathy. Intracoronary injection of allogeneic MPCs in sheep after acute MI produced a 40% decrease in scar size and a 50% increase in vascular density [57]. Similarly, using echocardiography to guide the catheter-based endomyocardial injection of allogeneic MPCs into sheep 4 weeks post-MI resulted in an increase in left ventricular ejection fraction (LVEF), wall thickness, and vascular density. In a model of non-ischemic cardiomyopathy, transendocardial administration of ovine allogeneic cells produced decreased left ventricular end-systolic volume, stabilization of LVEF, decreased myocardial fibrosis and increased myocardial regeneration [53].

2.1. Osteogenic, chondrogenic, and adipogenic differentiation

As mentioned above, MSCs can be readily expanded *in vitro* and can differentiate into osteoblasts, chondrocytes, and adipocytes [38, 39]. Various growth factors and molecules promote MSC differentiation. For instance, global gene expression profiling arrays were utilized to identify RNA transcripts, which led to the identification that TGF-β, platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) signaling pathways regulate MSC differentiation into adipogenic, osteogenic, and chondrogenic lineages [58, 59]. Adipogenic and osteogenic differentiation of MSCs were enhanced *in vitro* upon inhibition of TGF-β signaling but prevented chondrogenic differentiation. In contrast, inhibition of PDGF signaling decreased osteogenic differentiation, whereas inhibition of FGF receptor signaling completely blocked osteogenic differentiation and reduced chondrogenic differentiation. Moreover, inhibition of any one of these pathways decreased MSC proliferation. Differentiation thus depends substantially on the microenvironment [60].

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A key question regarding postnatal MSC function is the degree to which they participate in tissue homeostasis. For example, in the case of an osteogenic lineage, multiple investigators [61–63] have shown that exposure of MSCs to dexamethasone, β-glycerol phosphate, and ascorbic acid can lead to expression of alkaline phosphatase by the differentiated osteogenic cells with subsequent formation of a mineralized extracellular matrix [61]. Importantly, MSCs do retain the capacity for bone differentiation in vivo [38, 64]. For example, we have shown that subcutaneously implanting MSCs leads to osteoblast differentiation [38]. On the other hand, chondrogenic differentiation of MSCs can be achieved by treating MSCs with dexamethasone and TGF-β3 [58]. Similarly, dexamethasone together with insulin, indomethacin, and 1-methyl-3-isobutylxanthine can stimulate MSC differentiation into adipocytes, which express adipocyte-specific markers including peroxisome proliferator-activated receptor (PPAR)-γ [65].

Cao et al. [38] studied the regulation of MSC differentiation into adipocytes and osteoblasts with relation to PPAR-γ, an essential checkpoint regulator of the "adipogenesis-osteogenesis balance." The study showed that S-nitrosoglutathione reductase (GSNOR)-deficient mice have reduced adipogenesis and increased osteoblastogenesis compared to normal mice (**Figure 3**). Notably, GSNOR MSCs had improved differentiation capacity for bone and reduced propensity for adipocytes. This is due to higher levels of S-nitrosylated PPAR-γ protein with subsequent inhibition of its transcriptional activity, suggesting a negative feedback regulation by NO-mediated S-nitrosylation. In addition, S-nitrosylation of PPAR-y inhibits binding affinity to its downstream target fatty acid-binding protein 4 (FABP4) promoters (**Figure 4**). Importantly, the MSC differentiation affected the phenotype on the whole animal level. GSNOR deficient mice have lower body weight and fat mass, accompanied by elevated bone formation. In another study regarding osteogenic regulation, investigators found that modulation of specific microRNAs (-148b, -27a, and -489) plays a crucial role in MSC early osteogenic differentiation [66]. This has a tremendous corollary in bone diseases such as osteoporosis by providing both pathophysiological and therapeutic insights. Indeed, MSC differentiation into other cell lines of mesenchymal origin can offer further understanding into many other human disease processes, in support of future treatment strategies.

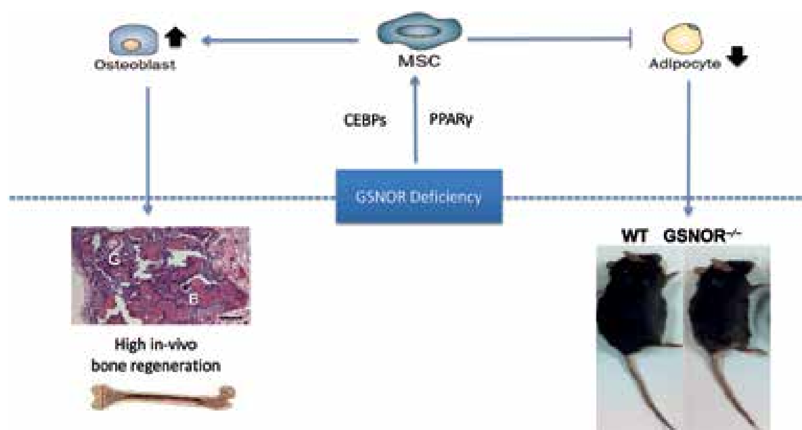

Figure 3. GSNOR deficient mice have reduced weight and body mass with increased bone formation.

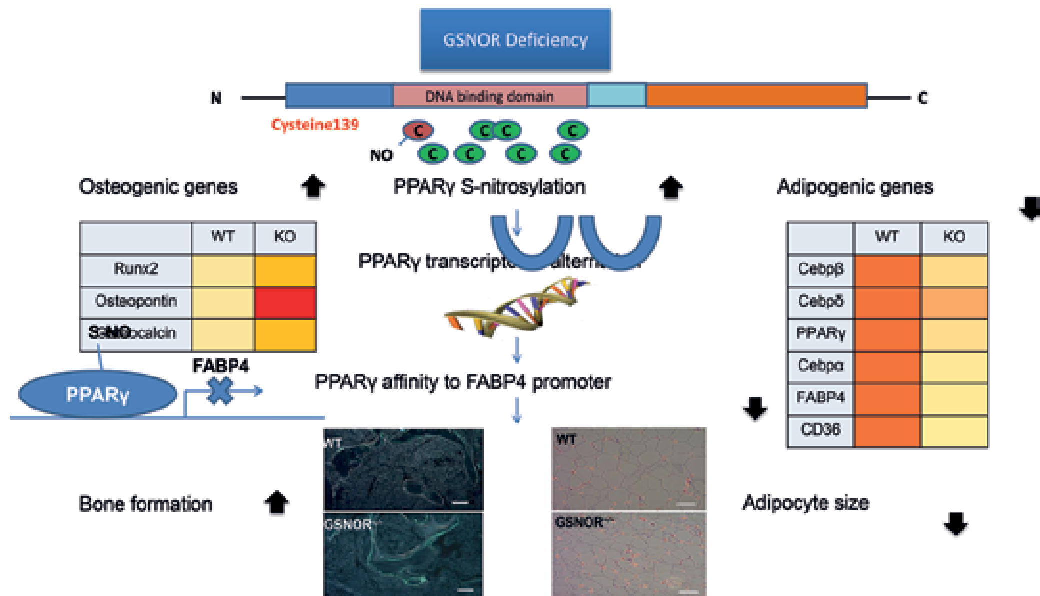

Figure 4. Regulation of adipogenesis-osteogenesis by MSCs. GSNOR deficiency with ensuing elevated levels of S-nitrosylated PPAR-γ leads to a decrease in PPAR- γ transcriptional activity and binding affinity to FABP4 promoter. This results in increased osteogenesis and decreased adipogenesis, which has strong implications in bone disease. *Reproduced from Cao Y et al., JCI, 2015.*

2.2. Cardiac differentiation

Cardiomyogenic differentiation of MSCs is of key interest for cardiac regenerative medicine, particularly ischemic and non-ischemic cardiomyopathy [40, 67, 68]. Treating MSCs with 5-azacytidine produces spontaneous, synchronous beating cells in culture with ventricular myocyte-like potentials, suggesting that MSCs are able to transdifferentiate into cardiomyocytes [43]. Alternative and potentially safer factors that induce differentiation into a cardiomyocyte phenotype include conditioned media containing bone morphogenetic protein-2 (BMP-2) and FGF-4 [69] as well as insulin, dexamethasone, and ascorbic acid [70]. The combination of these factors induces overexpression of cardiomyocyte-specific proteins, leading to cardiomyogenic differentiation for possible use in disease processes of injured myocardium [69–72]. Indeed, expression of myotubules, α-actinin, SERCA2 and other cardiac-related proteins in transdifferentiated cells may serve to attenuate cardiac infarct size and enhance perfusion, and regional function as suggested by early *in vivo* studies [73, 74]. Co-culture of mouse or rat MSCs with rat neonatal ventricular myocytes also stimulates MSC transdifferentiation into cardiomyocytes [75, 76]. The necessity of cell-to-cell contact [1, 75] versus secreted factors within the cardiac microenvironment [76] as a requirement for cardiomyogenic differentiation remains unclear.

MSC therapy promotes cardiomyogenesis not only by direct cardiomyocyte differentiation, but also by stimulating endogenous c-kit+ cardiac progenitors (CPCs) to proliferate, undergo lineage commitment, and form transient amplifying cells [1, 28, 29, 77–79]. We demonstrated

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that transendocardial injections of allogeneic MSCs in swine following myocardial infarction (MI) results in cardiogenic differentiation of MSCs accompanied by increased proliferation and enhanced lineage commitment of endogenous CPCs, and reconstitution of niche-like structures [1]. This stimulation of endogenous CPCs by MSCs requires a complex molecular interaction and is a crucial component of the beneficial cell therapeutic effects [1, 28, 29, 77–79]. Histologic examination revealed chimeric clusters (niches) comprised of adult cardiomyocytes, transplanted MSCs and CPCs expressing connexin-43 gap junctions, and N-cadherin mechanical connections between cells. These findings support the notion that MSCs act both as progenitors for certain cell lineages and through their participation in niches, as supporting cells for other lineages [80].

Stimulation of endogenous precursors may be a general mechanism underlying MSC bioactivity. We recently showed that in humans with endothelial dysfunction MSCs can trigger endogenous EPC activation increasing their number and functional quality [81]. Thus MSCs can serve as a powerful therapeutic tool by reconstituting endogenous stem cell niches as well as enabling and augmenting the reparative abilities of endogenous stem cells.

2.3. Anti-fibrotic and proangiogenic effects

The hypothesis that exogenously delivered stem cells would promote organ regeneration through transdifferentiation into tissue-specific cells sparked interest in stem cell research and cell-based therapy and was originally supported by studies in the heart [82] where MSCs become cardiomyocyte-like cells and endothelial cells [40, 41, 43]. However, subsequent studies have revealed that the MSC-mediated regenerative process is more complex than was initially envisioned, and that several mechanisms underlie the ability of MSCs to reduce scar size and improve left ventricular structure and function after myocardial injury [33, 83, 84]. MSCs engraft and persist for several months in myocardium when delivered by transendocardial injection [1, 33, 40] and they reduce cardiac fibrosis and promote neovascularization and cardiomyogenesis [40, 77, 85, 86]. Importantly, cardiac magnetic resonance imaging (MRI) documented a reduction of infarct size, improvement in left ventricular shape (measured as sphericity index of the left ventricle), and improvement in tissue perfusion and regional contractility [87]. Together, these preclinical studies support the anti-fibrotic and proangiogenic role of MSCs in the repair of the injured myocardium.

2.4. Immunomodulatory, anti-inflammatory, and anti-microbial effects

Preclinical studies have demonstrated that MSCs can differentiate into cardiomyocytes and/or vascular structures in both allogeneic [1, 40, 87] and xenotransplantation [88] models, contributing to cardiac functional improvement and reduction of infarct size. Remarkably, there has been no evidence of rejection in animals subjected to allogeneic transplantation of MSCs [1, 29, 40, 87]. These studies reveal that allogeneic MSCs represent a unique cell population for cellular therapy due to their anti-proliferative, immunomodulatory, and anti-inflammatory effects [2, 33, 89]. The absence of major histocompatibility class (MHC) II antigens [90–92] and the secretion of T helper type 2 cytokines characterize MSCs as both immunoprivileged and immunosuppressive [2, 92–94]. MSCs fail to induce proliferation

of allogeneic lymphocytes *in vitro* [90, 92], and suppress proliferation of T cells activated by allogeneic cells or mitogens [91]. This immunomodulatory capacity supports the feasibility of using allogeneic MSCs for cardiovascular regeneration as well as other clinical applications [2, 95]. Furthermore, MSCs have been used to treat severe graft-vs-host disease (GVHD) [13, 96], decreasing the potential of graft rejection and/or GVHD, and supporting the concept that MSCs are a unique cell population for regenerative medicine with minimal immune reactivity. Allogeneic MSCs have proven both safe and effective [5, 7, 11, 29, 89], highlighting that MSCs engrafted in the cardiac tissue despite potential HLA mismatching. An advantage of allogeneic MSCs is their potential use as an "off-the-shelf" therapeutic agent, precluding the need to obtain and expand bone marrow or another tissue source from the patient, and providing consistency to the cell product [97]. In addition, autologous cells may have functional deficiencies due to the underlying diseases, co-morbidities, lifestyle, concomitant medications, or age [98–105]. Although allogeneic MSCs may be cleared more rapidly than autologous cells after differentiation [106], immunologic clearance might also offer the advantage of reducing any long-term risks of cell implantation [8, 94, 107].

An important concern, and common exclusion criteria for participation in clinical trials is that the potential immunosuppressive effect of MSCs may lead to an increased risk of infection in patients who are already immunosuppressed due to medical therapy or concurrent chronic disease. In this regard, recent data has shown that MSCs exert significant anti-microbial effects through both direct and indirect mechanisms [108]. Indirect mechanisms include regulation of macrophages, neutrophils, phagocytes, and another pro- and anti-inflammatory cells of the immune system, whereas indirect mechanisms involve the secretion of anti-microbial peptides and proteins (AMPs) and the expression of indoleamine 2,3-dioxygenase, interleukin-17, and other molecules [94, 108]. Indeed, the anti-microbial effects of MSCs have been demonstrated in preclinical studies of sepsis, acute respiratory distress syndrome, and cystic fibrosis-related infections [108].

2.5. Enhancement of MSC therapy

Therapeutic interventions to optimize MSC function, such as growth factor administration [109–112], gene therapy [110], and modulation with small molecules or other pharmacologic approaches [110] are promising options under preclinical and clinical investigation to potentiate myocardial repair and regenerative capacity. For example, in the phase I cardiopoietic stem cell therapy in heart failure (C-CURE) trial and subsequent phase II/III congestive heart failure cardiopoietic regenerative therapy (CHART-1) study [72, 109, 113], autologous bone marrow-derived MSCs from patients with ischemic cardiomyopathy were treated ex-vivo with a cardiogenic cytokine cocktail to enhance their cardiac lineage commitment. In C-CURE, the authors reported significant improvement in cardiac function, physical performance, hospitalization, and event-free survival in the cell therapy group compared to controls [109]. However, the larger CHART-1 trial reported neutral results at 39 weeks of follow up with regards to composite and individual outcomes, including all-cause mortality, heart failure events, and surrogate cardiac structural and functional endpoints [113]. A sub-analysis of the CHART-1 study extended the follow-up period to 52 weeks at which point the anti-remodeling properties of the cardiopoietic MSCs became evident [72]. These findings are consistent with those of other clinical trials of MSC-based therapy for ischemic cardiomyopathy [7, 9, 114].

A potential approach to improve therapeutic potential is the combination of MSCs with c-kit+ CSCs [28, 29, 79]. Using a porcine model of chronic ischemic cardiomyopathy, the combination of autologous or allogeneic swine MSCs and c-kit+ CSCs provides greater reverse remodeling, scar size reduction, and functional improvements than MSCs alone [29, 79]. The demonstrated safety of cell-based therapy using MSCs [7, 9, 115, 116] and c-kit+ CSCs [117, 118] in patients with ischemic cardiomyopathy combined with these preclinical findings revealed important biological interactions between these two stem cell types that enhance therapeutic responses and led to the initiation of the Cardiovascular Cell Therapy Research Network (CCTRN)-sponsored, Combination of Mesenchymal and C-kit+ Cardiac Stem Cells as Regenerative Therapy for Heart Failure (CONCERT-HF; NCT02501811) clinical trial.

2.6. MSC senescence and potential malignant transformation

There is evidence that senescence impairs the capacity of MSCs for multi-lineage differentiation, homing, immune modulation and wound healing [102, 103]. As stem cells age, they undergo a "quiescence-to-senescence switch" that impairs their function [102, 104, 119, 120] (**Figure 5**). The mechanisms underlying the age-related declines in stem cell function involve intrinsic aging as well as age-related changes in their tissue microenvironment, including extracellular matrix components and the stem cell niche [101, 104, 121], thereby adversely impacting self-renewal and therapeutic potential. This has implications when considering the age and comorbidities of patients and donors. For example, dysfunctional stem cell niches

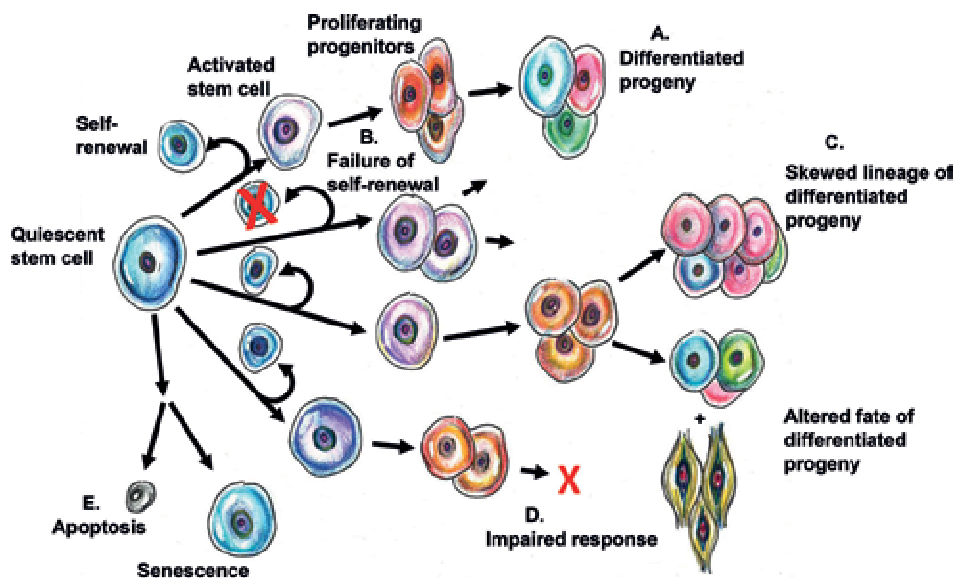

Figure 5. Proposed mechanisms of aging-induced stem cell dysfunction. (A). Normal stem cell function involves activation of a quiescent stem cell to divide asymmetrically giving rise to a new stem cell (self-renewal) and another daughter cell that undergoes proliferation and differentiation. (B). Failure of self-renewal involves differentiation of both daughter cells, leading to a gradual depletion of the stem cell pool. (C). Aberrant differentiation may result from the abnormal skewing of the distribution of progeny toward one fate instead of various potential fates. Another potential mechanism involves the daughter cells acquiring abnormal fates that are not part of the normal repertoire. (D). Impaired stem cell response may be due to a decline or impairment in extrinsic or intrinsic signals. (E). Senescence and apoptosis of the quiescent stem cell or among the progeny following activation has also been described in aging. Adapted from Jones DL et al., Nature Cell Biology, 2011.

have been implicated in the aging frailty syndrome, which is characterized by decreased strength, endurance, physiologic function, and reserve capacity in multiple organ systems [122, 123]. Moreover, aging, renal failure, C-reactive protein (CRP) levels, and other adverse health parameters have been shown to correlate significantly with poor angiogenic potency of bone marrow stem cells [105, 124]. These studies suggest that the therapeutic potential of autologous MSCs obtained from patients may be limited, whereas more robust repair and regeneration would occur by using allogeneic MSCs from young, healthy donors. Indeed, two clinical trials in patients with ischemic and dilated cardiomyopathy, respectively, compared autologous to allogeneic MSCs and found that although both provided benefits in cardiac structural endpoints, the allogeneic MSCs provided greater cardiovascular functional benefits [5, 7, 81]. On the other hand, a study on the impact of recipient age on the efficacy of MSC therapy found that older (>60 years of age) patients responded just as effectively as younger (<60 years of age) patients when administered either autologous or allogeneic MSC therapy for chronic ischemic cardiomyopathy [125]. This finding is highly significant since the majority of the population with cardiovascular disease requiring cell-based therapy is aged.

Although the evidence is conflicting [126–130], clinical trials of MSC therapy usually exclude patients with a history of cancer due to concerns regarding the MSCs' potential for carcinogenesis. It remains unclear whether MSCs have the potential to undergo spontaneous malignant transformation and/or whether they interact with surrounding tumor stromal elements [129–131]. Spontaneous malignant transformation of human bone marrow-derived MSCs has been shown in vitro during long-term cultures [127]. These MSCs underwent faster proliferation, failed to undergo complete differentiation, and exhibited altered morphology and phenotype. Moreover, when these altered MSCs were administered to immunodeficient mice rapid-growing tumors throughout the lung tissue were found. On the other hand, in a separate study [128], human bone marrow-derived MSCs were grown in culture and assessed at different time points for expression of various tumor-related proteins until they reached senescence or passage 25. A progressive decrease in proliferative capacity with shortened telomeres was observed in most cultured MSCs until they reached senescence. In addition, the MSCs did not express telomerase activity or telomerase reverse transcriptase transcripts, and no chromosomal abnormalities or alternative lengthening of telomeres were observed, supporting the safety of in vitro MSC expansion, and therapeutic use. Despite these encouraging findings, the functional, phenotypic, and genetic characterization of culture-expanded MSCs merits further careful study [129, 131, 132]. In addition, recent findings indicate that various direct (e.g., cell fusion) and indirect (e.g., exosome or vesicle-mediated) interactions between MSCs and cancer cells can produce functional interference and/or mutual acquisition of new cellular properties [130]. These functional and phenotypic cellular alterations can lead to changes in metastatic behavior and induce new cancer stem cell development. On the other hand, exosomes and vesicle-mediated mechanisms may be a promising therapeutic tool against cancer.

2.7. Sex differences in MSCs

Sex differences exist in many disease states as well as with respect to the role of MSCs in organ repair and regeneration after injury. There is evidence that female MSCs exhibit decreased apoptosis, interleukin-6, and tumor necrosis factor and increased endothelial growth factor and vascular endothelial growth factor expression compared to male donor MSCs [133].

Furthermore, in a mouse model of myocardial infarction, treatment with female MSCs produced greater improvement of cardiac functional endpoints than treatment with male MSCs [134]. Estradiol has been shown to contribute to these differences [135, 136]. A more complete understanding of how MSCs are influenced by donor sex and recipient hormonal environment is needed to address sex-related disparities in clinical outcomes as well as to optimize transplanted MSC function and survival.

3. MSCs as a regenerative therapeutic for cardiovascular diseases

The hypothesis that exogenously delivered stem cells would promote organ regeneration through transdifferentiation into tissue-specific cells sparked interest in stem cell research and cell-based therapy and was originally supported by studies in the heart [82] where MSCs become cardiomyocyte-like cells and endothelial cells [41, 43]. However, subsequent studies have revealed that the MSC-mediated cardiac regenerative process is more complex than was initially envisioned (**Figure 6**).

3.1. Clinical trials in cardiac disease

Multiple clinical trials suggest that MSCs can ameliorate left ventricular remodeling and improve cardiac function in patients with acute and chronic ischemic cardiomyopathy [7, 9, 11, 72, 84, 115, 116, 137–141]. The Transendocardial mesenchymal stem cells and mononuclear bone marrow cells for ischemic cardiomyopathy (TAC-HFT) trial demonstrated reverse remodeling and improved regional contractility of the scar as well as improved functional capacity and quality of life over 1 year in patients with chronic ischemic cardiomyopathy treated with transendocardial injection of autologous bone marrow-derived MSCs versus bone marrow mononuclear cells or placebo [9, 142]. The mesenchymal stromal cells in chronic ischemic Heart Failure (MSC-HF) trial showed that intramyocardial injection of autologous bone marrow-derived MSCs in patients with severe ischemic cardiomyopathy improved ventricular function and myocardial mass [140]. The same group showed that intramyocardial delivery of autologous MSCs into patients with coronary heart disease and refractory angina provided a sustained effect (3-year follow-up) in improving exercise capacity and ventricular function, and reducing hospitalization rates and revascularizations [143]. As mentioned previously, the CHART-1 study also demonstrated the anti-remodeling properties of cardiopoietic MSCs at the 1-year follow-up [72]. Encouraging results from preclinical studies with combination therapy [28, 79] have led to the initiation of the CONCERT-HF (NCT02501811) trial by the Cardiovascular Cell Therapy Research Network (CCTRN) in an effort to examine the effects of the transendocardial delivery of a combination of autologous bone marrow-derived MSCs and cardiac progenitor cells into patients with ischemic cardiomyopathy.

Autologous adipose tissue-derived MSCs are also undergoing investigation in the cardiovascular field. The adipose-derived stromal cells for treatment of patients with chronic ischemic heart disease (MyStromalCell) trial was a phase II, first-in-man, single-center, double-blind, randomized, and placebo-controlled study of intramyocardial injections of autologous adipose-derived MSCs in patients with chronic ischemic heart disease and refractory angina but preserved ejection fraction [111, 112]. The MSCs were obtained from abdominal adipose tissue,

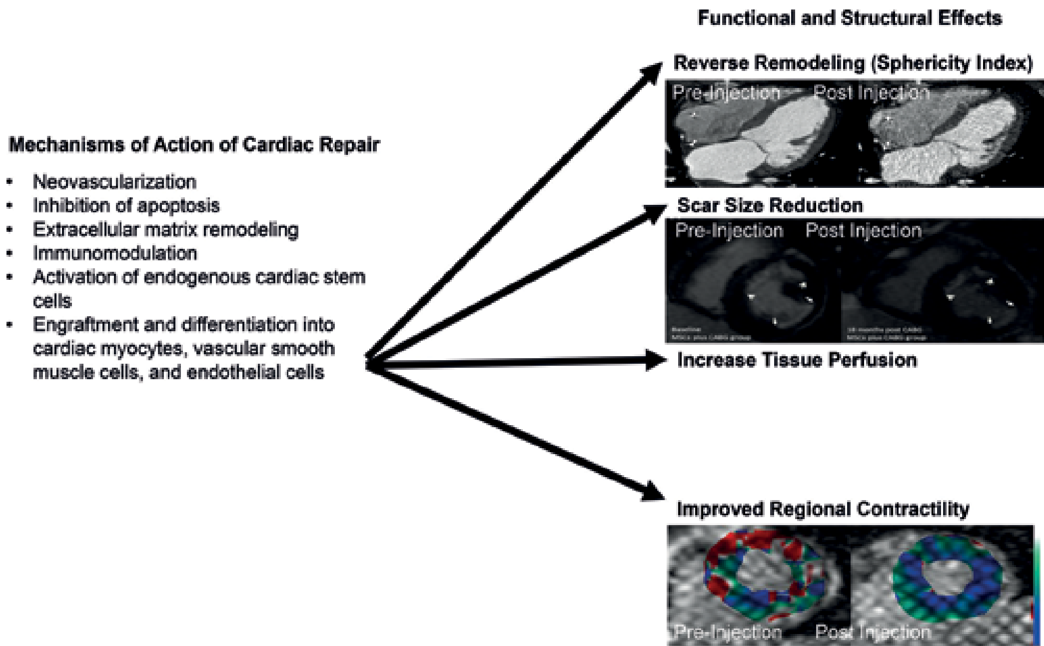

Figure 6. Effects Of mesenchymal stem cell therapy in heart disease.

culture-expanded in vitro and stimulated with vascular endothelial growth factor-A (VEGF-A) (165) the week before treatment. The six month follow-up results demonstrated safety, and although a significant increase in exercise capacity was observed in the patients treated with the MSCs but not with placebo, there was no statistically significant difference between the MSC and placebo treatment groups.

An important issue in this new field is whether MSCs can be used as an allograft [5, 7, 89], avoiding the need for bone marrow aspiration of patients and tissue culture delays prior to treatment. Furthermore, the function of autologous MSCs may be impaired in patients with comorbidities and/or advanced age [101–104]. A meta-analysis of 82 preclinical studies [144] demonstrated that allogeneic therapy is safe and at least as effective as autologous MSC therapy, suggesting that allogeneic MSCs are characteristically immunomodulatory, as discussed above.

The therapeutic benefit of allogeneic MSCs versus placebo delivered intravenously has been investigated in patients after acute MI [11, 145, 146]. Not only did these results show the safety of allogeneic MSC delivery to humans, but also moreover, echocardiography demonstrated a 6% increase in ejection fraction at 3 months for patients treated with MSCs. Moreover, the percutaneous stem cell injection delivery effects on neo-myogenesis (POSEIDON) trial compared allogeneic vs. autologous MSCs delivered by transendocardial stem cell injection in patients with chronic ischemic cardiomyopathy and showed that both MSC types are safe and clinically effective [7, 147]. Similarly, the percutaneous stem cell injection delivery effects on neo-myogenesis – dilated cardiomyopathy (POSEIDON-DCM) trial demonstrated safety and efficacy of transendocardial autologous vs. allogeneic MSC therapy in patients with non-ischemic, dilated cardiomyopathy, with a cardiac function efficacy preference toward allogeneic MSCs [5].

The transendocardial stem cell injection delivery effects on neomyogenesis study (TRIDENT) trial compared the safety and efficacy of two doses (20 million and 100 million) of allogeneic bone marrow-derived human MSCs delivered transendocardially in patients with ischemic cardiomyopathy [116]. Although both cell doses reduced scar size, only the 100 million doses increased LVEF, highlighting the crucial role of cell dose in the responses to cell therapy. In phase 2 dose-escalation study investigating immunoselected (Stro-1/Stro-3+ enriched), allogeneic bone marrow-derived MPCs (25, 75, and 150 million cells) delivered transendocardially in patients with ischemic and non-ischemic heart failure, no differences were observed in LVEF at 12 months of follow-up, although the 150 million MPC group had a significant reduction in left ventricular end-systolic and end-diastolic volumes, a measure of reverse remodeling, at 6 months and a non-significant decrease of both ventricular volumes at 12 months [56]. These and other ongoing studies determining the optimal dose and delivery are essential to advance the field, decipher mechanism(s) of action, and enhance planning of pivotal Phase III trials [148–152].

A recent trial assessed the safety and preliminary efficacy of intravenously administered, allogeneic, ischemia-tolerant MSCs in patients with non-ischemic cardiomyopathy [153]. Ischemia-tolerant MSCs are grown under chronic hypoxic conditions and have been shown to better migrate toward wound healing-related cytokines and cytokines found in ischemic tissues and express higher levels of hypoxia-inducible factor-1 [154]. These studies suggested that ischemia-tolerant MSCs may be therapeutically more effective than MSCs grown under normoxic conditions. An increase in LVEF and reductions in end-systolic and end-diastolic volumes were observed at three months of follow up in the treated group but was not significantly different from the placebo group. Functional capacity and health status were significantly improved in the MSC treated group compared to placebo.

MSCs derived from umbilical cord (UC-MSCs) have also been tested in patients with heart failure. The randomized clinical trial of intravenous infusion umbilical cord mesenchymal stem cells on cardiopathy (RIMECARD) trial is a randomized, double-blind, placebo-controlled trial that evaluated the safety and efficacy of UC-MSCs administered intravenously in patients with heart failure of ischemic or non-ischemic origin [141]. Infusion of allogeneic UC-MSCs was safe, with no development of alloantigen directed antibodies post-infusion, and effective in improving LVEF, functional status, and quality of life. Intramyocardial delivery of UC-MSCs in patients with heart failure has also been shown to produce improvements in LVEF and end-systolic volume in patients with severe heart failure [155].

Ongoing clinical trials are assessing the safety and efficacy of allogeneic MSC therapy in patients with acute myocardial infarction, chronic ischemic and non-ischemic cardiomyopathy, and left ventricular assist devices. These studies will continue to pave the way for the development of allogeneic cell-based regenerative therapies for structural and functional disorders of the myocardium. The results from cardiovascular stem cell clinical trials are so far promising, with recent trials highlighting the vast therapeutic potential of allogeneic over autologous stem cells. However, many challenges remain, such as addressing long-term safety, serial stem cell injections, and optimal cell type, dose, and delivery route [148–152].

3.2. Vascular disease

Endothelial dysfunction is characterized by impaired endothelial vasodilation, a proinflammatory and prothrombotic state, and impaired bioactivity of EPCs and contributes to the

pathophysiology of most forms of cardiovascular disease, including hypertension, coronary artery disease, heart failure, peripheral vascular disease, kidney disease, diabetes mellitus, and metabolic syndrome [156, 157]. Endothelial function is implicated in heart failure [158] and we have studied the therapeutic potential of MSCs in restoring endothelial function in patients with ischemic and non-ischemic cardiomyopathy [81]. As mentioned above, individuals with heart failure received either autologous or allogeneic MSCs, and those in the allogeneic MSC group exhibit increased EPC colony formation and improved flow-mediated vasodilation (FMD), both of which strongly correlate with improved endothelial function [158, 159] (**Figure 7**). Moreover, patients who received allogeneic MSCs had reduced levels of VEGF. Elevated VEGF is associated with heart failure progression [160]. The concordant restitution of these parameters to near normal after allogeneic MSC therapy has significant clinical implications for the heart failure population and may play a critical role in the advancement of cardiovascular treatment modalities.

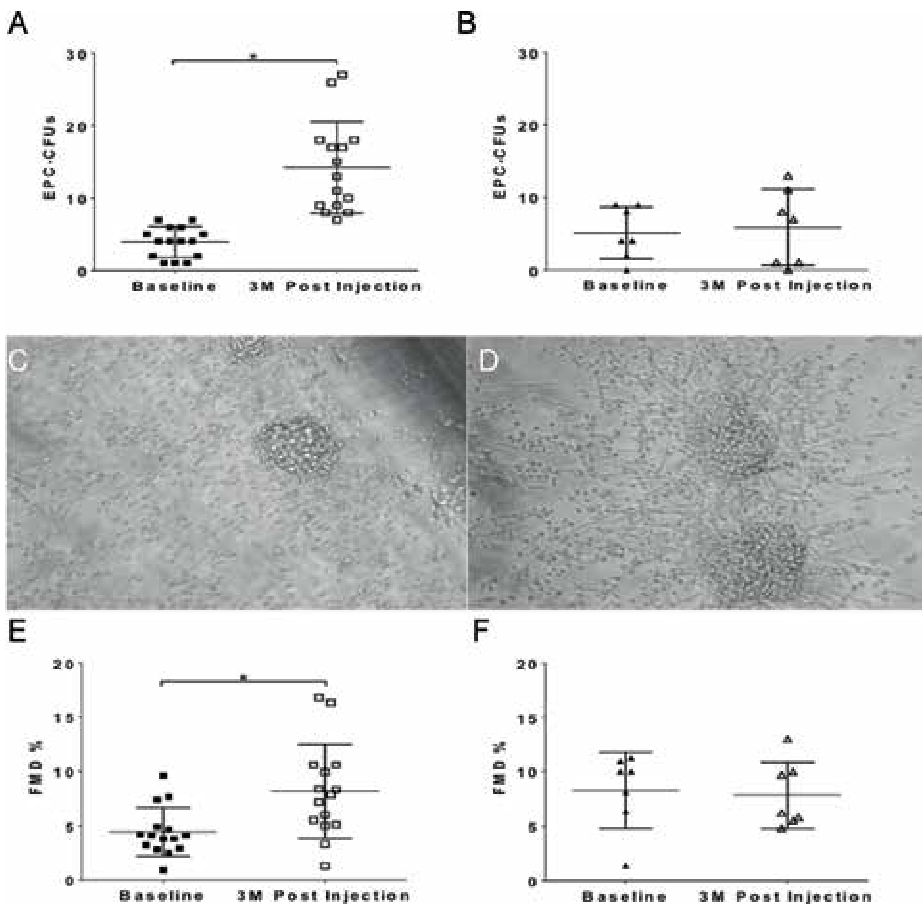

Figure 7. MSCs in vascular disease. Allogeneic mesenchymal stem cell therapy can help restore endothelial function in patients with cardiomyopathy by increasing EPC CFUs (A) and improving FMD (E) when compared to autologous therapy (B and F). Representative EPC-CFUs plated on fibronectin for 5 days before (C) and after (D) allogeneic MSC administration (magnification 20x). *Reproduced from Premer C et al., EBioMed, 2015.*

It is well established that cardiovascular disease is the leading cause of death and disability among people with type 2 diabetes mellitus [161] and has long been appreciated that endothelial dysfunction underlies the high rates of cardiovascular disease associated with long-term diabetes [162]. The persistent hyperglycemia and other metabolic abnormalities directly affect the endothelium, contributing to the pathophysiology of disease [163]. Based on our findings of improved endothelial function after allogeneic MSC treatment in patients with heart failure [81], we are conducting a clinical trial entitled, Allogeneic Mesenchymal Human Stem Cells Infusion Therapy for Endothelial Dysfunction in Diabetic Subjects (ACESO; NCT02886884) to investigate whether intravenously delivered MSCs restore endothelial function parameters, including FMD and EPC function, as well as decrease circulating inflammatory markers and improve clinical parameters of diabetes. Similarly, the Intravenous Infusion of Umbilical Cord Tissue (UC) Derived Mesenchymal Stem Cells (MSCs) Versus Bone Marrow (BM) Derived MSCs to Evaluate Cytokine Suppression in Patients With Chronic Inflammation Due to Metabolic Syndrome (CERES; NCT03059355) trial is testing MSC therapies to restore endothelial function.

Peripheral artery disease is generally caused by atherosclerosis in which cholesterol plaque builds up, ultimately weakening blood vessel walls and restricting blood flow, severely impairing endothelial function. The evaluation of cell therapy on exercise performance and limb perfusion in peripheral artery disease: The CCTRN patients with intermittent claudication injected with ALDH bright cells (PACE) Trial demonstrated safety but no improvement in peak walking time or capillary perfusion [164]. In patients with complete occlusion of femoral arteries, a post-hoc exploratory analysis suggested an improvement in the number of collateral arteries. Future clinical trials testing different cell types, doses, and administration routes are needed to optimize peripheral artery disease treatment.

4. MSCs as immunomodulatory, anti-Inflammatory, anti-fibrotic, and anti-rejection therapy

MSCs exhibit immune-privileged properties in vitro and in vivo [165] likely due to the absence of MHC II, B-7 costimulatory molecule, and CD40 ligand [90–92, 166] (**Figure 8**). The lack of costimulatory molecules prevents T-cell responses and also induces an immunosuppressive local microenvironment through the production of prostaglandins and other soluble mediators including nitric oxide, indoleamine 2,3-dioxygenase, and heme oxygenase-1 [92, 167–170]. MSCs reduce the respiratory burst that follows neutrophilic responses by releasing interleukin (IL)-6 [171]. They also inhibit the differentiation of immature monocytes into dendritic cells hence the antigen presentation to naïve T cells is greatly impaired [172]. In addition, MSCs release soluble factors, such as hepatocyte growth factor and transforming growth factor (TGF)-β1 [173], that suppress the proliferation of cytotoxic and helper T-(Th) cells. MSCs also stimulate Foxp3+ regulatory T cells with concurrent suppression of Th1, Th2, or Th17 responses [174]. These findings suggest that MSCs are an effective therapeutic strategy to induce tolerance in solid organ transplantation [175].

4.1. Transplantation

Le Blanc *et al.* first reported the clinical immunoregulatory response to MSCs in a case of severe, treatment-resistant grade IV acute graft-vs-host disease (GVHD) [13]. A multicenter phase 2 trials for steroid-resistant, severe acute GVHD confirmed this observation [12] and MSCs obtained from HLA-identical siblings, haploidentical third-party donors, or HLA-mismatched third-party donors were similarly effective. Recently, infusion of MSCs the day of hematopoietic cell transplantation (HCT) promotes engraftment and improves outcomes. A pilot study of allogeneic MSC infusion before nonmyeloablative HCT from HLA-mismatched donors showed sustained engraftment in 19 out of 20 patients, and the 1 year incidence of nonrelapse mortality, relapse, overall survival, progression-free survival, and death from GVHD was favorable compared to a historic control group [176]. In another pilot study evaluated the effect of infusion of MSCs at the time of dual transplant of cord blood and third-party donor mobilized hematopoietic stem cells regarding tolerance, cord blood engraftment, and effects on acute GVHD, both preventive and therapeutic [177]. MSC infusions were effective for treating severe acute GVHD, but no significant differences in cord blood engraftment and incidence of severe acute GVHD were observed. Although there is accumulating evidence of safety from these small pilot studies [96], randomized trials are necessary to establish efficacy.

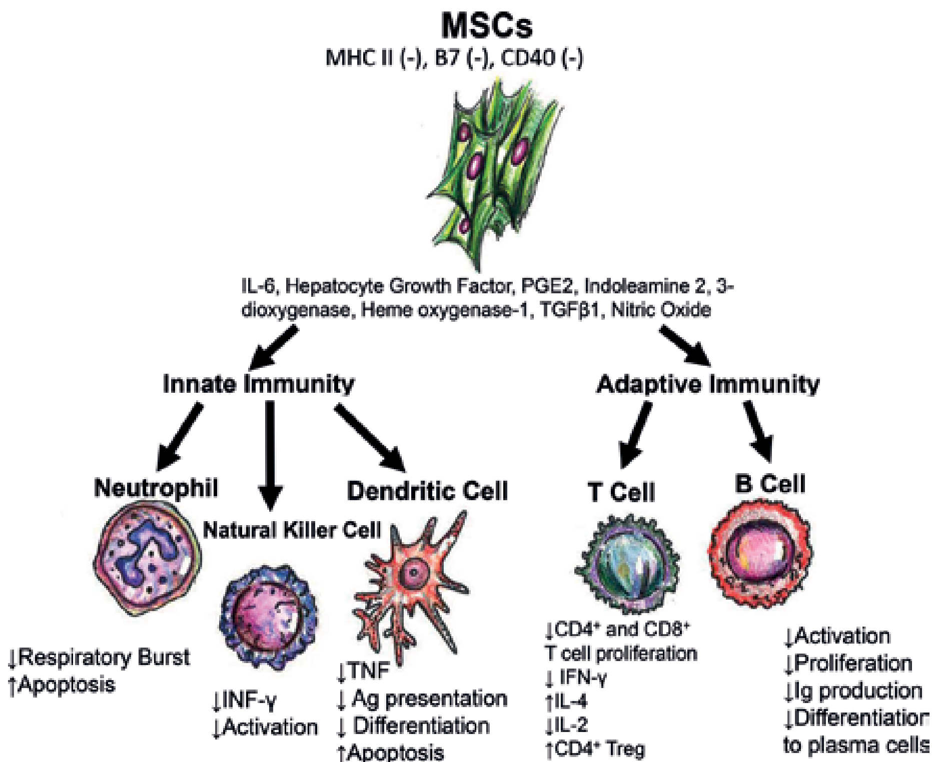

Figure 8. Immunomodulatory effects of mesenchymal stem cells. MSCs are immunoprivileged cells that inhibit both innate (neutrophils, dendritic cells, and natural killer cells) and adaptive (T cells and B cells) immune cells.

A single-site, open-label, randomized controlled clinical trial in 159 patients undergoing living-related donor kidney transplantation showed that induction therapy with autologous MSCs resulted in lower incidence of acute rejection, decreased the risk of opportunistic infection, and better estimated renal graft function at 6 months compared with anti-IL-2 receptor antibody induction therapy [16]. However, graft function and rejection rates were similar after 1 year [178]. Therefore, MSC therapy can safely replace induction immunotherapy, reducing opportunistic infections, without compromising graft function and survival [179].

Despite these encouraging results, the long-term safety of MSC transplants needs to be further investigated in chronically immunosuppressed patients that are at increased risk for opportunistic infections and tumors [132, 180]. In this regard, a clinical trial evaluated the safety and tolerability of third party MSC administration after liver transplantation. Patients enrolled in the experimental arm were infused with a single dose of 1.5 million MSCs/kg, 3(±2) days after the liver transplantation [181]. There was no impairment in liver transplant function and no increased rate of opportunistic infection or new cancer detected following MSC infusion. In addition, there was no difference in overall rates of rejection or graft survival. Weaning of immunosuppression in MSC recipients was not successful.

Issues needing further investigation include dose, timing and site of administration, interaction with immunosuppressive drugs, and whether MSCs are effective at preventing acute rejection and/or inducing tolerance. In a murine kidney transplant model, it was shown that MSC administration before (day -1) but not a few days after kidney transplantation avoided the acute deterioration of graft function while maintaining the immunomodulatory effect of MSCs [182]. Moreover, a clinical study found that autologous bone marrow-derived MSC infusion at day 7 post-kidney transplant induced acute kidney graft dysfunction, attributed to engraftment syndrome [183], although MSC infusion was associated with lower memory/effector CD8+ T cells, expansion of CD4+ regulatory T cells, and reduction of donor-specific CD8+ T-cell cytotoxicity compared with control kidney transplant recipients given the same induction therapy (basiliximab/low dose thymoglobulin) but not MSCs [184].

Islet cell transplantation combined with MSC therapy for type 1 diabetes in a cynomolgus monkey model provides clinical evidence for the anti-rejection effect of MSCs [185]. MSC treatment significantly enhanced islet engraftment and functions one month post-transplant, compared with animals receiving islets without MSCs. In addition, infusions of donor or third-party MSCs resulted in a reversal of rejection episodes and prolongation of islet function. Stable islet allograft function was associated with increased numbers of regulatory T cells in peripheral blood, suggesting that MSCs enhance islet engraftment, thereby decreasing the numbers of islets needed to achieve insulin independence.

4.2. Autoimmune diseases

Autologous MSC transplantation evaluated in clinical trials of amyotrophic lateral sclerosis [18] and multiple sclerosis [17, 186] is safe and associated with increased proportion of $CD4^+$ $CD25^+$ regulatory T cells, decreased proliferative responses of lymphocytes, and lower expression of co-stimulatory molecules ($CD40^+$, $CD83^+$, and $CD86^+$), and HLA-DR on myeloid dendritic cells within 24 hours of transplantation [17]. In a randomized, placebo-controlled, phase 2 trial of multiple

sclerosis, bone marrow-derived MSCs were also found to reduce inflammatory MRI parameters, supporting their anti-inflammatory and immunomodulatory properties [187]. Moreover, autologous and allogeneic MSC therapy showed evidence of benefit in other autoimmune disorders such as refractory Crohn's disease [188–191] and systemic lupus erythematosus [14, 192, 193], respectively. Although there are no clinical trial results in patients with rheumatoid arthritis (clinical trials are ongoing; NCT01851070), in vitro studies show that allogeneic MSCs or MSC-differentiated chondrocytes inhibit the proliferation and activation of collagen type II-stimulated T-cells and the secretion of proinflammatory cytokines, including IFN-gamma and TNF-alpha by CD4+ and CD8+ T cells, while increasing the secretion of IL-10 and restoring the secretion of IL-4 [194, 195]. These results suggest that the immunomodulatory and anti-inflammatory effects of MSCs offers an effective therapeutic modality for arthritic diseases [195], and several clinical trials are ongoing evaluating bone marrow, adipose, and UC-derived MSCs.

Transplanted MSCs exert a protective effect in type 1 diabetes mellitus [196]. MSCs localize to the pancreas after intravenous transplantation and lower blood sugar levels [197], similar to MSCs isolated from the Wharton's jelly of the umbilical cord, which differentiated into mature islet-like cell clusters and possessed insulin-producing ability in vitro and in vivo [198]. Transplanted MSCs lower blood sugar through secretion of trophic cytokines that promote endogenous pancreatic stem cells in the ductal epithelium to differentiate into new ß-cells and directly differentiate into functionally competent, new ß-cells [199]. Furthermore, MSCs produce a variety of cytokines and growth factors, which could promote survival of surrounding cells and improve the microenvironment of pancreas [200]. Based on these findings, clinical trials have been initiated to test safety and therapeutic efficacy. A pilot, randomized, controlled, and open-label trial investigated the potential benefits on metabolic control and safety of combined umbilical cord-derived MSCs and autologous bone marrow mononuclear cell transplantation without immunotherapy in patients with established type 1 diabetes [201]. The treatment was not only well tolerated, but at 1 year, metabolic measures, including hemoglobin A1C, fasting glycemia, and daily insulin requirements, improved in the treated patients, whereas it decreased in control subjects. In another clinical study, treatment with a single intravenous infusion of autologous MSCs was tested in new-onset type 1 diabetic patients and found to be safe and to show benefit in slowing disease progression and preserving β-cell function [202].

4.3. Pulmonary diseases

A recent randomized, double-blinded, placebo-controlled study demonstrated the safety of systemic administration of allogeneic MSCs in patients with moderate to severe chronic obstructive pulmonary disease (COPD) [15], however, there were no differences in the frequency of COPD exacerbations, pulmonary function tests, or quality of life after 2 years of follow up. A significant decrease in levels of circulating C-reactive protein (CRP) was observed in MSC-treated patients who had elevated CRP levels at study entry, suggesting a beneficial effect of MSC infusion on systemic inflammation [15].

Idiopathic Pulmonary Fibrosis (IPF) is a lung disease characterized by progressive interstitial fibrosis leading to hypoxemic respiratory failure for which no effective treatment exists [203]. Histologically, there is evidence of alveolar epithelial cell injury, interstitial inflammation,

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fibroblast proliferation, and extracellular matrix collagen deposition. Because MSCs home to sites of injury, inhibit inflammation and contribute to epithelial tissue repair, they offer a potential therapy for IPF [203]. The phase 1 clinical trial entitled allogeneic human mesenchymal stem cells in patients with IPF via intravenous delivery (AETHER) demonstrated the safety of bone marrow-derived MSCs in nine patients with mild to moderate IPF [10]. A 3.0% mean decline in percent predicted forced vital capacity, and 5.4% mean decline in percent predicted diffusing capacity of the lungs for carbon monoxide was observed by 60 weeks post-MSC infusion, suggesting potential for efficacy.

Of note, a study has provided evidence of a resident c-kit+ multi-potent stem cell in the human lung [204]. These lung c-kit+ stem cells were shown to have the capacity to develop into bronchioles, alveoli, and pulmonary vessels, supporting the notion that they play an important role in lung homeostasis and tissue regeneration after injury. Although the therapeutic implications of these findings have not been investigated, we can infer from findings in ischemic heart disease models that there is the potential for MSCs to stimulate endogenous c-kit+ lung stem cell proliferation and differentiation, thereby facilitating lung tissue repair and regeneration.

4.4. Cutaneous wounds

Chronic, non-healing cutaneous wounds are a major cause of morbidity. The ability of MSCs to differentiate into various cell types and their capacity to secrete factors important in accelerating wound healing have made cell therapy a promising strategy for tissue repair and regeneration [24, 205]. Although both autologous and allogeneic MSCs appear to be well suited as wound healing therapies, allogeneic MSCs derived from young healthy donors may have an advantage over autologous sources where age and systemic comorbidities, such as diabetes, chronic renal failure, and arterial or venous insufficiency, are a contributing factor. The effects of aging and systemic illness on MSCs include impaired cell migration, reduced growth factor production, and poor tissue remodeling [24]. A study evaluated MSCs and fibroblasts derived from normal donors and chronic wound patients to characterize the induction of mobilization when these cells are mixed as well as examine the effect of soluble factors on fibroblast migration [206]. These studies showed that MSCs participate in skin wound closure by affecting dermal fibroblast migration in a dose-dependent manner, but impairments were noted in chronic wound patient fibroblasts and MSCs as compared with those derived from normal donors. These results support the notion that allogeneic MSCs from "healthy" donors provide greater efficacy for wound healing compared to autologous MSCs. Such promising findings have supported the use of MSCs in animal models of burn wound healing [207–209]. Consequently, a clinical trial entitled "Stem Cell Therapy to Improve Burn Wound Healing" (NCT02104713) is currently underway and is examining the efficacy of allogeneic MSCs in burn wound closure for patients with a 2nd degree burn wounds of less than 20% total body surface area.

4.5. Neurological diseases

MSCs are also considered a promising therapeutic strategy for acute injury and progressive degenerative diseases of the central nervous system [210], such as spinal cord injury [211, 212] ischemic stroke [21, 22, 213, 214] Parkinson's disease [215, 216] traumatic brain injury [217, 218] multiple sclerosis [17, 186, 219, 220] and multiple system atrophy [23]. Studies suggest that the

neuroprotective effect of MSCs is mediated by the production of various trophic factors, including brain-derived neurotrophic factors, nerve growth factor, and insulin-like growth factor-1, which contribute to recovering neurobehavioral function and stimulating endogenous regeneration [210, 212, 221]. In addition, MSCs home to injured brain tissues and exert immunoregulatory properties, reduce apoptosis, and improve neuronal cell survival [215, 217, 221]. However, it is unclear if MSCs differentiate into neural cells in vivo [210, 212].

4.6. Liver diseases

The anti-fibrotic properties of MSCs may exert therapeutic effects in liver regeneration and disease. MSCs inhibit activated fibrogenic cells such as hepatic stellate cells [222]. Numerous preclinical studies on bone marrow [223–225]. adipose tissue [226], and UC-derived [227] MSC treatment for improvement of liver fibrosis have been conducted and have reported reductions in liver fibrosis as well as improvements in hepatic function. Indeed, MSC based therapies for patients with end-stage liver disease, have shown promise in phaseIand II clinical trials [19, 20, 228]. MSC transplantation was safe and well-tolerated and hepatic function improved in patients with liver fibrosis [20]. Moreover, the biochemical hepatic index and model for end-stage liver disease (MELD) score were markedly improved from 2 to 3 weeks post transplantation [19]. However, the long-term hepatic function was not significantly enhanced in patients with liver failure caused by hepatitis B [19]. Notably, many of these clinical trials differ in MSC source, and liver pathology [229–232] and perhaps certain type of MSCs may serve as better therapeutic options for specific liver pathologies. These early stage studies and more recent clinical trials suggest that MSC transplantation is safe and may confer benefit to patients with liver cirrhosis and various kinds of liver diseases [233].

4.7. Aging frailty

Frailty is a medical syndrome that increases in prevalence with age and augments the risk for adverse health outcomes, including mortality, hospitalization, fall, and institutionalization. Markers of frailty include age-associated declines in lean body mass, strength, endurance, balance, walking performance, and activity; and are accompanied by declines in physiologic reserve in most organ systems. Together, these symptoms lead to the loss of homeostasis and the capability to withstand stressors and resulting vulnerabilities. Notably, there is a robust correlation between frailty and biomarkers of inflammation. There is also evidence that endogenous stem cell production decreases with age, likely contributing to reduce ability to regenerate and repair organs and tissues. Therefore, a regenerative treatment strategy could ameliorate signs and symptoms of aging frailty. Currently, there are no approved treatments for frail patients and therefore no established standard of care. There are specific features of the frailty syndrome that support the hypothesis that MSCs will also ameliorate or improve frailty. Indeed, in a pilot study and subsequently in a randomized, double-blind, dose-finding study, we demonstrated safety of intravenous infusion of allogeneic MSCs into elderly, frail individuals and found significant improvements in physical performance measures and inflammatory biomarkers [6, 234–235]. These findings suggest that frailty can ultimately be prevented or attenuated, and the link between frailty and inflammation offers a potential therapeutic target, addressable by cell therapy

5. Conclusions

The promising cell-based therapy field has exploded in the past decade and currently, MSCs from various sources, mainly bone marrow and adipose-derived, are being evaluated in phase I and II trials for a myriad of chronic, disabling disorders with no currently effective therapies. Although preclinical studies provide mechanistic insights into therapeutic effects of MSCs and phase I/II studies provide evidence of safety in the short-term, questions regarding most effective dose, route of administration, interaction with other concurrent therapies, sustainability/durability of effect, and adverse effects, including opportunistic infections and tumor development or progression, remain to be resolved. Addressing these questions will require rigorously conducted, multicenter clinical trials with well-defined clinical outcomes, longer duration of follow up, and more patients [151, 236].

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JMH reported having a patent for cardiac cell-based therapy. He holds equity in Vestion Inc. and maintains a professional relationship with Vestion Inc. as a consultant and member of the Board of Directors and Scientific Advisory Board. JMH is the Chief Scientific Officer, a compensated consultant and advisory board member for Longeveron, and holds equity in Longeveron. JMH is also the co-inventor of intellectual property licensed to Longeveron. Longeveron LLC and Vestion Inc. did not participate in funding this work. The other author-reports no conflicts.

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Chapter 8

Therapeutic Strategies of Secretome of Mesenchymal Stem Cell

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Additional information is available at the end of the chapter

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Abstract

Great progress has been made in the therapeutic strategies of multiple diseases that lack curative treatments with the transplantation of mesenchymal stem cells (MSC), such as in onco-hematological diseases, myocardial infarction (MI), cerebrovascular diseases, degenerative diseases of the nervous system (multiple sclerosis, Alzheimer's disease), and diseases of the immune system, among others. Stem cells (SC) participate in the biological processes of tissue regeneration and repair through cell replication. Recently, the beneficial therapeutic effects of SCs that are generated by the release of proteins with paracrine actions and not by cell differentiation are more well known, and 80% of the therapeutic effect of SC is attributed to paracrine actions. The MSCs release large amounts of proteins and growth factors (GF), nucleic acids, proteasomes, exosomes, and microRNA, and membrane vesicles known as the secretome are released into the extracellular space, regulating multiple biological processes. Currently, the therapeutic strategies in tissue engineering (TE) and regenerative medicine (RM) are focused on the management of products derived from cells that act, both locally and remotely, in the affected tissue or organ, achieving regenerative actions. The application of new knowledge of the secretome initiates a change in the paradigm of regenerative therapy by knowing more about and using cell products derived from cells as a "factory" for biological drugs.

Keywords: stem cell, mesenchymal stem cells, cell therapy, paracrine activity, exosomes, extracellular vesicle, microvesicles, microRNA, miRNA, regenerative medicine, tissue engineering, growth factors, extra-cellular matrix, epidermal growth factor, endothelial cell, fibroblast growth factor, granulocyte colony stimulating factor, granulocyte-macrophage colony stimulating factor, interleukin, IL-1 receptor

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antagonist, interferon-γ, low-density lipoprotein, monocyte chemotactic protein-1, nuclear factor κB, nitric oxide, NO synthase, endothelial NOS, NYHA

1. Introduction

The mesenchymal stem cell (MSC) therapies offer new opportunities for confronting diseases that lack curative treatments through the properties of multipotentiality, self-renewal, and the secretion of paracrine factors derived from exosomes (cytokines, growth factors, microRNAs, and proteases), which act as mediators of intracellular communication and induce the repair and regeneration of organs and tissues [1].

Cell therapy with MSCs is safe and effective in the treatment of degenerative and traumatic diseases; they are found *in vivo* in minimal quantities throughout the body and they have the ability to differentiate into bone, cartilage, and adipose tissue through stimuli and in culture. The MSCs are located in the perivascular environment, activating and creating a regenerative microenvironment, with the secretion of molecules to regulate the immune response; however, the therapeutic effects through paracrine interactions of the MSCs are of short duration. The response to changes in the environment is attributed to MSCs through the transcriptional regulation of mediators that control inflammation, remodeling, repair, and cellular recruitment. The repair process involves the regulation of extracellular matrix (ECM) deposition, collagen synthesis, fibroblast proliferation, platelet activation, fibrinolysis, and angiogenesis; the immune process suppresses T-cells, activates macrophages, and recruits neutrophils [2].

Cell differentiation and replacement is attributed to cellular secretions that function as therapeutic inducers. The secretions of extracellular vesicles (EV) are both local and systemic. To determine the functions of the factors secreted by the MSCs in regeneration, it is necessary to identify precisely the molecular profile of the secretome of the MSC constituted by growth factors (GF), cytokines and chemokines, proteases, ECM, hormones, and lipid mediators, and so on [3].

The secretome of MSCs contains multiple overlapping elements that make it difficult to identify them. The *in vivo* examination of the secretome of MSCs and the strategies to modulate it and the result of the analysis are essential for the design of the next generation of regenerative therapies without cells. In this way, questions arise about the regulatory function of the secretome of the MSC, such as (i) what are the most effective approaches to study the secretome both *in vitro* and *in vivo* and are new technologies necessary to achieve it? (ii) how do the properties of the secretome change or become manageable, and after the transplant how does it evolve in the local microenvironment? (iii) what are the best methods to achieve the sustainability of the secretome and the control in the transplant? [4].

2. Stem cells and mesenchymal stem cells

More than 200 different types of cells make up embryonic and adult tissues and are regulated by local and systemic environmental factors. Embryonic stem cells (ESC) derived from the

internal cell mass of the blastocyst are constituted by ectoderm, endoderm, and mesoderm. Adult stem/progenitor cells, known as somatic SC, are undifferentiated cells located throughout the body. These cells have a high proliferative capacity and a differentiation potential limited to their lineage; they participate in regeneration, cell turnover, and homeostasis. The main function during life is to maintain the number of differentiated cells at a constant level and to replace dead cells or cells lost due to injury or disease [5].

SCs have a great capacity for self-renewal and the potential to produce a differentiated progeny. An SC can have the same phenotype but be less "mature" or less "differentiated" than its descendants and is classified into SCs/progenitors, "somatic", "adult", or "tissue" embryonic and nonembryonic cells. ESC are pluripotent, and most populations of progenitor cells arising during embryonic development cannot self-renovate and have common properties with adult SCs, such as the potential of differentiation and the capacity for asymmetric cell division [6].

SC can be differentiated into specific cell types. Their ability to self-renew is through indefinite replication, resulting in the creation of two identical SCs, and under appropriate conditions, differentiated into more specialized cells. The MSCs are spindle-shaped adherent plastic cells that can be isolated from the bone marrow (BM), adipose tissue, and other tissues; are multipotent; and have the ability to differentiate. *In vitro* they can differentiate into bone; a subset of the cells have a high proliferative potential colony-forming units (CFU-F) when they are grown in culture. Hematopoietic SCs regulate and maintain hematopoiesis in the microenvironment of BM [7].

The MSCs can produce blood cells, although they are derived from a different population called hematopoietic SCs. The MSCs are classified as nonhematopoietic multipotential SCs and have the ability to differentiate into mesenchymal as well as nonmesenchymal lineages. The MSCs have the capacity for self-renewal, colony formation, phenotypic expression pattern, and differentiation potential; they interact with cells of the innate and adaptive immune system in the modulation of immune response. They participate in physiological processes, such as tissue homeostasis and hematopoiesis, and in pathological processes such as diseases of aging, tissue damage, and degenerative, inflammatory, and autoimmune diseases. After administration *in vivo*, MSCs induce tolerance and migrate to injured tissues where they inhibit the release of proinflammatory cytokines and promote the survival of damaged cells [8].

The International Society of Cell Therapy has established the following minimum criteria to define multipotent MSCs: first, they must be adherent to the plastic, under standard culture conditions (minimal essential medium, plus 20% fetal bovine serum). Second, MSCs should express CD105, CD73, and CD90 and should not express surface molecules such as CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR. Third, they must be differentiated into osteoblasts, adipocytes, and chondroblasts *in vitro*. They can be isolated from many adult tissues, BM, and adipose tissue. They have the ability to differentiate and trans-differentiate into cells of different lineages and immunomodulation capacity. The term "mesenchymal stem cell" is used to refer to the subset of mesenchymal cells that demonstrate SC activity and meet these criteria [9, 10].

The main characteristics of MSCs are the potential for self-renewal, differentiation, and multipotency. Under appropriate microenvironmental conditions, they can proliferate and give

rise to other types of cells, they can be trans-differentiated in cells of other lineages, and exert proregenerative, immunomodulatory, and antiinflammatory functions. Because of these characteristics, they can be an ideal therapeutic strategy for the treatment of inflammatory and systemic autoimmune diseases and are essential in the tissue regeneration of congenital, degenerative, and traumatic diseases [5].

The origin of MSCs *in vivo* is controversial. They are located in the perivascular area of the adventitia from almost all vessels (arteries and veins). They are pericytes, which are in intimate contact with the basement membrane and the surrounding endothelial cells, forming the extensive network of the microvasculature. Phenotypic similarities are evident among microvessels, and pericytes can be isolated from any vascularized tissue, near smooth muscle cells of arterioles, venules, and larger vessels, and preserve the expression of pericyte markers such as NG2 and CD146 [11].

The immunomodulatory activity of MSCs is mediated by paracrine factors. Among these, the exosomes participate in the communication between the MSCs and the target tissue. To demonstrate this, one study investigated the effect of the exosomes derived from MSCs on peripheral blood mononuclear cells (PBMC), especially on T-cells. It was shown that the MSC-derived exosomes extracted from the BM of healthy donors suppressed the secretion of the proinflammatory factor TNF-α and IL-1β and, conversely, increased the concentration of the antiinflammatory factor TGF-β *in vitro*. Exosomes can induce the conversion of T helper type 1 (Th1) into T helper type 2 (Th2) cells and reduce the potential of the T-cells to differentiate into effector T-cells producing interleukin 17 (Th17). In addition, the levels of regulatory T-cells (Treg) and protein 4 associated with cytotoxic T lymphocytes were increased. The results suggest that the exosomes derived from MSCs possess immunomodulatory properties [12].

Inflammation is a response of the organism to self-evolutionary harmful stimuli to maintain homeostasis. In the process, MSCs secrete paracrine factors that influence immune cells, dendritic cells, and macrophages, polarizing them toward a tolerogenic phenotype. Regulatory immune cells accumulate and converge in their regulatory pathways and create a tolerogenic environment conducive to immunomodulation [13].

During tissue regeneration, the regulation of the inflammatory process is essential, as is the control of local and systemic inflammatory response without causing damage in the injured tissues. The MSCs possess immunomodulatory properties that facilitate the repair of tissues by releasing exosomes, which generate an appropriate microenvironment to modulate inflammation. The exosomes contain bioactive molecules, which act as a cell-cell communication vehicle and influence the activities of receptor cells. During this process, the horizontal transfer of exosomal microRNA to recipient cells regulates the expression of the target gene and is essential to control inflammation and tissue homeostasis to develop new therapeutic approaches [14].

In MSC therapy, the following points should be kept in mind: (i) arrival at sites of ischemia or injury, when administered systemically and (ii) modulation of the immune responses mediated by T-cells, which express chemokine receptors and ligands in the migration of the cells and the homing process.

The MSCs induce immunomodulatory effects, interact with innate immune cells (dendritic cells, monocytes, natural killer [NK] cells, and neutrophils) and cells of the adaptive immune system

(Th1, cytotoxic T lymphocyte and B lymphocyte), secreting factors such as TGF-β, IL-10, IDO, PGE-2, sHLA-G5. The MSCs are considered immune privileged cells due to the low expression of the major histocompatibility complex class II (MHC-II) and expressing costimulatory molecules on the cell surface and interfering with different pathways of the immune response. *In vitro*, MSCs inhibit cell proliferation of T-cells, B-cells, NK cells, and dendritic cells (DC), producing what is known as "division arrest anergy". On the other hand, MSCs can inhibit diverse key functions of the immune cells, such as the secretion of cytokines and the cytotoxicity of T-cells and NK cells; B-cell maturation and antibody secretion; DC maturation and activation; as well as antigen presentation. In inflammation, MSCs must be activated to generate immunomodulation by suppression of molecules such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ. On the other hand, MSCs recruit regulatory T lymphocytes (Tregs) from both lymphoid and graft organs [15].

In vivo studies have shown differences with respect to the immunomodulatory properties of MSCs. Currently, the effectiveness of MSC treatment to suppress the abnormal immune response in scenarios such as prevention, treatment of allograft rejection periods, and autoimmune and inflammatory diseases is being investigated. Clinical trials in humans are being developed in the treatment of autoimmune diseases such as Crohn's disease, ulcerative colitis, multiple sclerosis, diabetes mellitus type 1, prevention of allograft rejection, survival of bone marrow and kidney grafts, and treatment of resistant graft versus host disease [16].

In vitro the MSCs are able to differentiate to osteogenic, chondrogenic, adipogenic, and myogenic lineages, and they express markers of pericytes (CD146+, CD34-, CD45-, CD56). In vascular damage, released pericytes become MSCs, are activated by the lesion, and respond by secreting bioactive molecules that inhibit immune cells that produce tissue damage and prevent the development of autoimmune reactions. The secretion of these bioactive molecules establishes a regenerative microenvironment in the injured tissue [17].

Activated MSCs also locally produce antimicrobial peptides such as LL37, which eliminate bacteria and attract macrophages and hematopoietic cells. Together, these function as therapeutic elements in the affected site and stimulate and increase TH2 and regulatory T-cells through inhibitory effects on the immune system. Thus, MSCs function as "medical signaling cells" with healing actions at sites of injury or inflammation. These trophic and immunomodulatory activities suggest that MSCs can serve as "pharmacies" regulated *in situ*. The MSCs act as "sentinels" in acute and chronic injuries; they function as multidrug dispensaries *in situ*, with "pharmacy" functions that promote natural regeneration [18].

In addition to the secretion of cytokines/chemokines, the MSCs show a great capacity for mitochondrial transfer and microvesicle secretion (exosomes) in response to injury. On the other hand, MSCs are recruited to the lesion to repair damaged tissues, an event intimately associated with tumorigenesis. Tumors are made up of different types of cancer cells that contribute to heterogeneity. Among these populations are the cancer stem cells (CSC) that participate in its onset and progression. A CSC population consists of MSCs that differ in cells with mesodermal characteristics. Resident or migratory MSCs favor angiogenesis and increase tumor aggressiveness. This interaction between MSCs and CSCs is fundamental in the development of carcinogenesis, progression, and metastasis. In cancer, tumor cells aberrantly secrete large amounts of exosomes to transport paracrine signals that contribute to tumor and distance interaction [19–21].

The MSCs represent an opportunity in cell therapy because: (i) they are easily accessible; (ii) the isolation is simple, they can be expanded to clinical scales in a short period; (iii) they can be preserved with a minimum loss of potency and stored for administration; and (iv) so far they have not shown adverse reactions to allogeneic transplantation compared with auto-transplantation, and they can expand *in vitro*, without altering their main properties.

3. Therapeutic approach

The control of the growth, division, and differentiation of MSCs in a safe and predictable manner is essential in tissue regeneration. They should be used as bioreactors to achieve specific cell types in conjunction with soluble factors that lead to healing. A therapeutic strategy is the transplantation of differentiated functional cells to replace cells lost or damaged by disease. However, the strategy requires regulation of the differentiation of the SC toward specific cellular destinations, including those that are outside the mesenchymal lineage, by means of trans-differentiation, where genetic manipulation can promote it and the expression of certain transcription factors for cellular reprogramming.

Because of the plasticity of MSCs, in addition to generating bone, adipose tissue, cartilage, and other skeletal structures, differentiation can generate lineages of liver, kidney, muscle, dermal, nerve, and cardiac cells; regenerate damaged tissue; and treat inflammation in the MI, brain, spinal cord, cartilage, and bone lesions, Crohn's disease, graft-versus-host disease (GvHD) and BM transplantation. The mechanisms of orientation and immunomodulation, the potential for multiple differentiations, and paracrine actions contribute to tissue repair. Induced pluripotent stem cells (iPSC) are very promising for discovering new drugs in medicine regenerative (MR), for their ability to differentiate in any type of cell, and iPSC-induced technology will allow the development of new therapies based on cells and their products as new biological drugs [22].

Transcriptional and epigenetic regulations are essential mechanisms underlying pluripotency, are studied in ESCs, allowing them to give rise to lineages of the three germ layers, and are used in basic studies of tissue formation that provided the foundation for regenerative therapy. Continuous self-renewal is an essential requirement to maintain the transcriptional profile and pluripotent state. To differentiate themselves in other cell lineages, ESCs need to change the transcriptional profiles. On the other hand, new regulators of pluripotency and gene expression may emerge with the study of miRNAs [23].

The immunomodulatory properties of MSCs are related to paracrine factors whose expression varies in each pathology. These factors have a direct impact on cells of the adaptive immune system such as T-cells. However, in the inflammatory process, MSCs secrete paracrine factors that influence other subpopulations of immune cells, such as dendritic cells and macrophages, and polarize them toward a tolerogenic phenotype. *In vivo*, these immunomodulatory factors are increased in the serum of animal models with inflammatory diseases treated with MSCs. The manipulation of immune regulatory cells could improve the immunomodulatory therapeutic strategies of MSCs. Regulatory immune cells accumulate and converge in their regulatory pathways to create a tolerogenic environment [24].

The paracrine signals of the extracellular environment influence the microenvironment of MSCs, both in proliferation and in differentiation. Many therapeutic strategies try to increase the effectiveness of regenerative therapies by direct application in the affected tissue or by differentiation in mature tissues. The MSCs have phenotypic plasticity and harbor an arsenal of bioactive molecules that are released by detecting signals in the local environment or packaging in EVs [25, 26].

The rigidity and/or topography of the cellular environment controls the differentiation of the MSCs, the physical signals determining the target, and cellular differentiation, an environment with high rigidity that leads to osteogenic differentiation, while low rigidity induces lipogenic differentiation. These effects are independent of the chemical/biochemical inducers. Physical factors, such as tension, produce a reorganization of the cytoskeleton during the differentiation of the MSCs and affect the expression of the essential gene of the process. Physical signals control the lineage specification of the MSCs, reorganizing and adjusting the cytoskeleton, and the cells perceive physical signals and transform these into biochemical and biological signals. Specifically, biophysical signals can initiate and strengthen biochemical signaling for the determination and differentiation of the destination of MSCs. The physical properties of the cell environment direct the structural adaptation and functional coupling of the cells to their environment [27].

To facilitate the identification of terms that we use in the following section, we present here abbreviations and meaning of the terms:

“Extracellular vesicle” (EV), is synonymous with “membrane vesicle” (suggested for all populations of vesicles derived from cells);

“Exosomes” are vesicles of 50–100 nm in diameter, generated by exocytosis of multivesicular bodies (MVB), and are a macromolecular complex involved in the degradation of RNA;

“Ectosoma” is a microvesicle derived from neutrophils or monocytes;

“Microparticle” (MV) is any small particle, regardless of its origin, and is more appropriate to indicate membrane-bound structures;

“Microvesicles” (ExMV) are larger extracellular membrane vesicles (100–1000 nm in diameter) [28].

The EVs are classified into three main classes:

1. Microvesicles/microparticles/ectosomes: these are produced by the formation of buds and the fusion of the plasma membrane;
2. Exosomes: these form within the endosomal network and are released by fusing the multivesicular bodies with the plasma membrane; and
3. Apoptotic bodies: these are released as blisters of cells that undergo apoptosis.

The current nomenclature classifies the vesicles by their biogenesis. The criteria for classification are according to their origin, function, or biogenesis.

4. Mesenchymal stem cell extracellular microvesicles (ExMV)

Organ regeneration technologies attempt to restore the anatomical structure and original functionality of a damaged organ. Usually, the response is fibrosis and scar tissue formation and no regeneration. The strategies of IT and MR for the repair of organs/tissues allow restoration of normal functioning. The development of new products, derived from MSCs considered as active biological elements, has already started.

The mechanisms of action of therapies with MSC have focused on paracrine actions, for the ability to generate regeneration without the application of cells. The primordial component, which creates a regenerative medium, is exosomes: intraluminal vesicles of 40–100 nm that transfer proteins and nucleic acids between cells and establish intracellular communication. The exosomes participate in organogenesis and regeneration and repeat the bioactivity of the SCs [29].

The extracellular space of multicellular organisms contains metabolites, ions, proteins, and polysaccharides. In the extracellular environment, a large number of mobile vesicles participate, and the term "extracellular vesicles or EVs" is suggested, which includes exosomes, microvesicles, microparticles (MV) and apoptotic bodies. The EVs comprise a heterogeneous population of lipid vesicles derived from cells containing exosomes and microvesicles. They are the mediators of the intercellular information transfer and can be vehicles for the administration of drugs of autologous cellular products. The therapeutic effects of cell therapies are mainly attributed to the EVs secreted by cells and directly involved in tissue regeneration processes [30].

Currently, interest focuses on EVs (exosomes and MV). Vesicles similar to exosomes have a common origin; however, they lack lipid-based microdomains; their size and sedimentation properties distinguish them from exosomes; and the term refers to an extracellular vesicle with a diameter of 40–150 nm and a density of 1.09–1.18 g/ml, proteins, nucleic acids, and membrane vesicles that generate a regenerative environment [31, 32].

Eukaryotic cells communicate with each other through direct interaction (juxtacrine contact dependent signaling) or by the secretion of factors such as hormones, GF, and cytokines, when they act in the cell itself (autocrine signaling) or act in neighboring cells (paracrine signaling) and distant cells (endocrine signaling). Tissue regeneration is related to the release of paracrine and autocrine substances and not only by cellular replication and differentiation. Eighty percent of the therapeutic effect of adult SCs is through paracrine actions. The molecules released by the SCs, the secretome, contain molecules (100), proteins, microRNA, GF, proteasomes, and exosomes, which generate paracrine activities. The composition of the different types of molecules depends on the stage and varies according to cell type, age, and environment. The secretory activity of cell-derived byproducts acts at a distance and is the main regenerative mechanism [33, 34].

In multicellular organisms, cells exchange information with signals from molecules in packages included in the EVs, which contain proteins, lipids, and nucleic acids. When released in the extracellular environment, exosomes interact with receptor cells by adhesion to the cell surface, by lipid-ligand receptor interactions, by endocytic uptake, or by direct fusion of the vesicles to the cell membrane [35].

Preclinical and clinical studies with MSCs propose inducing endogenous repair, and this represents a new paradigm for the treatment of multiple diseases. The factors are produced from activated cells extracted from their physiological niches, aspirated BM or mobilized blood, such as peripheral blood mononuclear cells (PBMC), and these release biologically active paracrine factors that induce regeneration. The apoptotic secreted from PBMCs has been used successfully for the treatment of MI, chronic heart failure, spinal cord injury, stroke, and wound healing [36].

The EVs derived from the MSCs, exosomes, are powerful intercellular communication vehicles; composed of a lipid bilayer that contains transmembrane proteins, cytosolic proteins, and RNA; participate in diverse physiological and pathological functions of both receptor and parental cells; and transfer the information to other cells and influence the function of the recipient cell. The EVs transmit biomolecules (proteins, lipids, nucleic acids, and sugars) as a single information packet or deliver multiple messengers simultaneously to sites distant from the source EVs [37].

The EVs are between 100 and 1000 nm in diameter, and they are microvesicles, ectosomes, or microparticles, which sprout from the cellular plasma membrane. Other types of vesicles are exosomes, which are generated within multivesicular endosomes or multivesicular bodies (MVBs), secreted by fusing with the plasma membrane.) Exosomes are vesicles that are enriched with components derived from endosomes. They are targeted to the recipient cells and, once bound to a target cell, the EVs induce signaling through the receptor-ligand interaction or are internalized by endocytosis and/or phagocytosis, even fusing with the membrane of the target cell to release its cytosol content, which modifies the cellular receptor [38].

Exosomes of endocytic origin transmit different intercellular signals by surface interactions and by the displacement of functional RNA from one cell to another and are released by mast cells, dendritic cells, macrophages, epithelial cells, and tumor cells. Exosomes, released from mast cells exposed to oxidative stress, have the ability to communicate a protective signal to receptor cells exposed to oxidative stress to reduce cell death. Exosomes can influence the response of other cells to oxidative stress by providing resistance to oxidative stress to recipient cells and decreasing the loss of cell viability. The exosomal transfer of RNA is involved in cell-to-cell communication and influences the response of the recipient cells to an external stress stimulus. The mRNA content of the exosomes produced under oxidative stress differs both from the mRNA in the donor cell and in the exosomes produced by cells grown under normal conditions. [39]. Exosomes produced by cells exposed to oxidative stress have the ability to induce tolerance in other cells. This effect is associated with the change in the content of exosomal mRNA that is attenuated by the reduced activity of the RNA by exposure to ultraviolet light. This shows, for the first time, that exosomal RNA transfer can change the biological function of a recipient cell [40]. The encapsulation of biologically active ingredients of regeneration in carriers of nonliving exosomes offers advantages in the processing, manufacturing, and regulation of MSC-based therapies [41].

Exosomes and ExMVs derived from MSCs influence tissue responses to lesions, infections, and diseases, and the exosomes of MSCs are not static, since they are the product of origin of MSCs, and they have actions with intercellular immediate neighbors. The MSCs, through paracrine action, activate the endogenous repair pathways, and the horizontal transfer of this load induces therapeutic changes; meanwhile, it has been demonstrated that microvesicles/exosomes derived from MSCs repeat the therapeutic effects of the parental MSCs [42].

A new generation of drug delivery systems can be mediated by exosomes and ExMVs; because they have high administration efficiency and low immunogenicity, new therapies can be implemented, and standardization is achieved with isolation techniques, with high functional efficiency, solid performance, scalable production, adequate storage, and efficient loading methods, which do not damage its molecular integrity and the movement of the elements *in vivo* as novel "nano-vehicles" [43]. The elements derived from cells generate an endogenous mechanism for intercellular communication; they are vehicles with the capacity to transfer biological information and the potential use can be as means of drug delivery [44].

5. Exosomes as biomarkers and therapeutic targets

All cells in the body secrete ExMVs, a heterogeneous population of bilayer vesicles with membrane that transport and deliver loads of proteins and nucleic acids to the recipient cells, allowing cell-cell communication. The exosomes, of endosomal origin, regulate normal and pathological processes. Healthy subjects and patients with different diseases release exosomes with different RNA and protein contents into the circulation, which can serve as biomarkers [45]. Compared to conventional biomarkers in serum or urine samples, exosomal biomarkers have greater sensitivity and specificity due to their high stability. They are present in almost all body fluids that harbor molecular components, exosomal proteins, and miRNA, and they are carriers of genetic information, which can be used for diagnosis. Although most RNAs found in exosomes are nucleotide fragments of degraded RNA with a length of <200 nm, some full-length RNA may be present. For example, circulating exosomal miRNAs are equivalent to those of the cancer cells of origin [45].

The power of nanovesicles as biomarkers depends on the enrichment of the exosomal classification markers, which otherwise only represent a very small proportion (<0.01%) of the total proteome of body fluids. The enrichment of the exosomal biomarkers of diagnosis will help in the discovery of new biomarkers to provide more precise information related to the origin of each exosome. A proteomic analysis and characterization of the plasma exosomes is essential, and gel permeation chromatography has been used to purify TNFR1 exosomal-like vesicles from the low-density lipoprotein (LDL) fraction. With this multistage purification scheme, it was possible to identify the 66 proteins of the circulating healthy exosomes, in the plasma, including proteins, both cytosolic and membrane-associated, as extracellular secreted proteins and associated with the cells identified with vesicular trafficking. The advantage of this analysis is that it allows the separation of different populations of vesicles according to their size. This reduces complexity in the identification of proteins in the plasma sample that may contain more than 1 million different intermixed proteins, and the discovery of peroxisome proliferator-activated receptor gamma (PPARγ) as a component of plasma exosomes will allow identifying a new pathway for the paracrine transfer of nuclear receptors specific in each pathology [46].

5.1. Exosomal proteins as diagnostic biomarkers

The molecular content of exosomes is the fingerprint of the cell type that released it and its current status; most viable cells release extracellular environment, protein secretions of

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exosomes, and when fused with the plasma membrane, appear in the blood and urine, so they are easily accessible, and can be used as biomarkers, for the diagnosis and prognosis of malignant tumors and other pathologies [47, 48].

Due to their cellular origin, exosomes express protein markers specific to the endosomal pathway, such as tetraspanins (CD63, CD9 and CD81), heat shock proteins (Hsp70), proteins of the Rab family, Tsg101 and Alix, which are not found in other vesicles of similar size. Their function is to eliminate damaged or aged cellular molecules, to protect cells from the accumulation of waste or drugs, participate in physiological and pathological processes, and have a wide variety of clinical applications, ranging from biomarkers to cancer therapy [49]. Proteomic and biochemical analysis of the purified exosomes revealed that the bilayer membrane of phospholipids is embedded with various proteins and lipids originating in the parental cells. These can serve as surface markers for the characterization and differentiation of exosomes from other types of microvesicles [50]. Exosomes contain various proteins, which express specific cellular functions, so they can serve as biomarkers for the diagnosis of liver, kidney, and cancer diseases [51]. Proteins in urinary exosomes are easily available through nontoxic means, are invasive, and are useful in diagnosis, especially for diseases of the urinary tract [52].

5.2. Exosomal nucleic acids as diagnostic biomarkers

Exosomes contain exosomal RNAs, especially miRNAs that function as diagnostic biomarkers, are protected from RNase-dependent degradation, are detected in circulating plasma, and serve for the diagnosis of ovarian cancer [53, 54]. Nanostructure analysis and study of the transcriptome of exosomes that transport RNA are diagnostic and found in breast milk, saliva, blood, urine, malignant ascites, amniotic fluid, bronchoalveolar secretion, and synovial fluid [55, 56].

Urinary extracellular vesicles (uEV) are released in the nephron of the kidney and urinary tract. Specific proteomic and transcriptomic markers provide information on the cell of origin and are a reservoir for the discovery of biomarkers in kidney diseases. The uEV are a new means of cell signaling, renal tubular cells, and can provide exosomal markers not detectable in urine. Renal biopsy is an invasive technique with complications such as infection and hemorrhage. The analysis of proteomic and transcriptomic changes of uEV in different disease states as a biomarker can be a noninvasive alternative to biopsy [57].

In conclusion, the most important biomedical utility of exosomes is their application as biomarkers in clinical diagnosis. Compared with those detected in conventional samples, such as serum or urine, exosomal biomarkers provide comparable or superior sensitivity and specificity, attributed to their excellent stability, and the exosomal biomarkers of biofluids can be easily obtained. Recent technical advances in the isolation of exosomes will make diagnostics more beneficial.

6. Stem cell therapeutics; exosomes as biomarkers in cardiovascular diseases

The intercellular communication between cardiac, vascular, SCs, and progenitor cells with differentiated cardiovascular cells is a complex process, with a diversity of mechanisms in

cardiovascular disease and therapeutics. The EVs are produced through different pathways and are released and absorbed by most cells including cardiac, vascular, and progenitor stem cells [58].

The conventional treatment in obstructive coronary disease is percutaneous coronary revascularization, angioplasty, stent placement, or coronary revascularization graft. In patients where there was no improvement or these treatments were not indicated, it is necessary to limit the damage produced by MI, to restore blood flow, and supply blood to the ischemic region. Microcirculation is a therapeutic target for the treatment of ischemic disease. Several preclinical studies show that CD34+ cells can stimulate neovascularization in ischemic tissue by increasing capillary circulation and improving acute and chronic myocardial ischemia [59]. A double-blind study showed lower rates of amputation in patients with critical ischemia of the lower limb with the administration of CD34+ cells [60]. Other studies mention that transplantation of CD34+ cells into ischemic myocardium after MI is better than neovascularization with mononuclear cells [61].

Cardiovascular diseases (CVD) have a high prevalence, morbidity, and mortality. The identification of biomarkers with high sensitivity and specificity can evaluate the prognosis of CVD, optimize personalized treatment, and reduce mortality. Biomarkers based on exosomes may reflect the stage and progression of coronary artery obstruction, heart failure, cerebrovascular accidents, arterial hypertension, cardiac arrhythmia, cardiomyopathy, valvular heart disease, and pulmonary arterial hypertension. On the other hand, exosomes as immunomodulators can be used in cardiac ischemia, pulmonary hypertension and many other diseases, including cancer, and also be used as a biomarker of the disease [62, 63]. Some exosomes can inhibit cell apoptosis and increase cell proliferation, contain specific surface proteins and the like, such as CD9, CD63, CD81, and proteins can transfect cardiomyocytes, endothelial cells, SC fibroblasts, and smooth muscle cells, and induce beneficial cellular changes. The release of exosomes from the cell is mainly regulated by Rab GTPase (Rab27a/b and Rab35). After a MI, the exosomes work in local and systemic microcommunications, in the exosomal transport of miRNA, and in the contribution of signals for cardiac repair, the myocardium can secrete exosomes, especially those that emerge in the border area of MI, so control of the quality and quantity of exosomes can serve as biomarkers in the diagnosis and prognosis of MI and as a new therapeutic objective to regulate cardiac remodeling [64].

The EVs secreted by the cardiac progenitor cells (CPC) can improve the cardiac function after the lesion by the content of exosomes with angiogenic factors that generate the ischemic tissue repair and are cardioprotective agents; the exosomes are the active components of the CD34+ of the BM. Experimentally, exosomes secreted by MSCs and CPCs have been shown to decrease tissue damage and facilitate ventricular remodeling in animal models of myocardial ischemia and reperfusion injury. The EVs are the active paracrine component of the CPCs [65].

In a study of stability, the exosomes of adult cardiac myocytes were shown to release heat shock protein (HSP) 60 in exosomes. When this protein is not in the exosomes, apoptosis is generated through the activation of the Toll-like receptor 4 and the release of Hsp60 would damage the surrounding cardiac myocytes. On the other hand, fever and a change in the pH

or ethanol consumption increase the permeability of the exosomes, and different inducers of exosomes modify the content of the exosomal protein. The production of reactive oxygen species (ROS) is an underlying mechanism of increased production of exosomes and ethanol at "physiological" concentrations would trigger the release of exosomes. This work, as determined by Western blot analysis and mass spectrometry, mentions that exosomes retain their protein load in different physiological/pathological conditions; the protein content of the exosomes' cardiac etiologies differed from other types of exosomes due to their content of cytosolic, sarcomeric, and mitochondrial proteins. Ethanol did not affect the stability of the exosomes but increased the production of exosomes in cardiac myocytes; exosomes derived from ethanol and hypoxia/reoxygenation had a different protein content. Finally, inhibition of ROS reduced the production of exosomes [66].

Through circulating blood, circulating exosomes can reach distant tissues, allow direct communication with target cells, and regulate intracellular signals. Circulating exosomes and their exosomal charges participate in the hypertrophy of cardiomyocytes, apoptosis, and angiogenesis. Circulating exosomes enriched with various types of biological molecules can be modified in number and in loads of exosomes in cardiac lesions, such as MI, reperfusion injury, myocardial ischemia, atherosclerosis, hypertension, and cardiomyopathy due to sepsis, and can influence the function of the cardiomyocytes and contribute to the pathogenesis of CVD. A therapeutic strategy based on exosomes can be used to decrease myocardial injury and induce cardiac regeneration [67].

The CD34+ cells are a structural component in the formation of neovasculature in ischemic tissue, and secrete paracrine factors that stimulate the formation of new vessels, an element of proangiogenic paracrine activity associated with CD34+ cells secreted by exosomes, with a potent angiogenic paracrine activity both *in vitro* and *in vivo*. Exosomes stimulate mechanisms mediated by genetic receptors by transferring proteins, RNA, or microRNA directly to the cytoplasm of target cells [68].

All types of cardiac cells are able to secrete ExMVs, which are captured by the recipient cells and can alter gene expression or activate cascades of intracellular signals. A possible therapeutic intervention to reduce the ischemia/reperfusion injury (I/R) is the pre or post-conditioning, which allows the activation of the salvage recovery pathway by reperfusion salvage kinase (RISK), where transcription factors such as factor 1α-induced hypoxia (HIF-1α), mediators such as heat shock protein 70 kDa (Hsp70) and inducible nitric oxide synthase (iNOS), demonstrated in an in vitro preconditioning model, which does not influence the secretion of EV and its morphology, but it has an effect on EV size and particularly on its charge. EVs derived from fibroblasts enhanced cell migration and the effect was improved by means of in vitro preconditioning. An experimental model of *in vitro* preconditioning of cardiac cells concluded that it does not influence the concentration of ExMVs, but regulates their load and affects migration [69].

In conclusion, cardiac cells, such as cardiomyocytes, endothelial cells, and fibroblasts, release exosomes that modulate cellular functions. The exosomes released by CPCs are cardioprotective and improve cardiac function after MI, compared to that achieved by progenitor cells, and they have antiapoptotic, proangiogenic functions.

7. Cancer stem cells (CSC); exosomes

Malignant tumors arise from a small subset of cancer cells, have tumor heterogeneity, and small populations of cells with characteristics equivalent to SCs. These cells, called cancer stem cells (CSC) or cancer-initiating cells (CIC), have been identified in many malignancies and are thought to form the tumor clonogenic nucleus. The CSCs share many characteristics of ES and show activation of one or more signal transduction pathways, which are involved in tissue homeostasis and development, including Notch, Hedgehog (Hh), and Wnt pathways. Notch signaling, similar to the Wnt and Hh pathways, is a pathway for determining the fate of the evolutionarily conserved primordial cell, with great relevance in the biology of cancer, from CSC to angiogenesis and tumor immunity. The CSCs generally have slow growth rates and are resistant to chemotherapy and/or radiation therapy. The new treatment strategies seek to control the replication, survival, and differentiation of the CSCs [70]. These cells originate from a more differentiated cancer cell, with self-renewing properties, probably as a result of epithelial to mesenchymal transmission [71].

The most important and useful property of the CSC is that of self-renewal and characteristic differentiation, which is considered as a one-way specialization process as the cells develop the functions of their final destination and lose their immature characteristics, such as self-renewal. This property shows parallels between SCs and cancer cells. The tumors originate by the transformation of normal SCs by means of similar signaling routes, to which they regulate self-renewal, both of SCs and CSCs; the latter include the undefined potential of self-renewal that starts tumorigenesis. Otherwise, CSCs could be derived from a SC of normal tissue that undergoes a transformation as a result of oncogenic somatic mutations, due to the influence of extrinsic microenvironmental factors [72].

The CSCs are associated with tumor onset, metastasis, progression, invasion, recurrence, and resistance to therapies, and they play a central role in the biology of cancer cells; they interact with their surrounding cells inducing angiogenesis and metastasis. In the tumor microenvironment, multiple types of cells coexist, including adult SCs, CSCs, and stromal cells, and communicate with each other in modulating, tumor progression, functionally release exosomes that can be absorbed by CSCs or adult SCs, and modify their phenotype. Recent studies show that exosomes participate in interactions between cells within the tumor microenvironment by means of exosomal signals, modulating tumor progression [73].

We take Hannafon's approach in questions related to the function of the exosomes involved in the interaction of CSCs, adult SCs, and the surrounding cells within the tumor microenvironment, which are:

Do CSCs or adult SCs secrete exosomes that affect the function of the stromal cell? Are CSCs or adult SCs modified by the exosomes released from CSCs and surrounding stromal cells?

What are the possible molecular mechanisms and the biological consequences of exosome-mediated interactions between CSCs, adult SCs, and the cells that surround them? [74].

The SCs secrete a large number of exosomes, and in the extracellular environment, they function as intercommunicators in the tumor microenvironment and actively in tumorigenesis,

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angiogenesis, and tumor metastasis, and the mechanism of interaction between the cancer cells and the tumor cells involves the exchange of biological material through exosomes. On the other hand, exosomes induce the formation of the premetastatic niche, which regulates tumor metastasis. Mechanisms mediated by exosomes contribute to resistance to antitumor therapy. Certain exosomes have an influence on tumors to evade immune surveillance [75].

Exosomes derived from SCs provide information related to the regulation of genes to target cells, for cell growth and angiogenesis by the modulation of various signaling pathways. Exosomes derived from MSCs potentiate the expression of VEGF in tumor cells by activating the kinase 1/2 pathway regulated by extracellular signal regulated kinases (ERK1/2) that promote tumor growth [76].

The ExMVs released by adipose mesenchymal stem cells (ASC) may contribute to angiogenesis induced by ASCs. In CSC, exosomes derived only from CD105+ CSCs conferred an activated angiogenic phenotype to normal human endothelial cells, stimulating their growth and vessel formation. A specific source of the ExMVs derived from CSCs contributes to triggering the angiogenic process and metastatic diffusion during tumor progression. The effects of exosomes of different types of SCs on angiogenesis are similar to those of exosomes derived from SCs in tumor growth [77].

Exosomes released from SCs contribute to tumor metastasis. Several key steps in tumor invasion and metastasis are associated with MSCs, and include the epithelial-mesenchymal transition and the induction of SC-like properties that allow CSCs to increase their survival capacity through circulation [78].

Recently, CSCs have been used in the diagnosis and treatment of cancer. The exosomes released from prostate and breast cancers have specific biomolecular characteristics, including the expression of several exosomal markers such as CD9, CD63, CD81, ALIX, and TSG101. In addition, the exosomes derived from GC-MSC contain miR-221, which is a new biomarker for the diagnosis of several tumors. The finding of tumor biomarkers is a new diagnostic tool. The release of exosome cells provides valuable detailed molecular information about the cell of origin and the tumor characteristics, can be isolated from easily accessible body fluids, and can provide specific information for the predictive diagnosis of multiple tumors [79].

8. Future conclusions and addresses

Due to its complexity, the research and application of cell therapy with cells and cellular products should be considered with a multidisciplinary and translational approach and represents a great therapeutic potential for refractory diseases to conventional treatments such as noncommunicable chronic diseases: diabetes, cardiovascular ischemic diseases, cerebrovascular or renal diseases, degenerative diseases such as cancer, neurodegenerative such as Alzheimer's disease, multiple sclerosis, and aging.

Thus, cell therapy with MSCs emerges as a promising therapeutic tool, the main therapeutic objective of which is healing through trans-differentiation to repair and replace

damaged cells and generate new healthy cells. The rapid progress in MSC research and the primary function in cellular niches under normal and pathological physiological conditions and the management of cellular intercommunication of the microenvironment through the paracrine secretion and their biological products are being incorporated into clinical practice.

The initial paradigm of cellular therapy for tissue and organ repair and regeneration has been modified, with new knowledge from experimental, preclinical, and clinical studies related to the mechanism of action of MSCs both *in vivo* and *in vitro*, which have demonstrated to be processes fundamentally of paracrine action, by means of the generation of exosomes, microvesicles, and the horizontal transfer of proteins, mRNA, and microRNA.

Recently, a group of secreted vesicles, the "exosome", has been identified as the main mediator of the therapeutic efficacy of MSC. The ExMV participate in intercellular, local, and remote communication, which are translated into pleiotropic actions and generate a therapeutic potential by transferring biologically active molecules and which can be used as new biomarkers and potential regulators of inflammation and immune response to detect immune rejections. Exosome/microvesicle therapy derived from MSCs has potential advantages. First, it prevents the transfer of cells that may have mutated or damaged DNA. Second, the vesicles are small and easily circulated, while the MSCs are too large to easily circulate through the capillaries and many do not even reach the first capillary bed. Third, the dose of MSC decreases rapidly after transplantation, but the administration of the biological products of the cells allows higher therapeutic "doses". The disadvantage of using vesicles derived from MSCs is that they are static and cannot occur more when they are transplanted. The therapeutic efficacy of MSCs is based on their ability to respond in the microenvironment of the lesion, whereas the isolated exosomes are not expected to do so. The opportunity to exploit the potential therapy of MSCs and their products opens new scenarios for the identification of new molecules for the repair and regeneration of organs and tissues through proteome analysis of the secretome.

In the short term, the exosomes derived from MSCs will progress to clinical studies, and their usefulness and effectiveness will depend on establishing a series of critical parameters such as standardizing reproducible production methods for the manufacture of exosomes/microvesicles with precisely defined content, standardizing storage methods that maintain their potency, and evaluating therapeutic efficacy in controlled clinical trials, of appropriate power, designed with written criteria and with solid research foundations to generate scientific results that allow the translation of basic knowledge to create new regenerative therapies.

Conflict of interest

No conflicts of interest, financial or otherwise, are declared by the authors.

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Chapter 9

Clinical Applications of Mesenchymal Stromal Cells (MSCs) in Orthopedic Diseases

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Additional information is available at the end of the chapter

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Abstract

Mesenchymal stromal cells (MSCs) have the capacity for self-renewal and multi-lineage differentiation, have many advantages over other cells, and are thought to be one of the most promising cell sources for cell-based treatments. In fact, MSCs have already been widely applied in clinics as a treatment for numerous disorders, including orthopedic diseases, such as bone fracture, articular cartilage injury, osteoarthritis (OA), femoral head necrosis, degenerative disc, meniscus injury, osteogenesis imperfecta (OI), and other systemic bone diseases. With the progressions in R&D, the safety and efficacy of MSC-based treatments in orthopedic diseases have been largely recognized, but many challenges still exist. In this chapter, we intend to briefly update the recent progressions and discuss the potential issues in the target areas. Hopefully, our discussion would be helpful not only for the clinicians and the researchers in the specific disciplines but also for the general audiences.

Keywords: mesenchymal stromal cells (MSCs), orthopedic disease, cell therapy, tissue engineering, regenerative medicine

1. Introduction

Around 1960s, Friedenstein first found that there was a non-hematopoietic stem cell population in the bone marrow that could be differentiated into bone and fibrous tissue [1]; however, this population did not gain broad recognition until Caplan coined the term bone mesenchymal stem cells (MSCs) in 1991 [2]. This umbrella term did raise up the attention of this population, but this term is misleading and very controversial, and many investigators argue against to use this term loosely. As a result, many different terms have been proposed for this or the

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similar populations, including mesenchymal stromal cells (MSCs), mesenchymal progenitor cells, multipotent mesenchymal stromal cells, bone marrow stromal cells (BMSCs), bone marrow-derived MSCs, multipotent stromal cells, mesenchymal precursor cells, and skeletal stem cells [3]. Currently, most investigators prefer an alternative term, that is, multipotent mesenchymal stromal cells (MSCs). For this reason, we also use this term throughout this chapter.

Theoretically, self-renewal without significant loss of their characteristics (stemness) and multi-lineage differentiation potential are the two criteria that define MSCs as real stem cells, but in practice, this heterogeneous population proliferates in vitro as plastic-adherent cells, as fibroblast-like morphology, forms colonies in vitro and can at least differentiate into bone, cartilage and fat cells [4]. In addition, literatures also provided evidence that MSCs can differentiate into multiple other mesenchymal lineages or even non-mesenchymal cell types, including endothelial cells, osteoblasts, chondrocytes, fibroblasts, tenocytes, vascular smooth muscle cells, myoblasts, and neurons [5], though some of these capacities are controversial. The key caveat is that it is unlikely that all cells in the cultural meet the above-mentioned two criteria.

MSC-like cells can be obtained from almost all tissues, including the umbilical cord, amniotic fluid, placenta, adipose tissue, joint synovium, synovial fluid, dental pulp, endosteum, and periosteum [6]. Cultured MSCs have been characterized either by using cell surface antigens and/or by examining the cells' differentiation potential. The International Society for Cellular Therapy recommended that cells should fulfill the following criteria to be considered as MSCs: (1) the cells must be plastic adherent when maintained under standard culture conditions; (2) they must express CD73, CD90, and CD105 markers and should not express CD34, CD45, CD14, HLA-DR, CD11b, or CD19; and (3) they should be able to differentiate at least into osteoblasts, chondroblasts, and adipocytes in vitro [7]; however, this criteria has obvious problems therefore not been commonly accepted. For this reason, it is still challenging to consistently isolate or purify a well-defined clinical applicable MSC population.

On the other hand, the increasingly aging population has made the degenerative, non-traumatic and traumatic musculoskeletal diseases main socioeconomic issues, and MSCs seem to be a promising solution. In fact, MSCs have been widely used as a treatment for numerous orthopedic diseases, including bone defects, osteoarthritis (OA), femoral head necrosis, degenerative disc, spinal cord injury, knee varus, osteogenesis imperfecta, and other systemic bone diseases [8]. Currently, orthopedic researchers are still focusing on overcoming a variety of challenges so that they can fully realize the clinical therapeutic potential of MSCs, and the long-term goal is to change the main treatment strategy in the field of orthopedics from surgical replacement and reconstruction to bioregeneration and prevention [9].

In this chapter, we will briefly update the main advancements in these areas and discuss the major current and potential future applications pathways.

2. MSCs in nonunion bone fracture

Inadequate healing can lead to nonunion of the fractured bone. Clinically, about 5–10% of all fractures end up in persistent nonunion [10]; therefore, nonunion is one of the most troublesome complications. Since MSCs have the osteogenic differentiation ability, can secrete a

variety of cytokines and promote angiogenesis. It is reasonable to speculate that MSCs could accelerate fracture healing. In fact, there are experimental evidences to support the idea that MSCs treatment indeed promoted healing of nonunion fractures. For example, MSCs has been transplanted to animals to promote bone loss and fracture healing [11, 12]. Also, there are reports of BMSC treatment of bone nonunion caused by a bone defect, osteogenesis and local microenvironment disorders in patients [13–15]. It was reported that, after traumatic injuries, BMSCs could migrate from blood circulation to the lesion site, and then directly differentiate locally, and replace the injured cells. Consistently, the circulating BMSC can be detected in peripheral blood 39 to 101 hrs after fracture [16]. However, other data also suggested that these circulating cells account for only a small portion of cells in the fracture callus under normal circumstances, suggesting that the majority of the cells at the fracture site are migrated from the adjacent tissues [13]; nevertheless, therapeutic amplification of circulating MSCs through their mobilization could also represent a potential therapeutic opportunity in fracture repair [13].

Indirectly, BMSCs promote bone healing mainly through the secretion of bioactive molecules and extracellular membrane vesicles, which induce angiogenesis, regulate inflammation, inhibit apoptosis, and regulate osteogenesis differentiation. Since defective blood supply (ischemia) is an important cause of nonunion of bone, promoting blood vessel formation is beneficial to the healing of the nonunion bone. MSCs are known to secrete angiogenesis-related factors include angiopoietin Ang-1 and Ang-2, vascular endothelial growth factor (VEGF), FGF-2, and hepatocyte growth factor (HGF)-1 [17]. Furthermore, BMSCs have an anti-fibrotic effect and can limit that fibrosis progression of fracture zone and promote the regeneration of bone tissue. This is mainly accomplished by immunoregulating, inhibiting TGF-3 mediated differentiation of fibroblasts, inhibiting oxidative stress, and matrix reconstruction [18]. Interestingly, it was also found that HGF, VEGF, and microbubble secreted by BMSCs had an anti-apoptotic effect, which inhibits the apoptosis of transplant cells in the injured area [19, 20].

In addition, other conserved signaling pathways, such as the transformation growth factor (TGF)-β_3/bone morphogenetic proteins (BMP), Wnt, Hedgehogs, FGF, platelet-derived factor (PDGF), epidermal cell growth factor (EGF), and insulin-like growth factor (IGF), may also indirectly participate in the regulation of BMSCs and promote bone healing processes [21, 22]. Based on these observations, factors such as TGF-β_3 and its analogs, BMP, BMP-2, and BMP-7, have been used clinically to enhance and accelerate the bone repair or regeneration.

Technically, MSCs can be isolated from many different tissues. The iliac crest is the ideal position for bone marrow aspiration. In clinical practice, we indeed found that injection of bone marrow aspirate into the fracture space can promote the healing of fracture and shorten the healing time. For example, in one case with an open tibial fracture, which did not develop callus within the 6 months after surgery, and then we extracted marrow aspirate from the iliac crest and injected it into the fracture space. We found that the fracture healed well 6 months after the transplantation (**Figure 1**). Consistently, bone marrow aspirate injection has been shown to have a potential role in the treatment of aseptic, atrophic nonunions with acceptable alignment and minimal gap, or displacement between fracture fragments [15]. Generally, Tibial nonunion treatment with bone marrow aspirate has been well-documented and found to be successful in 75–90% of reported tibial nonunion case series [23, 24].

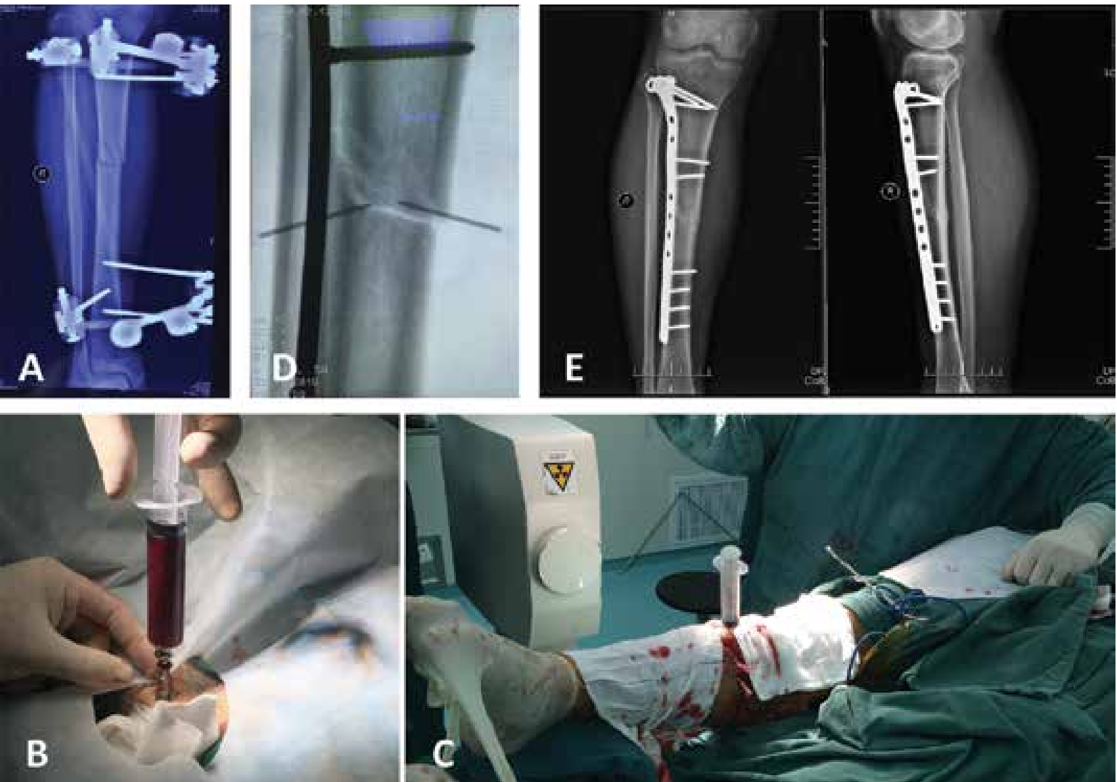

Figure 1. (A) A 32-year-old male underwent external fixation of an open tibial fracture. After surgery, no callus was observed in 6 months. (B, C) he underwent bone marrow aspiration and percutaneous grafting directly into the nonunion site. (D, E) 6 months after the transplantation, frontal and lateral X-ray images indicated the fracture healed well.

Researchers have described the purification and expansion of bone marrow MSCs from mice, rats, rabbits, dogs, and humans, and the ability of these cell populations to form bone when implanted ectopically with hydroxyapatite or an appropriate carrier has been established. To isolate MSCs from blood, mobilization of MSCs to the peripheral circulation with granulocyte colony-stimulating factor (G-CSF) is normally necessary. Data also suggests that the tissue-engineered constructs with MSCs (either genetic modified or not) brings us closer to a clinical application. However, nonunion still occurred in nearly half of the bone defects in a large animal model [25]. In fact, the commonly accepted idea that atrophic nonunion is due to lack of MSCs activities of MSCs might be not accurate. For example, an interesting study found the existence of MSCs (confirmed by their expression profile of CD105, CD73, HLA-DR, CD34, CD45, CD14, and CD19) in the site of atrophic nonunion, at a similar number and viability to those isolated from the iliac crest [26]. In another clinical study, Ismail et al. [14] also reported that iliac crest autograft with or without autologous MSCs (with 5 g/cm^3 hydroxyapatite granules, as scaffold carrier) had similar treatment efforts on atrophic nonunion.

3. MSCs in articular cartilage injury

Due to the limited ability of proliferation capacity of chondrocytes, articular cartilage injury often causes progressive degeneration of the joint and OA, which is a serious health and economic problem [27]. The typical current treatment for this disorder is microfracture, which is a surgical technique that was developed 20 years ago. This treatment uses the body's own healing abilities to regenerate the chondral surface. However, the regenerated fibrocartilage often has poor mechanical properties compared with normal cartilage.

Recently, the MSC-based autogenous transplantation treatment was proposed, since the potential of the MSCs to differentiate into chondrocytes has been well-recognized [28]. Compared with allogeneic cells, generally, autogenous cartilage progenitor cells are more effective in the treatment of articular cartilage defect [29]. The emerging typical paradigm to apply MSCs in this disorder is [30]: (1) during the first operation, a cartilage biopsy is taken from areas of damaged cartilage within the ankle or knee; (2) chondrocytes are isolated from the biopsy via enzymatic digestion and cultured in 2D monolayer cultures; (3) monolayer culture-expanded chondrocytes are seeded on a collagen type I–III membrane; and (4) in the second operation, the cartilage lesion is prepared and the collagen membrane is cut to size, placed in the lesion and secured with fibrin glue.

To clarify whether donor MSCs indeed contribute to cartilage regeneration in vivo via a progenitor-mediated mechanism [31], Zwolanek et al. describe a novel cell tracking system based on genetic transgenic donor and corresponding cell marker, and the results showed that MSC could contribute to cartilage regeneration via a progenitor - or nonprogenitor - mediated mechanism [31]. The study by Windt et al. in humans also produced similar results [32]. Further study found that chondrogenesis can be regulated by adjusting the time and concentration of TGF-β [33].

To further improve the efficiency of MSC-based treatment, combining bone marrow-derived MSCs with scaffold have been tried for the reconstruction of cartilage [34]. For example, Sadlik et al. reported that the scaffold-embedded MSC was implanted into the knee to repair cartilage through dry arthroscopy, and the tissue regeneration was successful [27]. In addition, other approaches, such as the stem cells cultured from the subpatellar fat pad of arthritis patients can also be induced to differentiate into chondrocytes, which are very similar to the normal chondrocytes [29]. Koga et al. also found that the transplantation of synovial MSCs (SMSCs) in a rabbit model resulted in a large number of cartilage matrix development, and they also observed that SMSCs differentiated into osteocytes deeper into the defect, but differentiated into chondrocytes on the surface [35].

4. MSCs in meniscus injury

Meniscus injury in the knee joint is probably the most frequent intra-articular damage. The typical treatment is a partial surgectomy, but it can lead to degeneration of articular cartilage, narrow joints, and early osteoarthritis. Intra-articular injection of MSCs could be a simple treatment with little damage since MSCs might promote the regeneration of meniscus. Indeed, it was found that when MSCs were injected directly into the articular cavity, they could migrate to the lesion site, directly participate in the tissue repair, and induce the repair of the host through the collateral secretion, and replace the injured tissue [36]. Murhpy et al. reported the first study of injection of BMSCs in sheep articular cavity [37], and observed the obvious repair of cartilage damage in meniscus injury, 6 and 12 weeks after injection. Whitehouse et al. also reported that undifferentiated MSCs/collagen-scaffold implant could provide a safe way to augment avascular meniscal repair in some patients [38]. Another study investigating the injection of allogenic MSCs in the context of post-subtotal meniscectomy found that there was evidence of meniscal regeneration in the two groups treated with MSCs [39]. However, Hong et al. used arthroscopic surgery to repair the meniscus of the posterior articular cavity with or without BMSCs after meniscus injury [40], and found that the meniscus and tibial plateau were not fully integrated, and the efficacy of MSCs treatment group was not significantly different from that of the control group. They argued that MSCs may differentiate into other tissue cells if they were not effectively induced to differentiate into specific cell types. Therefore, it is still a challenge to induce the cells into the meniscus cartilage phenotype in this context.

5. MSCs in the treatment of osteoarthritis

Osteoarthritis (OA) is a major cause of joint pain and loss of mobility in the elderly, which seriously affects the quality of life and causes huge social and economic burden. Many researchers have conducted a series of clinical studies on BMSCs transplantation to treat OA (**Table 1**), and these studies demonstrated that moderate confidence could be placed on the safety of MSCs therapy for knee OA, but the confidence in efficacy outcomes is low, mainly

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References	Location	BMCS[a]	Follow-up time	No. of cases	Pain subscale		Outcomes
					Pre-infusion values	Post-infusion values	
[41]	knee	Auto_ BM_MSC	1 year	12	24 ± 14[b]	6 ± 6[b]	pain improvement, higher cartilage quality (MRI)
[42]	knee	Auto_ AT_MSC	6 months	12	56 ± 19[b]	34 ± 23[b]	clinical improvements
[43]	knee	Allo_ BM_MSC	1 year	15	46 ± 15[b]	30 ± 16[b]	pain improvement, higher cartilage quality (MRI)
[44]	knee	Auto_ BM_MSC	24 weeks	2	4[c]	0.38[c]	pain improvement, higher cartilage quality (MRI)
[45]	Hip	Auto_ BM_MSC	3 years	10	34.5 ± 8.2[b]	19.2 ± 6.1[b]	pain improvement, improved function

[a]Auto_BM_MSC, Autologous Bone Marrow-derived MSCs. Auto_AT_MSC, Autologous Adipose Tissue-derived MSC. Allogeneic Bone Marrow-derived MSC.

[b]The WOMAC index (pain subscale) has been used; scale 0–100.

[c]The VAS index (pain subscale) has been used; scale 0–10.

Table 1. Summary of MSCs as the treatment of osteoarthritis.

due to limited clinical case number [46]. Therefore, further high-quality studies for OA with high internal and external validity are still required. In addition, Shi et al. compared the clinical results of platelet-rich plasma (PRP) and MSCs treatments for osteoarthritis of the knee in a systematic review and pointed out MSCS provide more significant disease therapeutic effect [47].

6. MSCs in femoral head necrosis

Avascular necrosis of the femoral head (ANFH) is a serious clinical problem. If untreated, about 80% of ANFH progresses to the collapse of the head within 1–4 years [48]. Numerous clinical methods have been tried, including core decompression (CD), a commonly used method for treating the early stages of ANFH. The presumption is that CD can reduce the intraosseous pressure and also stimulate stem cell regeneration. But the outcome of CD is variable and is still controversial.

With the development of non-biological materials, MSCs and tissue engineering techniques, the treatment of ANFH has been significantly improved recently [49]. For example, it was

reported that the efficacy of MSCs transplantation group was significantly better than that of the pure medullary decompression group [50]. In another study, 100 patients with early-stage ANFH were recruited and randomly assigned to BMMSC treatment or CD treatment only [51], a similar result was observed, that is, this intervention was proved to be safe and more effective in delaying or avoiding FH collapse. In another study of eight patients with bilateral femoral head necrosis, the researchers performed the medullary decompression on one side, while on the other side medullary decompression MSCs transplantation. The Harris hip score (HHS) and VAS score of the MSCs transplantation group were significantly improved, and the results of MRI quantitative analysis showed a significant decrease in necrosis area [52]. Consistently, another study found that the group of MSCs had a significantly superior recovery of the early stages of necrosis [53].

However, there have been reports of unsatisfactory success rates for end-stage osteonecrosis of the femoral head (ONFH), even with MSCs [54]. To improve the outcome, Zhao et al. describe a modified technique using BMSCs associated with porous tantalum rod implantation combined with vascularized iliac grafting for the treatment of end-stage ONFH, and they followed up for 5 years, and these authors found that Harris hip score was improved from 38.74 ± 5.88 points (range 22–50) to 77.23 ± 14.75 points (range 33–95) [55]. It is worthy to mention that, in this procedure, approximately 10 mL of bone marrow from the subtrochanteric region was directly aspirated once the decompression tunnel was established during the surgery, avoiding the need for bone marrow aspiration from the iliac crest.

7. MSCs in intervertebral disc degeneration

Intervertebral disc degenerative is a serious worldwide problem for the aging population. The apoptosis of nucleus pulposus cells could be the main cause of intervertebral disc degeneration, with a variety of manifestations, that is, reduced number of the cells, the changes of the mechanical structure, down-regulated synthesis of matrix components (such as proteoglycan), nucleus pulposus dehydration, and increased metabolic waste [56, 57]. Many treatment options have been proposed, including physical therapy, pain medication, epidural steroid drug injection, disc radiating, myeloid nucleation, intervertebral fusion, and intervertebral disc displacement. However, these therapeutic approaches aim only to relieve the symptoms of disc degeneration, not treat its underlying cause. MSCs transplantation provides a new therapeutic strategy for promoting proteoglycan synthesis, decelerating the course of disc degeneration, and stimulating disc regeneration.

For example, Sobajima et al. reported that BMSCs was injected into the lumbar intervertebral disc of the New Zealand white rabbit, and found that the transplanted BMSCs survived and migrated to the fibrous ring after 24 weeks [58]. Hee et al. confirmed that BMSCs implantation and axial distraction may have a synergistic effect in reversing degenerative disc disease in the rabbit model [59]. Some scholars have discovered that drug stimulation can regulate the differentiation of nucleus pulposus MSCs into nucleus pulposus cell

by promoting expression of hypoxia-inducible factor to repair and reconstruct degenerated intervertebral disc [60].

In a recent study, under fluoroscopic guidance, the BMCs were injected into the nucleus pulposus of 26 patients' with chronic (>6 months) discogenic low back pain [61]. These authors found the evidence of safety and feasibility in the non-surgical treatment of discogenic pain using autologous BMCs with durable pain relief (71% VAS reduction) and Oswestry Disability Index improvements (>64%) through 2 years.

Overall, the BMSCs as a treatment of degenerated intervertebral disc is successful both in an animal model and in clinical studies; however, there are no long-term follow-up results and the number of reports and the number of cases are still relatively low. Another concern is that, at least in theory, BMSCs may cause osteophyte formation in the vertebral isthmus when it is released from the nucleus. Further clinical trials are needed to clarify these concerns.

8. MSCs in osteoporosis

Osteoporosis is a common metabolic bone disease, characterized by loss of bone mass, bone density reduction, and bone structure damage, which leads to increased bone fragility and risks of bone fracture [62]. The exact underlying mechanisms of osteoporosis are still unclear, but a shift of the cell differentiation of MSCs to adipocytes rather than osteoblasts partly contributes to osteoporosis [63]. Furthermore, it was observed that osteoclast activity (bone resorption) was enhanced, while osteoblast function (bone formation) decreased. For this reason, the drugs that inhibit the activity of osteoclasts have been widely used in clinical practice; however, these drugs have many complications, such as mandibular necrosis, reflux esophagitis, and atypical fracture [62, 64]. Recently, it is found that the decrease of BMSC to osteogenic differentiation and the increase of lipid differentiation is an important factor in the pathogenesis of osteoporosis [65, 66], therefore, one of the new ways to inhibit osteoporosis is to promote osteogenesis differentiation of endogenous BMSCs. In the meantime, BMSCs transplantation can also effectively increase bone mass and density, increase bone mechanical strength, correct the imbalance in bone metabolism, and increase bone formation, and is expected to provide a new strategy and method for the treatment of osteoporosis [67].

Scholars have carried out a large number of studies, including signal transduction, gene transcription, and post-transcriptional level, and found that miRNA and epigenetic modifications are probably the main mechanisms for BMSC differentiation [63]. In addition, conserved signal regulation, mechanical stimuli, radiation, and diet also play important roles in regulating the differentiation fate of MSCs. Even though an MSC transplant could, at least in theory, provide a treatment for osteoporosis, the clinical trials of MSCs in osteoporosis have just begun; nevertheless, the animal studies have already found that autograft or allogeneic MSC transplantation can increase the bone mass of animal models of osteoporosis [66, 67]. Since

osteoporosis is a systemic disease, and the hormone levels and cytokines have changed dramatically, it is still unclear whether the simple local MSC transplantation can improve these changes in the long-term. In addition, the bone marrow homing efficiency of MSCs and the long-term survival of MSCs are still uncertain.

9. MSCs in genetic diseases

9.1. Osteogenesis imperfecta (OI)

Osteogenesis imperfecta is a rare congenital bone development disorder characterized by bone fragility, blue sclera, deafness, and joint relaxation. Horwitz et al. reported three cases of OI using allogeneic bone marrow cells [68]. Six months after the implantation, the new bone formation was observed with a reduced frequency of fractures, suggesting that bone marrow cells could be used to treat OI. The further study from the same group with BMSCs transplantation [69], obtained similar results, that is, donor BMSCs survived in 5/6 OI patients' which significantly improved the clinical symptoms of these patients.

9.2. Hypophosphatasia (HPP)

Hypophosphatasia is a rare, heritable, metabolic bone disease due to deficient activity of the tissue-nonspecific isoenzyme of alkaline phosphatase [70]. The disease is characterized by the disturbance of bone and tooth mineralization and reduced serum ALP activity. Tadokoro et al. used allogeneic MSCs obtained from the patient's father for an 8-month-old patient with hypophosphatasia [71], and they observed improved respiratory condition, and de novo bone derived from both donor and patient cells. Similarly, Cahill et al. reported an 8-month-old girl with worsening and life-threatening infantile HPP improved considerably after marrow cell transplantation [72]. More importantly, 4 months after treatment, radiographs demonstrated improved skeletal mineralization. The authors speculated that donor bone fragments and marrow may provide precursor cells for distribution and engraftment in the skeletal microenvironment in HPP patients to form tissue-nonspecific isoenzyme of alkaline phosphatase-replete osteoblasts that can improve mineralization.

10. Conclusions

BMSCs are easy to obtain, isolated and amplified, which provide a wide application prospect for the treatment of orthopedic diseases. Here, we briefly reviewed the progressions ions of MSCs in a variety of orthopedic diseases. Many studies have demonstrated the safety and efficacy of autologous bone marrow MSCs transplantation in animal models as well as in human clinical trials. However, there are still some issues to be solved, such as the reference standards of BMSCs, the regulatory mechanism of proliferation and differentiation of BMSCs, the time, route of administration, and dosages of the transplant. With the further BMSCs researches, we believe that these problems will be solved soon.

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Conflict of interest

The authors declare no competing interests.

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Chapter 10

Human Placenta-Derived Mesenchymal Stromal Cells: A Review from Basic Research to Clinical Applications

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Additional information is available at the end of the chapter

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Abstract

Placenta-derived mesenchymal stem/stromal cells (PMSC) present several aspects that make them more attractive as cellular therapy than their counterparts from other tissues, such as MSC from bone marrow or adipose tissue in regenerative medicine. Placenta-derived MSC have been used to treat a variety of disorders, such as, cancer, liver and cardiac diseases, ulcers, bone repair, and neurological diseases. Placenta-derived MSC are relatively new types of MSC with specific immunomodulatory properties and whose mechanisms are still unknown. Placenta-derived MSC secrete some soluble factors that seem to be responsible for their therapeutic effects, i.e., they have paracrine effects. On the other hand, Placenta-derived MSC can also serve as cellular vehicles and/or delivery systems for medications due to their migration capacity and their tropism for injury sites. Nanotechnology is an important field, which has undergone rapid development in recent years for the treatment of injured organs. Due to the special characteristics of placenta-derived MSC, the combination of these cells with nanotechnology will be a significant and highly promising field that will provide significant contributions in the regenerative medicine field in the near future.

Keywords: placenta, mesenchymal stromal cells, immunoregulation, regenerative medicine, nanotechnology, cancer, neurodegeneration, vascular, bone, cartilage, liver, urology, intestinal

1. Introduction

1.1. Structure and function of human placenta

Human placenta is an indispensable organ during pregnancy for supporting the development of the fetus. The placenta is a unique organ since it is a multicellular barrier, in which

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both maternal and fetal cells coexist. Placenta performs functions of metabolic exchange and endocrine regulation between two genetically distinct individuals, the mother and the fetus, while maintaining immunological tolerance between them [1, 2].

The term placenta derives from the latin and means “flat cake” because of its discoid shape. At the end of pregnancy, it is about 15–20 cm in diameter, 2–3 cm thick, and 500 g in weight, that is, 1/6 of the fetal weight.

The placenta is constituted by structures of fetal origin, such as, the placental disk, the fetal membranes, divided in amniotic and chorionic membranes, and the umbilical cord. The placenta is also composed by a membrane of maternal origin termed the decidua that originates from the endometrium. The functional unit of the placenta is the chorionic villosity that forms the border between maternal and fetal blood during pregnancy (**Figure 1**).

1.2. Placenta development

Placenta development is a continuous process that starts during early embryological stages, even before gastrulation occurs. Four to five days after fecundation, the morula (solid mass of cells called blastomers) has reached the uterus. The appearance of a fluid-filled inner cavity marks the transition from morula to blastocyst and is accompanied by cellular differentiation: the surface cells become the trophoblast (giving rise to extraembryonic structures, including the placenta and the umbilical cord) and the inner cell mass gives rise to the embryo [3]. Just before the implantation into the endometrium, the internal cell mass or embryoblast, goes through important changes such as cellular reorganization that gives place to a top layer, the epiblast and a bottom layer named hypoblast or primitive endoderm. Some extraembryonic tissues such as the amnion derive from the epiblasts that delimit the amniotic cavity that hosts the embryo during pregnancy. Because of the increase in production of amniotic liquid during gestation, the amnion will expand, and merge with the trophoblast to give rise to the amnion-chorionic membrane. Another of the earliest differentiation events in human embryogenesis takes place in the trophoblast with the development of the external syncytiotrophoblast

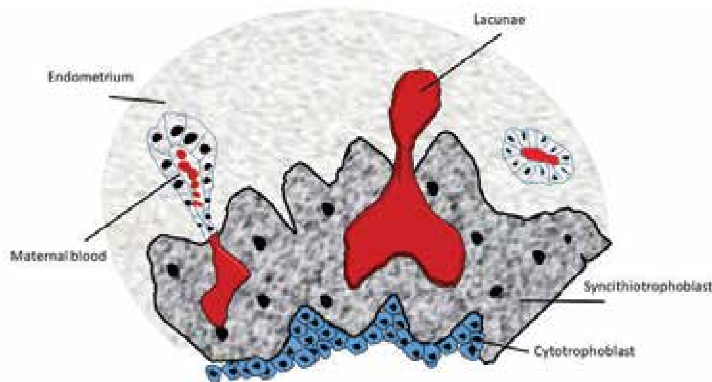

Figure 1. First stage in the interaction between fetal and maternal blood circulation. The syncytiotrophoblast erodes maternal vessels.

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and the internal cytotrophoblast. The cytotrofoblast is constituted by highly proliferative mononucleated cells. Syncytiotrophoblast is formed by fusion of cytotrophoblastic cells and has high invasive capacity. This syncytium is responsible for the implantation or anchorage of the blastocyst within the uterine walls.

The lytic activity of the syncytiotrophoblast, which is responsible for the degradation of the matrix of the endometrium, reaches the uterine capillaries, eroding them. As a result of vascular damage, maternal blood comes out to the syncytiotrophoblast where it forms lacunae; this lacunar stage is the first one toward a fetomaternal circulation. At the same time, the epithelial-like cells of the cytotrofoblast, which have continued proliferating, form accumulations that project toward the syncytiotrophoblast forming the chorionic villi that penetrate the decidua basalis [4]. These finger-like structures (cytotrophoblast covered with syncytiotrophoblast) are invaded by an extraembryonic mesoderm that, in the fourth week after fertilization, gives rise to blood vessels within each villi which makes possible the establishment of the interaction between the fetal circulation, in these embryo vessels, and the maternal blood contained in the trophoblastic lacunae (**Figure 1**). The different layers of the trophoblast (the cytotrophoblast and the syncytiotrophoblast), the basal membranes of the fetal vessels, and the vascular endothelium of these vessels constitute the placenta barrier that regulates the metabolite exchange between both circulations (fetal and maternal). It has been estimated that this exchange surface is about 5 m^2 at week 28 of gestation and reaches 10–11 m^2 at term [5]. Moreover, this barrier undergoes a progressive thinning throughout pregnancy going from 10 microns at the beginning to 1 or 2 microns at the end of the gestation [6]. The umbilical cord connects placenta to the fetus. It is a narrow tube that contains two arteries and one vein to transport metabolites between mother and fetus.

1.3. Regenerative medicine and placenta

Regenerative medicine is an interdisciplinary field within translational medicine whose purpose is to heal or replace damaged tissues or organs as a result of age, illness or trauma. It may involve the transplantation of stem cells that will repair the damaged tissue, stimulate the body’s own repair processes or serve as delivery-vehicles for therapeutic agents such as genes, cytokines, or therapeutic drugs.

Stem cells are unspecialized cells that have the capacity to renew themselves or differentiate toward more specialized cells. The proliferation of stem cells is indispensable for the maintenance of the stemness niche. The differentiation is the process by which, under certain physiological or experimental conditions, unspecialized cells are induced to become tissue- or organ-specific cells. The differentiation potential of stem cells is essential during the development of the embryo. In the adult, the main function of stem cells is the maintenance of the tissue homeostasis acting as an internal repair system.

Both embryonic and adult tissues are sources of stem cells with therapeutic potential. However, embryonic stem cells have some limitations in clinical practice, such as ethical concerns, difficulty in obtaining, and tumorigenicity. Adult stem cells have been identified in many organs and tissues, including brain, bone marrow, peripheral blood, adipose tissue, skeletal muscle, skin, teeth, heart, gut, liver, and placenta. Though the number of stem cells

is very small in many adult tissues, their isolation involves several risks and, once removed from the body, the cells have a limited capacity of proliferation and differentiation, making the generation of large quantities of stem cells difficult.

The placenta is a reservoir of stem cells with several advantages. What makes placenta such an interesting tissue for regenerative medicine? Placenta is spontaneously expulsed at birth, making the use of invasive methods unnecessary as in the case of other sources of adult stem cells. It is considered a medical waste and there are no ethical concerns in its use, unlike using embryonic stem cells [7]. Placenta is a high-yielding source of stem cells compared to other sources such as bone marrow and adipose tissue where the cell recovery decreases with donor age [8]. Versatility and differentiation potential of placental cells is very high probably due to their primitive origin [9]. Furthermore, pregnancy is an example of "tolerated allograft" and placenta is the immunoregulatory organ at the maternal-fetal interface [10]. Placenta is an immunoprivileged organ, and cells isolated from placenta display low immunogenicity in vitro [11] and in vivo [12] when xenotransplanted in immunocompetent animals. The feasibility of placental cells for allogeneic transplantation has been demonstrated [13].

In regenerative medicine, the effects of stem cells are not only restricted to cell or tissue restoration but also to transient paracrine actions. This paracrine action is related to factors produced and secreted by stem cells that will control the injury, modulate the immune responses, and promote self-repair in the surviving injured tissue [14]. Placenta plays a fundamental role in fetomaternal tolerance and this would explain why placenta-derived stem cells have an additional advantage over other stem cells in terms of immunomodulation [15].

Multiple mechanisms underlie maternal tolerance during pregnancy. Fetal and, in particular, placental tissues contribute to its immunoprivileged and immunoregulatory environment. Placental cells are characterized by the absence of MHC class II antigens that normally mediate graft rejection [16]. Placental cells not only express a low level of the highly polymorphic forms of the MHC class I antigens but also express the nonclassical form HLA-G that may play a role in the suppression of immune responses and contribute to maternal-fetal tolerance [17, 18]. Furthermore, through the release of hormones [19], cytokines [20], and soluble forms of MHC antigens, placental cells deviate maternal immune responses toward immune tolerance. Therefore, the cells of the innate immunity of the mother acquire a suppressive profile characterized by a diminished production of pro-inflammatory cytokines. In addition, the B cells and many T cells disappear, leaving the regulatory T cells (Tregs) as the major T-cell subpopulation, with both, immune suppressive and anti-inflammatory characteristics [21].

1.4. Placenta-derived stem cells

Different populations of cells with features of stem/progenitor cells have been isolated from placenta: hematopoietic, epithelial, trophoblasts, and mesenchymal cells.

Placenta is a hematopoietic organ since it harbors a large pool of hematopoietic stem cells (HSC) that possess functional properties of true HSC. Placenta-derived HSC can differentiate into all types of mature blood cells and are able to sustain the hematopoiesis during the life of the embryo. Placental HSC activity declines toward the end of gestation, possibly reflecting

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mobilization of placental HSC to the fetal liver and other developing hematopoietic organs within the embryo, such as thymus, spleen, and bone marrow [22].

The three layers of the placenta, such as the amnion, the chorion, and the decidua, are sources of stem cells. The amniotic layer is composed of a single-cell epithelial layer and a deeper mesodermal layer derived from the epiblast and hypoblast, respectively [23]. The chorion sheet is composed of the inner chorionic mesoderm similar to the mesenchymal region of the amnion and an outer layer of trophoblastic origin. The decidua, the uterine component of the placenta, is also a source of cells of mesodermal origin.

Amniotic epithelial cells (AEC) are very valuable stem cells for regenerative medicine. They have stem cell molecular markers such as OCT-4, Nanog, SOX-2, and Rex-1 (23). AEC do not have telomerase reverse transcriptase, show a stable karyotype, and do not originate tumors when injected. Amnion does not express MHC class II antigens, so AEC can elude the immune system. AEC can also modulate the immune system through an inhibition of the proliferation of T- and B-cells. In addition, AEC inhibit inflammation, as has seen in vitro [24].

Chorion trophoblastic cells (CTC) represent a mixed and still poorly characterized population of stem cells and there are no reliable methods to isolate them [25], and also, no consistent marking for identifying this population of cells [26].

Most of stem cells isolated from the placental tissues are cells of mesodermal origin and are named amnion mesenchymal stromal cells (AMSC), chorion mesenchymal stromal cells (CMSC), chorionic villi mesenchymal stromal cells (CV-MSC), and decidua mesenchymal stem cells (DMSC) [9, 27, 28] depending on the layer of origin. Inside the umbilical cord, there is a connective tissue that surrounds the umbilical vein and the two umbilical arteries. This tissue, also known as Wharton's jelly, is a rich source of mesenchymal stromal cells called umbilical cord mesenchymal stem cells (UC-MSC) [29]. They are all considered true mesenchymal stromal cells (MSC), as they meet the three minimal criteria proposed by the International Society for Cellular Therapy [30]. First, placenta-derived MSC exhibit plastic adherence in culture. Second, they express a specific set of cell surface markers, such as CD105, CD73, and CD90, and do not express hematopoietic markers including CD34, CD45 and CD14 or CD11b, CD79a or CD19, and HLA-DR. Third, they have the ability to differentiate in vitro into different mesodermal cell lineages including adipocytes, chondrocytes, and osteoblasts. In addition, AMSC and CMSC are from fetal origin according to the first international workshop on placenta-derived stem cells [31].

Cells with properties of mesenchymal stromal cells have also been isolated from the amniotic fluid (AF) which is used to perform the evaluation of karyotyping and prenatal diagnostic testing. AF is a source of MSC that could be used as autologous cellular therapy for perinatal disorders [32]. These AF-MSC can be easily isolated, have minimal ethical objections, high renewal activity, multiple differentiation capacity, and maintain genetic stability in culture [33].

In this chapter, we will refer to placenta-derived mesenchymal stromal cells as placenta mesenchymal stromal cells (PMSC) regardless of the placenta region where they were isolated.

1.5. Placenta-derived mesenchymal stromal cells

Mesenchymal stromal cells (MSC) can be isolated from virtually all adult tissues in the body, although not always in large quantities. They are thought to be a precursor cell population capable of reconstituting all the cellular elements that comprise the supportive stromal tissue in each organ [34]. First described in bone marrow as a subset of non-hematopoietic cells [35], they have become the paradigm cell in regenerative medicine. MSC are the most widely studied cell type in both preclinical and clinical trials. The advantages of MSC include ease of isolation and subsequent maintenance in culture, high expansion capacity, high plasticity, and tissue repair activity. The restorative activity of MSC is not necessarily by the replacement of dead or damaged cells, but also, by paracrine actions that mediate immune-regulation and promote cell growth and/or differentiation (**Figure 2**). Besides, MSC do not form teratomas after transplantation, ensuring safety to the host and, their low immunogenicity makes them suitable for allogeneic transplantation. Furthermore, these cells have the ability to migrate to inflammatory microenvironments [36] and tumors [12, 37], where they play an active role inducing many processes, such as angiogenesis and wound healing, mainly in a paracrine manner [38]. This feature provides an important therapeutic advantage to MSC since they can be injected via systemic infusion and can be used as vehicles for the delivery of drugs such as anticancer agents to the tumor site.

The use of placenta as a source of MSC has several advantages with respect to other adult MSC. Besides the ease of extraction of MSC from the placenta without invasive methods, the isolated MSC represent a more homogeneous and primitive population [9, 39]. The last feature is associated with a higher proliferative rate in culture compared to bone marrow MSC [40]. This fact makes it possible to achieve a greater number of cells in fewer passages

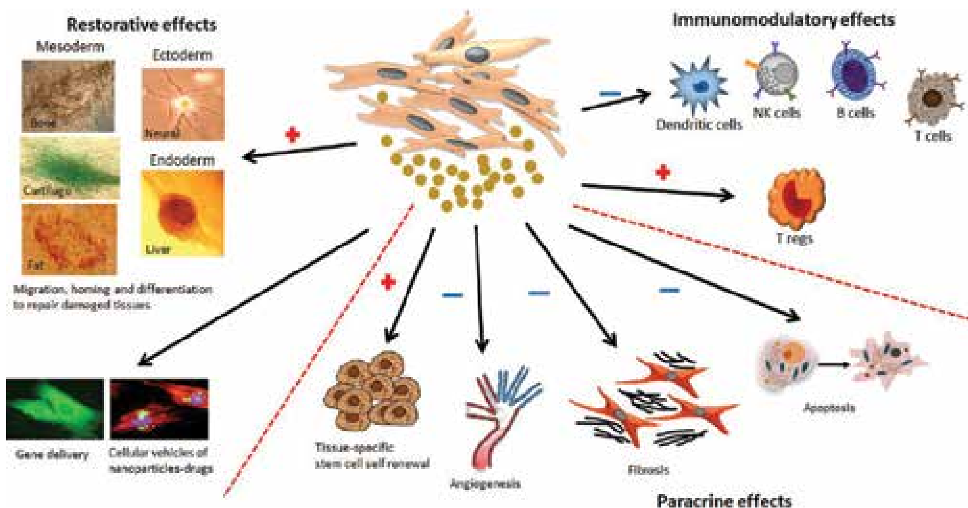

Figure 2. PMSC mechanisms of action. PMSC can migrate, home, and differentiate into tissue specific cells to repair injured tissue, transport restorative genes and used as a cellular vehicles of therapeutic agents. PMSC also exert their actions through paracrine effects and have immunomodulatory properties.

reducing the risk of ex vivo senescence influencing gene expression and resulting in aging phenotype [41, 42]. The senescent state needs to be taken into account for quality control of PMSC in cellular therapy. In addition, the clinical efficacy and safety of PMSC could be higher, compared to other sources of MSC, since PMSC are younger cells that have been exposed less time to harmful agents, such as reactive oxygen species (ROS), chemical and biological agents, and physical stressors [43]. Also, PMSC have a limited capacity to grow in culture related to low telomerase activity, which is also lost during proliferation, making them a safe product to be used in regenerative medicine [9]. Moreover, PMSC could be advantageous with respect to migratory properties and homing capacities into damaged tissues. Homing of MSC is basically dependent on the release of chemoattractants by the injured tissue and the expression of chemokine receptors on the MSC membrane. For extravasation into tissue, MSC have to attach to and migrate through the endothelium. Several integrins and other adhesion molecules are known to be expressed on MSC. Dependence on the VLA-4/VCAM-1 (very late antigen-4/vascular cell adhesion molecule-1) axis for MSC adherence to endothelial cells has been demonstrated [44]. PMSC have a higher expression of VLA-4 compared to bone marrow MSC suggesting that PMSC may have enhanced properties for homing to damaged tissue [45].

2. Therapeutic applications of placenta mesenchymal stromal cells (PMSC) in preclinical models

Stem cell therapies are expected to provide substantial benefits to patients suffering a wide range of pathologies. The plasticity and pleiotropic properties of PMSC that include immunomodulation and inflammation control, angiogenesis, neuroprotection, and antiapoptosis, among others, have been widely evaluated at the preclinical level [9, 46, 47].

2.1. Use of placental mesenchymal stem/stromal cells in cardiovascular diseases

2.1.1. Myocardial infarction

Myocardial infarction (MI) is a major cause of death and disability worldwide. MI occurs when there is an interruption in blood flow to the heart muscle followed by heart ischemia. Since regeneration of heart muscle is virtually absent, damaged myocardium after infarct is replaced by scar tissue leading to reduced cardiac function. PMSC transplantation is a promising strategy to restore cardiac function and reduce myocardial fibrosis in MI due to their angiogenic and immunosuppressive properties.

PMSC have the potential to differentiate into cardiomyocytes, and exhibit spontaneous beating under in vitro conditions suggesting that they can therapeutically act in the cardiac repair process [9, 48, 49]. Several groups have investigated the effects of PMSC when transplanted in animal models of MI. PMSC injected into rat hearts after the induction of a MI showed integration into cardiac tissues and in vivo transdifferentiation into cardiomyocytes [48]. The CXCR4 chemokine receptor and its ligand, stromal cell-derived factor (SDF-1)

axis (CXCR4-SDF1) is the main pathway mediating migration of MSC toward injured tissues. Since it has been shown that chemokine receptor type 4 (CXCR4) is greatly induced in PMSC by hypoxia, a high chemotactic response of PMSC to the ischemic microenvironment of the infarcted heart is expected [50]. Intravenous injection of PMSC in a rat model of infarct showed a sustained cardiac function over 32 weeks from injury [51]. Preconditioning PMSC by hyaluronan mixed ester of butyric and retinoic acid (HBR) potentiates their reparative capacity. Transplantation of preconditioned PMSC in pigs produced a significant reduction in scar size, higher myocardial perfusion and glucose uptake, enhanced capillary density, and decreased fibrous tissue [52]. The paracrine potential of conditioned medium (CM) of PMSC has also been evaluated. Injection of PMSC-CM limited infarct size and cardiomyocyte apoptosis, while promoting capillary density in the infarct border area in a rat model of ischemia/reperfusion [53].

2.1.2. Critical limb ischemia

Critical limb ischemia (CLI) is the advanced stage of peripheral artery disease (PAD) with progressive stenosis, and ultimately the obstruction of peripheral arteries. The consequences of the markedly reduced blood flow to the lower limbs are pain at rest, nonhealing ulcers, and gangrene. The risk factors of PAD are advanced age, hyperlipidemia, hypertension, and mainly diabetes. Unfortunately, amputation, in many cases, is the only therapeutic option for CLI as blood capillaries cannot be corrected, and restenosis of vessels is produced.

Preclinical studies have reported benefits of cell therapy in neovascularization in several mouse models of hindlimb ischemia. PMSC have demonstrated pro-angiogenic effects when intramuscularly injected into the ischemic region of the affected limb, improving blood flow and promoting new vessel formation [54–56]. Similar results have been described in a diabetic nude rat model [57]. Moreover, CM from the PMSC also had pro-angiogenic action in a mouse hindlimb ischemic model, comparable to the PMSC transplanted group in the same study, revealing that PMSC action resulted primarily from a paracrine action of the angiogenic factors released from the PMSC [55]. However, in another study, cells were more efficacious than cell lysate in rescuing blood flow, probably indicating the importance of prolonged paracrine effect for maximal blood flow recovery [57].

2.1.3. Stroke

Stroke is an acute focal injury of the central nervous system (CNS) by a vascular cause, including cerebral infarction, intracerebral hemorrhage (ICH), and subarachnoid hemorrhage (SAH), and is a major cause of disability and death worldwide. Thrombolysis is the most commonly used therapeutic approach although most patients fall outside of the clinical time window for effective treatment.

Experimental data show that stem cell therapy can limit neuronal degeneration and improve the functional outcome. The neuroprotective action of PMSC has been demonstrated in a rat model of stroke. Intravenous administration of PMSC, 4 hours after the injury, resulted in a significant improvement of functional outcome and significant decrease of lesion volume, correlating with increased vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and brain-derived neurotrophic factor (BDNF) levels in the ischemic brain compared to controls [58].

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2.2. Use of placental mesenchymal stem/stromal cells in cancer

Cancer is one of the main problems in public health worldwide. Despite great progresses having been made in understanding the molecular basis of cancer, and the rapid advances in diagnosis, the efficacy of current treatment strategies is limited and mortality is still high. Stem cell-based treatments have been extensively explored for their possible potential to treat various cancers. Tumor microenvironment resembles a wound environment as tumors are considered as unhealed wounds [60]. Inflammatory and wound microenvironments induce migration of PMSC [36, 61]. Due to the characteristic of placenta-derived MSC, these cells represent an important tool for their use in anticancer therapies. First, PMSC can migrate and engraft into the tumor site and directly affect tumor biology through paracrine signaling. Second, PMSC could be used for the specific delivery of drugs to tumors thus reducing the doses administered and the side effects. Third, PMSC can also be genetically modified to give a stable expression of antitumor factors specifically in the tumor.

Placenta-derived MSC have an intrinsic tropism for sites of injury regardless of tissue or organ. Furthermore, it has been shown that PMSC and CM from PMSC are able to inhibit the proliferation of several tumor cell lines [62]. Moreover, PMSC have an antitumor effect in vivo, inhibiting tumor progression when were intravenously injected in a rat model of mammary cancer [12]. Similarly, PMSC showed antitumor effects in vivo when previously expanded in the presence of tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) [63] and when engineered to deliver growth factors to the tumor site, such as, pigment epithelium-derived factor [64], or endostatin [65].

2.3. Use of placental mesenchymal stem/stromal cells in neurological diseases

Neurodegeneration involves a progressive and irreversible loss of neurons. Alzheimer's, Parkinson's, and multiple sclerosis are some of the more studied neurodegenerative syndromes. The neuromuscular disorder amyotrophic lateral sclerosis (ALS) is a degenerative process caused by motor neuron loss. To date, there is no cure for these diseases. Cell therapy with stem cells arises as a therapeutic alternative based, either on the replacement of the lost neurons, or on a neuroprotective action through release of neurotrophic factors. PMSC are able to differentiate in vitro into several neural lineages, including neurons [9, 66], oligodendrocytes [66], glial cells [67], and dopaminergic neurons [68].

2.3.1. Parkinson's disease

Parkinson's disease (PD) is a progressive neurodegenerative disease associated with a specific loss of dopaminergic neurons in the substantia nigra and depletion of dopamine levels in the striatum. The main therapeutic objective in PD is the recovery of dopaminergic neurotransmission in the striatum. Cellular replacement has been emerged as a suitable therapeutic strategy. First-trimester human PMSC differentiated to neural progenitors and transplanted into the striatum of a rat model of PD, underwent dopaminergic differentiation and showed an attenuation of the symptoms [69]. PD motor pathology is also accompanied by other disabilities, such as, mood disorders, constipation, and hyposmia. It is expected that besides the regenerative effects of PMSC, the secretion of trophic factors, their anti-inflammatory and antiapoptotic effects, could also alleviate these nonmotor symptoms.

2.3.2. Alzheimer's disease

Alzheimer's disease (AD) pathogenesis is characterized by a deposition of β-amyloid peptide and hyperphosphorylation of tau causing loss of the synaptic and neuronal activities and neuroinflammation. It has been demonstrated that PMSC, transplanted into an Alzheimer's disease mouse model, modulated the inflammatory response. Moreover, mice injected with PMSC presented higher levels of β-amyloid degrading enzymes, reduced levels of pro-inflammatory cytokines, and increased levels of anti-inflammatory cytokines (TGF-β and IL-10). The effect of PMSC injection resulted in an improvement of memory function [70].

2.3.3. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by loss of nerve cells in the brain and spinal cord, leading to muscle weakness, paralysis, respiratory problems, and eventually, death. Multiple intravenous injections of PMSC in a mouse model of ALS, resulted in a protection of motor neurons from inflammatory effectors delaying functional deterioration and increasing lifespan [71].

2.3.4. Multiple sclerosis

Multiple sclerosis (MS) is a chronic disease of the central nervous system characterized by demyelinated areas in the brain and spinal cord that heal forming a glial scar (sclerosis). It is believed that MS is caused by T cell-mediated autoimmune reaction against proteins of the myelin sheath inducing oligodendrocytes and neuronal loss. Most of therapies in MS patients target the immune system or the inflammatory process. Since the pathogenic process of MS can be divided into inflammatory and degenerative phases, PMSC-based cell therapy seems appropriate since it may be able to specifically regulate immune responses and also induce neuronal regeneration. The animal model that closely resembles the MS symptoms is the experimental autoimmune encephalomyelitis (EAE) in mice where the animals are injected with myelin antigens that initiate an immune response. Several preclinical trials based on the treatment of EAE animals with PMSC have been published. Intracerebroventricular (ICV) transplantation of PMSC at day 5 (pre-symptomatology) or day 14 (at the beginning of the disease) after immunization, significantly reduced the severity of the disease and prolonged survival without delaying the onset of the disease [72]. Several intraperitoneal injections of PMSC in EAE mice delayed the onset of the symptoms and decreased disease incidence in the treated group respect to control, as well as inhibiting T cell proliferation and downregulating the production of pro-inflammatory factors while increasing the production of anti-inflammatory cytokines [73]. Likewise, ICV or intrathecal (ITH) injection of PMSC in EAE rats, also delayed the onset of motor symptoms, reduced inflammation, prevented axonal loss, and reduced disease severity [74].

2.4. Use of placental mesenchymal stem/stromal cells in bone and cartilage diseases

Bone regeneration is the physiological process of bone formation, which is involved in continuous remodeling throughout adult life, and can be observed during bone healing after damage. However, there are large lesions created by traumatism, infection, tumor resection or skeletal

abnormalities in which physiological bone regeneration is not sufficient. There are also other conditions, such as osteoporosis, in which regeneration is compromised. PMSC have the potential to differentiate into osteogenic lineage, and seem to be an appropriate therapeutic option for bone regeneration. The use of 3D scaffolds that support cell differentiation and improve engraftment has become habitual in PMSC-mediated bone regeneration therapy. Several published studies confirm that PMSC have potent in vivo bone-forming capacity and may be worthwhile candidates for in vivo bone tissue repair. So, when PMSC were subcutaneously injected into severe combined immunodeficiency (SCID) mice with hydroxyapatite/tricalcium phosphate particles as a vehicle, new bone formation was found throughout all implants [75]. Another study showed that PMSC administered in combination with nanobiphasic calcium phosphate ceramics in a rat model of femur bone defects produced complete healing of the defect in 3 months without evidence of fibrosis [76].

Osteoarthritis (OA) is a degenerative process of the cartilage in joints. There is still no treatment available to improve or reverse the degenerative process and current pharmacological treatments are only palliative. Given the potential of PMSC to differentiate into musculoskeletal lineages including bone and cartilage, MSC have been proposed as an optimal regenerative cellular therapy for degenerative musculoskeletal conditions as OA. There are numerous data that support this hypothesis in preclinical models. PMSC embedded in a collagen I gel and transplanted in a rat model of femoral cartilage defect appeared to cover the tissue defects with soft tissue positive for toluidine blue suggesting in vivo differentiation of transplanted cells [77]. Also PMSC grown on silk fibroin and transplanted into the knee in rabbits with knee osteochondral defects resulted in newly created hyaline cartilage without inflammatory response [78]. Similarly, PMSC seeded onto poly lactic-co-glycolic acid (PLGA) and preconditioned in chondrogenic medium were well tolerated and found in the reparative tissue of OA rabbit knees 8 weeks after transplantation [79].

2.5. Use of placental mesenchymal stem/stromal cells in liver diseases

Cirrhosis is the common end-stage of most of the injuries affecting the liver such as virus infections, chronic alcoholism, metabolic diseases, or acute liver failure. A scar is formed by extracellular matrix, making the normal function of the liver difficult. Cirrhosis is an irreversible state that can become life-threatening and, frequently, liver transplantation is the only alternative for healing. Donor shortage and continuous need for immunosuppression are the main limitations to liver transplant and cell transplantation appears as a suitable alternative. In addition to fetal and adult hepatocytes, stem cells are considered for cell transplantation. PMSC can be helpful since their potential capacity to differentiate to hepatic-like cells and form functional three-dimensional structures have been reported [80].

Transplanted into animal models of disease, PMSC induced a significant reduction of fibrosis and of serum levels of transaminases. Liver regeneration has been proposed to be promoted by the induction of autophagy process [81], stimulation of liver cell proliferation [82], decreased apoptosis, and suppression of stellate cells activation [83]. Although no evidence of differentiation of the transplanted cells into hepatocytes was reported in a CCl_4-induced fibrosis rat model [82], in other models, PMSC engraftment and expression of human albumin and α-fetoprotein have been reported [83–85].

2.6. Use of placental mesenchymal stem/stromal cells in intestinal inflammatory diseases

Crohn's disease (CD) and ulcerative colitis (UC) are chronic conditions caused by a sustained inflammation of the intestinal epithelium that ends in tissue destruction throughout the gastrointestinal tract. It is believed that these disorders are the result of an abnormal host immune response to intraluminal antigens in genetically predisposed individuals. Several genetic variants of nucleotide-binding oligomerization domain 2 (NOD2) are associated with the development of Crohn's disease [86]. Both pathologies have a major impact on the quality of life and there is no curative treatment. Furthermore, many patients are not responsive to current therapy.

Intraperitoneal administration of conditioned medium from PMSC ameliorated clinical parameters in a mouse model of dextran sulfate sodium (DSS)-induced colitis [87]. Intraperitoneal injection of PMSC also prevented the loss of body weight and decreased the mortality of mice. These benefits were greater when NOD2-activated PMSC were used [88].

2.7. Use of placental mesenchymal stem/stromal cells in urological diseases

Stress urinary incontinence (SUI) is a widespread disorder, commonly associated with childbirth, with a detrimental impact on the quality of life. SUI triggers a weakening of muscles and ligaments causing involuntary leakage of urine during physical activity, sneezing, or coughing. Surgical intervention to place a tissue sling that provides support to the urethra is the usual therapeutic action.

Animal models of SUI have been employed to prove the benefits of cell therapy in this pathology. Periurethral injection of myogenic differentiated PMSC in SUI mice restored the urethral sphincter to apparently normal histology and function [89].

3. Use of placenta mesenchymal stem/stromal cells (PMSC) and nanotechnology for tissue regeneration

The goal of cell-based regenerative medicine is to repair, replace, or regenerate cells, tissues, or organs when damaged. However, there are still some unresolved issues such as engraftment of transplanted cells onto the injured tissue and the survival for the time needed to repair the damage. Nanotechnology can be very helpful since nanomaterials can be used as scaffolds to improve the engraftment of stem cells onto the damaged tissue. In addition, the use of nanoparticles (NPs) for gene/drug delivery can complement the therapeutic benefits of transplanted stem cells, and allow the tracking of the cells inside the body [90].

Several reports described the therapeutic application of PMSC combined with biomaterials. PMSC proliferation and differentiation into myocardial and neuronal cells improved when the cells were grown on top of gold-coated collagen nanofibers (GCNFs) [91]. The peptide hydrogel PuraMatrix® (PM; 3-D Matrix, Ltd) was used to support PMSC in rat models of both acute MI and post-MI ischemic cardiomyopathy. The peptide hydrogel and the PMSC create a film to coat the heart. The epicardial "coating" method has advantages with respect to intramyocardial injection such as higher survival of the transplanted cells and lower complications [92].

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In bone regenerative medicine, the RKKP glass ceramic has been proposed as a biocompatible support for PMSC. RKKP exhibits a higher osteointegration rate compared to other ceramic materials mainly in osteopenic bone. Additionally, the biology of PMSC is not affected when grown over this support while maintaining their osteogenic potential [93] PMSC seeded over poly-L-lactic acid (PLLA) nanofibrous scaffolds and subjected to osteogenic conditions have been successfully grafted in a rabbit model of sternal defect closure [94].

Some systems have shown suitable behaviors as recipients of PMSC for cartilage regeneration. Collagen sponge allowed the formation of a cartilage-like tissue both, in vitro and in vivo, under chondrogenic-inducing conditions [95]. Similarly, PMSC embedded in alginate incorporating nanosized calcium-deficient hydroxyapatite (nCDHA) and/or a recombinant protein containing arginine-glycine-aspartate (RGD) and seeded over poly(D,L-lactide-co-glycolide) (PLGA) gave rise to cartilage formation [96].

The use of nanoparticles for gene/drug delivery can significantly contribute to the advance of regenerative medicine. The use of stem cells as carriers of NPs containing biologically active molecules (e.g., pro-survival, anti-inflammatory) or chemicals such as anticancer drugs is very promising. PMSC have been employed as a platform to load mesoporous silica nanoparticles. NP loading did not affect the chemotactic ability of PMSC toward tumors in vitro and in vivo. When carrying doxorubicin-loaded NP, PMSC promoted breast cancer cells death in a co-culture system [97]. In a proof of concept, ultrasound-responsive NPs loaded with antitumor drugs were transported to tumor tissues by PMSC, and the cargo was released by NPs only after ultrasound application [98].

In vivo monitoring of cells, after transplant, is needed and NP-based probes are useful for this purpose. They offer the possibility of tracking the bio-distribution and engraftment of cells into the body with minimally invasive techniques. However these probes have to ensure minimal changes in cell phenotype [97]. PMSC have been efficiently labeled with albumin-conjugated fluorescent nanodiamonds (FNDs) [99], with silica-coated magnetic nanoparticles incorporating rhodamine B isothiocyanate, MNPs@SiO2(RITC) [100], with rhodamine B labeled mesoporous silica nanoparticles [98] and with human serum albumin coated iron oxide nanoparticles (HSA-IONPs) [101] without any detrimental effect.

4. Therapeutic applications of placenta mesenchymal stem/stromal cells (PMSC) in clinical trials

Based on the benefits produced by transplanted PMSC in different animal models resembling human diseases, some clinical studies have been carried out and there are also an increasing number of ongoing clinical trials. The web pages http://www.clinicaltrialsregister.eu and http://www.clinicaltrial.gov offer up-to-date information on clinical trials giving current status. There are a good number of completed trials of which no results have yet been published. Other completed studies and clinical trials have published reports with the results obtained demonstrating the safety of the use of PMSC. In general, therapeutic benefits have been found.

Intracoronary infusion of UC-MSC in MI patients resulted in safe and significantly improved myocardial viability and the perfusion within the infarcted area. Improvement in some

parameters such as the increase in the left ventricular ejection fraction (LVEF) and decreases in end-diastolic volumes and LV end-systolic volumes were observed up to 18 months after treatment [102]. RIMECARD is a phase I/II clinical trial that has demonstrated the safety and efficacy of the intravenous infusion of UC-MSC in patients with chronic heart failure and reduced ejection fraction. Improvements in left ventricular function, functional status, and the quality of life were observed in treated subjects [103].

Cell therapy has been introduced as a new therapeutic attempt to restore blood flow and attenuate ischemia promoting collateral vessel formation in CLI. In January 2017, a Phase III study of PLX-PAD cells[1] in the treatment of critical limb ischemia (CLI) has been cleared by the U.S. Food and Drug Administration (FDA). Data from previous studies have shown that by increasing tissue perfusion, PMSC may improve the healing of wounds in CLI patients, and could allow for significant delays in events of amputation and death.

Safety and efficacy of UC-MSC infusion in patients with decompensated liver cirrhosis have been reported in a 1-year follow-up study. There were no significant side effects or complications and there was a significant reduction in the volume of ascites and improvement in liver function, as indicated by the increase of serum albumin levels and a decrease in total serum bilirubin levels [104].

Therapeutic effects of PMSC transplantation in MS patients have been evaluated in different studies. Intravenous infusion of UC-MSC appears to be safe and well tolerated in patients with MS, and the overall symptoms of treated patients remained stable or improved compared to the control group [105]. In another clinical trial, patients with relapsing-remitting MS or with secondary progressive MS randomly received PMSC (PDA-001)[2] and most treated subjects had stable or decreasing Expanded Disability Status Scale scores [106].

OA affecting the hip can mean, in many cases, the need for a total hip replacement (THR). A frequent side effect of THR is a gluteus medius injury. PMSC administered directly to the injured muscle during surgery have demonstrated their safety and efficacy inducing a greater increase in the gluteus medius muscle strength than placebo, and a significant improvement in muscle volume based on MRI. EudraCT Number: 2011-003934-16.

Safety of the intravenous administration of PMSC (PDA001) to moderate-to-severe Crohn's disease patients unresponsive to other therapies has been demonstrated and some remission rates of the disease have been reported [107]. Likewise, in a randomized controlled clinical trial, intravenous injection of PMSC patient condition improved significantly allowing a significant reduction in steroid dosage. Additionally, several patients with anal fistula showed remarkable improvement [108].

[1] PLX-PAD – Placenta eXpanded adherent stromal cells produced by PluriStem Ltd. PLX-PAD cells are derived from the decidua of human placenta and are expanded using the company's 3D proprietary technology.

[2] PDA-001 (previously cenplacel-L) is a placenta adherent cells-based therapy developed by Celgene Cellular Therapeutics (CCT, a subsidiary of Celgene Corporation) to treat autoimmune diseases. It is administered as an intravenous injection.

5. Conclusions

PMSC are promising candidates for use in regenerative medicine in humans. Cell therapy using PMSC is based mostly on three important characteristics of these types of cells: (i) their inherent reparative capacities or by secretion of paracrine factors; (ii) their homing and engraftment abilities; and (iii) their immune modulation capacities. However, clinical use of PMSC is still in its infancy and most of the trials are, to date, under development. Most studies of cellular therapy have been realized with autologous cells. Nevertheless, the use of patient's own cells has several limitations. First, there is a time-limiting factor as the expansion and quality control of autologous cells may require several weeks. Furthermore, the cells can show less potency due to inherent aging aspects and, even, certain characteristics of the subject may render autologous transplantation unfeasible as occurs in the case of elderly patients and those having a specific systemic disease such as diabetes. In contrast, allogeneic MSC have the potential to be mass-produced rapidly so they can be readily available and administered immediately. They can be obtained under more standardized and strictly validated conditions and probably reduce costs. To date, published data regarding reliability of treatment with PMSC indicate that the use of PMSC is safe and therefore there are already products "off-the-shelf." Although most clinical trials are ongoing or have no published results, there are some favorable data regarding to the efficacy of treatments with PMSC.

Stem cell nanomedicine is a very promising field that at the preclinical level has yielded very encouraging results. Treatment of certain pathologies can benefit from the use of scaffolds that provide a three-dimensional structure to give support to the cells, promoting their adhesion and growth, so definitely improving the engraftment and therefore the therapeutic results. Besides the use of cells as carriers of nanoparticles to deliver drugs inside the injured tissue and, even more, the possibility of stimulus-controlled release of the drug appears exciting.

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Conflict of interest

The authors declare no conflict of interest.

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Stromal cells are connective tissue cells of any organ, and they support the function of the parenchymal cells of that particular organ. Stromal/stromal stem cells are fundamentally a heterogeneous population of cells with contradictory differentiation potential depending upon their environmental niche. Stromal cell biology is not only intriguing, but equally stromal cell ontogeny *in vivo* remains challenging. In recent years there has been substantial advances in our understanding of stromal cell biology, especially stromal cell isolation, characterization, differentiation, and interactions in physiological (epithelial–stromal interactions) as well as pathophysiological (stromal–cancer interactions) contexts. In addition, stromal cells are also utilized more and more as a therapeutic tool not only in the field of gene therapy but also in the translational field of tissue engineering and regenerative medicine. Therefore, the goal of this book is to consolidate the recent advances in the area of stromal/stromal stem cell biology covering a broad range of interrelated topics in a timely fashion and to disseminate that knowledge in a lucid way to a greater scientific audience. This book will prove highly useful for students, researchers, and clinicians in stem cell biology, developmental biology, cancer biology, pathology, oncology, as well as tissue engineering and regenerative medicine. This quick reference will benefit anyone desiring a thorough overview of stromal cell structure, function, and its therapeutic implications.

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