Human Bone Marrow and Adipose Tissue Mesenchymal Stem Cells: A User's Guide

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Mesenchymal stem cells (MSCs) are adult stem cells that hold great promise in the field of regenerative medicine. They can be isolated from almost any tissue of the body and display, after expansion, very similar properties and minor differences, probably due to their microenvironment of origin. Expansion in vitro can be obtained in cytokine-free, serum-enriched media, as well as in serum-free, basic fibroblast growth factor–enriched media. A detailed immunophenotypic analysis is required to test the purity of the preparation, but no unique distinguishing marker has been described as yet. Functional assays, that is, differentiation studies in vitro, are needed to prove multilineage differentiation of expanded cells, and demonstration of pluripotency is necessary to identify most immature precursors. MSCs show powerful immunomodulative properties toward most of the cells of the immune system: this strengthens the theoretical rationale for their use also in an allogeneic setting across the major histocompatibility complex (MHC) immunological barriers. Systemic intravenous injection and local use have been tried: after systemic injection, MSCs show a high degree of chemotaxis based on pro-inflammatory cytokines, and localize at inflamed and neoplastic tissues; local regeneration has been improved using synthetic, as well as organic scaffolds. On the other hand, inadequate heterotopic in vivo differentiation and neoplastic transformation are potential risks of this form of cell therapy, even if evidence of this sort has been collected only from studies in mice, and generally after prolonged in vitro expansion. This review tries to provide a detailed technical overview of the methods used for human bone-marrow (BM)-derived and adipose-tissue (AT)-derived MSC isolation, in vitro expansion, and characterization for tissue repair. We chose to use BM-MSCs as a model to describe techniques that have been used for MSC isolation and expansion from very different sources, and AT-MSCs as an example of a reliable and increasingly common alternative source.

Introduction

MESENCHYMAL STEM CELLS (MSCs) ARE adult stem cells originally isolated from the bone marrow (BM) as precursors of stromal elements [1–3] and currently thought as part of a whole complex system diffused throughout the body. This idea comes first from the evidence that they can be isolated from almost every tissue of the body [4,5], including peripheral blood [6], and, second, from the occurrence of mobilization from the BM toward distant tissues at physiological conditions or in case of damage and neoplasia [7,8]. However, the real in vivo counterpart of culture-expanded MSCs is still unknown. In a unifying attempt to explain their origin, it has been hypothesized by some authors that MSCs are a subgroup of vessel-lining pericytes that may contribute to vessel homeostasis by reacting to tissue damage with regenerative processes, by locally modulating the inflammatory reaction, and by entering systemic circulation to relocate ac-

cording to different cytokine gradients [9,10]. Still, no definitive answer on the real origin of MSCs has been obtained so far.

Despite this, a consensus on the definition and properties of MSCs after in vitro expansion has been recently reached by the International Society for Cellular Therapy [11]: a specific immunophenotype, ex vivo plastic-adherent growth, and multilineage differentiation are defined as the minimal prerequisites needed. Given the high level of heterogeneity existing in isolated MSCs [12], the term "stem cells" should be limited on this basis to cases with proven pluripotency [11].

It may be difficult for the unexperienced scientist to enter the ever-growing field of research on MSCs without previous knowledge: to help new enthusiasts in their initial steps, we tried to summarize a detailed technical overview of the methods used for human MSC isolation, expansion, and characterization for tissue repair, using BM-derived MSCs, the first to be isolated and thoroughly evaluated, as a model.

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We based on both personal experience and the available literature: whenever unspecified, the suggested protocols and methods are among the ones currently in use in our laboratories.

Isolation

Bone marrow

MSCs have been first isolated from the BM. The ease of collection and the relatively high frequency of MSCs [$1/10^4$–$1/10^5$ BM-mononuclear cells (MNCs)] [1,2,13] make it still a commonly used source of MSCs. BM is usually collected by aspiration from the iliac crest or sternum through a Jamshidi needle. Small volumes (<4 mL) are preferred, to prevent hemodilution: vertebrae have provided an additional potential site of collection, while femoral heads are a further alternative, even though they do not always seem reliable sources [14].

Thanks to their density (1.073 g/dL), MSCs are isolated in the mononuclear ring of a density gradient centrifugation (1.077 g/dL) [14]. Centrifugation is carried out at 600 g for 20–30 min with no brakes, and the ring of BM-MNCs is collected manually with a Pasteur pipette and then washed twice with phosphate-buffered saline (PBS) (centrifuged at 300 g for 10 min, at room temperature) [13,14].

After resuspension in a growth medium (GM), cells are counted in an hemocytometer using a vital color—for example, Acridine Orange/Bromure Ethidium—and plated at a density of 1.2×10^6 BM-MNCs/cm^2 [passage 0 (P0)] [15–18]. Other authors have suggested a slightly lower cell density (8×10^5) for initial plating [19]; a technical study testing different media proved alpha modified Eagle's medium (α-MEM) + Glutamax + fetal bovine serum (FBS) 10% and α-MEM + L-glutamine + FBS 10% to be the most efficient in providing good cell expansion ($103.65\pm23.0\times10^6$ and $85.07\pm35.0\times10^6$ per 1×10^6 BM-MNCs, respectively) [20].

After 72 h the plates should be washed with PBS and the GM entirely changed [15]. Adherent cells organize in small clusters of spindle-shaped cells within 7–10 days. Subsequent changes of GM, usually twice a week, should be limited to 40%–50% of total volume.

Adipose tissue and other sources

MSCs have been isolated from almost every tissue of the body [4,5], including lymphoid organs (thymus and spleen) [21], subcutaneous fat [22,23], periodontal ligament [24], scalp tissue [25], endometrium and menstrual blood [26], peripheral blood [6], umbilical cord blood [27] and Wharton's jelly [28,29], as well as fetal tissues [30]. The amount of material needed depends on the abundance of MSCs (eg, 40–50 mL in the case of subcutaneous lipoaspirate) [22].

In almost all cases, careful surgical removal of the connective tissue surrounding the parenchyma is needed as a first step, followed by enzymatic digestion, made by a solution of collagenase type II (0.075% in PBS or HBSS, 30 min at 37°C, shaken at 120–150 rpm) [22,23] or equivalent protease (eg, collagenase type I 0.1% + bovine serum 1%, 60 min at 37°C, in continuous agitation) [31,32]. The cell-enriched supernatant is then collected and the enzyme neutralized by adding GM in a 1:2 ratio. In the case of subcutaneous fat, after centrifugation (200 g, 10 min at room temperature) the

supernatant will contain low-density floating adipocytes, whereas the pellet will contain the vascular-stromal fraction [22,23,31,33]. The pellet should then be washed 3–4 times in PBS. Contaminating erythrocytes, which are most evident in the case of splenic tissue, are then lysed using 160 mM NH$_4$Cl solution (10 min at room temperature), and the cellular suspension is passed through a cell strainer of 70 μm (100 μm in the case of subcutaneous fat) to remove small tissue debris and cellular aggregates. The final pellet is resuspended in GM and the cells are counted and plated. Initial density depends on the abundance of MSCs in the tissue: 10×10^6 cells/cm^2 for cells isolated from the thymus or lymphonodes, 5×10^6 cells/cm^2 from the spleen, and 1×10^6 cells/cm^2 in the case of subcutaneous fat [21].

After, at 72 h the plates are washed with PBS and GM entirely changed. As previously stated, subsequent changes, twice a week, should be limited to 40%–50% of total volume, to preserve medium conditioned by soluble factors released by MSCs. After 6 days in culture, adipose-derived stem cells usually reach early confluence, and need to be detached and replated at lower density (1.8–3.1×10^3 cells/cm^2) to improve the purity of the preparation [22,23,33]. Usually, 3–5 weeks of in vitro culture is sufficient to generate a homogeneous cell population.

Even though MSCs share common features regardless of their source, there is evidence of a role of different specific microenvironments in determining small differences in their immunophenotype (eg, CD34 expression early after isolation) and in vitro differentiation [13,31,32].
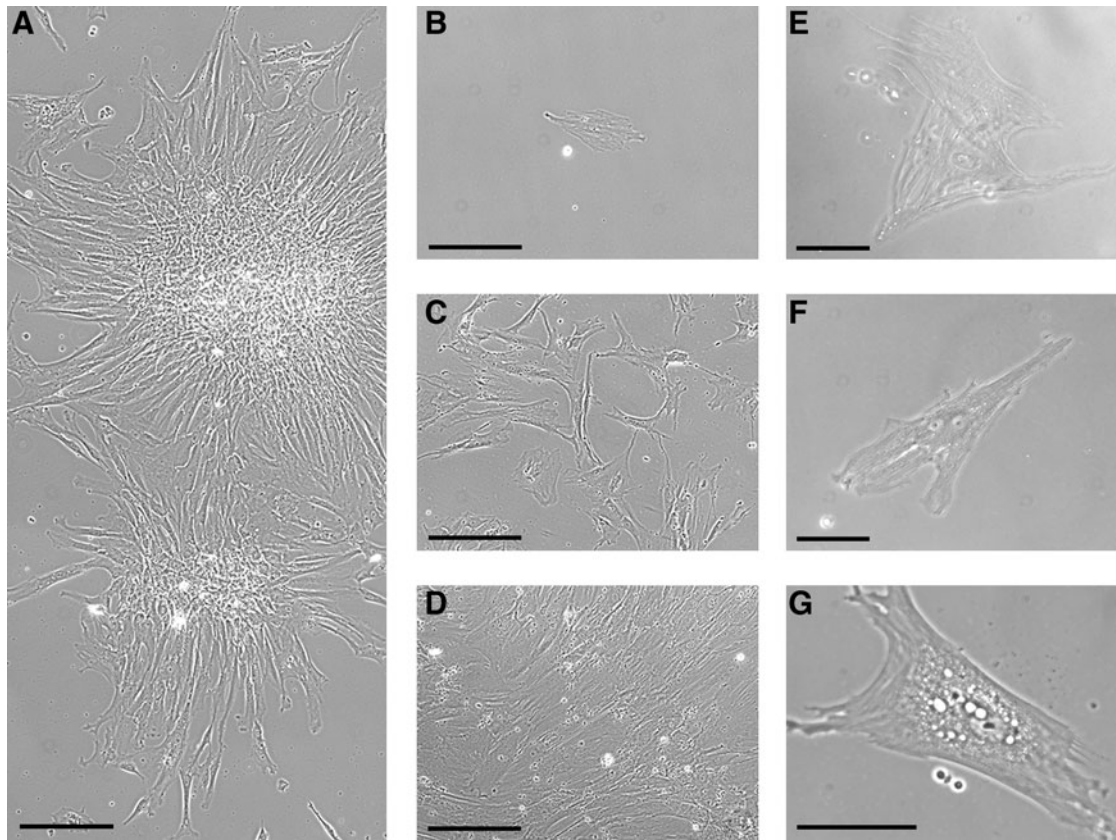
Expansion

Adhesion to the substrate

As previously stated, early separation of adherent elements is carried out in the first 72 h of culture. After 7–14 days of culture, clusters of spindle-shaped, fibroblast-like adherent cells can be detected on the plastic surface of the flask [1–3] (Fig. 1); although this feature suggests the clonogenic potential of cells in the cultured population, it does not permit the quantification of clonogenicity, which requires limiting dilution methods.

Normally, <0.01% of BM adherent cells are ultimately MSCs [3,13]. Therefore, homogeneity can be achieved only after prolonged culture, usually after 2–3 weeks of culture (P3–P4) [3,15,16].

Role of GM

MSCs expand in basic GM, usually in the absence of cytokines, with the addition of 10%–20% fetal calf serum (FCS)/FBS [3,14–16,19,20,34–36] to provide essential proteins for cell adhesion and growth and to prevent spontaneous differentiation. The addition of antibiotics is often useful (penicillin 100 U/mL + streptomycin 100 μg/mL) [15,16]. α-MEM and Dulbecco's MEM (DMEM) are mostly used [20]. Composition may vary, from high-glucose DMEM supplemented by 15% FCS [15,16] to low-glucose DMEM + 10% FBS [14,17,18], to α-MEM + 10% FBS [19], to DMEM/F-12 Ham's medium + 10% FBS [31,32]. A technical study has been conducted comparing different media and protocols to expand BM-MSCs from the same donors [20]: α-MEM + Glutamax + FBS 10% and α-MEM + L-glutamine + FBS 10%

FIG. 1. MSC culture in vitro. (**A**) Clusters of BM-MSCs. Magnification $100\times$. Scale bar $= 200\,\mu m$. (**B–D**) Under expansion by growth media, MSCs grow in vitro spreading throughout the plate until reaching full confluence. Magnification $100\times$. Scale bars $= 200\,\mu m$. (**E, F**) Higher magnification of MSCs in vitro ($40\times$; scale bars $= 40\,\mu m$). (**G**) Accumulation of fatty vacuoles is an early sign of cellular senescence. Magnification $640\times$. Scale bar $= 40\,\mu m$. BM, bone marrow; MSCs, mesenchymal stem cells.

were the most powerful media to achieve quick in vitro expansion while preserving multipotency; in addition, lower initial plating densities ranging from 1,000 to 25,000 BM-MNCs/cm^2 allowed superior numbers of adherent cells at P0 as compared with the group plated at cell density higher than 50,000 BM-MNCs/cm^2 [20]. Despite this, the overall proliferative potential of isolated cells did not apparently correlate with the initial plating density, but rather with passaging density (ranging from 50 to 1,000 cells/cm^2), again with lower densities after P0 resulting in higher proliferation rates [20].

The minimal growth conditions allow for the selection of cultured cells and preserve their uncommitted status. In fact, early immunophenotypical analysis can usually detect contaminating lineage-committed cells, such as hematopoietic precursors, resting lymphocytes, monocytes, and macrophages [13,36]. Preferential in vitro expansion of BM-MSCs is therefore based on three key properties of MSCs: their ability to adhere to plastic surfaces, their ability to continuously grow in poor media, and their high sensitivity to trypsinization as compared with monocytes [14]; therefore, to increase homogeneity after replating especially at earlier passages, trypsinization should never exceed 5–7 min at 37°C. When cultures reach near-confluence (70%–80%), cell-contact growth inhibition should be prevented by detaching, for example, by means of prewarmed 0.05% trypsin–ethylenediaminetetraacetic acid solution, and replating the cells at a lower density (from 1×10^4 to 8×10^4 cells/cm^2) [15–19,21]. This additionally enhances the preferential expansion of MSCs.

Cytokine-induced growth

MSCs are highly sensitive to basic fibroblast growth factor (bFGF, FGF-2), which increases MSC expansion and maintains their uncommitted state [37]. FGF-2 has also been used to replace bovine/fetal serum in FBS-free media [38]. Similarly, other related cytokines increase MSC proliferation and prevents their differentiation, such as FGF-4 [39] and heparin-binding epidermal growth factor–like growth factor (through binding to human EGFR related receptor tyrosine kinase [HER-1]) [15]. On the other hand, there is at least some evidence that FGF-2 may alter in vivo generation of bone [40], and when combined with other cytokines [ie, FGF-2 5 μg/mL + bone morphogenetic protein-2 (BMP-2) 2 μg/mL], FGF-2 promoted osteoblastic differentiation and bone growth when used for lumbar arthrodesis [41]. On the contrary, at higher doses ($\geq$5 ng/mL) FGF-2 appears to induce neuronal differentiation [42] and possibly enhance MSC immunosuppressive ability [20].

MSCs express fetal liver kinase (FLK1) [43] and vascular endothelial growth factor receptor 2 (VEGF-R2) and VEGF-R3 [44]. Moreover, the vascular fate of MSCs can be modulated indirectly, through platelet-derived growth factor receptors (PDGF-R) that regulate MSC migration and proliferation [45].

The search for serum-free media

The appropriate medium is critical to obtain a sufficient amount of MSCs, while retaining all their differentiation potential, and keeping a safety level in accordance with clinical needs. The use of serum-free media containing only well-defined factors is one of the main goals for future developments in MSC expansion. The first step in culturing still requires proteins already present in the medium or exogenously added, to make native MSCs adhere to their support. Culture in total serum deprivation (basic medium without cytokines or growth factors) allows the establishment of an MSC subpopulation that shows features of early progenitors with increased expression of embryonic stem cell genes [46]. However, serum-free media usually require the addition of growth factors (eg, PDGF, FGF-2) [47] to substain growth. A good expansion can be obtained by using serum substitutes (eg, ULTROSER®) [48]; however, these media still contain serum components that may not be highly purified, well defined, or characterized.

The use of serum-free media is important also for the clinical safety: the use of FCS/FBS has raised some concerns, due to the theoretical transmissions of prions and potentially unidentified zoonoses with the cells, as well as the possibility to induce immune reactions by the host. A possible approach to avoid such risks, although with some practical problems, would require autologous or allogeneic human serum for in vitro expansion: in one study, autologous serum has proved to be superior to both FCS and allogeneic serum in inducing long-term proliferation and expansion of MSCs [49].

MSC expansion for clinical use

The production of MSCs for clinical intervention needs to comply with good manufacturing practice (GMP) to ensure the final delivery of a safe, reproducible, and efficient "cell-drug." All steps of the process must be defined, from the source for isolation to culture methods, to the procedures, materials and methods used for cell culture, and quality controls. Even if MSCs are expandable from virtually all tissues [10], to date the preferred source remains the BM. In fact, hundreds of millions MSCs can be expanded in vitro starting from 10 to 20 mL of BM aspirate, although cell yield may vary depending on age and condition of the donor [35,50] and on the expansion techniques [20]. Some other tissues, such as trabecular bone [51], cord blood, or amniotic membrane [52] could have clinical interest in the future. Moreover, adipose tissue (AT) may represent an important alternative to easily obtain a large number of MSCs [23]. The parameters of the culture process must be optimized to reach GMP goals; the first critical parameter is the plating density, which could be involved in the maintenance of early progenitors [35]. Additionally, the time in culture may also change the quality of MSCs. In humans, after 3 weeks and 12–15 population doublings, MSCs decrease their proliferation rate and progressively loose their multipotency [53].

At present, a real serum-free medium is currently not available for clinical-grade MSCs. The main alternative to FCS/FBS-supplemented medium is the use of human serum enriched with platelet growth factors, namely, platelet lysate [54,55], which increases safety by excluding xenogeneic proteins. Totally closed systems for MSC cultures for the pro-duction of large amounts of MSCs are currently under development [56]. To ensure that MSCs expanded in different culture conditions retain all the required characteristics, careful quality controls of the processed MSCs must be established: these controls must not only analyze the microbiological safety and phenotype of the cells by flow cytometry, but also critically test the genetic stability of expanded cells.

Cryopreservation

Cryopreservation is carried out in a cell preservation fluid consisting of FBS + 20% dimethylsulfoxide (DMSO; Me$_2$SO), which should be added to the cells in GM in a 1:1 ratio, to reach a final concentration of 10^6 cells/mL in 10% DMSO [57–60]. Similar media are effective also for AT-MSCs [31], while commercial preparations based on the same principles are also available [61]. Cells should be frozen with a slow and progressive drop in temperature ($-1°C$/min). This can be achieved by passing the cells at increasingly lower temperature [58], by means of a freezing box containing room-temperature isopropylic alcohol, or, more accurately, by using automated freezing devices based on liquid nitrogen in closed circuits [59,61,62]. The latter proved superior to conventional slow freezing and 2 different vitrification protocols in terms of preservation of viability and growth after thawing of MSCs isolated from fetal liver [62]. The cells are then rapidly stored at $-196°C$ in liquid nitrogen vapors. Freezing is generally thought to diminish cell growth capability after thawing, even though some studies, cultivating both frozen and unfrozen freshly isolated MSCs in parallel, proved the persistence of comparable cell growth after cryopreservation [57,63]. Differentiation of MSCs is scarcely affected by cryopreservation [57,59,63].

The thawing medium should contain high FBS concentration: this can be achieved by rapidly thawing the cryovials in a 37°C water bath while adding fresh culture media in doubling volumes [58], or by increasing FBS concentration in GM. We use a thawing medium made of 60% FBS + 40% GM, which should be prewarmed at 37°C and added immediately to the cells in a 10:1 ratio right after initial thawing in the water bath, to dilute DMSO as rapidly as possible and re-establish chemical homeostasis; after centrifugation, the pellet is plated in normal GM, and a complete change of GM is made after 24 h.

Immunophenotype

MSC immunophenotypic characterization

No unique single marker has been found for MSCs so far. Therefore, the combination of markers necessary to identify a homogeneous cell population should include CD105, CD73, CD90, CD44, CD29 (all expressed by MSCs), and CD34, CD45, CD11c, CD14, CD31/PECAM-1, and other endothelial markers (all not expressed by MSCs) [3,13,31,32,64]. Although most MSCs, including BM-MSCs and AT-MSCs, are negative for CD117 (c-kit) [13,31], this marker has been shown to be expressed by MSCs isolated from Wharton's jelly [29]. Adipose-derived MSCs are CD34^{pos} at the first stage of culture, and CD34 expression decreases on the cells over time [13,65], while never becoming completely abolished [31]. MSCs do not express the costimulatory molecules CD80 (B7-1), CD86 (B7-2), CD40, or CD40L, even after in-

terferon gamma (IFN-γ) stimulation [16,66]. An example of immunophenotypic characterization of expanded BM-MSCs is provided in Fig. 2. Although hematopoietic precursors are expected to fully differentiate after initial passages (P3–P4), MSCs are capable of maintaining long-term hematopoietic precursors [67]. In one study [19] a significant contamination by blood precursors (up to 15%) was present at earlier passages (P1), and more common in MSCs expanded from young adult donors as compared with pediatric donors (15.45% vs. 3.66% at P1). These hematopoietic colonies were then lost during culture, and accounted for 0.5% and 4.32% of the entire cell population, respectively, at P10 [19].

Immunophenotype of MSCs is heterogeneous and dynamic: slight differences exist between MSCs expanded in vitro and their putative in vivo counterpart, as detected by a panel of commonly expressed mesenchymal markers right after tissue sampling. This may be also due to the difficulty of unequivocally detecting the in vivo counterpart of expanded MSCs, which might be different in relation to different sources [68]. Other changes in the immunophenotype also happen during culture: for instance, in BM-MSCs CD29 seems to upregulate [69]; CD146 [40] and CD271 [12,70] gradually disappear during in vitro culture. Similarly, studies comparing the immunophenotype of freshly isolated human AT-derived cells from the stromal vascular fraction (SVF) and serial-passaged AT-MSCs show a progressive increase of mesenchymal markers (CD13, CD29, CD44, CD63, CD73, CD90, and CD166) during culture; as previously noted, CD34 expression is maximal on SVF cells right after tissue digestion and at P1, while persisting at lower levels also at later passages (P4); the same is true for CD146, a marker of pericytes [31,32]. Although at low levels, endothelial markers, such as CD31, CD144, or VE-cadherin, remain detectable in SVF cells and in AT-MSCs throughout the culture period [31]. Sometimes, changes in immunophenotype can be related to a significant switch in behavior of the cells (eg, the loss of CD90 seems to be linked to neoplastic transformation of MSCs) [71], or to the effect of specific cytokines in the medium (eg, IFN-γ can upregulate major histocompatibility complex-II [MHC-II]) [11]. In most cases, cultures tend to become more homogeneous as they progress over time [1,2,31].

In any case, immunophenotype alone cannot specifically distinguish between MSCs and more differentiated mesenchymal progeny; functional assays, demonstrating clonogenicity and in vitro multilineage differentiation, are always needed to complement the immunophenotyping and to fully characterize the expanded cell population.

Use of immunophenotypic sorting to ameliorate MSC isolation

Several markers have been tested to improve the efficiency of MSC isolation and expansion, mostly in the case of BM-MSCs. None of them have provided definitive advantages as compared with isolation from whole BM-MNCs. However, the negative selection of committed lineages by sorting with a cocktail of markers of differentiation can significantly increase the rate of MSCs isolated from a sample by retaining the late-adhering MSCs that are normally lost after the initial change of the medium. In specific conditions, immunomagnetic sorting based on CD105 improves the isolation efficiency when MSCs are expected to be very rare, for example, in the samples obtained by washing the empty BM collection bags [72].

Initial selection based on STRO-1 increases 10–100-folds the formation of colony-forming units fibroblasts (CFU-F) according to the separation method (immunomagnetic vs. dual color cell sorting) and the coexpression of other markers [73]. In other cases, however, no difference in CFU-F number has been noted [74].

CD133 was used for positive selection in 1 study by Tondreau and colleagues [75]. Isolated adherent cells expressed the embryonal transcription factor Oct-4, showed pluripotent differentiation potential, and were endowed with higher proliferative potential than those from the CD133^{neg} population. Interestingly, they appeared CD73^{pos} and CD105^{pos}, and negative for CD14, CD34, CD45, and HLA-DR. CD49a (α1 integrin subunit) is another important marker of native MSCs, as all the multipotential CFU-F are included in the CD49a^{pos} fraction of BM-MNCs, and a double selection using CD49a and CD133 can be effectively used to amplify the number of early progenitors [76].

CD146 has been suggested as a highly specific marker of MSCs, as only CD146^{pos} MSCs were capable of supporting hematopoietic repopulation of a subcutaneously transplanted bone chip by CD34^{pos} hematopoietic stem cells (HSCs) and their progeny [40]. Further, CD146 also identifies STRO-1^{neg} cells with MSC features from human endometrium [77]. Similarly, CD200 has been used to purify native MSCs at very high efficiency [78]. CD271 was also used as a selective marker of MSCs, and cells isolated from a CD271^{pos} population showed higher clonogenicity and lower hematopoietic contamination than MSCs, either isolated from whole BM-MNCs or from CD105^{pos} cells [79]. Recently, CD271 has been proposed as one of the most specific markers for BM-derived MSCs and used as the gold standard to compare putative new identifiers [80].

A potential side effect of sorting is the activation of unknown molecular pathways due to cross-linking of surface antigens. To avoid this, aptamers have been recently proposed as alternative sorting tools. These single-stranded DNA or RNA molecules can fold into a 3-dimensional structure and bind to a variety of targets, including peptides, enzymes, and various cell surface receptors, with high affinity and no immunogenicity [81]. Aptamers can be up to 100 bases long; they have been custom-generated from combinatorial libraries through an in vitro selection process and have also been recently applied in MSC isolation with consistent improvement in the frequency of clonogenic MSCs in the expanded population. However, the target of the aptamers remains unclear [70].

Despite these potential advantages, any kind of selection increases the potential risk of isolating a biologically distinct subpopulation; it has been recently shown in a population of MSCs derived from CD271^{pos}, MSCA-1^{pos}, and CD56^{pos} (39D5 epitope) cells that MSCs tend to differentiate into chondrocytes and pancreatic-like islets [82].

MSCs and their in vivo counterpart

Although the phenotype of cultured MSCs is well known, the phenotype of in vivo, or native, MSCs still remains elusive. New data show that MSCs seem to be located within the vicinity of vessel walls: CD146 is a marker of multipotent
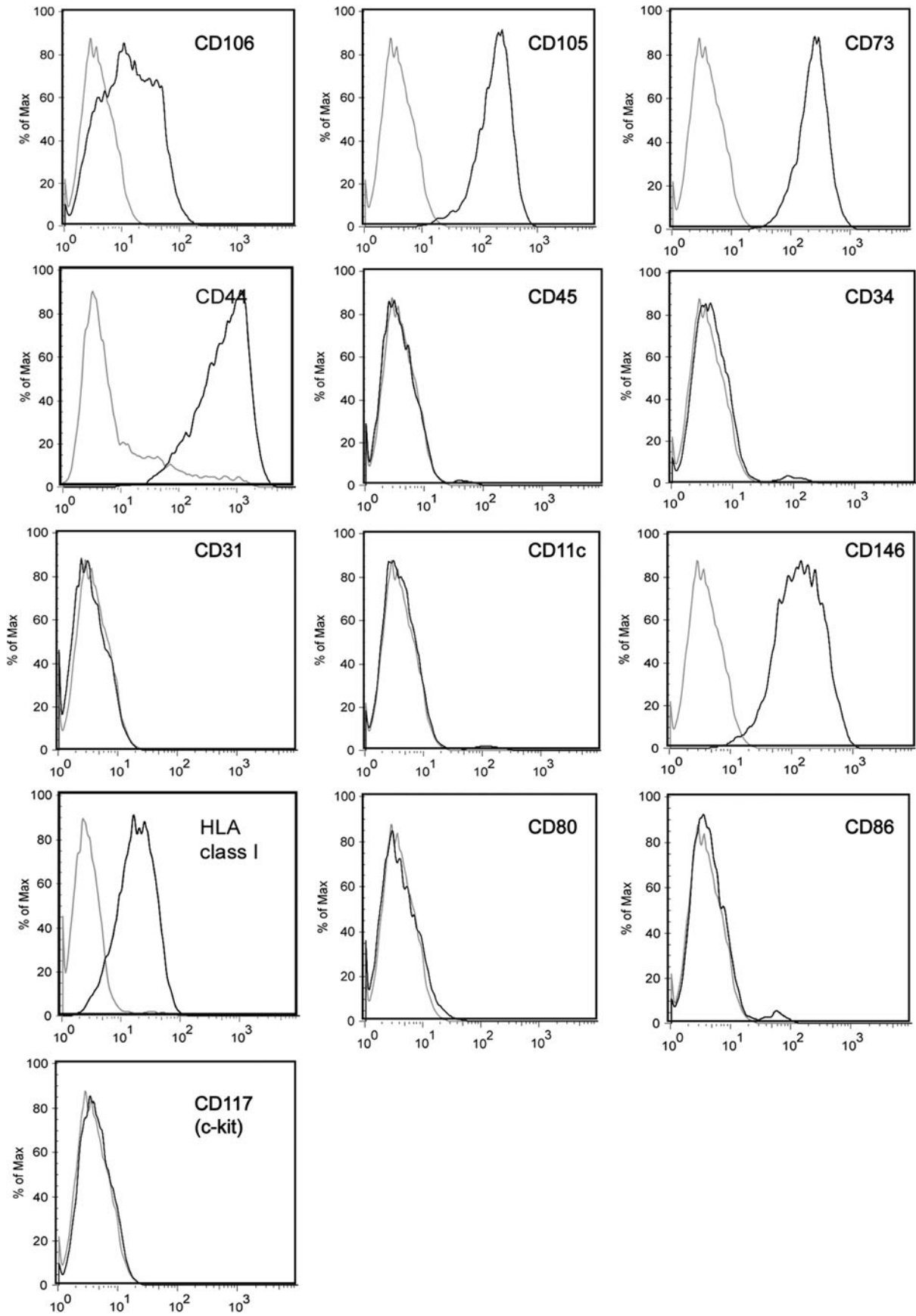
FIG. 2. Immunophenotype of human BM-MSCs (P3) expanded in vitro. Expression of specific markers (black) as compared with isotype controls (gray).

native MSCs; these latter also express other molecules typical of perivascular cells [40]. It has been argued by some authors that all the cells with properties of MSCs derive from pericytes [10]: when cultivated using the same conditions routinely used for MSCs, whatever their tissue origin, pericytes grow, display an MSC phenotype, and are capable of differentiating into osteoblastic, chondrocytic, and adipocytic lineages. In the case of BM-MNCs, enrichment in native MSCs may be obtained by cell selection according to different surface antigens, such as STRO-1, CD146, CD49a, CD200, or CD271. However, expression of some of them, for example, STRO-1, CD146, and CD200, is downregulated during culture [78].

All considered, one may question whether MSCs are indeed a unique and homogeneous population; to this aim, the embryonal development and the tissue origin of MSCs need to be considered. For a long time, MSCs were considered a unique cell population originating from the mesoderm. Actually, it has been recently demonstrated that a first wave of MSCs arises from neuroepithelium during embryogenesis [68]. These cells are transiently found during ontogenesis and their real persistence after birth as part of the MSC pool is currently unknown. Their origin could partially explain the different differentiation behavior of MSCs expanded from different tissues. Alternatively, MSCs may be subjected to the influence of specific microenvironments, thus explaining the preferential differentiation profiles. For instance, while BM-MSCs do not easily differentiate into cardiomyocytes, adipose-derived MSCs show a greater tendency to develop into endothelial and cardiac cells both in vivo and in vitro [83,84].

Functional Assays

Self-renewal and clonogenicity (CFU-F assay)

CFU-F assays are made at best by single-cell seeding after FACS [1]. Alternatively, it is possible to plate at limiting dilution, for example, 1×10^6 cells in a T25 flask (4×10^4/cm^2), and then counting cell clusters with >50 cells as colonies at day 10 [18,85]. The possibility to obtain clones at every passage is a necessary condition to claim the maintenance in culture of uncommitted, self-renewing progenitor cells; however, a more detailed analysis of their ability to self-renew and to differentiate into multiple lineages at the clonal level is needed to actually demonstrate the persistence/expansion of such cells in vitro [1,2].

Differentiation in vitro

Although multipotent in vitro differentiation is a fundamental property of MSCs [11], this is actually true only for a minority of expanded MSCs [86]; in vitro culture leads to a substantial loss of multipotentiality, due to cellular senescence [87], and the real differentiation of MSCs highly depends on the tissue source [88]. For instance, adipose-derived MSCs tend to be less sensitive to osteoblastic differentiation, and are prone to differentiate more easily into adipocytes; stromal cells isolated from BM are capable of supporting hematopoiesis in vivo upon de novo bone formation [40], whereas dental pulp stromal cells give rise to dentin rather than bone in vivo [24]. Moreover, MSCs expanded from antigen-sorted populations may show a tendency toward specific lineages [82]. Finally, plating density also influences differentiation; for instance, a plating density around 10^4 cells/cm^2 at 70%–80% confluence is suggested for adipo-blastic and osteoblastic differentiation [34,89] (Table 1).

Signs of induction are detectable a few hours after stimulation, in the case of morphological changes in the cell shape and mRNA expression of specific genes, or after several days, in the case of the accumulation of neutral lipid vacuoles (Fig. 3), de novo protein production or positivity to specific cytochemical stainings (Table 2). However, in vitro multipotent differentiation does not imply by definition the effective in vivo differentiation in the same lineages: a few studies demonstrated long-term and functional in vivo differentiation of MSCs; in addition, neural differentiation in vivo of MSCs is still debated [90].

Notes on adipogenic differentiation.

- 3-isobutyl-L-methylxanthine (IBMX) (Table 1) is either a competitive nonselective phosphodiesterase inhibitor [91] and a nonselective adenosine receptor antagonist [92]. Its final action on cell metabolism is to raise intracellular cyclic adenosine monophosphate (cAMP), activating protein-kinase A. IBMX has shown to promote the conversion of fibroblasts into adipocytes, apparently with no effect on cell proliferation [93]. Its importance in inducing early adipogenic differentiation has been also established on a preadipocytic cell line, 3T3-L1, where the addition of IBMX and dexamethasone to the medium leads to a significant increase in the production of two species of chondroitin 4-sulfate proteoglycans (CSPG-I and -II) and heparan sulfate proteoglycan [94], and promotes their differentiation into adipocytes. Subsequently, it has become part of induction media of most differentiation media used also for MSCs [95]. Its action may be negligible once activation of the signaling pathways involved in adipogenic commitment has been established, especially in the presence of insulin [96].
- For an early evaluation of differentiation, the analysis of de novo mRNA expression of genes related to adipocytic differentiation, can be performed. The accumulation of neutral lipid vacuoles usually does not become visible before 4–5 days from the start of the induction and may require 2–3 weeks to occur (Fig. 3).
- Suggested genes typical of adipogenic differentiation include peroxisome proliferative activated receptor $\gamma 2$ (PPAR-$\gamma 2$), fatty acid-binding protein 4 (FABP4), and lipoprotein lipase (LPL) [97] (Table 1).

Notes on osteogenic differentiation.

- Von Kossa staining reveals calcium salts using substitution with oxidized (black) silver nitrate [98–100] (Table 2; Fig. 3). After the addition of the silver nitrate solution, the sample should be exposed to the light of an electric bulb for 30–60 min. Caution is advisable when examining black areas, because of the occurrence of false positive signals.
- Genes of osteocytic commitment include alkaline phosphatase (ALP), runt-related transcription factor 2 (RUNX2/Cbfa1/Pebp2αA), integrin-binding bone sialoprotein (IBSP), osteocalcin (BGLAP), osteopontin (SPP1), osteoprotegerin (tnfrsfIIb-OPN), and dual-specific phosphatase 1 (DUSP1) [97] (Table 1).

TABLE 1. INDUCTION AND EVALUATION OF IN VITRO DIFFERENTIATION OF BONE MARROW–MESENCHYMAL STEM CELLS

Differentiation	Induction medium	Notes	IHC demonstration	Gene expression analysis	References
Adipogenic	IBMX 100 µg/mL Insulin 10 µg/mL DMS 10^{-6} M DMEM high glucose FBS 15%	Plate the cells at density 10^4/cm² 70%–80% confluence [34]	Oil Red O [3,101] At 2–3 weeks	PPAR-γ2 FABP4 LPL FABPaP2	Pittenger et al. [3] Krampera et al. [15] Menicanin et al. [97]
Variants (1)	IBMX 0.5 mM Insulin 5 µg/mL DMS 10^{-6} M Indomethacin 60 µM DMEM low glucose FBS 10%				Lu-Lu et al. [18]
(2)	IBMX 0.5 mM Insulin 170 nM DMS 10^{-6} M Indomethacin 0.2 mM DMEM low glucose FBS 5%				Janderova et al. [95]
(3)	DMS 10^{-9} M Insulin Transferrin Selenium 1× Linoleic acid BSA 1× Ascorbic acid 10^{-4} M DMEM low glucose 60% MCDB-201 40% Horse serum 10%				Reyes et al. [101]
(4)	Insulin 100 ng/mL IBMX 0.5 mM DMS 0.5 µM Indomethacin 50 µM α-MEM +l-glutamine 1% FBS 16.5%				Reger et al. [34]
Osteogenic	DMS 10^{-7} M β-Glycerophosphate 10 mM Ascorbic acid 50 µg/mL DMEM high glucose FBS 10%	Plate the cells at density 10^4/cm² 80%–90% confluence [34]	Von Kossa [3,101] ALP at 3 weeks	ALP RUNX2/Cbfa1/Pebp2αA IBSP Osteocalcin (BGLAP) Osteopontin (SPP1) Osteoprotegerin (tnfrsfllb-OPN) DUSP1	Pittenger et al. [3], Krampera et al. [15] Menicanin et al. [97]
Variants (1)	DMS 10^{-7} M β-Glycerophosphate 10 mM				Reyes et al. [101], Lu-Lu et al. [18] Quiroz et al. [89]

|	Medium	Notes	Markers	Reference
(2)	Ascorbic acid 0.2 mM DMEM low glucose FBS 10% DMS 10^{-8} M β-Glycerophosphate 20mM Ascorbic acid 50 μM α-MEM + l-glutamine 1% FBS 16.5%			Reger et al. [34]
Chondrogenic	DMS 10^{-7} M	Work on cell pellets, eg, 2×10^5 MSCs [34]; start induction without TGF-β1, and add 24-h later	Toluidine blue	Krampera et al. [15]
	Sodium pyruvate 1 mM Ascorbic acid 170 μM l-proline 350 μM ITS PREMIX Culture Supplement 0.4% DMEM low glucose TGF-β1 10ng/mL		agc1 Col II a1 Col IX a1 Col X a1	Menicanin et al. [97]
Variants (1)	DMS 10^{-9} M Insulin Transferrin Selenium 1× Linoleic acid BSA 1× Ascorbic acid 10^{-4} M TGF-β1 100 ng/mL DMEM low glucose 60% MCDB-201 40%			Reyes et al. [101]
(2)	Ascorbic acid 50 μg/mL L-PROLINE 40 μG/mL Sodium pyruvate 100 μg/mL ITS PREMIX Culture Supplement 1% TGF-β3 10ng/mL DMS 10^{-7} M BMP-2 500ng/mL DMEM high glucose			Reger et al. [34] Mackay et al. [102]
Myogenic (cardiomyocytes)	5-Azacytidine 3 μM IMDM FBS 20%	5-Azacytidine is a demethylating agents with broad unspecific action	Myosin heavy and light chain α-actin	Makino et al. [105]
Variants (1)	DMEM low glucose 60% of volume MCDB-201 28% Insulin 1.0mg/mL Transferrin 0.55 mg/mL Sodium selenite 0.5 μg/mL BSA 50 mg/mL Linoleic acid 0.47 μg/mL Ascorbate phosphate 10^{-4} M		MEF-2A MEF-2D Nkx2.5 GATA4 MEF-2C	Shim et al. [107]

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(*Continued*)

TABLE 1. (CONTINUED)

Differentiation	Induction medium	Notes	IHC demonstration	Gene expression analysis	References
(2)	DMS 10^{-9} M FBS 10% FGF-4 BMP-2				Yoon et al. [106]
Myogenic (skeletal muscle)	DMS 10^{-9} M	Plate cells at 2×10^4/cm^2	MYO-D myogenin transcription factors At 4–7 days	MYO-D	Reyes et al. [101]
	Insulin Transferrin Selenium 1× Linoleic acid BSA 1×	Suspend 5-azacytidine after first 24h		MEF-2C	
	Ascorbic acid 10^{-4} M DMEM low glucose 60% MCDB-201 40% FBS 2% EGF 10 ng/mL PDGF-BB 10 ng/mL 5-Azacytidine 3 μM (first 24h only)	Change medium every 3–4 days	Fast-twitch myosin α-sarcomeric actin At 15 days		
Myogenic (smooth muscle)	D-erythro-SPC 2 μM	Plate cells at 3,500 cells/cm^2	α-Smooth muscle actin phospho-Smad2 phospho-ERK	α-Smooth muscle actin	Jeon et al. [110]
	TGF-β3 2 ng/mL α-MEM FBS 10%			h$_1$-Calponin SM22α	
Neurogenic	Butylated-hydroxyanisole 200 μM	Preincubate with bFGF 5 ng/mL for 24h, then switch to induction medium	Use IHC for specific antigens (eg, NF-L, β3-tubulin, PMP-22, GFAP, NeuN)	NF-L	Krampera et al. [111], Anghileri et al. [112]
	Valproic acid Forskolin KCl N2 supplement DMEM high glucose FBS 15%			B3-tubulin PMP-22 GFAP NeuN	
Variants (1)	bFGF 25 ng/mL Glutamine 2 mM DMEM low glucose FBS 10%				Tropel et al. [42]

Abbreviations: agc1, aggrecan1; ALP, alkaline phosphatase; bFGF, basic fibroblast growth factor; BMP-2, bone morphogenetic protein-2; BSA, bovine serum albumin; Col II a1, type II collagen; Col IX a1, type IX collagen; Col X a1, type X collagen; DMEM, Dulbecco's modified Eagle's medium; DMS, dexamethasone; DUSP1, dual-specific phosphatase-1; EGF, epidermal growth factor; FABP4, fatty acid-binding protein 4; FBS, fetal bovine serum; IBMX, 3-isobutyl-L-methylxantine; IBSP, integrin-binding bone sialoprotein; LPL, lipoprotein lipase; MSCs, mesenchymal stem cells; PDGF, platelet-derived growth factor; PPARγ2, peroxisome proliferative activated receptor γ2; RUNX2, runt-related transcription factor 2; SPC, sphingosyl-phosphoryl-choline; TGF-β1, transforming growth factor β1; IMDM, Iscove's modified Dulbecco's medium; IHC, immunohistochemistry.
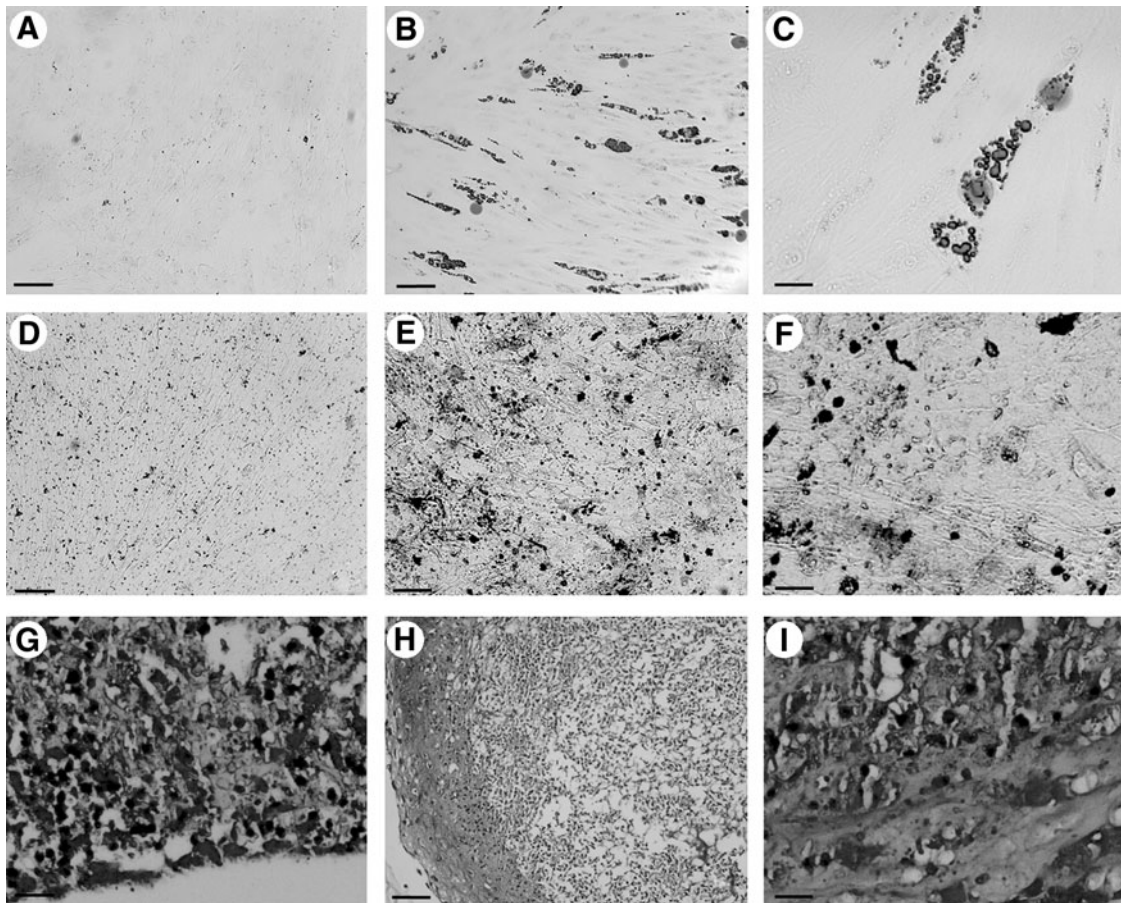
FIG. 3. In vitro multipotent differentiation of MSCs. (**B, C**) Adipogenic induction, Oil Red O staining. (**E, F**) Osteogenic differentiation, von Kossa staining. (**H, I**) Chondrogenic differentiation, Toluidine blue staining. (**A, D, G**) MSCs kept in the growth medium and used as controls. (A, B, D, E, G, H) Magnification 100×; scale bars = 80 μm. (C, F, I) Magnification 400×; scale bars = 20 μm.

Notes on chondrogenic differentiation.

- Chondrogenic differentiation is usually performed with cell pellets. Although several authors prefer DMEM with low glucose for chondrogenic differentiation [15,97,101], media with high-glucose concentrations have been used as well: as pointed out by Mackay and colleagues, high-glucose concentration may help increase the micromass pellets before chondrogenic differentiation [102] (Table 1).

- At the start of the induction, 1 mL of the induction medium without transforming growth factor (*TGFβ1*) is added to the top for the first 24 h; then, the supernatant is replaced with the induction medium containing TGF-β1 for the next 2–3 weeks.

- Evaluation of chondrogenic differentiation is carried out by staining with Toluidine-blue, Alcian-blue, or Safranin-O [98–100]. The first 2 methods are probably more indicated to highlight proteoglycan [103]; by contrast, Toluidine blue has been found in some cases less accurate than Safranin O in the detection of cartilage glycosaminoglycans due to its stoichiometric staining characteristics [104] (Table 2 and Fig. 3).

- Gene expression analysis should include aggrecan1 (*agc1*), collagen type II (*Col II a1*), type IX (*Col IX a1*), and type X (*Col X a1*) [97].

Cardiomyogenic differentiation. After 24-h exposure to 5-azacytidine (3 μM), an unspecific demethylating agent, MSCs can acquire in 1 week markers for myocyte and smooth muscle cells, an elongated morphology and sarcomeric striations; after 2 weeks, these cells show reciprocal connections and can begin spontaneous beating [105] (Table 1). Sarcomeres can be observed by phalloidin staining, myocytic proteins, and transcription factors by immunohistochemistry and reverse transcriptase (RT)-polymerase chain reaction (PCR). Analysis should include Nkx2.5, GATA-4, MEF-2, α-sarcomeric actinin, α-sarcomeric actin, and β-myosin heavy chain.

Alternative approaches for myogenic differentiation are based on the treatment with BMP-2 and FGF-4 [106]; insulin, dexamethasone, and ascorbic acid [107]; or coculture with adult cardiomyocytes [108]. MSCs also appear to differentiate into myocytes spontaneously, although at a very low rate [83].

Differentiation into smooth muscle cells. Differentiation of MSCs into smooth muscle cells seems to depend on TGF-β1 signaling, either directly [109] or after stimulation with TGF-β3 or D-erythro-sphingosyl-phosphoryl-choline [110] (Table 1). In both cases, expression of specific markers such as α-smooth muscle actin, calponin 1, SM22α, and myocardin is significantly increased, as a consequence of ERK and

TABLE 2. HISTOCHEMICAL METHODS TO HIGHLIGHT IN VITRO MULTILINEAGE DIFFERENTIATION OF BONE MARROW–MESENCHYMAL STEM CELLS

Tissue/cells	IHC staining	Identifies	Materials	Methods	References
Adipocytes	Oil Red O	Neutral lipid vacuoles	Oil Red O 0.5 g PG, 100% 100 mL	1. Deparaffinize and hydrate to ethanol 70% 2. Place in PG 100% for 2–5 min 3. Stain in prewarmed Oil Red O 0.5% solution (in PG), 8–10 min at 60°C 4. Move slides in PG 85% (in ddH₂O), 2–5 min 5. Rinse in ddH₂O 6. Stain in hematoxylin, 30 s 7. Wash in running tap water, 3 min 8. Mount in glycerin jelly	Sheehan and Hrapchak [98] Bancroft and Stevens [99] Carson [100] Reger et al. [34]
Osteoblasts	Alizarin red S	Calcium deposits	Alizarin red S 2 g ddH₂O 100 mL Adjust pH to 4.1–4.3 Acetone 100% 25 mL Xylene 100% 25 mL	1. Deparaffinize and hydrate to ethanol 70% 2. Rinse in ddH₂O 3. Alizarin red solution, 0.5–5 min 4. Shake off excessive dye, blot sections 5. Fix with acetone, followed by aceton-xylene 50% 6. Clear in xylene and mount	Carson [100] Quiroz et al. [89] Reger et al. [34]
	Von Kossa (silver nitrate)	Calcium deposits	Silver nitrate 25 g ddH₂O 500 mL Stable 1 year at 4°C 5% Hypo nuclear FR	1. Deparaffinize and hydrate to ddH₂O 2. Add 3.5% silver nitrate solution, 30–60 min exposed to a 60-watt lamp 3. Rinse in ddH₂O 4. Add 5% Hypo, 5 min 5. Wash in tap water, rinse in ddH₂O 6. Nuclear staining (nuclear FR, 5 min) 7. Wash in ddH₂O 8. Dehydrate and mount	Sheehan and Hrapchak [98]
	Alkaline phosphatase	Alkaline phosphatase activity	Tris/HCl buffer: 0.2 M Tris (2.42 g/100) + 0.1 N HCl (0.85/110 mL) Naphthol ASBI stock:	1. Deparaffinize and hydrate to ddH₂O 2. Incubate 5–15 min in Naphthol ASBI stock solution (10 mL) + Fast Red TR (10 mg freshly added. Filter before use). 3. Wash in ddH₂O	Sheehan and Hrapchak [98]

Stain	Target	Solution	Procedure	Reference
Chondroblasts	Heparin, histamine, mucopolysaccharides, proteoglycan, mast cell granules	Naphthol ASBI phosphate 25 mg + N:N dimethyl formamide 50% (in ddH$_2$O) 20 mL Add 300 mL ddH$_2$O and Tris/HCl buffer 180 mL; pH 8.0	4. Stain in hematoxylin, 30 s 5. Wash in ddH$_2$O 6. Mount in glycerin jelly	Sheehan and Hrapchak [98]
Toluidine blue		Stock solution: Toluidine blue O 1 g 70% ethanol (in ddH$_2$O) 100 mL 1% sodium chloride (in ddH$_2$O), pH 2.3 Make fresh each time and check pH Working solution: Toluidine blue stock solution 10% in 1% Sodium chloride, pH 2.3–2.5	1. Deparaffinize and hydrate to ddH$_2$O 2. Stain sections in Toluidine blue working solution, 2–3 min 3. Wash in ddH$_2$O 4. Dehydrate quickly in 95% and 100% ethanol, 3 min each 5. Clear in xylene, 3 min×2 changes 6. Mount in resinous medium	Bancroft and Stevens [99] Carson [100] Shepard and Mitchell [103] Hyllested et al. [104] Reger et al. [34]
Alcian blue	Acid mucopolysaccharides, proteoglycan, acid mucins	Alcian blue 1% (1 g/100 mL) in 3% acetic acid solution (in ddH$_2$O) Adjust to pH 2.5 0.1% nuclear FR (in ddH$_2$O + 5 g/100 mL aluminum sulfate)	1. Deparaffinize and hydrate to ddH$_2$O 2. Stain in Alcian blue solution, 30 min 3. Wash in running tap water, 2 min, rinse in ddH$_2$O 4. Counterstain in nuclear FR solution, 5 min 5. Wash in running tap water, 1 min 6. Dehydrate quickly in 95% and 100% ethanol, 3 min each 7. Clear in xylene, 3 min×2 changes 8. Mount in resinous medium	Sheehan and Hrapchak [98] Bancroft and Stevens [99] Carson [100] Hyllested et al. [104]
Safranin O	Mucin, proteoglycan, glycosaminoglycans, mast cell granules	Hematoxylin solution: Hematoxylin 1% in 95% ethanol (in ddH$_2$O)	1. Deparaffinize and hydrate to ddH$_2$O 2. Stain with Weigert's solution, 10 min	Sheehan and Hrapchak [98] Bancroft and Stevens [99]

(Continued)

TABLE 2. (CONTINUED)

Tissue/cells	IHC staining	Identifies	Materials	Methods	References
			Iron stock solution:	3. Wash in running tap water, 10 min	Carson [100]
			4 mL 29% ferric chloride + 95 mL ddH$_2$O + 1 mL HCl Weigert's solution:	4. Stain with 0.001% Fast Green (FCF) solution (in ddH$_2$O), 5 min	Hyllested et al. [104]
				5. Rinse quickly with 1% acetic acid solution, 10–15 s	
			50% Hematoxylin solution + 50% iron stock solution	6. Stain in 0.1% safranin O solution (in ddH$_2$O), 5 min	
			Stable 4 weeks at 4°C	7. Dehydrate quickly in 95% and 100% ethanol, 3 min each	
				8. Clear in xylene, 3 min×2 changes	
				9. Mount in resinous medium	

Abbreviation: PG, propylene glycol; Fast Green FCF, C37H34N2Na2O10S3, C.I. 42053, E143; FR, fast red solution; IHC, immunohistochemistry.

Smad2 phosphorylation [110]. Notch signaling may be additionally involved in the differentiation process, as the knockdown of Notch-ligand Jagged1 may revert the effects of TGF-β on overexpression of these smooth muscle markers.

Neural differentiation. MSCs can be induced to differentiate into neural lineages (Table 1): in one method [42] MSCs were grown at low density (3,000 cells/cm^2) on poly-lysine-coated plates [use 10 μg/mL poly(lysine) in PBS overnight] for 7 days; the amplification medium comprised low-glucose DMEM, 10% FCS, glutamine 2 mM, and bFGF 25 ng/mL.

In another approach [111,112] MSCs were incubated with 5 ng/mL bFGF for 24 h, followed by the complete substitution of the medium for 2–16 h with DMEM, N2 supplement, butylated-hydroxyanisole, KCl, valproic acid, and forskolin. With either methods, MSCs show a dramatic change in morphology after 24–48 h, and they develop elongated neurite-like branches and axon-like structures, significantly increase their basal nestin expression (from 50.3% ± 8.2% to 94.5% ± 3.4%), and start to express neuronal and glial markers, such as *NF-L*, β3-tub [38], *PMP-22*, *GFAP*, and *NeuN* [111]. Functional assays show the appearance on the cells of functional neural receptors and pharmacologically sensitive voltage-dependent calcium channels [42]. However, with both differentiating media MSCs revert to basic conditions when induction is suspended. In contrast, coculture with Schwann cells seems to lead the cells to express an irreversible neural phenotype [111].

Senescence and transformation

Senescence, defined as irreversible growth arrest, is the ultimate physiological condition of human MSCs expanded ex vivo. It is characterized by the gradual disappearance of telomerase in the cells, decreased telomeric length, and increasing appearance of p16^{INK4a}-positive MSCs, a marker for upregulation of signal pathways related to senescence and apoptosis in several stem cell types [113]. Senescent MSCs accumulate internal fatty vacuoles, lower their growth rate, and eventually stop growing and become apoptotic. The absence of senescence phenomena after prolonged expansion suggests the neoplastic transformation of MSCs, which should be tested by different approaches (in particular, [114]):

- Cytogenetic analysis: normally at the end of the first (P1) and second (P2) passages, by means of standard karyotyping according to the International System for human Cytogenetics Nomenclature, and fluorescent in situ hybridization according to standard protocols [114].
- Expression of genes involved in transformation: assessment by means of real-time RT-PCR of *c-myc, p21, p53,* and *p16*^{INK4a} gene expression and the presence of functional full-length hTERT transcript, and the integrity of *Ink4a/ARF locus* by PCR analysis of p16 exons 2 and 4 [114].
- Culture in soft agar and β-galactosidase (β-Gal) expression: in vitro testing of anchorage-independent colony development is carried out by culturing MSCs in soft agar at 2 time points, end of P3 and end of last passage. Senescence of cultivated MSCs may be studied by β-Gal staining using specific senescence detection kits [114].
- In vivo tumorigenesis in mice: female SCID mice are sublethally γ-irradiated and infused intravenously with

10^6 MSCs; animals are monitored for 8 weeks and then sacrificed for organ analysis [114].

Through these different approaches, it has been recently shown that clinical-grade human MSCs expanded in vitro for tissue engineering or immunoregulatory purposes show poor susceptibility for neoplastic transformation [114], although 2 previous studies had described the capacity of human MSCs to accumulate chromosomal instability and give rise to carcinoma in immunocompromised mice after long-term culture [115,116]. Tarte and colleagues have shown that some transient and donor-dependent recurring aneuploidy may be detected in vitro, independently of the culture process. However, MSCs with or without chromosomal alterations show progressive growth arrest and enter senescence without evidence of transformation either in vitro or in vivo [114].

Experimental Evidence for Potential Use of MSCs for Tissue Repair

A proper expansion and the use of a large amount of homogeneous MSCs are often not sufficient to obtain a significant improvement in a clinical setting. The use of MSCs alone has not led to clear-cut results in terms of tissue regeneration yet. MSCs were initially used in osteoarthritis, with poor results [117]. More promising results were obtained in a mouse model of osteogenesis imperfecta, where intravenously injected MSCs engrafted and gave origin to de novo osteogenesis [118]. Further, the use of MSCs in three affected children led to new lamellary bone formation and improved laboratory and clinical findings [119]. However, the effective role of MSCs in inducing de novo osteogenesis has been questioned; in fact, the conditioning regimen used to achieve BM transplantation might have directly favored this phenomenon.

Similarly, the inoculation of human MSCs in a mouse model of Duchenne's muscular dystrophy (*mdx* mouse) was associated with scarse evidence of formation of new functionally active myoblasts and of significant sarcolemmatic expression of dystrophin [120].

Thus, it has become clear that the microenviroment in which MSCs are transplanted (ie, growth factors and local cellular interactions) plays a pivotal role in determining both MSC biology (survival, proliferation, and specific differentiation) and eventually a clinical measurable improvement. For instance, in tendon repair MSCs alone led to a partial recovery of tendineal function (20%; 37% at 12 months in a model of lesions of Achilles' tendon); nevertheless, in this study ectopic bone formation was detected in 30% of cases [121]. Growth factors [117], biomaterials like poly-L-lacitide-co-glycolyde (PLGA) [122], and even moderate mechanical stress all seem to improve these results [123]. For all these reasons, orthopedic regenerative medicine started to employ different kinds of biological scaffolds, switching from biologically inert materials, such as ceramics or titanium, to porous biomaterials, capable of bone integration, like hydroxyapatite and tricalcic phosphate [124] and biodegradable polymers (poly-L-lactide and PLGA). This approach has been successfully used to repair extensive bone and mandibular defects [125]. Ialuronate-based biomatrices have been used in animal models of meniscal and cartilage regeneration

[126]. Foci of incomplete regeneration have been observed, together with a decreased osteophyte formation and secondary arthritis [126]. An alternative ialuronate-based biomatrix, with the addition of type I collagen and calcium phosphate, has been used for extensive osteochondral defects of the knee [127]. Biologically active elements have been successfully introduced into the biomatrices, like recombinant human BMP-2 [128].

In cardiac regeneration, there are several animal studies pointing to effective improvement of cardiac function after intramyocardial injection or intracoronary infusion of MSCs, either by transdifferentiation [129] or by positive paracrine effects exerted by the cells on various targets, including resident cardiac progenitors [130]. In fact, even if MSC-derived myocytes connect with preexisting residual myocardium through connexin-43 GAP-junctions in several animal models [8,129], the significance and the long-term in vivo persistence of MSC-derived cardiomyocytes in humans is still under debate [130]. Two clinical trials have been concluded, so far, with positive results on very short follow-up (3–6 months) [131,132], whereas several others are ongoing. Another potential approach would involve in vitro predifferentiation of MSCs into cardiomyocyte (CMCs): after exposure to 5-azacytidine, MSCs react to electrical currents [105] and to adrenergic and cholinergic stimuli; in mice, they integrate in preexisting tissue and survive for up to 4 weeks [133]. Nevertheless, no clinical trial using these cells has been conducted so far.

MSCs have also been used to engineer transplantable immunocompatible heart valves starting from decellularized homograft [134] or xenografts [135]. Currently, in vitro engineering using decellularized porcine valves seem somehow more promising, as residual cellularity and calcification of the valves before MSC implantation can hamper subsequent tissue formation and function. Still, like in bone engineering, moderate stress, for example, the one provided by cyclic flexure or laminar flow artificially reproduced in some types of bioreactors, seems important to drive tissue formation [136].

In vivo neural differentiation of MSCs is even more problematic. Adipose-derived MSCs can migrate through the hemato-encephalic barrier after adhesion to the endothelium by VLA-4 [112], and they can differentiate into neuroglial lineages after intraventricular in utero injection in rats [137]. They provide a certain level of functional recovery in animal models of Parkinson and hypoxic-ischemic neural damage [138]. To date, however, there is no evidence about the neural regenerative potential of MSCs in vivo in humans.

In summary, the question is: to fully characterize MSCs expanded in vitro we have to carry out all the functional assays that have been previously described; but are we really sure that in vivo differentiation of MSCs is absolutely required to achieve positive effects in treated patients? On the basis of the current experimental evidence, the answer could be no: only a few studies managed to demonstrate the long-term persistence of MSC-derived differentiated cells. Paracrine effects by a large number of biologically relevant molecules and cytokines produced by MSCs have been often advocated to explain the functional benefits achieved in animal models and treated patients [119,120,130,138]. It is likely that in most cases MSCs may simply help tissue repair without directly contributing to it, by acting on resident progenitor cells or boosting recipient tissue activity.

In other words, we are capable of thoroughly characterizing what we recognize as MSCs, but nowadays we have only partial knowledge about the precise mechanisms by which MSC-based therapy seems to work.

Potential Risks of the Use of MSCs in Clinical Trials

MSCs have been successfully used in phase-II and -III clinical trials for the prevention and treatment of graft-versus-host disease after allogeneic HSC transplantation, and they have a number of promising applications in regenerative medicine, especially in the orthopedic clinical setting. However, their short- and long-term safety needs to be addressed, not only in preclinical animal models, but also with a long-term follow-up of MSC-treated patients. For instance, the engraftment of MSCs in a specific tissue may result in inadequate differentiation, as shown by the appearance of undesired ectopic bone within the heart of MSC-treated animals in a mouse model of myocardial infarction [139]. This type of unexpected differentiation needs to be further investigated in different relevant animal models before starting large-scale human trials. Further, the possible neoplastic transformation during prolonged MSC expansion must be always taken into consideration, as previously discussed. Its occurrence was demonstrated during long-term culture of adipose-derived MSCs: the cells acquired chromosomal abnormalities, then became capable of growing persistently, and finally expressed human telomerase and formed tumors when injected in immunocompromised mice [140]. Actually, neoplastic transformation of MSCs seems to be generally a long process that requires sequential steps [141]; as recently shown, it does not occur even in long-term cultures [114, 142]; however, as long-term-expanded MSCs show progressive growth arrest and enter senescence [114], it is always preferable not to use these cells for clinical use.

Finally, it has to be reminded that theoretically MSCs injected into patients might promote the growth of preexistent tumors, even without evidence of their direct neoplastic transformation [143]; this event is related either to the immunosuppressive activity of MSCs or the production of pro-angiogenic soluble factors, for example, angiopoietin 1 and VEGF [144]. However, preclinical experimental data are not clear-cut: in some cases (Kaposi sarcoma) MSCs inhibited tumor growth [145], while they led to faster tumor development when coinjected with melanoma cells [146]. Therefore, the careful pretransplant evaluation and long-term follow-up of all patients treated with MSCs are required.

Conclusions

After Friedenstein et al.'s article in 1976 [1] and almost 20 years of relatively poor interest, MSCs have experienced a Renaissance period in the last decade of the 20th century, as the improvement of isolation and expansion methods for stem cells permitted to obtain large numbers of cells from small starting samples. Thus, MSCs have now entered the clinical setting. Large international consortia have been formed to develop effective and reproducible protocols for MSC isolation and expansion, while meeting high standards of clinical safety.

Considering the increasing interest toward regenerative medicine based on MSCs and the heterogeneity of isolation,

expansion, and differentiation protocols employed by different groups worldwide, a sort of handbook concerning biological features, immunophenotype, culture techniques, functional assays, and quality controls used for BM-MSCs and AT-MSCs (most commonly used so far) may be of potential interest for new scientists entering this intriguing field.

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References

1. Friedenstein AJ, JF Gorskaja and NN Kulagina. (1976). Fibroblast precursors in normal and irradiated mouse hemopoietic organs. Exp Hematol 4:267–274.
2. Prockop DJ. (1997). Marrow stromal cells as stem cells for nonhemopoietic tissues. Science 276:71–74.
3. Pittenger MF, AM Mackay, SC Beck, RK Jaiswal, R Douglas, JD Mosca, MA Moorman, DW Simonetti, S Craig and DR Marshak. (1999). Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147.
4. da Silva ML, PC Chagastelles and NB Nardi. (2006). Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci 119(Pt 11):2204–2213.
5. Kern S, H Eichler, J Stoeve, H Kluter and K Bieback. (2006). Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood or adipose tissue. Stem Cells 24:1294–1301.
6. Roufosse CA, NC Direkze, WR Otto and NA Wright. (2004). Circulating mesenchymal stem cells. Int J Biochem Cell Biol 36:585–597.
7. Devine SM, J Cobbs, M Jennings, A Bartholomew and R Hoffman. (2003). Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. Blood 101:2999–3001.
8. Kawada H, J Fujita, K Kinjo, Y Matsuzaki, M Tsuma, H Miyatake, Y Muguruma, K Tsuboi, Y Itabashi, Y Ikeda, S Ogawa, H Okano, T Hotta, K Ando and K Fukuda. (2004). Nonhematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction. Blood 104:3581–3587.
9. da Silva L, AI Caplan and N Nardi. (2008). In search of the in vivo identity of mesenchymal stem cells. Stem Cells 26:2287–2299.
10. Crisan M, S Yap, L Casteilla, CW Chen, M Corselli, TS Park, G Andriolo, B Sun, B Zheng, L Zhang, C Norotte, PN Teng, J Traas, R Schugar, BM Deasy, S Badylak, HJ Buehring, JP Giacobino, L Lazzari, J Huard and B Péault.

(2008). A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell 3:301–313.
11. Dominici M, K Le Blanc, I Mueller, I Slaper-Cortenbach, F Marini, D Krause, R Deans, A Keating, DJ Prockop and E Horwitz. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8: 315–317.
12. Schaefer R, M Dominici, I Muller, F Dazzi, K Bieback, K Godthardt, K Le Blanc, R Meisel, R Pochampally, R Richter, T Skutella, G Steinhoff, M Mitterberger, H Wendel, J Wiskirchen, R Handgretinger and H Northoff. (2007). Progress in characterization, preparation and clinical applications of non-hematopoietic stem cells. Cytotherapy 9:397–405.
13. Bernardo ME, F Locatelli and WE Fibbe. (2009). Mesenchymal stromal cells: a novel treatment modality for tissue repair. Ann N Y Acad Sci 1176:101–117.
14. Lennon DP and AI Caplan. (2006). Isolation of human marrow-derived mesenchymal stem cells. Exp Hematol 34:1604–1605.
15. Krampera M, A Pasini, A Rigo, MT Scupoli, C Tecchio, G Malpeli, A Scarpa, F Dazzi, G Pizzolo and F Vinante. (2005). HB-EGF/HER-1 signalling in bone marrow mesenchymal stem cells: inducing cell expansion and reversibly preventing multi-lineage differentiation. Blood 106:59–66.
16. Krampera M, L Cosmi, R Angeli, A Pasini, F Liotta, A Andreini, V Santarlasci, B Mazzinghi, G Pizzolo, F Vinante, P Romagnani, E Maggi, S Romagnani and F Annunziato. (2006). Role for IFN-γ in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells 24:386–398.
17. Le Blanc K, L Tammik, B Sundberg, SE Haynesworth and O Ringden. (2003). Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic response independently of the major histocompatibility complex. Scand J Immunol 57:11–20.
18. Lu-Lu L, L Yong-Jun, Y Shao-Guang, Z Qin-Jun, W Xin, G Wei, H Zhi-Bo, X Zhen-Shu, L Yong-Xin, L Delong, C Zhi-Zhe and H Zhong-Chao. (2006). Isolation and characterization of human umbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials. Haematologica 91:1017–1026.
19. Mareschi K, I Ferrero, D Rustichelli, S Aschero, L Gammaitoni, M Aglietta, E Madon and F Fagioli (2006). Expansion of mesenchymal stem cells isolated from pediatric and adult donor bone marrow. J Cell Biochem 97:744–754.
20. Sotiropoulou PA, SA Perez, M Salagianni, CN Baxevanis and M Papamichail. (2006). Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. Stem Cells 24:462–471.
21. Krampera M, S Sartoris, F Liotta, A Pasini, R Angeli, L Cosmi, A Andreini, F Mosna, B Bonetti, E Rebellato, MG Testi, F Frosali, G Pizzolo, F Tridente, E Maggi, S Romagnani and F Annunziato. (2007). Immune regulation by mesenchymal stem cells derived from adult spleen and thymus. Stem Cells Dev 16:797–810.
22. Zuk PA, M Zhu, H Mizuno, J Huang, JW Futrell, AJ Katz, P Benhaim, HP Lorenz and MH Hedrick. (2001). Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7:211–228.
23. Zuk PA, M Zhu, P Ashjian, DA De Ugarte, J Huang, H Mizuno, ZC Alfonso, JK Frazer, P Benhaim and MH Hedrick. (2002). Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13:4279–4295.

24. Trubiani O, G Orsini, S Caputi and A Piatelli. (2006). Adult mesenchymal stem cells in dental research: a new approach for tissue engineering. Int J Immunopathol Pharmacol 19:451–460.

25. Shih DT, DC Lee, SC Chen, RY Tsai, CT Huang, CC Tsai, EY Shen and WT Chiu. (2005). Isolation and characterization of neurogenic mesenchymal stem cells in human scalp tissue. Stem Cells 23:1012–1020.

26. Hida N, N Nishiyama, S Miyoshi, S Kira, K Segawa, T Uyama, T Mori, K Miyado, Y Ikegami, C Cui, T Kiyono, S Kyo, T Shimizu, T Okano, M Sakamoto, S Ogawa and A Umezawa. (2008). Novel cardiac precursor-like cells from human menstrual blood-derived mesenchymal cells. Stem Cells 26:1695–1704.

27. Erices A, P Conget and JJ Minguel. (2000). Mesenchymal progenitor cells in human umbilical cord blood. Br J Haematol 109:235–242.

28. Troyer DL and ML Weiss. (2008). Concise review: Wharton's jelly-derived cells are a primitive stromal cell population. Stem Cells 26:591–599.

29. La Rocca G, R Anzalone, S Corrao, F Magno, T Loria, M Lo Iacono, A Di Stefano, P Giannuzzi, L Marasà, F Cappello, G Zummo and F Farina. (2009). Isolation and characterization of Oct-4+/HLA-G+ mesenchymal stem cells from human umbilical cord matrix: differentiation potential and detection of new markers. Histochem Cell Biol 131:267–282.

30. In't Anker PS, SA Scherjon, C Kleijburg-van der Keur, GM de Groot-Swings, FH Claas, WE Fibbe and HH Kanhai. (2004). Isolation of mesenchymal stem cells of fetal and maternal origin from human placenta. Stem Cells 22:1338–1345.

31. Mitchell JB, K McIntosh, S Zvonic, S Garrett, ZE Floyd, A Kloster, YD Halvorsen, RW Storms, B Goh, G Kilroy, X Wu and JM Gimble. (2006). Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. (2006). Stem Cells 24:376–385.

32. McIntosh K, S Zvonic, S Garrett, JB Mitchell, ZE Floyd, L Hammill, A Kloster, YD Halvorsen, JP Ting, RW Storms, B Goh, G Kilroy, X Wu and JM Gimble. (2006). The immunogenicity of human adipose-derived cells: temporal changes in vitro. Stem Cells 24:1246–1253.

33. Boquest AC, A Shahdadfar, JE Brinchmann and P Collas. (2006). Isolation of stromal stem cells from human adipose tissue. Methods Mol Biol 325:35–46.

34. Reger RL, AH Tucker and MR Wolfe. (2008). Differentiation and characterization of human MSCs. In: *Mesenchymal Stem Cells: Methods and Protocols.* Prockop DJ, DG Phinney and BA Bunnel, eds. Humana Press, Totowa, NJ, pp 93–107.

35. Sekiya I, BJ Larson, JR Smith, R Pochampally, JG Cui and DJ Prockop. (2002). Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. Stem Cells 20:530–541.

36. Prockop DJ, I Sekiya and DC Colter. (2001). Isolation and characterization of rapidly self-renewing stem cells from cultures of human marrow stromal cells. Cytotherapy 3:393–396.

37. Benavente CA, WD Sierralta, PA Conget and JJ Minguell. (2003). Subcellular distribution and mitogenic effect of basic fibroblast growth factor in mesenchymal uncommitted stem cells. Growth Factors 21:87–94.

38. Battula VL, PM Bareiss, S Treml, S Conrad, I Albert, S Hojak, H Abele, B Schewe, L Just, T Skutella and HJ Buehring. (2007). Human placenta and bone marrow derived MSCs cultured in serum-free, b-FGF-containing medium express cell surface frizzled-9 and SSEA-4 and give rise to multilineage differentiation. Differentiation 75:279–291.

39. Farrè J, S Roura, C Prat-Vidal, C Soler-Botija, A Llach, CE Molina, L Hove-Madsen, JJ Cairò, F Gòdia, R Bragos, J Cinca and A Bayes-Genis. (2007). FGF-4 increases in vitro expansion rate of adult bone marrow-derived mesenchymal stem cells. Growth Factors 25:71–76.

40. Sacchetti B, A Funari, S Michienzi, S Di Cesare, S Piersanti, I Saggio, E Tagliafico, S Ferrari, P Gehron Robey, M Riminucci and P Bianco. (2007). Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. Cell 131:324–336.

41. Minamide A, M Yoshida, M Kawakami, M Okada, Y Enyo, H Hashizume and SD Boden. (2007). The effects of bone morphogenetic protein and basic fibroblast growth factor on cultured mesenchymal stem cells for spine fusion. Spine 32:1067–1071.

42. Tropel P, N Platet, JC Platel, D Noel, M Albrieux, AL Benabiol and E Barger. (2006). Functional neuronal differentiation of bone-marrow derived MSCs. Stem Cells 24:2868–2876.

43. Ritter E, A Perry, J Yu, T Wang, L Tang and E Bieberich. (2008). Breast cancer cell-derived fibroblast growth factor 2 and vascular endothelial growth factor are chemoattractants for bone marrow stromal stem cells. Ann Surg 247:310–314.

44. Conrad C, H Niess, R Huss, S Huber, I von Luettichau, PJ Nelson, HC Ott, KW Jauch and CJ Bruns. (2009). Multipotent mesenchymal stem cells acquire a lymphendothelial phenotype and enhance lymphatic regeneration in vivo. Circulation 119:281–289.

45. Ball SG, CA Shuttleworth and CM Kielty. (2007). Mesenchymal stem cells and neovascularization: role of platelet-derived growth factor receptors. J Cell Mol Med 11:1012–1030.

46. Pochampally RR, JR Smith, J Ylostalo and DJ Prockop. (2004). Serum deprivation of human marrow stromal cells (hMSCs) selects for a subpopulation of early progenitor cells with enhanced expression of OCT-4 and other embryonic genes. Blood 103:1647–1652.

47. Gronthos S and PJ Simmons. (1995). The growth factor requirements of STRO-1 positive human bone marrow stromal precursors under serum-deprived conditions in vitro. Blood 85:929–940.

48. Meuleman N, T Tondreau, A Delforge, M Dejeneffe, M Massy, M Libertalis, D Bron and L Lagneaux. (2006). Human marrow mesenchymal stem cell culture: serum-free medium allows better expansion than classical α-MEM medium. Eur J Haematol 76:309–316.

49. Shahdadfar A, K Fronsdal, T Haug, FP Reinholt and JE Brinchmann. (2005). In vitro expansion of human mesenchymal stem cells: choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptosome stability. Stem Cells 23:1357–1366.

50. DiGirolamo CM, D Stokes, D Colter, DG Phinney, R Class and DJ Prockop. (1999). Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. Br J Haematol 107:275–281.

51. Tuli R, S Tuli, S Nandi, ML Wang, PG Alexander, H Haleem-Smith, WJ Hozack, PA Manner, KG Danielson and RS Tuan. (2003). Characterization of multipotential mesen-

chymal progenitor cells derived from human trabecular bone. Stem Cells 21:681–693.

52. Bieback K, S Kern, H Klüter and H Eichler. (2004). Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells 22:625–634.

53. Banfi A, A Muraglia, B Dozin, M Mastrogiacomo, R Cancedda and R Quarto. (2000). Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: implications for their use in cell therapy. Exp Hematol 28:707–715.

54. Doucet C, I Ernou, Y Zhang, JR Llense, L Begot, X Holy and JJ Lataillade. (2005). Platelet lysates promote mesenchymal stem cells expansion: a safety substitute for animal serum cell-based therapy application. J Cell Physiol 205: 228–236.

55. Schallmoser K, C Bartmann, E Rohde, A Reinisch, K Kashofer, E Stadelmeyer, C Drexler, G Lanzer, W Linkesch and D Strunk. (2007). Human platelet lysate can replace fetal bovine serum for clinical-scale expansion of functional mesenchymal stromal cells. Transfusion 47:1436–1446.

56. Godara P, CD McFarland and RE Nordon. (2008). Design of bioreactors for mesenchymal stem cell tissue engineering. J Chem Technol Biotechnol 83:408–420.

57. Bruder SP, N Jaiswal and SE Haynesworth. (1997). Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. J Cell Biochem 64:278–294.

58. Liu G, C Shu, L Cui, W Liu and Y Cao. (2008). Tissue-engineered bone formation with cryopreserved human bone marrow mesenchymal stem cells. Cryobiology 56:209–215.

59. Xiang Y, Q Zheng, B Jia, G Huang, Y Xu, J Wang and Z Pan. (2007). Ex vivo expansion and pluripotential differentiation of cryopreserved human bone marrow mesenchymal stem cells. J Zhejiang Univ Sci B 8:136–146.

60. Casado-Diaz A, R Santiago-Mora, R Jimenez, J Caballero-Villarraso, C Herrera, A Torres, G Dorado and JM Quesada-Gomez. (2008). Cryopreserved human bone marrow mononuclear cells as a source of mesenchymal stromal cells: application in osteoporosis research. Cytotherapy 10:460–468.

61. Gonda K, T Shigeura, T Sato, D Matsumoto, H Suga, K Inoue, N Aoi, H Kato, K Sato, S Murase, I Koshima and K Yoshimura. (2008). Preserved proliferative capacity and multipotency of human adipose-derived stem cells after long-term cryopreservation. Plast Reconstr Surg 121:401–410.

62. Todorov P, E Hristova, R Konakchieva, A Michova and Y Dimitrov. (2010). Comparative studies of different cryopreservation methods for mesenchymal stem cells derived from human fetal liver. Cell Biol Int 34:455–462.

63. Haack-Sorensen M, L Bindslev, S Mortensen, T Friis and J Kastrup. (2007). The influence of freezing and storage on the characteristics and functions of human mesenchymal stromal cells isolated for clinical use. Cytotherapy 9:328–337.

64. Caplan AI. (1994). The mesengenic process. Clin Plast Surg 21:429–435.

65. Traktuev DO, S Merfeld-Clauss, J Li, M Kolonin, W Arap, R Pasqualini, BH Johnstone and KL March. (2008). A population of multipotent CD34+-positive adipose stromal cells share pericyte and mesenchymal surface markers, reside in a periendothelial location, and stabilize endothelial networks. Circ Res 102:77–85.

66. Le Blanc K, C Tammik, K Rosendahl, E Zetterberg and O Ringden. (2003). HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. Exp Hematol 31:890–896.

67. Kadereit S, LS Deeds, SE Haynesworth, ON Koc, MM Kozik, E Szekely, K Daum-Woods, GW Goetchius, P Fu, LA Welniak, WJ Murphy and MJ Laughlin. (2002). Expansion of LTC-ICs and maintenance of p21 and BCL-2 expression in cord blood CD34+/CD38- early progenitors cultured over human MSCs as a feeder layer. Stem Cells 20:573–582.

68. Takashima Y, T Era, K Nakao, S Kondo, M Kasuga, AG Smith and SI Nishikawa. (2007). Neuroepithelial cells supply an initial transient wave of MSCs differentiation. Cell 129:1377–1388.

69. Aggarwal S and MF Pittenger. (2005). Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 105:1815–1822.

70. Guo KT, R Schaefer, A Paul, A Gerber, G Ziemer and HP Wendel. (2006). A new technique for the isolation and surface immobilization of mesenchymal stem cells from whole bone marrow using high-specific DNA aptamers. Stem Cells 24:2220–2231.

71. Miura M, Y Miura, HM Padilla-Nash, AA Molinolo, B Fu, V Patel, BM Seo, W Sonoyama, JJ Zheng, CC Baker, W Chen, T Ried and S Shi. (2006). Accumulated chromosomal instability in murine bone marrow mesenchymal stem cells leads to malignant transformation. Stem Cells 24:1095–1103.

72. Mageed AS, DW Pietryga, DH DeHeer and RA West. (2007). Isolation of large numbers of mesenchymal stem cells from the washings of bone marrow collection bags: characterization of fresh mesenchymal stem cells. Transplantation 83:1019–1026.

73. Dennis JE and P Charbord. (2002). Origin and differentiation of human and murine stroma. Stem Cells 20:205–214.

74. Stenderup K, J Justesen, EF Eriksen, SI Rattan and M Kassem. (2001). Number and proliferative capacity of osteogenic stem cells are maintained during aging and in patients with osteoporosis. J Bone Miner Res 16:1120–1129.

75. Tondreau T, N Meuleman, A Delforge, M Dejeneffe, R Leroy, M Massy, C Mortier, D Bron and L Lagneaux. (2005). Mesenchymal stem cells derived from CD133-positive cells in mobilized peripheral blood and cord blood: proliferation, Oct4 expression, and plasticity. Stem Cells 23:1105–1112.

76. Ginchaux F, Z Selmai, L Obert, S Davoni, P Tiberghien, P Hervé and F Deschaseaux. (2007). Human and rodent bone marrow mesenchymal stem cells that express primitive stem cell markers can be directly enriched by using the CD49a molecule. Cell Tissue Res 327:471–483.

77. Schwab KE and CE Gargett. (2007). Co-expression of two perivascular cell markers isolates mesenchymal stem-like cells from human endometrium. Hum Reprod 8:739–751.

78. Delorme B, J Ringe, N Gallay, Y Le Vern, D Kerboeuf, C Jorgensen, P Rosset, L Sensebé, P Layrolle, T Haupl and P Charbord. (2008). Specific plasma membrane protein phenotype of culture-amplified and native human bone marrow mesenchymal stem cells. Blood 111:2631–2635.

79. Jarocha D, E Lukasiewicz and M Majka. (2008). Advantage of mesenchymal stem cells (MSCs) expansion directly from purified bone marrow CD105+ and CD271+ cells. Folia Histochem Cytobiol 46:307–314.

80. Buhring HJ, VL Battula, S Treml, B Schewe, L Kanz and W Vogel. (2007). Novel markers for the prospective isolation of human MSCs. Ann N Y Acad Sci 1106:262–271.

81. Nimjee SM, CP Rusconi and BA Sullenger. (2005). Aptamers: an emerging class of therapeutics. Annu Rev Med 56:555–583.

82. Battula VL, S Treml, PM Bareiss, F Gieseke, H Roelofs, P de Zwart, I Muller, B Schewe, T Skutella, WE Fibbe, L Kanz and HJ Buhring. (2009). Isolation of functionally distinct mesenchymal stem cell subsets using antibodies against CD56, CD71, and mesenchymal stem cell antigen-1 (MSCA-1). Haematologica 94:173–184.

83. Planat-Bénard V, C Menard, M André, M Puceat, A Perez, JM Garcia-Verdugo, L Pénicaud and L Casteilla. (2004). Spontaneous cardiomyocyte differentiation from adipose tissue stroma cells. Circ Res 94:223–229.

84. Planat-Benard V, JS Silvestre, B Cousin, M Andre, M Nibbelink, R Tamarat, M Clergue, C Manneville, C Saillan-Barreau, M Duriez, A Tedgui, B Levy, L Penicaud and L Casteilla. (2004). Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. Circulation 109:656–663.

85. Castro-Malaspina H, RE Gay, G Resnick, N Kapoor, P Meyers, D Chiarieri, S McKenzie, HE Broxmeyer and MA Moore. (1980). Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. Blood 56:289–301.

86. Muraglia A, R Cancedda and R Quarto. (2000). Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J Cell Sci 113(Pt 7):1161–1166.

87. Di Girolamo CM, D Stokes, D Colter, DG Phinney, R Class and DJ Prockop. (1999). Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. Br J Haematol 107:275–281.

88. Ho AD, W Wagner and W Franke. (2008). Heterogeneity of mesenchymal stromal cell preparations. Cytotherapy 10:320–330.

89. Quiroz FG, OM Posada Estefan, D Gallego Perez, N Higuita Castro, CA Sarassa Velasquez, DJ Hansford, P Agudelo Florez and LE Lopez Rojas. (2008). Isolation of human bone marrow mesenchymal stem cells and evaluation of their osteogenic potential. Rev Ingenieria Biomed 2:48–55.

90. Croft AP and SA Przyborski. (2006). Formation of neurons by non-neural adult stem cells: potential mechanism implicates an artifact of growth in culture. Stem Cells 24:1841–1851.

91. Essayan DM. (2001). Cyclic nucleotide phosphodiesterases. J Allergy Clin Immunol 108:671–680.

92. Daly JW, KA Jacobson and D Ukena. (1987). Adenosine receptors: development of selective agonists and antagonists. Prog Clin Biol Res 230:41–63.

93. Russell TR. (1979). Differentiation of 3T3-L2 fibroblasts into adipose cells in bromodeoxyuridine-suppressed cultures. Proc Natl Acad Sci U S A 76:4451–4454.

94. Calvo JC, D Rodbard, A Katki, S Chernick and M Yanagishita. (1991). Differentiation of 3T3-L1 preadipocytes with 3-isobutyl-1-methylxanthine and dexamethasone stimulates cell-associated and soluble chondroitin 4-sulfate proteoglycans. J Biol Chem 266:11237–11244.

95. Janderova L, M McNeil, AN Murrell, RL Mynatt and SR Smith. (2003). Human mesenchymal stem cells as an in vitro model for human adipogenesis. Obes Res 11:65–74.

96. Green H and O Kehinde. (1975). An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. Cell 5:19–27.

97. Menicanin D, PM Bartold, ACW Zannettino and S Gronthos. (2009). Genomic profiling of mesenchymal stem cells. Stem Cell Rev Rep 5:36–50.

98. Sheehan D and B Hrapchak, eds. (1980). Theory and Practice of Histotechnology. Battelle Press, Columbus, OH.

99. Bancroft J and A Stevens, eds. (1982). Theory and Practice of Histological Techniques. Churchill Livingstone, New York City, NY.

100. Carson F, ed. (1990). Histotechnology: A Self-Instructional Text. ASCP Press, Chicago, IL.

101. Reyes M, T Lund, T Lenvik, D Aguiar, L Koodie and C Verfaillie. (2001). Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood 98:2615–2625.

102. Mackay AM, SC Beck, JM Murphy, FP Barry, CO Chichester and MF Pittenger. (1998). Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. Tissue Eng 4:415–428.

103. Shepard N and N Mitchell. (1976). Simultaneous localization of proteoglycan by light and electron microscopy using Toluidine Blue O: a study of epiphyseal cartilage. J Histochem Cytochem 24:621–629.

104. Hyllested JL, K Veje and K Ostergaard. (2002). Histochemical studies of the extracellular matrix of human articular cartilage—a review. Osteoarthritis Cartilage 10:333–343.

105. Makino S, K Fukuda, S Miyoshi, F Konishi, H Kodama, J Pan, M Sano, T Takahashi, S Hori, H Abe, J Hata, A Umezawa and S Ogawa. (1999). Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 103:697–705.

106. Yoon J, BG Min, YH Kim, WJ Shim, YM Ro and DS Lim. (2005). Differentiation, engraftment and functional effects of pre-treated mesenchymal stem cells in a rat myocardial infarct model. Acta Cardiol 60:277–284.

107. Shim WS, S Jiang, P Wong, J Tan, YL Chua, YS Tan, YK Sin, CH Lim, T Chua, M Teh, TC Liu and E Sim. (2004). Ex vivo differentiation of human adult bone marrow stem cells into cardiomyocyte-like cells. Biochem Biophys Res Commun 324:481–488.

108. Wang T, Z Xu, W Jiang and A Ma. (2006). Cell-to-cell contact induces mesenchymal stem cell to differentiate into cardiomyocytes and smooth muscle cell. Int J Cardiol 109:74–81.

109. Kurpinski K, H Lam, J Chu, A Wang, A Kim, E Tsay, S Agrawal, D Schaffer and S Li. (2010). TGF-β and Notch signaling mediate stem cell differentiation into smooth muscle cells. Stem Cells 28:734–742.

110. Jeon ES, HJ Moon, MJ Lee, HY Song, YM Kim, YC Bae, JS Jung and JH Kim. (2006). Shingosylphosphorylcholine induces differentiation of human mesenchymal stem cells into smooth-muscle-like cells through a TGF-β-dependent mechanism. J Cell Sci 119:4994–5005.

111. Krampera M, S Marconi, A Pasini, M Galiè, G Rigotti, F Mosna, M Tinelli, L Lovato, E Anghileri, A Andreini, G Pizzolo, A Sbarbati and B Bonetti. (2007). Induction of neural-like differentiation in human mesenchymal stem cells derived from bone marrow, fat, spleen and thymus. Bone 40:382–390.

112. Anghileri E, S Marconi, A Pignatelli, P Cifelli, M Galié, A Sbarbati, M Krampera, O Belluzzi and B Bonetti. (2008). Neuronal differentiation potential of human adipose-derived mesenchymal stem cells. Stem Cells Dev 17:909–916.

113. Janzen V, R Forkert, HE Fleming, Y Saito, MT Waring, DM Dombkowski, T Cheng, RA DePinho, NE Sharpless and DT Scadden. (2006). Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16[INK4a]. Nature 443:421–426.

114. Tarte K, J Gaillard, JJ Lataillade, L Fouillard, M Becker, H Mossafa, A Tchirkov, H Rouard, C Henry, M Splingard, J Dulong, D Monnier, P Gourmelon, NC Gorin, and L Sensebé. (2010). Clinical-grade production of human mesenchymal stromal cells: occurrence of aneuploidy without transformation. Blood 115:1549–1553.

115. Rubio D, J Garcia-Castro, MC Martin, R de la Fuente, JC Cigudosa, AC Lloyd, and A Bernad. (2005). Spontaneous human adult stem cell transformation. Cancer Res 65:3035–3039.

116. Rubio D, S Garcia, T De la Cueva, MF Paz, AC Lloyd, A Bernad, and J Garcia-Castro. (2008). Human mesenchymal stem cell transformation is associated with a mesenchymal-epithelial transition. Exp Cell Res 314:691–698.

117. Wakitani S, K Imoto, T Yamamoto, M Saito, N Murata and M Yoneda. (2002). Human autologous culture expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in osteoarthritic knees. Osteoarthritis Cartilage 10:199–206.

118. Pereira RF, KW Halford, MD O'Hara, DB Leeper, BP Sokolov, MD Pollard, O Bagasra and DJ Prockop. (1995). Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. PNAS 92:4857–4861.

119. Horwitz EM, DJ Prockop, LA Fitzpatrick, WW Koo, PL Gordon, M Neel, M Sussman, P Orchard, JC Marx, RE Pyeritz and MK Brenner. (1999). Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med 5:309–313.

120. Goncalves MA, AA de Vries, M Holkers, MJ van de Watering, I van der Velde, GP van Nierop, D Valerio and S Knaan-Shanzer. (2006). Human mesenchymal stem cells ectopically expressing full-length dystrophin can complement Duchenne muscolar dystrophy myotubes by cell fusion. Hum Mol Genet 15:213–221.

121. Awad HA, GP Boivin, MR Dressler, FN Smith, RG Young and DL Butler. (2003). Repair of patellar tendon injuries using a cell-collagen composite. J Orthop Res 21:420–431.

122. Ouyang HW, JC Goh, XM Mo, SH Teoh and EH Lee. (2002). The efficacy of bone marrow stromal cell seeded knitted PLGA fiber scaffold for Achilles tendon repair. Ann N Y Acad Sci 961:126–129.

123. Juncosa-Melvin N, GP Boivin, MT Galloway, C Gooch, JR West, AM Sklenka and DL Butler. (2005). Effects of cell to collagen ratio in mesenchymal stem cell-seeded implants on tendon repair biomechanics and histology. Tisse Eng 11:448–457.

124. Vats A, NS Tolley, JM Polak and JE Gough. (2003). Scaffolds and biomaterials for tissue engineering: a review of clinical applications. Clin Otorinolaringol 28:165–172.

125. Quarto R, M Mastrogiacomo, R Cancedda, SM Kutepov, V Mukhachev, A Lavroukov, E Kon and M Marcacci. (2001). Repair of large bone defects with the use of autologous bone marrow stromal cells. N Engl J Med 344:385–386.

126. Murphy JM, KW Kavalkovitch, D Fink and FP Barry. (2000). Regeneration of meniscal tissue and protection of articular cartilage by injection of mesenchymal stem cells. Osteoarthritis Cartilage 8(Suppl. B):S25.

127. Wakitani S, T Goto, SJ Pineda, RG Young, JM Mansour, AI Caplan and VM Goldberg. (1994). Mesenchymal cell-based repair of large, full thickness defects of articular cartilage. J Bone Joint Surg Am 76:579–592.

128. Reddi AH. (1998). Role of morphogenetic proteins in skeletal tissue engineering and regeneration. Nat Biotechnol 16:247–252.

129. Toma C, MF Pittenger, KS Cahill, BJ Byrne and PD Kessler. (2002). Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation 105:93–98.

130. Mazhari R and JM Hare. (2007). Mechanisms of action of mesenchymal stem cells in cardiac repair: potential influences on the cardiac stem cell niche. Nat Clin Pract Cardiovasc Med 4:S21–S26.

131. Chen SL, WW Fang, J Qian, F Ye, YH Liu, SJ Shan, JJ Zhang, S Lin, LM Liao and RC Zhao. (2004). Improvement of cardiac function after transplantation of autologous bone marrow mesenchymal stem cells in patients with acute myocardial infarction. Chin Med J (Engl) 117:1443–1448.

132. Mohyeddin-Bonab M, MR Mohamad-Hassani, K Alimoghaddam, M Sanatkar, M Gaseemi, H Mirkhani, H Radmehr, M Salehi, M Eslami, A Farhing-Parsa, H Emami-Razavi, MG al-Mohamad, AA Solimani, A Ghavamzadeh and B Nikbin. (2007). Autologous in vitro expanded mesenchymal tem cell therapy for human old myocardial infarction. Arch Iran Med 10:467–473.

133. Hattan N, H Kawaguchi, K Ando, E Kuwabara, J Fujita, M Murata, M Suematsu, H Mori and K Fukuda. (2005). Purified cardiomyocytes from bone marrow mesenchymal stem cells produce stable intracardiac grafts in mice. Cardiovasc Res 65:334–344.

134. Bin F, L Yinglong, X Nin, F Kai, S Laifeng and Z Xiadong. (2006). Construction of tissue-engineered homograft bioprosthetic heart valves in vitro. ASAIO J 52:303–309.

135. Knight RL, C Booth, HE Wilcox, J Fischer and E Ingham. (2005). Tissue engineering of cardiac valves: reseeding of acellular porcine aortic valve matrices with human mesenchymal progenitor cells. J Heart Valve Dis 14:806–813.

136. Engelmayr GC Jr., VL Sales, JE Jr. Mayer and MS Sacks. (2006). Cyclic flexure and laminar flow synergistically accelerate mesenchymal stem cell-mediated engineered tissue formation: implications for engineered heart valve tissues. Biomaterials 27:6083–6095.

137. Munoz-Elias G, D Woodbury and IB Black. (2003). Marrow stromal cells, mitosis, and neuronal differentiation: stem cell and precursor functions. Stem Cells 21:437–448.

138. Corti S, F Locatelli, S Strazzer, M Guglieri and GP Comi. (2003). Neuronal generation from somatic stem cells: current knowledge and perspectives on the treatment of acquired and degenerative central nervous system disorders. Curr Gene Ther 3:247–272.

139. Breitbach M, T Bostani, W Roell, Y Xia, O Dewald, JM Nygren, JWU Fries, K Tiemann, H Bohlen, J Hescheler, A Welz, W Bloch, SEW Jacobsen and BK Fleischmann. (2007). Potential risks of bone marrow cell transplantation into infarcted hearts. Blood 110:1362–1369.

140. Rubio D, J Garcia-Castro, MC Martin, R de la Fuente, JD Cigudosa, AC Lloyd and A Bernad. (2005). Spontaneous human adult stem cell transformation. Cancer Res 65:3035–3039.

141. Serakinci N, P Guldberg, JS Burns, B Abdallah, H Schroedder, T Jensen and M Kassem. (2004). Adult human mesenchymal stem cells as a target for neoplastic transformation. Oncogene 24:5095–5098.

142. Bernardo ME, N Zaffaroni, F Novara, AM Cometa, MA Avanzini, A Moretta, D Montagna, R Maccario, R Villa, MG Daidone, O Zuffardi and F Locatelli. (2007). Human

bone marrow–derived mesenchymal stem cells do not undergo transformation after long-term in vitro culture and do not exhibit telomere maintenance mechanisms. Cancer Res 67:9142–9149.

143. Djouad F, P Plence, C Bony, P Tropel, F Apparailly, J Sany, D Noel and C Jorgensen. (2003). Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. Blood 102:3837–3844.

144. Kinnaird T, E Stabile, MS Burnett, M Shou, CW Lee, S Barr, S Fuchs and SE Epstein. (2004). Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res 94:678–685.

145. Khakoo AY, S Pati, SA Anderson, W Reid, MF Elshal, II Rovira, AT Nguyen, D Mlide, CA Combs, G Hall, J Zhang, M Raffeld, TB Rogers, W Stetler-Stevenson, JA Frank, M Reitz and T Finkel. (2006). Human mesenchymal stem cells exert potent antitumorigenic effects in a model of Kaposi's sarcoma. J Exp Med 203:1235–1247.

146. Djouad F, C Bony, F Apparailly, P Louis-Plence, C Jorgensen and D Noel. (2006). Earlier onset of syngeneic tumors in the presence of mesenchymal stem cells. Transplantation 82:1060–1066.

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