

Methodology, biology and clinical applications of mesenchymal stem cells

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1. ABSTRACT

Mesenchymal stem cells (MSCs) are adult stem cells able to give rise to mature mesenchymal cell types. Plastic-adherent cells are operationally defined as MSCs based on their ability to proliferate and differentiate into cells such as osteoblasts, adipocytes and chondrocytes. In the past ten years, cultured MSCs have been shown to

exhibit great plasticity in culture, as they can differentiate into cells with ectodermal and endodermal characteristics, suggesting their use as a source of cells to treat different diseases. More recently, cultured MSCs were found to secrete various bioactive molecules that display anti-apoptotic, immunomodulatory, angiogenic, anti-scarring, and chemoattractant properties, providing a basis for their use as tools to create local regenerative environments *in*

Table 1. Phenotypic markers of mesenchymal stem cells. Molecules present (positive) or absent (negative) on MSCs

Positive	Negative
3G5 antibody-defined ganglioside	vWF
Calponin	CD4
Desmin	CD11a
Nestin	CD11b
NG-2 proteoglycan, HMW-MAA	CD14
Sca-1 (mouse only)	CD15
Stro-1	CD18
Vimentin	CD25
α -SMA	CD31
CD9	CD34 ¹
CD10	CD45
CD13	CD49d ²
CD29	CD50
CD44	CD62E
CD49a	CD62P
CD49b	CD117 ²
CD49c	CD133
CD49e	CD144
CD51	
CD54	
CD58	
CD61	
CD62L	
CD71	
CD73	
CD90	
CD102	
CD104	
CD105	
CD106	
CD109	
CDw119	
CD120a	
CD120b	
CD121a	
CD123	
CD124	
CD126	
CD127	
CD140a	
CD140b	
CD146	
CD164	
CD172a	

¹Some results show presence in adipose-derived mesenchymal stem cells, ² Positive in some animal species

vivo. Whereas the properties of cultured MSCs have been studied for a long time, their exact location *in vivo* is slowly becoming apparent as evidence indicates that pericytes behave as stem cells throughout the organism. In this review, we discuss some aspects of MSC basic biology, the methodology involved in MSC culture, and some clinical and pre-clinical applications of cultured MSCs.

2. INTRODUCTION

A mesenchymal stem cell (MSC) may be defined as a type of adult stem cell (ASC) with an intrinsic potential to give rise to different mesenchymal cell types such as osteoblasts, chondrocytes, adipocytes, tenocytes, and others. This definition of MSC can be found in nearly every article focusing on this ASC since the introduction of its concept by Dr. A. I. Caplan in 1991 (1). Historically, research involving the cells currently referred to as MSCs dates back to the 60s and 70s, when Dr. Alexander J. Friedenstein and colleagues started researching fibroblastic

cells from bone marrow (BM) of rodents and rabbits (reviewed in (2)). At first, these cells were not called MSCs, not even were termed stem cells, but were considered to be fibroblastic precursors derived from an entity with unknown anatomical location in BM termed the colony-forming unit-fibroblast (CFU-F). Experiments involving transplantation of BM cells into ectopic sites including the renal capsule resulted in ectopic bone formation, and brought up the notion that BM houses osteogenic precursors (reviewed in (2)). Later, the fibroblastic colonies derived from BM cells were found to be able to differentiate into cells with characteristics of osteoblasts, chondrocytes, and adipocytes (reviewed in (2)). By that time, Dr. T. M. Dexter and colleagues developed a cultured system to study hematopoiesis *in vitro* (3). In this system, cells bearing hematopoietic stem cell (HSC) characteristics were found to be non-adherent to the culture vessels, and to be dependent on the establishment of a layer of adherent cells that were viewed as representative of the BM stromal environment. The notion that CFU-F was derived from the stromal compartment of BM became established, and the term bone marrow stromal cell used in reference to these culture-adherent BM cells (4).

The term MSC was devised by analogy with the model proposed for hematopoiesis, where a stem cell gives rise to progenitor cells from different lineages that will ultimately differentiate into the different hematopoietic cell types (1). While many adhered to the MSC nomenclature, others preferred to continue using the term marrow stromal cell, defined by the same acronym (5). The term marrow stromal cell, in essence, encompasses any cell belonging to the BM stroma, e.g., fibroblasts, adipocytes, and even macrophages (called histiocytes in BM). The term, however, became used in reference to adherent BM cells able to proliferate *in vitro*, and to differentiate into osteoblasts, chondrocytes, and adipocytes under appropriate culture conditions. On the other hand, the term MSC refers exactly to this specific BM cell population.

As shown above, MSCs are mainly defined by means of *in vitro* assays. Although MSCs can be further characterized by their ability to form bone (and cartilage, to a lesser extent) when inserted into porous ceramic vehicles and subcutaneously implanted into mice (6), the implanted cells are derived from cultures. *In vitro* manipulation provides a means for the obtainment of MSCs; therefore, different culture techniques have been developed for this purpose. The consequence of applying different methodologies to isolate and culture cells bearing MSC characteristics is that different cell populations may be selected by the culture conditions. Also, phenotypic differences between cell populations maintained under different conditions may simply reflect differences in their response to different environments. To date, MSC-related stem cells include MAPCs, MIAMI cells, and rapidly self-renewing (RS) cells (7). Since no study thus far has analyzed each of these different cell types when cultured under the conditions described for the others, it is not possible to precisely say if they constitute intrinsically different entities, or if they can be arranged in a hierarchy. Consequently, these MSC-related cell types cannot be considered equivalent.

3. CONDITIONS EMPLOYED FOR MSC ISOLATION, CULTURE EXPANSION, AND DIFFERENTIATION

Currently, the culture of MSCs from human BM most invariably starts with the centrifugation of BM aspirates on a density gradient formed by Ficoll-Paque or Percoll to separate the nucleated cells from the red blood cells (8, 9). In rodents, BM is obtained by flushing the BM out of the long bones, and the tissue obtained is usually disaggregated by flushing it in and out a syringe with needle (10, 11). MSCs obtained from solid tissues can be obtained by digestion using collagenase type I with or without the use of other proteolytic enzymes (12-14). The dissociated tissue may be subjected to additional purification steps such as filtering through nylon meshes or sedimentation for the obtainment of a single cell suspension. The cells are then counted and placed in plastic culture vessels with culture-treated surfaces in culture medium. Positive selection (e.g., of CD49a⁺ cells (15)), or depletion of cells positive for hematopoietic markers such as CD45 and CD11b (16) can be performed for BM- or solid-tissue-derived cells prior to plating. However, MSCs are mainly selected based on their adherence to plastic and proliferation. This is due to the absence of specific cell markers for MSCs through the past years. To date, some molecules are becoming established as reliable MSC markers, at least in some specific contexts (reviewed in (17)).

The most commonly employed media for MSC culture are low-glucose Dulbecco's Modified Eagle's Medium (DMEM) and the alpha modification of Eagle's Minimum Essential Medium (α -MEM), and proprietary formulations can be also found. Fetal bovine serum (FBS) is the most used supplement, usually added at a final concentration of 10% (v/v). Other MSC-related cell populations may require elaborate culture conditions in addition to a competent medium composition. Marrow-isolated adult multilineage inducible (MIAMI) cells, for example, are initially cultured in DMEM containing 5% FBS, and FBS concentration is later reduced to 2% (18). MSCs are most of the times cultured in humidified incubators containing a mixture of 95% environmental air and 5% CO₂. These conditions represent non-physiological O₂ concentrations (20%). MIAMI cells, on the other hand, are cultured in hypoxic conditions (3% O₂).

Cells in primary culture proliferate and eventually occupy the whole culture area, i.e., become confluent. At this point, or a little before confluence, the adherent cells are treated with trypsin, and inoculated in new culture vessels at densities high enough to allow for cell survival, and low enough to maximize cell yield at each passage. Human MSC expansion in culture is highly variable (9). A study on MSC expandability found MSCs could reach 38±4 population doublings in culture (19). Murine MSCs, in contrast, have been shown to be able to proliferate beyond 100 population doublings (12). Some works have focused on the conditions maximizing human MSC production in culture (20, 21). However, data indicating that prolonged expansion in culture may lead to

genetic alterations add a word of caution for the use of long-term expanded MSCs in clinical trials (22).

Human MSCs can be characterized as early as at the end of the second passage, as most other cell types are lost due to passaging. Murine MSC populations from BM, on the other hand, take longer to become nearly devoid of hematopoietic cells (11). MSC characterization consists of the analysis of their proliferation, differentiation, and surface molecule profile. Osteogenic differentiation is usually induced by culture in medium containing dexamethasone, β -glycerolphosphate, and ascorbic acid (23). Adipogenic differentiation can be performed by adding dexamethasone and insulin to the culture medium (4, 11), which may or may not have high glucose content and additional adipogenic inducers such as indomethacin and isobutylmethylxanthine (12, 24). Chondrogenesis can be assayed in a fully chemically defined medium containing transforming growth factor- β , ascorbic acid, and β -glycerolphosphate (25). The surface molecules expressed by cultured MSCs, reviewed in detail elsewhere (7), are summarized in Table 1. To date, the consensus is that MSCs are positive for CD29, CD44, and CD90 and do not express the hematopoietic markers CD45 and CD34, although some studies contradict this view (15, 26, 27). When murine MSCs derived from diverse organs were analyzed by flow cytometry after some passages in culture, CD34 was found to be expressed in some of the cell populations; in one of the populations studied, CD34 was detectable at an early passage, and its expression decreased to control levels after long-term culture (12). In some cases, CD45 levels seemed to be slightly higher than controls; however, these results were not consistent enough to define the cells as positive for CD45 expression (L. da Silva Meirelles, unpublished observations). According to the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy, minimal criteria to define human MSCs include the expression of CD105, CD73 and CD90, lack of expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules in addition to the ability to proliferate as plastic-adherent cells in standard culture conditions, and to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro* (28).

4. MSCs FOUND IN SEVERAL DIFFERENT ORGANS/TISSUES

Cells bearing MSC characteristics have been derived from different locations of the body including BM, adipose tissue, tendon, skin, bone, muscle, brain, liver, kidneys, lungs, spleen, pancreas, thymus, synovial membrane, and umbilical cord (12, 29-36). We have established MSC cultures from several organs of mice, and found no evidence that MSCs are present in circulating blood under physiological conditions (12). In the same study, the MSC populations derived from the different organs exhibited many characteristics in common, in spite of some differences regarding differentiation potential. The successful derivation of MSC cultures from decapsulated glomeruli was regarded as evidence that cultured MSCs are derived from pericytes *in vivo*, as previously suggested (37-

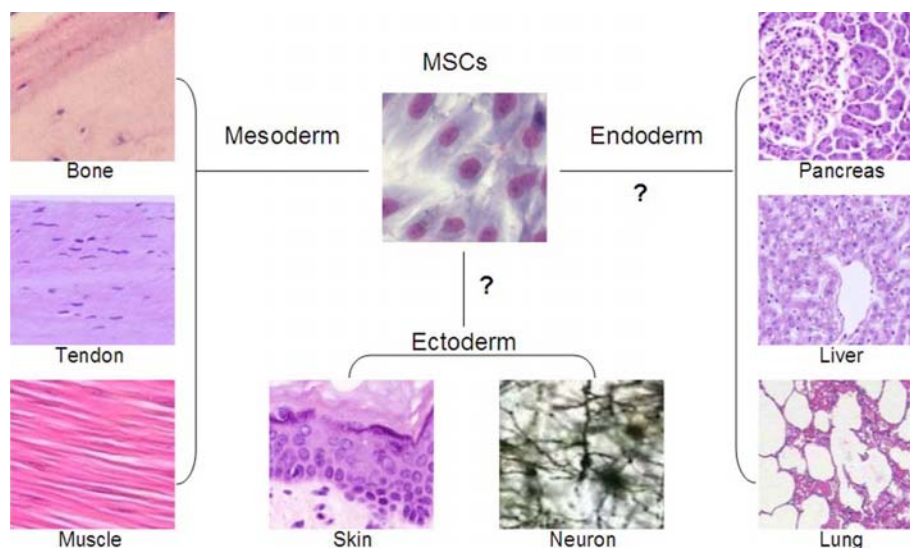


Figure 1. Plasticity of mesenchymal stem cells. *In vitro* and *in vivo* studies have shown that MSCs differentiate into many mesodermal cell types. Other studies have described the potential of MSCs to differentiate into ectodermal and endodermal lineages, but these results have been questioned and may represent experimental artifacts.

39). This evidence in addition to data concerning the behavior of pericytes during tissue repair obtained from the literature (40, 41) and reports showing the broad differentiation capabilities of MSCs, especially when in contact with mature cell types (16, 32, 42-45), provided a basis for the proposition of a model in which pericytes are stem cells throughout the vasculature, contributing for the replenishment of lost cells under physiological conditions, and possibly assuming a more active role during tissue injury (12).

5. MSC DIFFERENTIATION POTENTIAL AND PLASTICITY

As shown above, differentiation of MSCs into mesenchymal cell types is expected and confirmed. However, from 1999 on, a number of studies found that MSCs can give rise to non-mesenchymal cell types such as glial cells (16), neurons (45, 46), hepatocytes (43, 47), and even insulin-producing cells (42, 48). In addition, MSC-related cells termed MAPCs were shown to be able to give rise to all types of cells when injected in murine blastocysts (49). However, some of these results were later challenged: the results found by Kopen *et al.* (16) could be attributed to the transfer of the label to the surrounding cells (50); the reproducibility of obtainment of MAPCs was questioned (51); cell fusion was described as a possible cause for the observed differentiation of BM cells infused *in vivo* (52). Mature mesenchymal cells were shown to dedifferentiate when cultured, and to bear many of the characteristics, including differentiation potential, attributed to MSCs (53). Further, circulating monocytic cells were shown to differentiate along osteogenic, chondrogenic and adipogenic pathways *in vitro* (54). In view of these facts, the apparent plasticity of MSCs (Figure 1) is questionable, as it could be an artifact introduced by cell culture. Likewise, the differentiation of circulating monocytes into

multiple cell types *in vitro* could be a consequence of *in vitro* manipulation. In considering cell fusion, however, there is at least one study demonstrating MSC differentiation into hepatocytes *in vivo* where no evidence of cell fusion was found (44). Furthermore, the low frequency of fused cells found by Alvarez-Dolado *et al* (52) raises doubts about the biological significance of cell fusion in experiments involving cell transplantation.

Apart from the problems exposed above, which are mainly theoretical, differentiation of MSCs into non-mesenchymal cell types is a useful characteristic for therapies. Neuronal differentiation of MSCs, for example, could provide cells to replace neurons lost due to neurodegenerative diseases. Neuronal differentiation of MSCs *in vitro* has been suggested to be transient (55); however, other studies have shown that neurons differentiated from MSCs exhibit functional neuronal properties (56), including negative action potential (- 60 mV) observable for at least one week in culture (57). This suggests that transplanted MSC-derived neurons may become electrophysiologically integrated with the host neural tissue, as suggested by the results of experiments employing a rat model of Parkinson's disease (58).

6. MSC IDENTITY TRACED TO PERICYTES *IN VIVO*

In view of the confusion regarding the identity of the MSC *in vivo* and the apparent MSC plasticity observed *in vitro*, we have recently analyzed the evidence supporting the hypothesis that pericytes behave as stem cells *in vivo* (59). As a result, we found data indicating that perivascular cells behave as stem cells in bone (40), cartilage (60), adipose tissue (41), testis (61), periodontal ligament (62), endometrium (63), and brain (64). However, the behavior of pericytes as stem cells in the testis and brain does not

reflect that expected for a *mesenchymal* stem cell. This leads to a broader perspective, where perivascular stem cells are distributed throughout adult tissues, and these can be viewed as MSCs in mesenchymal tissues. This view does not necessarily imply that perivascular stem cells from different tissues are equivalent, in spite of their similarities.

7. MSCs AS TROPHIC MEDIATORS

In addition to their differentiation potential, which can be used as the basis for tissue engineering and other therapeutic purposes, MSCs have recently been proposed to exert trophic effects that are also of therapeutic interest (65). Some studies have found that the beneficial effects of MSCs applied to experimental models of injury are due to secretion of bioactive factors rather than to differentiation (66-69). Indeed, MSC's trophic activities provide a basis for the generation of local regenerative environments, in contrast with their use as a source of cells for tissue engineering (70).

Since activated endothelium in injured sites express CD106 and CD62E, it is possible to devise therapeutic strategies where cultured MSCs are retained in injured sites by binding to these molecules via CD29/CD49d integrin and CD44, respectively (reviewed in (59)). There, the secretion of bioactive factors by MSCs may accelerate healing and minimize tissue death.

8. IMMUNOREGULATORY FUNCTION OF MESENCHYMAL STEM CELLS

Evidence demonstrating that MSCs have unique immunomodulatory properties and are capable of exerting a powerful immunosuppressive effect on the immune system (71, 72) has accumulated in recent years. MSCs seem to affect all cells of the immune system, inducing an arrest of cell division in the G0/G1 phase of the cell cycle, associated with inhibition of cyclin D2 expression (73). This effect is well controlled, and although MSCs mainly suppress immune responses, they may also activate the immune system, depending on the stimulus to which they are exposed (reviewed in (74)).

The immunosuppressive effect exerted by MSCs on T cells is seen in *in vitro* experiments in which they inhibit the activation of mature T cells by alloantigens or non-specific mitogens in a dose-dependent, non-HLA-restricted, manner (73). *In vivo* studies show that MSCs suppress the response of naïve and memory antigen-specific T cells in mice (75), prolong skin engraftment in nonhuman primates (76), and reduce (77) or cure (78) graft-versus-host disease (GVHD) after allogeneic transplantation in humans. Furthermore, *in vitro* experiments suggest that MSCs can induce the generation of regulatory T cells, a key element on the regulation of the immune response (79).

As already mentioned above, MSCs affect all cells of the immune system. B lymphocytes are also inhibited by MSCs, after T-cell-dependent or T-cell-independent stimulation; similarly, human MSCs have been shown to

inhibit IL-2- or IL-15-driven NK proliferation (reviewed in (80)). MSCs also have an inhibitory effect on the differentiation and function of dendritic cells (81). This effect could be related to their capacity to produce anti-inflammatory cytokines, which inhibit the *in vitro* activation and maturation of dendritic cells (82).

The mechanisms responsible for these effects are not fully elucidated yet, but they do not appear to involve the induction of apoptosis of proliferating cells (75, 83), and are mediated by both direct cell-to-cell interactions and soluble factors. The main factors involved in the process include transforming growth factor- β 1 (TGF- β 1), hepatocyte growth factor (73), prostaglandin E2 (84), indoleamine dioxygenase (IDO)-mediated tryptophan metabolites (85), and nitric oxide (86).

MSCs express MHC class I determinants. MHC class II molecules, constitutively expressed in MSC subsets (87), are present intracellularly and can be expressed at the surface after exposure to low levels (10 U/ml) of interferon- γ (IFN- γ) (88). High levels (100 U/ml) of IFN- γ , on the other hand, result in downregulation of MHC class II (89), so that MSCs may function as antigen-presenting cells (APC) during a narrow window of time before IFN levels are increased during the development of the immune response. The role of IFN- γ in increasing MHC II expression in murine MSCs was shown to be enhanced by high cell density or serum deprivation, suppressed by TGF- β , and regulated by the activity of the type IV MHC class II transactivator (CIITA) promoter independently of STAT1 activation (90). Furthermore, the bimodal response of MHC-II expression by MSCs in response to IFN- γ was shown to be dependent at least in part on cytoplasmic retention of CIITA (91). The expression of MHC class II molecules on MSCs has also been shown to depend on the concomitant presence of any of three other proinflammatory cytokines, TNF- α , IL-1 α , or IL-1 β (92). These cytokines lead to the expression of high levels of inducible nitric oxide synthase (iNOS) by MSCs, resulting in suppression of T cell responsiveness. An understanding of the control of MHC class II expression could provide insights on BM homeostasis, with implications for protection against infection and exacerbated inflammatory responses, as well as MSC dysfunction in hematological disorders.

The powerful immunoregulatory capacity of MSCs plus their widespread distribution in the organism suggests their possible role in the maintenance of immune homeostasis (59). Thus, MSCs are proposed to have an active role in the prevention of self-responses in both physiological and pathological conditions.

9. PERIVASCULAR STEM CELLS AND TISSUE REGENERATION

Based on the evidence supporting the fact that cultured MSCs derive from perivascular stem cells *in vivo*, and on the trophic and immunomodulatory properties of these cells, we have further proposed a model for the action of pericytes/MSCs during tissue repair (59). In this model,

Table 2. Clinical trials involving MSCs as of April 2008

Condition	Number of entries
Graft-versus-host disease	12
Skeletal repair	7
Heart diseases	6
Chron's Disease	4
Liver repair	3
Multiple Sclerosis	1
Co-transplantation with pancreatic islets	1
Not applicable	3

Clinical Trials database (www.clinicaltrials.gov) was queried using the term “mesenchymal stem cell” (quotation marks included). Please note that this analysis does not include data from clinical trials where bone marrow mononuclear cells, an MSC-containing cell population, were employed.

tissue injury leads to local cell death, with consequent activation of endothelial cells. Pericytes/MSCs loose contact with endothelial cells and the basement membrane, and proliferate (become activated). The increased number of pericytes/MSCs secreting a large number of molecules with trophic, immunomodulatory, angiogenic and chemoattractive effects leads to increased survival of local cells, including tissue-intrinsic progenitors, and to modulation of the immune response. At the end of this process, some pericytes/MSCs may undergo apoptosis or differentiate into tissue-specific cells.

10. CLINICAL AND PRE-CLINICAL APPLICATIONS OF MSCS

The differentiation capabilities of cultured MSCs, as well as their trophic, immunomodulatory and homing properties, make them potential tools for the treatment of different types of conditions. Importantly, these different properties allow for the design of different types of therapeutic approaches. For example, biological structures aiming at mimicking tissues such as bone, cartilage or fat can be developed by combining MSCs with appropriate scaffolds and signaling molecules in order to induce MSC differentiation. A different strategy could consist in delivering MSCs to injured sites where they would act as therapeutic agents through the secretion of anti-apoptotic, angiogenic and immunomodulatory molecules. In another scenario, MSCs can be genetically modified to correct genetic defects, or to forcedly express specific molecules, such as anti-tumoral or anti-apoptotic cytokines.

A search at ClinicalTrials.gov performed at the time this review was written returned 37 results (Table 2). Three entries did not represent clinical trials involving the direct application of MSCs; these were not included in the analysis, and were classified under “Not applicable”. Twenty-one of the trials found are/were sponsored by academic institutions, whereas eight are sponsored by industry, and five of them sponsored by academic institutions in partnership with the industry. Most of the entries retrieved represent approaches relying on the paracrine effects of MSCs rather than on their differentiation capabilities, although MSC differentiation properties still show importance, especially in skeletal repair. Considering this trend, we discuss below some aspects of selected conditions, with especial emphasis on

those where MSCs are expected to exert therapeutic effects through the secretion of bioactive factors. Reviews detailing the use of MSCs as components for tissue engineering can be found elsewhere (7, 93, 94).

10.1. Myocardial infarction

The first work on the use of BM cells to treat myocardial infarction come from a study where Lin[−]c-kit⁺ cells were shown to differentiate into cardiomyocytes in a murine model of myocardial infarction (95). The results of this work were later challenged by other studies that found that Lin[−]c-kit⁺ cells actually give rise to mature hematopoietic cells when injected in the surroundings of infarcted areas (96, 97). It is likely that the problems with the reproducibility of these results are a consequence of the use of surface markers to isolate the cell population used, since the specificity of marker molecules is highly context-sensitive. In mice, for example, hematopoietic stem cells can be found in a variety of cell populations sorted based on the expression of surface molecules; however, different sets of surface markers do not define the entire hematopoietic stem cell population (98).

The cell populations employed in the studies above were not subjected to the regular *in vitro* tests that define MSCs and, thus, the consequent results do not constitute evidence that MSCs regenerate the injured myocardium. However, MSCs have been shown to differentiate into cardiomyocytes *in vitro* (99, 100), and other studies have shown that MSCs do improve cardiac function when injected shortly after infarction (101-103). By the time these studies were conducted, MSCs were thought to contribute to cardiac function by means of differentiation into cardiomyocytes. This paradigm later evolved, incorporating the notion that the soluble factors secreted by MSCs exert trophic effects at sites of injury. Dai *et al.* (104) found that the therapeutic effects of the injected MSCs were evident before cells expressing cardiac-specific markers could be detected, and that such effects actually declined by the time cardiomyocyte-like cells derived from the injected MSCs were observed, indicating that paracrine factors locally secreted by MSCs are responsible for the cardiac improvement observed. This view is supported by data from experiments in which serum-free medium conditioned by genetically modified MSCs under hypoxic conditions was shown to improve cardiac performance after infarction (105). Considering these facts, it seems that long-term engraftment of MSCs is not the key factor when aiming at treating acute myocardial infarction.

10.2. Fibrosis

One of the mechanisms underlying the beneficial effects of MSCs on myocardial infarction is the release of anti-scarring factors (106, 107); for this reason, myocardial repair is maximized if cell administration takes place shortly after infarction, before the establishment of a fibrous scar (108). This anti-fibrotic effect of MSCs has been previously described for other organs such as lung (109) and liver (110). In these cases, MSCs could prevent scarring if administered in a short time after experimental injury, but were ineffective if delivered after the

development of fibrosis. Recently, a pro-fibrotic behavior was proposed for MSCs infused in an experimental model of liver fibrosis (111); however, in that study, the animals received radiation in addition to the chemical used to induce the fibrotic lesions. Consequently, these results are not readily comparable to others in the literature. A recent phase I clinical trial of decompensated liver cirrhosis has found that the infusion of culture-expanded autologous MSCs is safe (112). Cirrhosis is characterized by the presence of strong inflammation; for this reason, administering MSCs may be beneficial to the patient even if the infused cells contribute, to a little extent, to fibrosis as the benefit of their immunomodulatory properties may overcome this drawback. Actually, the anti-scarring effects of MSCs may be a consequence of their immunomodulatory effects, as fibrosis is consequent to inflammation (113).

10.3. Immunological diseases

The bimodal aspect of the immune functions exerted by MSCs, which are able to exert both immunosuppressive and immunostimulatory effects depending on the type and magnitude of the immune challenge, makes these cells extremely important for the treatment of different types of disorders involving the immune system. Preclinical and clinical studies investigating the effects of MSCs in tissue and organ transplantation as well as for the treatment of autoimmune diseases are helping to define the optimal conditions for their use (reviewed in (71, 114)).

GVHD is a condition in which immune system cells present in allogeneic donor BM recognize the recipient's cells as foreign, and attack them. This represents a nearly normal, but extremely exacerbated, immune response, which, as such, involves the different components of immune system (115). In 2004, a paper reporting the successful treatment of a patient affected by graft-versus-host disease (GVHD) using third-party MSCs (78) attracted the attention of researchers worldwide as it represented a proof of the concept that the immunosuppressive effects of MSCs previously observed *in vitro* (as discussed earlier in this paper) can be applied to a clinical setting.

Again, it is important to emphasize that immunosuppression is but one of the immunomodulatory effects of MSCs, and that immunomodulation provided by these cells is dynamic rather than static. In other words, the manner how MSCs influence the immune response is context-sensitive. The upregulation of the expression of anti-inflammatory molecules by cultured MSCs upon stimulation with pro-inflammatory cytokines (116, 117) exemplify this concept. In addition, dendritic cells generated in presence of high concentrations of MSCs show suppressive effects on T lymphocytes, whereas those generated in presence of low MSC concentrations actually stimulate T cell activation (88).

The expectation that the administration of cultured MSCs will be a valuable therapeutic approach to treat immunological diseases is reflected in the number of clinical trials testing the effects of these cells on different

forms of GVHD and also other immunological disorders such as Chron's Disease and Multiple Sclerosis (Table 2). Conversely, data from experiments in which B-lymphocytes from Lupus Erythematosus patients were co-cultured with MSCs indicate that MSCs might actually worsen the symptoms by promoting B cell proliferation and differentiation into antibody-secreting plasma cells (118). These findings are in contrast with those obtained by co-culturing MSCs and B cells from healthy human donors (119), and with data from a murine model that mimics some aspects of Lupus Erythematosus (120). Given that the different cell types present in the *in vivo* context influence the immunomodulatory effects of MSCs, infusing MSCs in animal models of Lupus Erythematosus is a necessary step to evaluate the safety and efficiency of MSC administration for the treatment of this disease.

10.4. Stroke

Results from pre-clinical studies indicate that cultured BM-derived MSCs exhibit the potential to efficiently ameliorate the consequences of ischemic lesions in the brain when infused intravenously (121). There is evidence that this therapeutic effect is mediated by neuroprotective factors released by the infused cells (122), and that it is augmented when MSCs are genetically modified to forcedly express neurotrophic factors (123). The factors secreted by MSCs have been shown to reduce apoptosis in the injured site, stimulate the proliferation of endogenous progenitors in the infarcted area (124), and favor angiogenesis in the surroundings of the lesion (125). In addition, a phase I/II clinical trial has shown that administration of cultured MSCs to stroke patients is safe (126). However, the improvement caused by MSCs was only moderate in that study, possibly because the cells were administered to the patients more than a month after stroke was diagnosed. It is possible that efficiency of MSC treatment for stroke can be increased if cells are administered soon after diagnosis. Since MSC frequency in BM is low, the use of MSCs obtained from other tissues, and even from allogeneic donors may represent an alternative to reduce the time needed to achieve the high cell numbers necessary for infusion.

10.5. Angiogenesis and cancer therapy

As briefly mentioned above, MSCs show the ability to favor vascularization of the surroundings of injured sites. Soluble molecules mediate this action, and these include basic fibroblast growth factor, vascular-endothelial growth factor, placental growth factor, and monocyte chemoattractant protein-1 as assessed in a murine model of unilateral hind limb ischemia (68). Whereas homing of MSCs to injured sites by chemotactic and adhesion molecule-mediated mechanisms (reviewed in (59)) is a desirable feature in the context of cell therapy, this characteristic makes these cells likewise attracted to tumors (127, 128), where they support tumoral vascularization by secreting soluble factors and becoming physically incorporated into the tumor stroma.

Vascular support provided by MSCs can be regarded, thus, as a two-edged sword: on one side, it is useful to accelerate the healing of ischemic lesions; on the

other hand, it may facilitate the establishment of solid tumors (129). This situation can be reverted by turning MSCs into Trojan horses by genetically modifying them to forcibly express anti-tumoral molecules such as interferon- α (129), interleukin-12 (130), herpes simplex virus thymidine kinase (131) or tumor necrosis factor-related apoptosis-inducing ligand (132).

In the context of tissue engineering, the angiogenic properties of MSCs add to their potential use as a source of differentiated cells since they significantly improve vascularization of biological structures, which is a key factor for the survival of engineered tissues when implanted. Such improvement can be maximized if MSCs are genetically modified to constitutively express angiogenic molecules, e.g., vascular-endothelial growth factor (133, 134). In addition, MSC's ability to interact with endothelial cells and stabilize blood vessels (135, 136) can be used to produce vascularized tissue equivalents. Recently, vascular grafts lined with MSCs were shown provide conditions for the establishment of a host-derived endothelial cell layer (137), indicating that MSCs can be used to line cardiovascular grafts in order to avoid platelet aggregation and activation.

11. METHODS FOR THE *IN VITRO* STUDY OF MESENCHYMAL STEM CELLS

This section describes experimental methods for the isolation, culture and differentiation of MSCs. Few references are mentioned, as the protocols have been developed based on multiple studies available in the literature and modified according to our own experience. Similarly, suppliers of the materials mentioned in the protocols are not specified and, for most of them, we have tested different brands with similar results. We describe the main reagents, systematic procedures, and, in some cases, specific observations for each of the methods.

11.1. Isolation and culture of murine bone marrow MSCs

Although other organs and tissues may be an adequate source of MSCs for basic and pre-clinical studies (see below), BM is still the most widely used source of cells. The protocol below may be used for murine or rat BM. However, it is important to emphasize the establishment of MSC cultures from murine BM by means of serial passaging in basal medium supplemented with fetal bovine serum (FBS) is far more difficult as compared to other species, even though the same does not apply to other tissues. While discrete fibroblastic colonies develop when BM from rats or humans are plated and cultured, primary cultures of murine BM are highly heterogeneous and contain high levels of hematopoietic contaminants (138). Murine MSCs can be obtained by means of serial passaging in Dulbecco's Modified Eagle's Medium containing 10 % FBS (11); see below). Nevertheless, this process is lengthy, and allows for the obtainment of long-term cultured cell populations with MSC characteristics, which may become altered due to the extended time in culture. We have defined conditions in which murine BM cells form discrete fibroblastic colonies as the basis for a

CFU-F assay for this species (11). Although this method provides short-term cultured murine MSCs with relatively low amounts of hematopoietic contaminants, its efficiency is low. In this regard, depletion of hematopoietic cells from murine BM has been reported to allow for the obtainment of a population of cells with MSC characteristics (16), and constitutes an alternative to selection by serial passaging; however, the cells obtained by these two methodologies cannot be considered equivalent as no study has directly compared them.

11.1.1. Reagents

- Standard culture medium (SCM): Low-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 3.7 g/l sodium bicarbonate, 10 - 15 mM HEPES (free acid), and 10% fetal bovine serum (FBS). Antibiotics and antimycotics may be added.
- $\text{Ca}^{2+}\text{Mg}^{2+}$ -free Hank's Balanced Salt Solution (HBSS)
- Trypsin/EDTA

11.1.2. Procedure

Euthanize the mouse by cervical dislocation and excise the femurs (tibias may also be used). Remove epiphyses and, using a syringe coupled to a needle, flush marrow out with HBSS or medium. After washing by centrifugation at 400 g for 10 min and counting viable cells with Trypan blue in a Neubauer chamber, resuspend the cells in medium to a final concentration of 5×10^6 cells/ml. To initiate an MSC culture, cells are plated in six-well tissue culture dishes, at 3.5 ml/well (1.94×10^6 cells/cm²). The culture is kept in a humidified 5% CO₂ incubator at 37°C for 72 h, when most non-adherent cells are removed by changing the medium. Primary cultures generally become confluent within around 6 to 7 days, and they should be subcultured at this time. For that, the cultures are washed once with HBSS and incubated with a 0.25% trypsin solution containing 0.01% ethylenediaminetetraacetic acid (EDTA) for 10 min at 37°C. After detachment, the cells are resuspended in medium to a final volume of 10.5 ml, and the resulting suspension is split into three new wells. Subsequent passages are performed when cultures reach around 90% confluence. Split ratios are defined empirically so that subcultures are performed twice a week, and should be modified as needed. Split ratios may generally be set to 1:6 at passage 5 or 6, 1:9 at around passage 11 and, if necessary, ratios of up to 1:24 may be used, especially for cell populations subjected to extensive subcultivation (more than 20 passages). Culture medium is changed every 3–4 days.

11.1.3. Observations

Cultures must be observed with phase-contrast microscopy. The initially heterogeneous aspect of the cultures is gradually replaced by a homogeneous culture with the typical aspect of MSCs (Figure 2) along the passages. During the first passages, it may take over one month for the cultures to reach confluence. Later on, cells divide more rapidly and confluence is reached in a few days. Overconfluence should be avoided to prevent cell death or spontaneous differentiation and to facilitate subculture

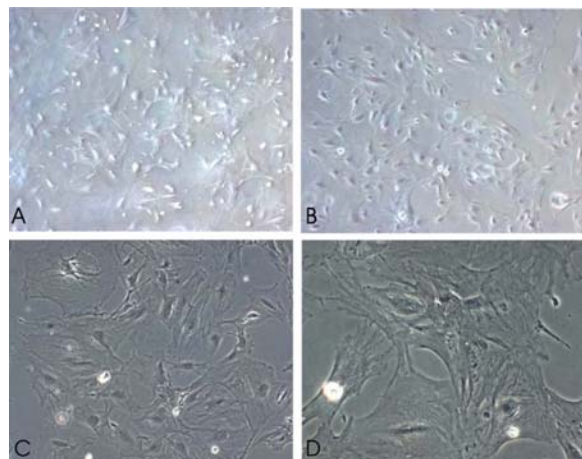


Figure 2. Phase-contrast images of a culture established from the bone marrow of a C57Bl/6 mouse, on days 1, 3, 5 and 7 (A to D, respectively). Original magnification: x100.

11.2. Isolation and culture of MSCs from other murine organs and tissues

As already documented in the literature (12), MSCs can be isolated from virtually any tissue of the body. Cultures are very similar in morphology, immunophenotype and function, but seem to exhibit some differences which have not been yet fully investigated. Depending on the basic or applied study to be conducted, it may be interesting to explore the characteristics of cells isolated from specific organs or tissues.

11.2.1. Procedure

Organs/tissues such as liver, spleen, pancreas, lung, kidney, aorta, vena cava, thymus, brain and muscle are collected from perfused or non-perfused animals, rinsed in HBSS, transferred to a Petri dish and cut into small pieces. The dissected pieces (around 0.2-0.8 cm³) are washed with HBSS, cut into smaller fragments, and subsequently digested with collagenase type I (0.5 mg/ml in DMEM containing 10 mM HEPES) for 30 minutes to 3 hours at 37°C. Whenever gross remnants persist after collagenase digestion, they are allowed to settle for 1 to 3 minutes, and the supernatant is transferred to a new tube, which is then filled up with the addition of SCM. Cell suspensions may be further cleared from debris by centrifugation on Ficoll-Hypaque, followed by an additional washing step. After centrifugation at 400 g for 10 min at room temperature (RT), the pellets are resuspended in 3.5 ml of SCM, seeded in six-well dishes (3.5 ml/well), and incubated at 37°C in a humidified atmosphere containing 5% CO₂. Three days later, if the cultures are not confluent, the whole volume of SCM is replaced, and the adherent layer is maintained and expanded as described above. Split ratios are empirically determined to allow for two subcultures a week at the most.

To establish glomeruli-derived MSC cultures, kidneys are placed in a 15-ml centrifuge tube containing 5 ml SCM, and mechanically disrupted by several rounds of aspiration/expulsion using a 10 ml pipette. Single glomeruli devoid of the Bowman's capsule are isolated from the cell suspension by micromanipulation, and transferred either

individually or collectively to 12-well dishes containing fresh SCM. Subsequent passages are performed as described above.

11.3. Isolation and culture of MSCs from human bone marrow

Most studies focusing on human stem cells use BM as a source of either hematopoietic or mesenchymal ("stromal") stem cells. The basic procedure involves the isolation of mononuclear cells by density gradient centrifugation. The isolation of adherent cells results in the establishment of MSC cultures, similar to the protocols described above for murine cells. The protocol below is suitable for the isolation of MSCs for basic or pre-clinical studies. Clinical-grade protocols may demand further precautions, such as the use of human AB or autologous serum instead of FBS.

11.3.1. Additional reagents

- Phosphate-buffered saline (PBS)

11.3.2. Procedure

Heparinized BM is generally obtained by iliac crest aspiration from normal volunteer donors or as an aliquot of material collected for transplantation. BM is diluted 4:3 (v:v) with PBS, layered on the top of an equal volume of Ficoll-Hypaque previously dispensed in a centrifuge tube, and centrifuged at 400 g for 30 min at room temperature. The layer of mononuclear cells is collected, washed in HBSS, and viable cells are counted with Trypan blue and resuspended in SCM at 3×10^6 viable, nucleated cells/ml. Cells are plated in six-well tissue culture dishes, at 3.5 ml/well (1.2×10^6 cells/cm²), and incubated in a humidified incubator with 5% CO₂ at 37° C for 72 h, when non-adherent cells are removed by changing the medium. Cultures are maintained and expanded as described above for murine cells.

11.4. Isolation and culture of MSCs from human liposuction material

Although human BM is still the most frequently used source of stem cells for basic studies or clinical trials, autologous BM procurement has potential limitations. Adipose tissue obtained by liposuction has been shown to contain mesenchymal stem-like, multipotent cells (36), and has been being increasingly used as an alternative stem cell source to BM-derived MSCs.

11.4.1. Additional reagents

- Collagenase type I
- Lysis buffer (160 mM NH₄Cl)

11.4.2. Procedure

The liposuction sample is extensively washed with PBS. This can be easily done with a separatory funnel (Figure 3). The extracellular matrix is digested by incubation of the washed aspirates with a 3-fold volume of 1 mg/ml collagenase type I, at 37°C for 30 min with gentle agitation. Excess collagenase is washed out, and residual tryptic activity is inhibited, by addition of an equal volume of DMEM/10% FBS and centrifugation at 800 g for 10 min at RT. The pellet, containing the stem cell fraction among

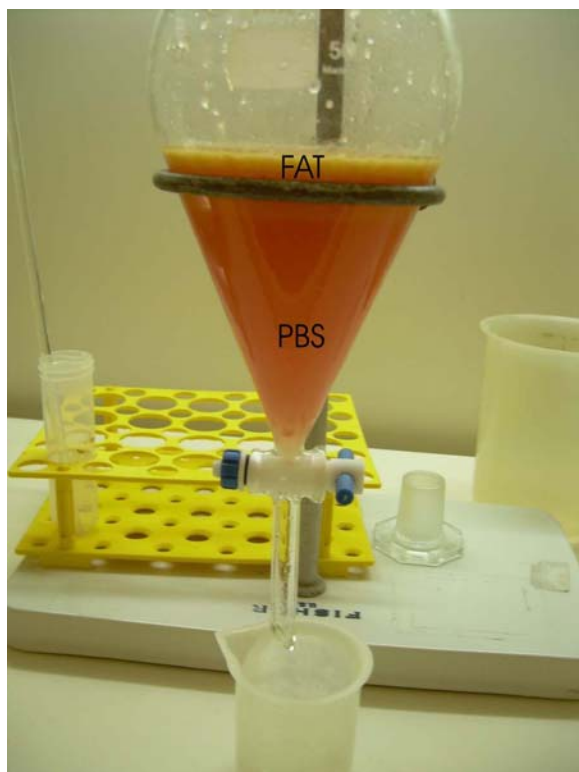


Figure 3. Separatory funnel used to wash liposuction material for isolation of adipose-derived stem cells. Fat, liposuction material; PBS, phosphate-buffered saline used for washing.

other non-adipocytic cells, is incubated with lysis buffer for 10 min at RT to lyse contaminating red blood cells. After washing and counting with Trypan blue, cells are resuspended in SCM at 2×10^6 viable cells/ml, and plated in six-well tissue culture dishes at 3.5 ml/well. The plates are incubated at $37^\circ\text{C}/5\% \text{ CO}_2$, and non-adherent cells are removed 24 h later. From then on, the cultures are maintained as described for murine cells.

11.5. CFU-F assay

As reviewed above, the frequency of CFU-Fs has been considered indicative of the frequency of MSCs. Recently, based on the hypothesis that MSCs are a subset of perivascular cells, the number of CFU-Fs detected in equine adipose tissue has been found to positively correlate with the vascular density of the specimens (139), validating the CFU-F assay as tool to estimate MSC frequency. The protocol below has been designed for murine BM cells and is similar to the establishment of a conventional MSC culture, except for the fact that the cells are plated at a low density. It is important to highlight that this assay should be adapted to tissues/organs other than BM in order to avoid overlapping of the fibroblastic colonies, as CFU-F numbers are expected to be proportional to the degree of vascularization of the sample assayed. The stromal-vascular fraction of human adipose tissue, for example, contains around 500-fold more CFU-Fs than BM mononuclear cells plated at the same cell density (140).

11.5.1. Procedure

Murine BM is collected as described above, and a cell suspension containing 2.25×10^5 viable nucleated cells/ml is prepared with DMEM/10% FBS. Two milliliters of this cell suspension are dispensed into each well of a six-well plate (4.98×10^4 viable nucleated cells/cm²). The culture is kept in a humidified incubator with 5% CO₂ at 37°C , and the medium is replaced on days 3 (with consequent removal of most non-adherent cells) and 8. On day 13, the medium is removed and the cultures are stained with Giemsa. The number of colonies displaying five or more cells is scored on the inverted microscope. Colonies with four cells are counted when one of them presents two nuclei. Colonies whose morphology clearly differ from the characteristic mMSC morphology are excluded from the results.

11.6 Differentiation assays

As part of the minimal criteria proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy to define human MSCs (28), cells must be able to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro*. The protocols to induce differentiation of cultured MSCs into these lineages are presented below. Figure 4 presents the final aspect of cultures differentiated into the three lineages and stained as described.

11.6.1. Osteogenic differentiation

11.6.1.1. Additional reagents

- Osteogenic medium: DMEM supplemented with 10% FBS, 15 mM HEPES, 10^{-8} M dexamethasone, 5 - 50 µg/ml ascorbate-2-phosphate, and 10 mM β-glycerophosphate. Antibiotics and/or antimycotics may be added.

11.6.1.2. Procedure

Osteogenic differentiation is induced by culturing confluent cultures for up to 4 weeks in osteogenic medium. The medium is changed twice a week. The mineralized extracellular matrix produced by osteoblasts is usually observable at around one week (mMSCs) or two weeks (hMSCs) after the start of osteogenic induction. Calcium deposition is revealed by washing the cultures once with PBS, fixing with 4% paraformaldehyde in PBS for 15-30 minutes at RT, and staining for 5 minutes at RT with Alizarin Red S stain at pH 4.2 (Figure 4B).

11.6.2. Adipogenic differentiation

11.6.2.1. Additional reagents

- Adipogenic medium: high-glucose DMEM supplemented with 10% FBS, 15 mM HEPES, 10^{-8} M dexamethasone, 2.5 µg/ml insulin, and 100 µM indomethacin. Other protocols include further supplementation of the adipogenic medium with 0.5 mM isobutylmethylxanthine (IBMX), 3.5 µM rosiglitazone or 5 µM 15-deoxy-D^{12,14}-prostaglandin J₂. Antibiotics and/or antimycotics may be added.
- Oil Red O solution: mix 3 volumes of 3.75% (w/v) Oil Red O in isopropanol plus two volumes of distilled water and filter

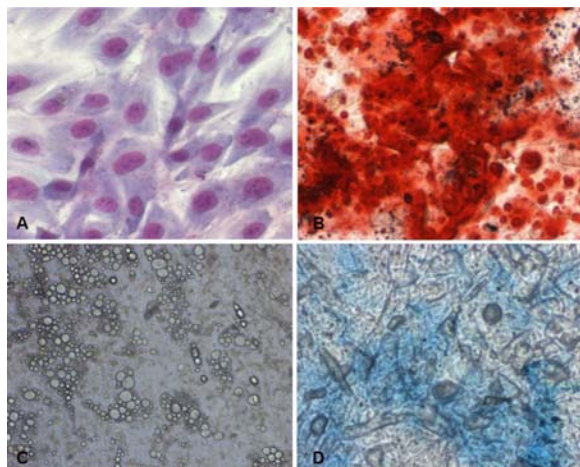


Figure 4. Differentiation of MSCs. A. Normal MSC culture, stained with Giemsa. B. Osteogenic differentiation, stained with Alizarin Red S. C. Adipogenic differentiation, phase-contrast image evidencing fat vacuoles. D. Chondrogenic differentiation, culture stained with Alcian blue. Original magnification: x100.

- Sudan Black B solution: mix three volumes of 2% Sudan Black B in isopropanol plus two volumes of distilled water and filter

11.6.2.2. Procedure

Nearly confluent MSC cultures are maintained for up to 4 weeks in adipogenic medium, with two medium changes every week. Alternatively, MSCs may be cultured in the adipogenic induction medium for two weeks, and the differentiated adipocytes switched to a maintenance medium consisting of high-glucose medium supplemented with 10% FBS and 5 – 10 mg/ml insulin, to allow for increased adipocytic maturation. Adipocytes are easily discerned from the undifferentiated cells by phase-contrast microscopy (Figure 4C). To further confirm their identity, cells are fixed with 4% paraformaldehyde in PBS for 1 hour at RT, and stained with either Oil Red O solution or Sudan Black B solution for 5 minutes at RT. When stained with Oil Red O, the cultures are counterstained with Harry's hematoxylin (1 minute at RT) to make nuclei evident.

11.6.3. Chondrogenic differentiation

11.6.3.1. Additional reagents

- Chondrogenic medium: high-glucose DMEM supplemented with 1% FBS, 6.25 µg/ml insulin, 10 ng/ml TGF-β1, and 50 µM ascorbate-2-phosphate. Antibiotics and/or antimycotics may be added.

11.6.3.2. Procedure

Confluent MSC cultures are maintained for up to 4 weeks in chondrogenic medium, with two medium changes every week. The extracellular matrix produced by chondrocytes is rich in sulfated glycosaminoglycans, which are stained with Alcian blue: cultures are washed with PBS, fixed with 4% paraformaldehyde in PBS for 15-30 minutes at RT, and stained for 5 minutes at RT with Alcian blue

(Figure 4D). Chondrogenic differentiation of three-dimensional cell aggregates (25, 141) constitutes an alternative to this method, and can be performed in a fully chemically defined medium.

12. CONCLUSION

The relative ease with which mesenchymal stem cells may be isolated from multiple sites and expanded in culture, their high plasticity and important biological properties have drawn great attention to these cells. The concept that cultured MSCs show a tendency to home to injured sites where they can exert therapeutic effects through the secretion of bioactive molecules that play antiapoptotic, immunomodulatory, anti-scarring, angiogenic and chemoattractant roles dramatically increases their range of potential clinical applications, which had been previously thought to be limited to cell and tissue replacement. In addition, the notion that MSCs are distributed throughout the organism in a perivascular location allows for the development of therapeutic protocols aimed at the subendothelial compartment in order to treat different disorders. Further research on perivascular stem cell biology and their behavior in different physiological and non-physiological backgrounds is required in order to establish new therapeutic strategies. The outlook is that therapies based on the paracrine effects of MSCs may become standard clinical procedures in a few years.

13. ACKNOWLEDGEMENTS

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Abbreviations: MSCs: mesenchymal stem cells; ASC, adult stem cell; BM, bone marrow, HSC; hematopoietic stem cell, CFU-F; colony-forming unit-fibroblast, DMEM; Dulbecco's Modified Eagle's Medium, FBS; fetal bovine serum; GVHD, graft-versus-host disease; TGF- β 1, transforming growth factor- β 1; MIAMI, marrow-isolated adult multilineage inducible; iNOS, inducible nitric oxide synthase

Key Words: Mesenchymal stem cells, Adult stem cells, Mesenchymal stem cell isolation, Perivascular stem cells, Tissue regeneration, Differentiation and plasticity, Immunological diseases, bone marrow cells, chondrogenic, adipogenic, osteogenic Clinical applications

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