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Bone marrow- and subcutaneous adipose tissue-derived mesenchymal stem cells

Differences and similarities

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Bone marrow (BM) and subcutaneous adipose tissue (Ad) are both considered prospective sources of MSC for therapeutic applications. However, functional properties and therapeutic efficacy of MSC derived from different tissues of the same patient are still poorly investigated. In our study, BM-MSC and F-MSC cultures from 43 adult donors were evaluated in successive passages for immunophenotype; secretion of VEGF, SDF1, MCP1, IL6 and TGFβ1; frequency of colony-forming units (CFU-F); frequency of adipo- and osteoprogenitors (CFU-Ad, CFU-Ost) and for onset of in vitro replicative senescence. We have demonstrated that at early passages (P2-P3 or up to 14–15 in vitro population doublings), BM- and Ad-derived MSC cultures are comparable in such important characteristics as proliferation rate (population-doubling time: 3.4 ± 0.2% in BM-MSC, 3 ± 0.3% in F-MSC), clonogenity (CFU-F frequency: 32 ± 5% in BM-MSC, 31 ± 5% in F-MSC) and differentiation potential (CFU-Ad frequency: 10.4 ± 2% in BM-MSC, 13 ± 3% in F-MSC; CFU-Ost frequency: 18.5 ± 5.5% in BM-MSC, 18 ± 5% in F-MSC) but differ significantly in abundance of $CD146^+$ fraction within the sample (25 ± 5% in BM-MSC, 7 ± 3% in F-MSC) and in levels of VEGF, SDF-1, MCP1 and TGFβ1 secretion. We have also demonstrated that BM-MSC enter senescence after P3–4, while most F-MSC did not show senescence features up to P6–8. Together, these data demonstrate that specific properties of MSC from different sources should be always taken into account when developing and optimizing the specific protocols for MSC expansion and evaluation for each particular clinical application.

Introduction

Stem cells found in adult tissues are an attractive stem cell source in clinical applications for the regeneration of damaged tissues,[1-9] immunomodulation[2,10] and ex vivo hematopoietic stem cell (HSC) expansion (reviewed in ref. 11). Bone marrow (BM) has been the main source for the isolation of multipotent mesenchymal stem cells (MSC), and, to date, BM-MSC are best characterized. However, alternative sources for MSC isolation are subject to intensive investigation (reviewed in ref. 10). Bone marrow and fat tissue are both considered to be a prospective source of MSC for therapeutic applications.[8] Despite the rapid growth of the field, the ideal cell type has not yet been found, and few studies have directly compared different stem cell types.[12] Uncertainties remain with respect to the defining characteristics of these cells, including their therapeutic potential (reviewed in ref. 13). Additional characterization and demonstration of efficacy in animal models are needed before a clinical role for these cells can be established.[13,14]

Finally, it is also critical to standardize cell expansion procedures and to develop the MSC sample evaluation criteria so that cells with similar properties are delivered to each patient and/or used in experimental models in order to make possible the comparison of clinical and/or experimental results.

It is also essential to understand the mechanism of stem cell-induced physiological effects, since the different characteristics of the MSC may account for the different mechanisms of tissue repair. It is accepted now that regeneration by MSC differentiation is taking place only in a few situations; for example, MSC implanted in bone or cartilage lesions may repair by direct differentiation into osteoblasts or chondrocytes.[15] In other disease models, including cardiovascular diseases, benefit from MSC injection might result from the secretion of paracrine factors, which could stimulate angiogenesis, control inflammation, improve metabolic modulation and reduce apoptosis, resulting in increased reparative and cardioprotective responses.[10] To date, there is convincing evidence suggesting that MSC from different tissues are not the same (reviewed in ref. 13). However, there are no comprehensive comparative data on the specific differences in functional properties of MSC derived from different tissues of the same donor. Moreover, to date, there is no widely accepted opinion concerning which source

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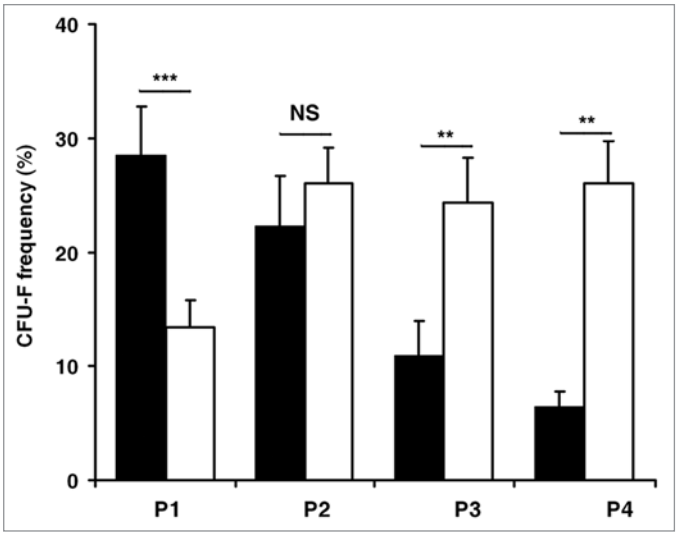

Figure 1. Frequency of CFU-F declined with successive passages in BM-MSC cultures (black bars) but not in F-MSC cultures (open bars). Seven to 10 MSC samples were used for each experimental point. P1-P4 passage numbers. *p < 0.001, **p < 0.05, ***p < 0.01.

of cells is better to use for therapeutic applications in each particular case.

The purpose of this study was to compare the properties of cultured bone marrow and subcutaneous adipose tissue derived MSC from the same donor in order to reveal the potential differences in regenerative capacity in models of human disease. We also aimed to develop our in-lab MSC sample evaluation criteria and standardize cell expansion procedures so that cells with similar properties were used in each experimental protocol to make possible the comparison of experimental results.

To achieve these goals, we examined proliferative activity, frequency of colony-forming units (CFU), frequency of adipo- and osteoprogenitors (CFU-Ad, CFU-Ost), secretory profiles, immunophenotype and onset of senescence in cultured MSC derived from bone marrow (BM-MSC) and subcutaneous adipose tissue (F-MSC) from the same group of adult donors.

Results

Progenitor frequency, cumulative population doublings (PD). Bone marrow mesenchymal stem cells (BM-MSC) and/or adipose-derived stem cells (F-MSC) are defined as adherent culture-amplified cells giving rise, when cultured in appropriate conditions, to adipocytes, osteoblasts and chondrocytes.[16] Thus, MSC samples could be examined and compared for clonogenity (CFU-F), frequency of adipo- and osteoprogenitors (CFU-Ad, CFU-Ost).

In order to normalize our results and compare BM- and Ad-derived samples of MSC, the calculation of the number of in vitro population doublings (PD) was performed for each MSC sample. First, a limiting dilution assay was done to estimate a fraction of clonogenic cells (CFU) in the initial sample of bone marrow mononuclear cells (BMMC) and Ad-derived stromal fraction (ASC) samples. In our study, CFU frequency was 0.0029 ± 0.0008% in BMMC samples and 0.12 ± 0.096% in ASC samples, which is in accordance with data from literature.[16-18] With these initial numbers, we were able to calculate cumulative PD at every subsequent passage for every MSC culture.

Table 1. BM-MSC and F-MSC in vitro cumulative doublings at passages P1–P3

Sample	P1 (n)	P2 (n)	P3 (n)
F-MSC	8,87 ± 0,58 (15)	11,84 ± 0,66 (15)	15,06 ± 0,77 (15)
BM-MSC	11,52 ± 0,34 (15)	14,14 ± 0,49 (15)	16,22 ± 0,63 (15)

The results are summarized in **Table 1**, and it is clear there that while BM-derived MSC cultures underwent 14.14 ± 0.49 PD in vitro by passage 2 (P2), Ad-derived MSC samples had about the same number of PD by P3 (15.06 ± 0.77), which means that F-MSC at P3 in our study have about the same "in vitro replication" status as BM-MSC at P2.

Clonogenity, PD time and replicative senescence. Both clonogenity and multipotency are the defining characteristics of MSC. A subset of MSC is designated as clonogenic if it is able to grow in a density-insensitive fashion. To estimate a fraction of clonogenic cells (CFU-F) in MSC samples, a limiting dilution assay was performed for each sample at every passage (**Fig. 1**). We have found that dynamics of changes in CFU-F frequency were different in BM-MSC and F-MSC: while the frequency of CFU-F in the BM-derived MSC population declined as much as 4-fold by passage 4, CFU-F in F-MSC remained practically unchanged up to passage 7. Simultaneously, population-doubling time was calculated for every passage, and results are shown in **Figure 2**. Interestingly, that PD time at passage P0 was about 1 d in both Ad- and BM-derived MSC samples and remained at the 2.5 level 3 d up to passage 7 in F-MSC. However, PD time in BM-MSC samples increased dramatically by passage 3, which indicates BM-MSC senescence after 14–16 in vitro population doublings. Indeed, by passage 4, BM-MSC but not F-MSC started to show significant signs of senescence (**Fig. 3**). As expected, PD time correlated strongly ($p < 0.004$) with CFU-F frequency in MSC population (data not shown).

Lineage progenitor frequency. It was demonstrated earlier in MSC transplantation studies that only a fraction of CFU-Fs are multipotent.[18,19] Therefore, after CFU-F frequency was calculated, the cells were induced to undergo adipogenesis and osteogenesis to estimate the frequency of lineage progenitors (CFU-Ost and CFU-Ad) in MSC samples. This approach allowed us to estimate the fraction of CFU-Fs that is able to differentiate either into adipocytes or into osteoblasts in each population of MSC and to compare the general differentiation potential of different samples. In order to identify the difference between BM-MSC and F-MSC, these experiments were performed at P2 and P4 (**Fig. 4**). Frequency of CFU-Ost lineage progenitors was slightly higher than frequency of CFU-Ad progenitors in all groups of samples, especially at P2; however, that difference was not statistically significant.

Cytokine secretion profile. To assess MSC cytokine secretion profile, MSC at P2-P3 were used when BM-MSC and F-MSC did not differ significantly in their clonogenity (CFU-F frequency), differentiation potential (frequency of CFU-Ad and CFU-Ost) and senescence score (**Figs. 1, 2 and 4**). We have found that while

the level of IL6 secretion did not differ significantly between BM- and F-derived MSC, the level of secretion of VEGF, SDF1, MCP1 and TGFβ1 was significantly higher in BM-derived MSC cultures (**Fig. 5**).

Immunophenotype. All cells used for this experiment were analyzed for expression of stromal cell-associated markers CD105, CD90, CD166 and CD73 and for hematopoietic lineage cells markers CD34, CD19, CD14, CD45 to make sure that cells meet minimal criteria for defining multipotent mesenchymal stromal cells.[20] Both BM-MSC and F-MSC were CD105/CD90/CD166/CD73 positive and negative for CD34, CD19, CD14 and CD45 at all passages (data not shown). Additionally, the analysis of CD146 expression by BM- and Ad-derived MSC was performed. CD146 (also known as MCAM) was chosen for this study, because it appears to be one of the markers that are considered candidate markers for MSCs "stemness,"[15,20] and it was shown recently that clonogenic BM-MSCs can be enriched by using this surface marker.[21,22] We have found that CD146$^+$ cells were more abundant in BM-MSC than in F-MSC at early passages, but CD146$^+$ fraction in BM-MSC declined significantly with subsequent passages. CD146 expression in BM-MSC correlated with CFU-F frequency in MSC sample (**Fig. 6**).

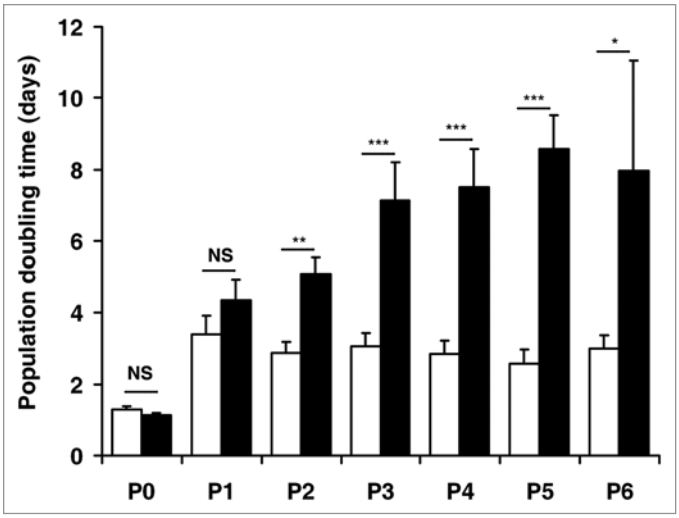

Figure 2. Cell population-doubling times of BM-MSC (black bars) increased significantly between second and fourth passages, while population-doubling times of F-MSC (open bars) remained unchanged up to passage six. n = 30 or more for each experimental point. P0–P6 passage numbers. *p < 0.05, **p < 0.001, ***p < 0.0001.

Discussion

In the present study, a systematic comparison of MSC samples derived from two tissues of the same group of donors was performed in order to compare MSC samples derived from bone marrow and subcutaneous adipose tissue for onset of in vitro replicative senescence, frequency of colony-forming units, frequency of adipo- and osteoprogenitors, immunophenotype and cytokine secretion, and reliable MSC sample evaluation criteria were developed.

We have found that BM- and Ad-derived MSC cultures are very similar at early passages (up to about 14 in vitro populations doublings) in such important "stemness" properties as proliferation activity and clonogenity (PD time, CFU-F frequency, **Figs. 1 and 2**), differentiation potential (lineage progenitor frequency, **Fig. 4**) and expression of stromal markers CD105, CD90, CD166 and CD73. However, we have demonstrated here that BM-MSC and F-MSC also differ in some key biological properties. First, the cytokine secretion profile in BM- and F-MSC was different: level of VEGF, SDF1, MCP1 and TGFβ1 secretion at early passages in BM-MSC was significantly higher than in F-MSC. Second, there was a BM-specific CD146$^+$ fraction of MSC detected at early passages, which was 41.4 ± 8% of the whole population at P1 and 24.7 ± 4.8% at P2. Finally, the early onset of replicative senescence during in vitro expansion was detected in BM-MSC but not in F-MSC.

All these differences might be crucial if cells intended to be used for specific therapeutic applications. For example, it is currently believed that VEGF is an important stem cell paracrine mediator providing postischemic myocardial protection,[23-26] and there is evidence that stem cells from adipose and bone marrow promote angiogenesis via distinct cytokine and protease expression mechanisms.[27] Additionally, there are data in the literature that the benefit of MSC therapy is due to preservation of cardiac tissue, and that at least one factor mediating this effect is SDF-1. Indeed, the infusion of SDF-1-overexpressing MSC in the infarct zone led to a significant decrease in cardiac myocyte apoptosis.[14]

Further functional studies are required to evaluate the role of VEGF, SDF1 and other BM- and Ad-specific secretory factors before a clinical role for these cells can be established. Further evaluation of the functional efficiency of the BM-specific CD146$^+$ MSC subpopulation is also required. CD146$^+$ BM-derived MSC population is known to be extensively proliferative, able to differentiate into osteoblasts, chondrocytes and adipocytes and to support hematopoiesis through a complex adhesion molecule expression and cytokine secretion pattern, including VEGF, placental-derived growth factor, angiopoietin (Ang)-1/2, FLT-3 ligand (FL) and stem cell factor (SCF) in both soluble and membrane-bound isoforms.[22,23] It is likely that this population of MSC might be exceptionally useful in therapeutic applications in the field of hematology, e.g., in ex vivo hematopoietic stem cell (HSC) expansion strategies that employ MSC and HSC co-culture.[11]

It must be emphasized that our study has also revealed an important problem: the early onset of replicative senescence in BM-MSC but not in F-MSC might limit BM-MSC usefulness in therapeutic applications when BM sample volume is small, and a high degree of MSC expansion is required for research and clinical purposes. Therefore, while some BM-MCS-specific properties might increase the therapeutic potential of MSC samples, F-MSC samples are easier to obtain and expand. This set of our data are in accordance with data available in the literature: recent studies have indicated that murine and human MSC exhibit reduced differentiation potential upon prolonged in vitro culture,[28-31] and it was shown for equine MSC that BM-MSC became senescent much earlier than F-MSC and umbilical cord tissue-derived MSC.[12] It is unclear, however, if the early onset of

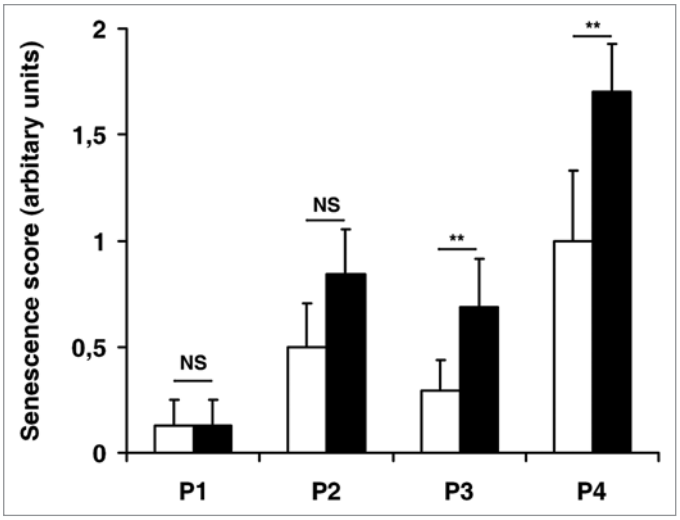

Figure 3. By passage 4, BM-MSC (black bars) but not F-MSC (open bars) cultures started to show significant signs of replicative senescence (β-galactosidase staining). n = 10 or more for each experimental point. P1–P4 passage numbers. **p < 0.05.

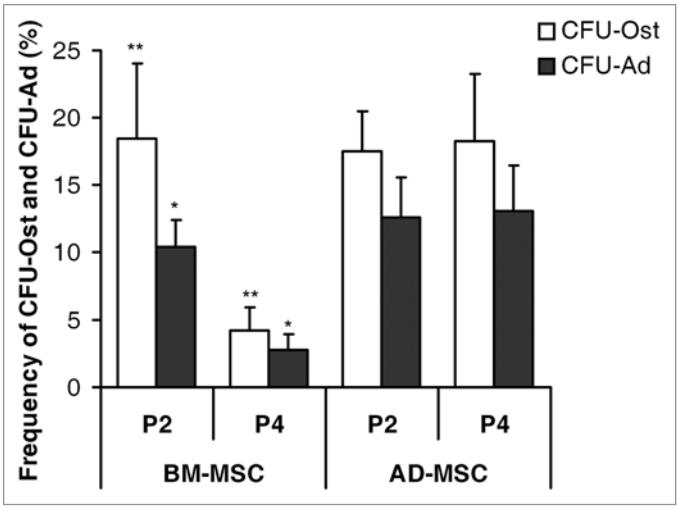

Figure 4. Dynamics of frequency of CFU-Ad and CFU-Ost in BM-MSC and F-MSC cultures with successive passages. n = 9. P2, P4 passage numbers. *p < 0.01; **p < 0.05.

the senescence program is in the nature of BM-MSC or whether the standard culture conditions (specifically, high oxygen level) could contribute to acceleration of in vitro replicative senescence. It was demonstrated that hypoxic culture prevented early onset the senescence in expanded BM-MSC through downregulation of E2A-p21 by the HIF-TWIST pathway.[32] Indeed, bone marrow tissue is hypoxic in nature, with the oxygen tension approximately 1–7%,[33] and BM-derived MSC, when cultured under normoxic conditions, might experience severe stress that could result in early onset of senescence. In contrast, Ad-derived MSC might not be as sensitive to high oxygen levels as BM-MSC are and might be more comfortable with standard culture conditions, resulting in stable F-MSC properties upon extended in vitro culturing. Therefore, further development, optimization and evaluation of MSC expansion protocols are important in order to obtain MSC samples with predicted cellular and functional properties essential for particular clinical application.

Materials and Methods

Donors. A total of 10 healthy adult donors and 33 patients with chronic heart failure were enrolled. The bone marrow sample and subcutaneous adipose tissue sample were collected from each donor/patient. The study was conducted in compliance with current Good Clinical Practice standards and in accordance with the principles set forth under the Declaration of Helsinki (1989). Institutional review board approval of the study protocol was obtained before the initiation of patient enrollment. All patients and donors entering the program agreed to and signed an institutional review board-approved statement of informed consent.

Bone marrow derived cell cultures (BM-MSC). BM aspirates were obtained from the iliac crest or sternum. Standard BM-MSC cultures were established from plastic adherent BM cell fractions as described elsewhere with some modifications.[16] Briefly, a density gradient was used in the isolation procedure to eliminate unwanted cell types that were present in the marrow aspirate. A portion of cells isolated from the density interface (bone marrow mononuclear cells, BMMC) was used in CFU assay to estimate a frequency of colony-forming units (CFU, see below) in each sample. The remaining cells were suspended and plated immediately in T75 flasks in culture medium at a density of about 300,000 cells/cm^2 of surface area for expansion and culture. HSC and nonadherent cells were removed with changes of medium. This initial passage of the primary cell culture was referred to as passage 0 (P0).

Adipose-derived cell cultures (F-MSC). Small volume liposuction aspirates were obtained from paraumbilical subcutaneous adipose tissue sites. F-MSC cultures were prepared as described in reference 34. Briefly, tissues were washed three to four times with phosphate-buffered saline (PBS) and suspended in an equal volume of PBS supplemented with 0.1% collagenase type III prewarmed to 37°C. The tissue was placed in a shaking water bath at 37°C with continuous agitation for 30 min and centrifuged for 5 min at 300 g at room temperature, referred to further as adipose stromal cells (ASC). A portion of the ASC was used in CFU assays to estimate a frequency of colony-forming units (CFU, see below) in the cell population. The remaining cells were suspended and plated in T75 flasks in culture medium at a density of about 200,000/cm^2 of surface area for expansion and culture. This initial passage of the primary cell culture was referred to as passage P0.

Calculation of the number of cumulative population doublings (PD) and the time of PD. Though the most convenient parameter for documentation of long-term culture is counting of the number of cell passages, the normalization of results to passage numbers only may lead to deceptive results. In this respect, calculation of PD is more accurate.[16] MSC cultures are isolated by plastic adherent growth, and the initial MSC number can only be estimated by accounting colony-forming unit (CFU)

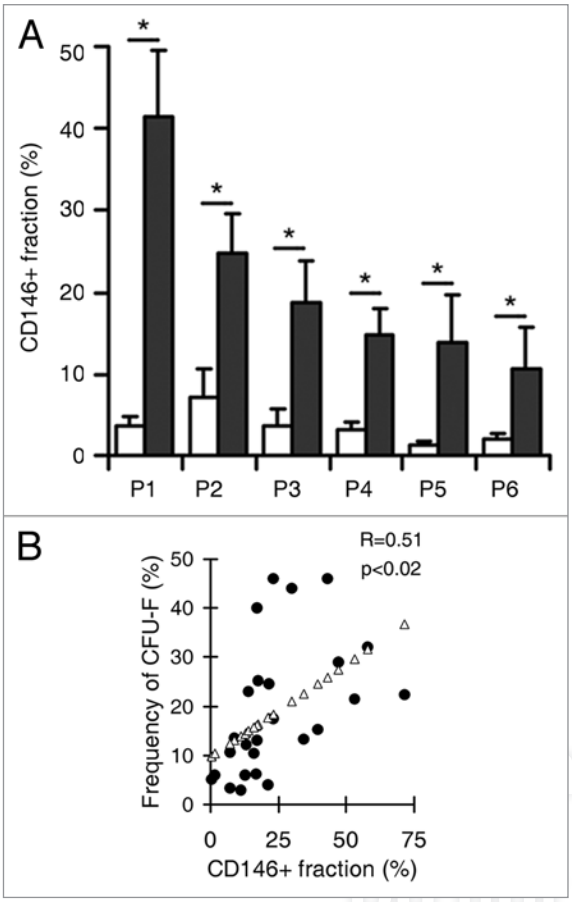

Figure 6. (A) The population of CD146$^+$ cells was more abundant in BM-MSC (black bars) than in F-MSC (open bars) at early passages and declined significantly by passage four. (B) The frequency of CD146$^+$ cells in the population correlates with the frequency of CFU-F in BM-MSC culture. P0–P6 passage numbers. n ≥ 10. *p < 0.01.

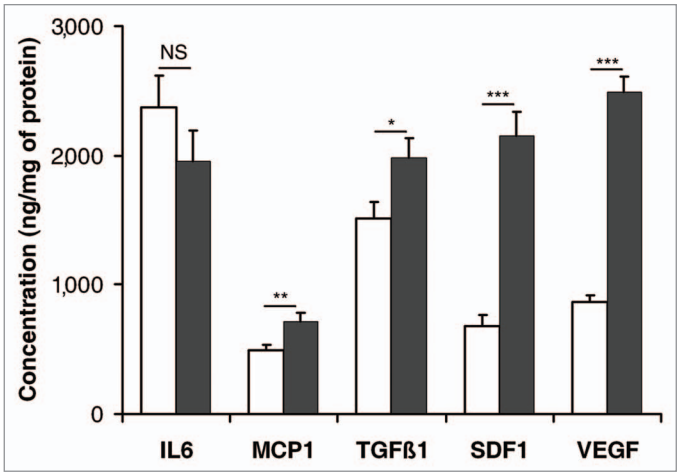

Figure 5. The secretion of IL6, TGFβ1, MCP1, SDF1 and VEGF by BM-MSC (black bars) and F-MSC (open bars) cultures. n = 43. *p < 0.01; **p < 0.02; ***p < 0.001.

frequency based on the assumption that every colony has been derived from a single clonogenic MSC. CFU in BMMC and ASC was estimated as described in reference 35. Briefly, cell suspension was serially diluted 2-fold across the six columns of 96-well plates, resulting in columns containing from 20,000-625 cells per well. After 10–14 d of culture, the number of positive and negative wells was determined for each cell concentration, and CFU frequency in initial BMMC or ASC population was calculated. Thereafter, cell number was determined at all consecutive passages, and time of PD and number of cumulative PD were calculated using an online tool.[36]

Limiting dilution assay application for CFU-F, CFU-Ad and CFU-Ost determination. Determination of frequency of colony-forming units (CFU-F) and the frequency of osteogenic and adipogenic lineage progenitors (CFU-Ad and CFU-Ost) in BM-MSC and F-MSC populations was performed at every subsequent passage as described: cell suspension was serially diluted 2-fold across the eight columns of 96-well plates, resulting in columns containing from 50-0.39 cells per well. After 10 d of culture, the number of positive and negative wells was determined for each cell concentration, and CFU-F frequency in MSC population was calculated. After CFU-F frequency was calculated, cells were induced to undergo adipogenesis and osteogenesis to estimate the frequency of lineage progenitors in MSC population. CFU-Ad-positive wells were determined by Oil O Red staining, and CFU-Ost-positive wells were determined by Alizarin Red staining after 14 and 21 d, respectively. CFU-Ad and CFU-Ost frequency estimated as described.[35]

Stimulation of adipogenesis. Adipogenesis was induced by replacing the culture media with adipocyte induction medium composed of culture medium supplemented with 1 μM insulin, 1 μM dexamethasone and 0.5 μM 3-isobutyl-1-methylxanthine.

Stimulation of osteogenesis. Osteogenesis was induced by replacing the culture medium with osteogenic induction medium composed of culture medium supplemented with 10 mM β-glycerophosphate, 10 nM dexamethasone and 0.16 mM sodium ascorbate 2-phosphate.

Senescence assay. Senescence-associated β-galactosidase activity was used as a biomarker for assessing replicative senescence in MSC. This enzymatic activity was measured by staining cells with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal) at pH 6.0, a reaction condition that suppresses lysosomal β-galactosidase activity sufficiently to ensure that most nonsenescent cells will appear unstained. Number of β-galactosidase-positive cells in three fields (x100) was counted. The score was calculated as follows: score 0 = less than three β-galactosidase-positive cells, score 1 = less than six β-galactosidase-positive cells, and score 2 = greater than six β-galactosidase-positive cells in three fields.

Flow cytometry. Cells were analyzed for hematopoietic lineage cell markers CD34, CD19, CD14 and CD45 and for stromal cell-associated markers CD105, CD90, CD73, CD166 and CD146 by using directly conjugated phycoerythrin (PE), ñuorescein isothiocyanate (FITC) or allophycocyanin (APC) antibodies (Becton-Dickinson BioSciences) on a BD FACS Calibur using CELLQuest acquisition software.

VEGF, IL6, MCP1, TGFβ1 and SDF1 secretion. In order to obtain a profile of cytokine secretion, 50,000 MSC were seeded

in 24-well plates in duplicate; after 24 h, the culture medium was replaced and samples of media from each well were collected after 48 h of culturing and frozen in aliquotes prior to analysis. Cytokines were analyzed using the DuoSet ELISA Development Systems for human VEGF, MCP1, TGFβ1, IL6 and CXCL12/SDF1 (R&D Systems). Analysis was performed according to manufacturer's instructions. Results were normalized to protein amount.

Disclosure of Potential Conflicts of Interest

The authors certify that they have no affiliation with or financial involvement in any organization or entity with a direct financial interest in the subject matter or materials discussed in this manuscript.

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