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Characterization of the Optimal Culture Conditions for Clinical Scale Production of Human Mesenchymal Stem Cells

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ABSTRACT

Mesenchymal stem cells (MSCs) are multipotent cells defined by multilineage potential, ease to gene modification, and immunosuppressive ability, thus holding promise for tissue engineering, gene therapy, and immunotherapy. They exhibit a unique in vitro expansion capacity, which, however, does not compensate for the very low percentage in their niches given the vast numbers of cells required for the relative studies. Taking into consideration the lack of a uniform approach for MSC isolation and expansion, we attempted in this study, by comparing various culture conditions, to identify the optimal protocol for the large-scale production of MSCs while maintaining their multilineage and immunosuppressive capacities. Our data indicate that, apart from the quality of fetal calf serum, other culture parameters, including basal medium, glucose concentration, stable glutamine, bone marrow mononuclear cell plating density, MSC passaging density, and plastic surface quality, affect the final outcome. Furthermore, the use of basic fibroblast growth factor (bFGF), the most common growth supplement in MSC culture media, greatly increases the proliferation rate but also upregulates HLA-class I and induces low HLA-DR expression. However, not only does this upregulation not elicit significant in vitro allogeneic T cell responses, but also bFGF-cultured MSCs exhibit enhanced in vivo immunosuppressive potential. Besides, addition of bFGF affects MSC multilineage differentiation capacity, favoring differentiation toward the osteogenic lineage and limiting neurogenic potential. In conclusion, in this report we define the optimal culture conditions for the successful isolation and expansion of human MSCs in high numbers for subsequent cellular therapeutic approaches. STEM CELLS *2006;24:462–471*

INTRODUCTION

Mesenchymal stem cells or marrow stromal cells (MSCs) are rare multipotent stem cells, residing mainly in bone marrow (BM), but they have also been isolated from other tissues [1, 2]. They are capable of differentiating in vitro and in vivo along multiple pathways that include bone, cartilage, cardiac and skeletal muscle, neural cells, tendon, adipose, and connective tissue [3].

Systemic administration of autologous or allogeneic MSCs in healthy animals has been reported to lead to the migration and engraftment of them in a plethora of nonhematopoietic tissues [4], whereas in injury models, they migrate specifically to the site of damage and undergo tissue-specific differentiation patterns [2, 5]. Expression of a variety of adhesion molecules on MSCs may account for their potential multiorgan homing capacity [6]. MSCs' multilineage differentiation ability, together with their relatively easy isolation from BM and their extensive capacity for in vitro expansion, led to important approaches of utilizing MSCs for tissue engineering as well as for gene therapy for a variety of congenital and acquired diseases [2]. Clinical

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trials involving MSCs concern distinct disorders, such as facilitation of hematopoietic recovery in hematopoietic stem cell transplantation [7], osteogenesis imperfecta [8], metabolic diseases [9], amyotrophic lateral sclerosis [10], and myocardial infarction [11].

MSCs are not inherently immunogenic, being unable to be recognized by allogeneic T or natural killer cells [12]. They express negligible levels of major histocompatibility complex (MHC) class II and intermediate levels of MHC class I molecules, whereas they do not express costimulatory molecules, such as B7-1, B7-2, CD40, or CD40 ligand [13]. Induction of MHC class II on MSCs by interferon-γ (IFN-γ) does not stimulate alloreactivity [14]. Moreover, MSCs seem to be natural immunosuppressive elements, being able to inhibit in vitro T cell proliferation and function of both naive and memory T cells [15–17] or to suppress the development of monocyte-derived dendritic cells in an in vitro system [18]. Additionally, using a baboon model, Bartholomew et al. [19] have shown that the immunosuppressive effect of MSCs results in moderate but significant prolongation of histoincompatible skin graft survival.

The ability of MSCs to modulate immune responses implies their potential role in cellular immunoregulatory therapy by facilitating engraftment in organ transplantation [19] and reintroducing tolerance in autoimmune diseases [20]. To this end, MSCs have already been used in a clinical trial for the effective treatment of acute graft-versus-host disease [21]. Furthermore, their susceptibility to gene modification enables them to serve as delivery vehicles for intratumoral production of anticancer agents, such as IFN-β [22] and interleukin-2 [23, 24].

Safety, feasibility, and efficacy of MSC transplantation for a variety of pathological conditions are currently under thorough investigation. There are several studies that use extremely high numbers of MSCs, sometimes up to 10^9 [11]. Despite the need for vast numbers of human MSCs as therapeutic means, there is only limited information on the optimization of culture conditions required for their production, specifically regarding passaging density [25, 26], fetal calf serum (FCS) lot selection [27], population selection [28–30], use of basic fibroblast factor (bFGF) for proliferation enhancement [31–34], and effect of donor age and cryopreservation [35]. Currently, there is extensive inconsistency among laboratories concerning the media, the starting and passaging cell-plating density, the culture surfaces, and the addition of supplementary factors for the successful isolation and expansion of MSCs that ends up with heterogeneous cell populations both in in vitro experiments and in clinical trials. Therefore, in this study we attempted to pinpoint the optimal culture conditions for the effective clinical-scale production of vast numbers of MSCs to serve for cellular therapy in transplantation, immunotherapy, and regenerative medicine.

Materials and Methods

Isolation of Human MSCs

BM aspirates (3 to 5 ml) were obtained from the iliac crests of normal adult donors under local anesthesia, after informed consent, and were diluted 1:2 in Hanks' balanced salt solution (HBSS) (Life Technologies, Paisley, Scotland, http://www.invitrogen.com). BM mononuclear cells (MNCs) were isolated by Ficoll-Hypaque (Biochrom AG, Berlin, http://www.biochrom.de) centrifugation using standard procedures. BM MNCs were washed thoroughly in HBSS, resuspended, and cultured under the conditions described in each experiment. On day 7, nonadherent cells were discarded and adherent cells were washed twice with HBSS. Cells were detached by 5- to 10-minute incubation in 0.05% trypsin/EDTA (Life Technologies), harvested, and counted in duplicate by two blinded individuals with a hematocytometer. Thereafter, cells were passaged in weekly intervals under the specified culture conditions.

Culture of Human MSCs

For the culture of human MSCs, the following media were tested: Dulbecco's modified Eagle's medium (DMEM) with 1,000 mg/ml glucose and L-glutamine (DMEM/LG/L-G), DMEM with 4,500 mg/ml glucose and L-glutamine (DMEM/HG/L-G), DMEM with 1,000 mg/ml glucose and Glutamax (DMEM/LG/GL), DMEM with 4,500 mg/ml glucose and Glutamax (DMEM/HG/GL), Iscove's modified Dulbecco's medium (IMDM) with L-glutamine, MEM alpha (aMEM) with L-glutamine (aMEM/L-G), aMEM with Glutamax (aMEM/GL), and Optimem (all from Life Technologies), all supplemented with 10% FCS (Biochrom AG), selected from 20 different lots for optimal growth of human MSCs (using DMEM as basal medium and testing proliferation, colony-forming ability, and osteogenic/adipogenic potential) and 50 μg/ml Gentamicin (Life Technologies), whereas media with L-glutamine were also supplemented with additional L-glutamine (Life Technologies) to reach a final concentration of 4 mM. Results obtained were compared with those produced with mesenchymal stem cell medium (MSCGM) (Cambrex BioScience, Nottingham, U.K., http://www.cambrex.com), which is developed especially for the optimal growth of MSCs but is restricted for in vitro use only.

BM MNCs were plated at 1,000, 5,000, 10,000, 25,000, 50,000, 100,000, or 200,000 cells/cm^2. Adherent cells isolated by each plating density were passaged at 50, 100, 250, 500, or 1,000 cells/cm^2.

Cultures were performed in 10 ml of the respective medium, in 75-cm^2 flasks obtained from Nunc (Nunc A/S, Kamstrup, Denmark, http://www.nuncbrand.com), Greiner (Greiner Bio-One, Frickenhausen, Germany, http://www.gbo.com), Costar (Corning, Corning, NY, http://www.corning.com), or Falcon (Becton, Dickinson, and Company, Mountain View, CA, http://www.bd.com).

Monoclonal Antibodies and Immunophenotyping

Monoclonal antibodies against human CD44 conjugated with phycoerythrin (PE) and CD105 conjugated with fluorescein isothiocyanate (FITC) were purchased from Serotec (Oxford, http://www.serotec.com). Anti-CD34 conjugated with PE was obtained from Becton, Dickinson, and Company. Anti-CD29 conjugated with antigen-presenting cell and isotype controls for FITC and PE were purchased from PharMingen (San Diego, CA, http://www.pharmingen.com). PEcy5-conjugated anti-CD45, anti-HLA-DR, and anti-mouse immunoglobulin G, PE-conjugated anti-CD71, and FITC-conjugated anti-HLA-ABC were obtained from Immunotech (Beckman Coulter, Paris, France, http://www.beckman.com). The unlabelled antibodies against galactocerebroside, neurofilament M, NeuN, synapto-

physin, tubulin beta III, and MAB1470 were obtained from Chemicon (Temecula, CA, http://www.chemicon.com). For the determination of SH2, SH3, and STRO-1 expression, culture supernatants from the respective hybridoma cell lines (obtained from American Type Culture Collection, Manassas, VA, http://www.lgcpromochem.com/atcc for SH2 and SH3 and from Developmental Studies Hybridoma Bank, Iowa City, IA, http://www.uiowa.edu/~dshbwww for STRO-1) were used.

Cells were washed twice with ice-cold phosphate-buffered saline (PBS), supplemented with 1% bovine serum albumin (BSA) followed by incubation with saturating concentrations of the appropriate antibodies for 15 minutes at room temperature. Staining with unlabeled antibodies was followed by a second cycle of immunostaining with goat anti-mouse Ig antibody conjugated with PE (DAKO A/S, Glostrup, Denmark, http://www.dakocytomation.com). Thereafter, cells were washed twice in ice-cold PBS/1% BSA and fixed with 1% paraformaldehyde in PBS. Samples were analyzed using FACSCalibur (Becton, Dickinson, and Company) and CellQuest analysis software.

Colony-Forming Assay

Colony-forming unit fibroblast assays have been performed as described previously [36]. Briefly, MSCs to be tested were plated on 35-mm-diameter Petri dishes (Sarstedt, Newton, NC, http://www.sarstedt.com) at 10 cells/cm^2 in the respective media. After 14 days of culture, medium was removed and cells were stained with 0.5% Crystal Violet (Merck, Darmstadt, Germany; http://www.merck.com) in methanol for 5 minutes. The dishes were washed twice with distilled water and dried, and the numbers of colonies with diameter greater than 2 mm were scored. Results are presented as number of colonies formed per 100 cells seeded.

In Vitro Differentiation Assays

MSCs were plated at 10,000/cm^2, and the relevant differentiation medium was added. Neurogenic differentiation was induced by culture in DMEM/L/GL supplemented with 2% FCS, 10^{-7} M dexamethasone (Decadron, Merck & Co Inc, Whitehouse, NJ, http://www.merck.com), 0.5 μM linoleic acid (Sigma-Aldrich, St. Louis, http://www.sigma-aldrich.com), 10 ng/ml platelet-derived growth factor (R&D Systems, Abingdon, U.K., http://www.rndsystems.com), 10 ng/ml epidermal growth factor (R&D Systems), and 50 μg/ml gentamicin [37]. After 2 weeks of culture, cells were immunostained with antibodies against tubulin BIII, synaptophysin, galactocerebroside, neurofilament M, and NeuN. The cells staining positive for each one of these five markers did not vary within each group more than $\pm$10%; thus, for the interest of brevity, neurogenic potential was expressed as percentage of cells positive for the marker with the lowest percentage.

To promote adipogenic differentiation, cells were cultured for 3 weeks in DMEM/H/GL supplemented with 2% FCS, 0.5 μM dexamethasone, 0.5 mM isobutylmethylxanthine (Sigma-Aldrich), 60 μM indomethacin (Sigma-Aldrich), and 50 μg/ml gentamicin [38]. Lipid droplets in the generated adipocytes were visualized by staining with Sudan Black IV (Sigma-Aldrich). The percent of adipocytes was estimated by counting 500 total cells in multiple fields.

The osteogenic differentiation was stimulated in a 3-week culture in DMEM/L/GL, supplemented with 10% FCS, 10^{-7} M dexamethasone, 10 mM b-glycerol phosphate (Fluka, Buchs, Switzerland, http://www.sigmaaldrich.com), 50 μM L-ascorbic acid-2 phosphate (Sigma-Aldrich), and 50 μg/ml gentamicin [39]. Assessment of Ca accumulation and alkaline phosphatase (AP) activity was visualized by AP/Von Kossa staining (http://stemcell.ibme.utoronto.ca/protocols), monitored under stereomicroscope, and analyzed using Image ProPlus software (Media Cybernetics, San Diego, CA, http://www.mediacy.com). Osteogenic differentiation is presented as percent of the mineralized area in the total culture area.

Allogeneic Mixed Lymphocyte Reaction and Mitogen Stimulation Assay

A standard mixed lymphocyte reaction (MLR) assay was performed using peripheral blood MNCs obtained from normal adults, which were plated in 96-well flat-bottomed plates at 100,000/well in RPMI-1640 medium (Life Technologies) supplemented with 10% FCS, 2 mM L-glutamine, and 50 μg/ml gentamicin. MNCs were stimulated with equal numbers of irradiated (20-Gy) peripheral blood MNCs from an HLA-mismatched donor. Phytohemagglutinin (Sigma-Aldrich) addition at 5 μg/ml was used as a control. In MLR/MSC microcultures, MLRs were performed on a layer of 10,000 MSCs seeded 1 day before. On day 5, 1 μCi/well [^{3}H]TdR (Amersham Pharmacia Biotech, Buckinghamshire, U.K., http://www4.amershambiosciences.com) was added for the last 18 hours of culture. Cells were then harvested and [^{3}H]TdR uptake was measured in a microbeta counter (Wallac, PerkinElmer Inc, Jügesheim, Germany, http://www.perkinelmer.com). All cultures and controls were performed in triplicates.

In Vivo Assays

For in vivo assays, BALB/C and C57 BL/6 mice were purchased from the animal breeding facility of the Hellenic Pasteur Institute (http://www.pasteur.gr) and B6.CB17-Prkdc^{scid} (severe combined immunodeficient [SCID]) mice from Jackson Laboratory (Bar Harbor, ME, http:/www.jax.org). Mice were all age-matched (6 to 8 weeks) and sex-matched, and experiments were performed according to animal experimental ethics committee guidelines.

MSCs were tested for their ability to support hematopoietic stem cell engraftment following the protocol used by Maitra et al. [40]. In brief, 4×10^6 human umbilical cord blood–derived MNCs, alone or mixed with 10^6 MSCs, were intravenously injected in 100 μl PBS in SCID mice 24 hours after sublethal total body irradiation (250 cGy). Mice were killed 6 weeks after cell infusion, and human cell engraftment was detected in BM and spleen by flow cytometry using anti-human CD45 antibody.

In vivo bone formation was detected after the protocol used by Kuznetsov et al. [41]. Briefly, 10^6 MSCs were suspended in 60 μl medium, loaded on 50 to 70 mm^3 Collagraft blocks (Zimmer Inc, Los Gatos, CA, http://www.zimmer.com), incubated for 1.5 hours at 37°C, and transplanted in subcutaneous pockets on the dorsal surface of SCID mice under general anesthesia. Transplants were recovered 8 weeks after transplantation, fixed, partially decalcified, paraffin-embedded, and stained with hematoxylin and eosin. Bone formation was detected under stereomicroscope, analyzed using Image ProPlus

software, and expressed as score 0 through 4 as previously described [41].

MSC in vivo immunosuppressive ability was tested by potential to enhance tumor growth and ability to suppress graft-versus-host-disease (GvHD). The first assay was performed modifying a formerly used protocol [42]. Briefly, 0.1×10^6 CT26 cancer cells (BALB/C-originated colon carcinoma cell line, grown in RPMI-1640 supplemented with 10% FCS) were injected intravenously, alone or mixed with 0.1×10^6 MSCs, in 100 μl PBS in BALB/C and C57 BL/6 mice. Mice were killed 21 days after cell infusion, and lungs were removed, paraffin embedded, and stained with hematoxylin and eosin. Pulmonary infiltration with cancer cells was estimated as percent of total pulmonary area using Image ProPlus software. Scoring was estimated as follows: [3] when tumor cells infiltrated more than 80% of pulmonary tissue, (2) when infiltration was 50%–80%, (1) for infiltration up to 50%, and (0) for noninfiltrated lungs (Fig. 1). For the second assay, 5×10^6 spleen cells from C57 BL/6 mice were injected intravenously in 100 μl PBS in BALB/C mice 24 hours after total body irradiation at 300 cGy, alone or together with 10^6 or 0.3×10^6 MSCs. Mice injected with spleen cells alone developed GvHD, characterized by weight and hair loss [43], and died within 8 to 9 days, whereas animals coinjected with 10^6 MSCs did not develop GvHD. The immunosuppressive potential of different culture condition–derived MSCs was estimated by monitoring the lifespan of mice coinjected with 0.3×10^6 MSCs.

Statistical Analysis

Experimental data were analyzed by SPSS statistical software (SPSS Predictive Analytics, Chicago, http://www.spss.com). One-way analysis of variance multiparameter analysis was applied to assess statistically significant differences and correlations. The cutoff value for significance was .05.

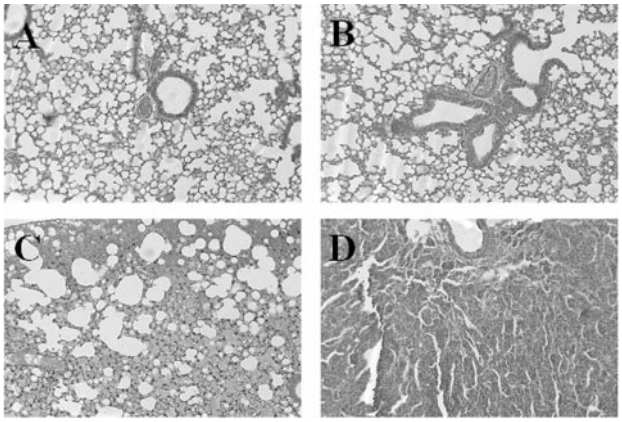

Figure 1. Scoring of pulmonary infiltration by CT-26 cancer cells. Lungs from BALB/C or C57BL/6 mice injected with 0.1×10^6 CT-26 BALB/C-originated cancer cells, alone or mixed with 0.1×10^6 mesenchymal stem cells. Scoring was estimated as follows. **(A)**: Score 0, noninfiltrated lungs. **(B):** Score 1, infiltration up to 50%. **(C):** Score 2, infiltration 50%–80%. **(D):** Score 3, infiltration greater than 80% of pulmonary tissue.

Results

Selection of Optimal Basal Medium for Isolation and Expansion of Human MSCs

To define the optimal basal medium for the isolation of MSCs, BM MNCs were plated at 25,000 cells/cm^2 in Nunc flasks and cultured for 7 days in the presence of different media, as described under Materials and Methods. Subsequently, adherent cells (passage 0 cells [P0]), which represent a MSC-enriched population, were trypsinized and counted. Results depicted in Figure 2A show the medium-dependent effect on the size of the initial MSC-enriched population: adherent cells isolated from 10^6 MNCs ranged between 7,111 $\pm$ 12,317 for IMDM and 120,099 $\pm$ 19,269 for aMEM/GL (overall p value was less than .01), whereas the number of adherent cells for the MSC-specific medium MSCGM was 120,349 $\pm$ 34,695 (data not shown).

Next, P0 cells were plated at 100 cells/cm^2 in Nunc flasks in the respective media. Cells were counted and passaged at the same cell density at weekly intervals (Fig. 2B). IMDM failed to support MSCs' culture further than P1. For the rest of the media,

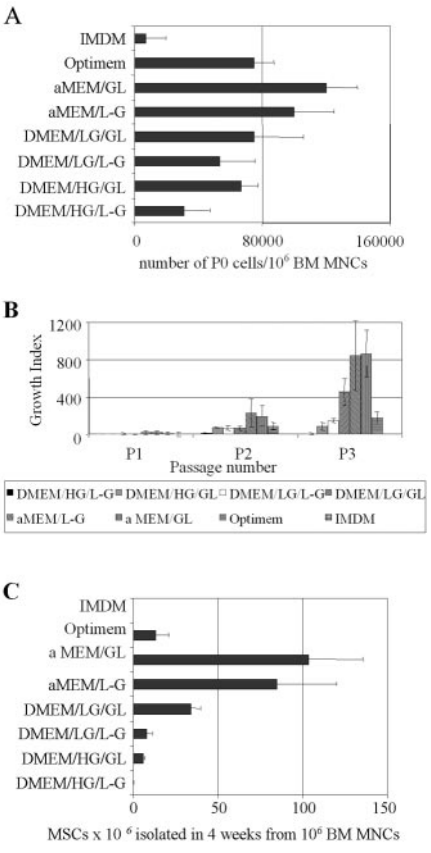

Figure 2. Dependence of human mesenchymal stem cell (MSC) isolation and expansion upon the culture medium. **(A):** Number of adherent cells isolated after 7 days of culture from 10^6 bone marrow (BM) mononuclear cells (MNCs) plated in Nunc culture flasks at 25,000/cm^2. **(B):** Growth index of MSCs cultured in the respective media at passages 1, 2, and 3, performed at weekly intervals. Cells were passaged at 100 cells/cm^2. **(C):** The final number of MSCs isolated at passage 3 (4 weeks in culture) from 10^6 BM MSCs in the media used. Data represent the mean $\pm$ standard deviation from five independently performed experiments.

the growth index (number of cells at the actual time point divided by the initial number of P0 cells) was calculated for each passage and ranged at passage 3 from 8 ± 6.4 for DMEM/HG/L-G to 865 ± 251 for aMEM/GL (compared with 1,600 ± 384 for MSCGM).

Combining the data from isolation and expansion of MSCs cultured in the presence of different media, we estimated the number of MSCs generated after 4 weeks in culture (Fig. 2C). The optimal media seem to be aMEM/GL and aMEM/L-G ($103.65 \pm 23.0 \times 10^6$ and $85.07 \pm 35.0 \times 10^6$ per 10^6 BM MNCs, respectively; $p = .03$). Concerning the rest of the media tested, MSCs generated ranged between $0.25 \pm 0.22 \times 10^6$ per 10^6 BM MNCs for DMEM/HG/L-G and $34.1 \pm 5.9 \times 10^6$ per 10^6 BM MNCs for DMEM/LG/GL, whereas, as mentioned above, IMDM failed to support MSC growth. When MSCGM was used, $192.56 \pm 38.7 \times 10^6$ MSCs were generated per 10^6 BM MNCs (data not shown).

After 1 month in culture, we assessed the quality of cells acquired. MSCs cultured in the distinct basal media were all able to suppress T cell proliferation in MLR, whereas comparable results were also obtained with respect to their in vivo bone formation, CD34 cell-engraftment support, ability to suppress GvHD, and enhancement of tumor growth. Minor differences were observed in phenotype and in vitro differentiation potential, whereas aMEM/GL, aMEM/L-G, and DMEM/LG/GL demonstrated higher colony-forming ability ($p < .01$; Table 1). When the MSC-specific medium MSCGM was used, colony-forming ability was significantly higher (92% ± 7%, $p < .01$), whereas in vitro differentiation potential slightly varied (65% ± 7% of cells under adipogenic-inducing medium were Sudan Black IV$^+$, the lowest expressed neural-specific marker used was present on 55% ± 8% of the cells when cultured in neurogenic medium, and 71% ± 4% of the culture area was mineralized under osteogenic-inducing conditions).

Among the various basal media tested, aMEM/GL was selected for the subsequent experiments in terms of numbers and quality of MSCs acquired.

Effect of Initial and Passage Plating Density on Isolation and Expansion of Human MSCs

BM MNCs were plated on Nunc tissue culture flasks in aMEM/GL-based culture medium at 1,000, 5,000, 10,000, 25,000, 50,000, 100,000, and 200,000 cells/cm^2. One week later, cells were trypsinized and counted (Fig. 3A). The data suggest that initial plating density of BM MNCs has great impact on the size of the MSC-enriched population derived, with maximum numbers of adherent cells isolated at lower plating densities. Statistical analysis separates the concentrations used into three groups. The optimal condition is represented by the density of 1,000 cells/cm^2 (192,766 ± 35,050 cells/10^6 BM MNCs), followed by the densities of 5,000, 10,000, and 25,000 cells/cm^2 (134,938 ± 20,444, 133,921 ± 22,490, and 108,057 ± 17,173 cells/10^6 BM MNCs, respectively), whereas the last group consists of the densities of 50,000, 100,000, and 200,000 cells/cm^2

Table 1. Phenotypic characterization, colony-forming ability, and differentiation potential of P3 cells cultured in different media

	Optimem	aMEM GL	aMEM L-G	DMEM LG/GL	DMEM LG/L-G	DMEM HG/GL	DMEM HG/L-G
SH2	+	+	+	+	+	+	+
SH3	+	+	+	+	+	+	+
CD29	+	+	+	+	+	+	+
CD34	−	−	−	−	−	−	−
CD44	+	+	+	+	+	+/−	+/−
CD45	−	−	−	−	−	−	−
CD71	+	+	+	+	+	+	+
CD105	+	+	+	+	+	+	+
MAB1470	+	+	+	+	+	+/−	+/−
STRO-1	+	+	+	+	+	+	+/−
HLA-ABC	+	+	+	+	+	+	+
HLA-DR	−	−	−	−	+/−	−	+/−
Colonies (% cells seeded)	40 ± 7	65 ± 5	63 ± 8	68 ± 9	30 ± 2	35 ± 3	38 ± 1
In vitro adipogenic differentiation[a]	58 ± 6	49 ± 4	71 ± 8	63 ± 2	59 ± 3	52 ± 12	61 ± 6
In vitro osteogenic differentiation[b]	72 ± 5	59 ± 8	63 ± 5	68 ± 4	58 ± 5	70 ± 8	61 ± 6
In vitro neurogenic differentiation[c]	63 ± 5	52 ± 8	49 ± 5	71 ± 1	56 ± 4	67 ± 4	63 ± 2
Percent human CD45$^+$ cells in bone marrow	3.06 ± 0.6	3.66 ± 0.7	3.48 ± 0.6	3.76 ± 0.8	3.22 ± 0.3	3.04 ± 0.5	2.96 ± 0.6
Human CD45$^+$ cells in spleen (%)	2.50 ± 0.8	2.76 ± 0.7	3.28 ± 0.6	3.04 ± 0.9	2.92 ± 0.8	2.32 ± 1.0	2.56 ± 0.7
In vivo bone formation[d]	3.3	2.9	3.2	3.4	2.9	3.3	3.1
GvHD survival (days)	16.1 ± 1.3	16.2 ± 2.5	15.6 ± 1.5	15.8 ± 2.4	15.0 ± 1.6	14.4 ± 1.7	14.0 ± 2.3
CT-26 lung infiltration[e]	2.1	3.0	2.5	2.4	2.6	2.9	2.2

Fluorescence-activated cell sorter analysis: (+), most cells positive; (+/−), some cells positive; (−), negative.

[a]Percent Sudan Black IV$^+$ cells out of 500 total cells counted.

[b]Percent mineralized area.

[c]Percent of cells stained positive (of 500 total cells counted) for the lowest expressed neural-specific marker used.

[d]Mean score from seven animals, each one using mesenchymal stem cells (MSCs) from independent experiments. Scoring according to Kuznetsov et al. [41]: (4) bone spreads over more than 50% of the sections, (3) bone occupies less than 50% of the sections, (2) bone structures occupy a small part of each section or of some sections, (1) bone found only in a few sections, (0) no bone is formed.

[e]Mean score from 10 animals, each one using MSCs from independent experiments. Scores as described in Figure 1.

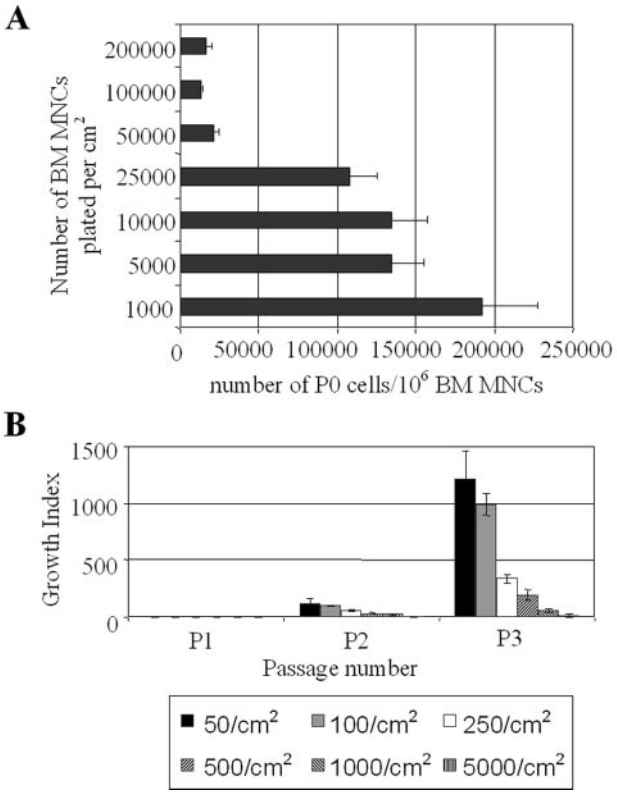

Figure 3. Effect of initial plating density and passaging density on isolation and proliferation of human mesenchymal stem cells (MSCs). **(A):** Number of adherent cells isolated from 10^6 bone marrow (BM) mononuclear cells (MNCs) after 1 week of culture in aMEM with Glutamax in Nunc flasks at the plating densities described. **(B):** Growth index of MSCs, derived from an initial plating density of 10,000 BM MNCs/cm^2, passaged at the indicated cell densities at weekly intervals. Data are expressed as mean ± standard deviation from four independently performed experiments.

(22,080 ± 2,629, 13,406 ± 1,078, and 17,086 ± 3,023, respectively). The three groups differ statistically significantly ($p < .02$), whereas within each group, p value varied between 0.6 and 1.0.

P0 cells from each initial plating density were passaged at 50, 100, 250, 500, or 1,000 cells/cm^2 to evaluate whether cells isolated from the initial cultures differ at their proliferative potential, as well as the effect of cell density at MSCs' passaging. Results indicated no apparent correlation between initial plating density and proliferative potential of isolated cells (data not shown). Nevertheless, passaging density critically affects MSCs' proliferation potential, yet again with lower densities resulting in higher proliferation rates (Fig. 3B for initial plating density of 10,000 cells/cm^2), which is in keeping with other studies [25, 26, 44]. However, passaging densities of 50 and 100 cells/cm^2 do not differ significantly concerning growth index at passage 3 (1,214 ± 240 and 988 ± 94, respectively, $p = .18$), while remaining higher compared with all other passaging densities tested (growth index ranging from 341.6 ± 40.0 to 12.1 ± 15.0, $p < .01$).

The phenotype of the cells described above and their immunosuppressive capacity, colony-forming ability, and in vitro multilineage differentiation potential remained unaffected. Their in vivo immunosuppressive ability, osteogenic capacity, or CD34-engraftment facilitation also had no apparent differences (data not shown).

Impact of Adherence Surface Quality on Isolation and Expansion of Human MSCs

BM MNCs were plated at 10,000 cells/cm^2 in aMEM/GL-based culture medium in 75-cm^2 tissue culture flasks obtained from Greiner, Nunc, Costar, or Falcon. One week later, adherent cells were trypsinized and counted (Fig. 4A). Subsequently, P0 cells were passaged at 100 cells/cm^2 in the same medium and the respective brand's flask. Cells were passaged three times at weekly intervals, being counted at each passage (Fig. 4B).

As indicated by our results, the quality of plastic affects to a great extent the isolation as well as the proliferative potential of human MSCs (ranging from 120,554 ± 44,312 to 250,690 ± 21,213 isolated adherent cells/10^6 BM MNCs, $p < .01$, and growth index ranging from 1,145 ± 486 to 2,321 ± 745 for P3, $p < .01$). The final outcome of the 4-week culture resulted in significantly higher numbers of MSCs when Falcon flasks were

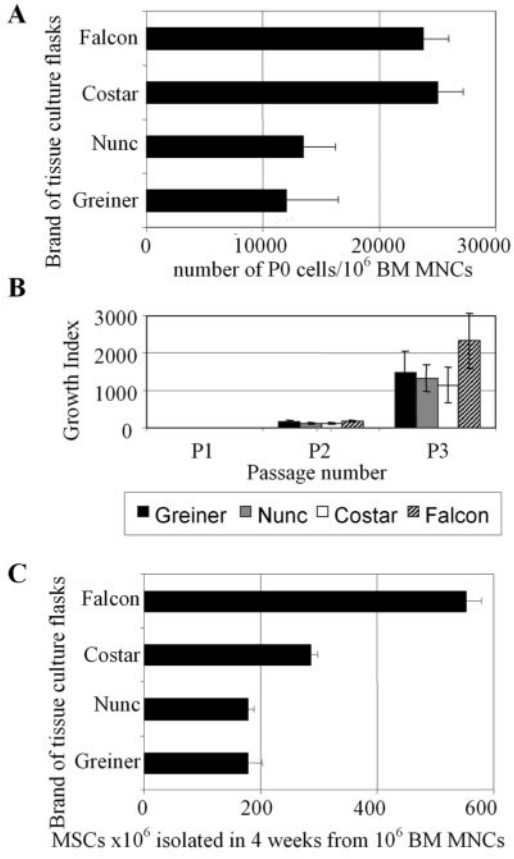

Figure 4. Relation between the quality of plastic adherence surface and the isolation and proliferation of human mesenchymal stem cells (MSCs). **(A):** Number of adherent cells isolated from 10^6 bone marrow (BM) mononuclear cells (MNCs) plated at 10,000/cm^2 after 7 days of culture in aMEM with Glutamax. **(B):** Growth index of MSCs constitutively passaged at weekly intervals at 100 cells/cm^2. **(C):** Total number of cells acquired after 4 weeks of culture from 10^6 BM MNCs, cultured in the indicated branches' flasks. Data represent mean ± standard deviation from five independently performed experiments.

used (553.07 ± 26.26 cells/10^6 BM MNCs) as opposed to the rest of the flasks (178.06 ± 24.90 to 287.04 ± 10.31 cells/10^6 BM MNCs, $p < .01$, Fig. 4C).

Phenotypically, P3 cells isolated from different tissue culture flasks did not statistically significantly vary, whereas no differences were detected in all in vitro and in vivo assays performed (data not shown).

Effect of bFGF-Supplemented Media on Isolation and Expansion of Human MSCs

The use of bFGF as potent mitogen for MSCs, while maintaining their differentiation potential and increasing their telomere length, has previously been reported in various culture systems [31, 32, 34, 45]. However, others have suggested that bFGF addition favors their osteogenic potential [46, 47]. Since the addition of growth factors to stem cell culture media tends in general to induce differentiation and loss of multilineage capacity, we sought to test the impact of bFGF supplementation on MSC properties.

BM MNCs were plated at 10,000 cells/cm^2 in Falcon tissue culture flasks in aMEM/GL-based culture medium, plain or supplemented with 0.01, 0.1, 1, 5, 10, or 20 ng/ml bFGF. One week later, P0 cells were enumerated (Fig. 5A) and passaged consecutively three times at 100 cells/cm^2 under the respective culture conditions (Fig. 5B). Evidence suggests that supplementation of MSC culture media with bFGF clearly affects isolation and proliferation, resulting in vast numbers of cells within 1 month of culture ($p < .01$). Colony-forming ability of such cells does not differ significantly among the groups (data not shown).

However, MSC morphology and phenotype are critically altered when bFGF is added in the system. Predominantly, cells tend to shorten, as already demonstrated by Solchaga et al. [34], and lose their spindled shape (Fig. 5F). Regarding their phenotype, they upregulate HLA-DR, reaching in some experiments even 82% of cells staining positive. The expression depends on bFGF concentration, with greatest effects seen at 5 ng/ml (Fig. 5G). Still, the mean fluorescence intensity (MFI) remains low (198 to 295) compared with dendritic cells, the professional antigen-presenting cells, which, under the same instrumental settings, express HLA class II with MFI 382 ± 28 (data not shown). Furthermore, HLA class I molecules expressed on the surface of each individual cell are amplified, as determined by the increased MFI (2,100 to 3,900 compared with 1,129 seen in control cultures without bFGF). This effect is also concentration-dependent, with the highest expression achieved at 5 ng/ml (Fig. 5C). Other differences relate to CD44 expression, which decreases in a dose-dependent fashion in the presence of bFGF (Fig. 5D).

Since HLA-DR molecules are substantial for antigen presentation to T cells and immunorecognition, we tested the ability of bFGF-cultured MSCs to elicit T cell responses. Peripheral blood mononuclear cells (PBMCs) were cultured over irradiated MSCs from HLA-mismatched donors, developed in the absence or presence of 5 ng/ml bFGF (the concentration resulting in the higher HLA-DR expression), at PBMC:MSC ratios of 1:1, 1:10, 1:100, and 1:1,000. As a control, irradiated PBMCs autologous to the MSCs used as stimulators at 1:1 ratio were tested. Before the experimental procedure, percentages and expression levels of HLA-DR on MSCs were tested by flow cytometry (65%–78% of MSCs expressed HLA-DR with MFI 242–267). As depicted in Figure 5E, MSCs cultured in the presence of bFGF elicit some proliferation to allogeneic PBMCs (stimulation in-

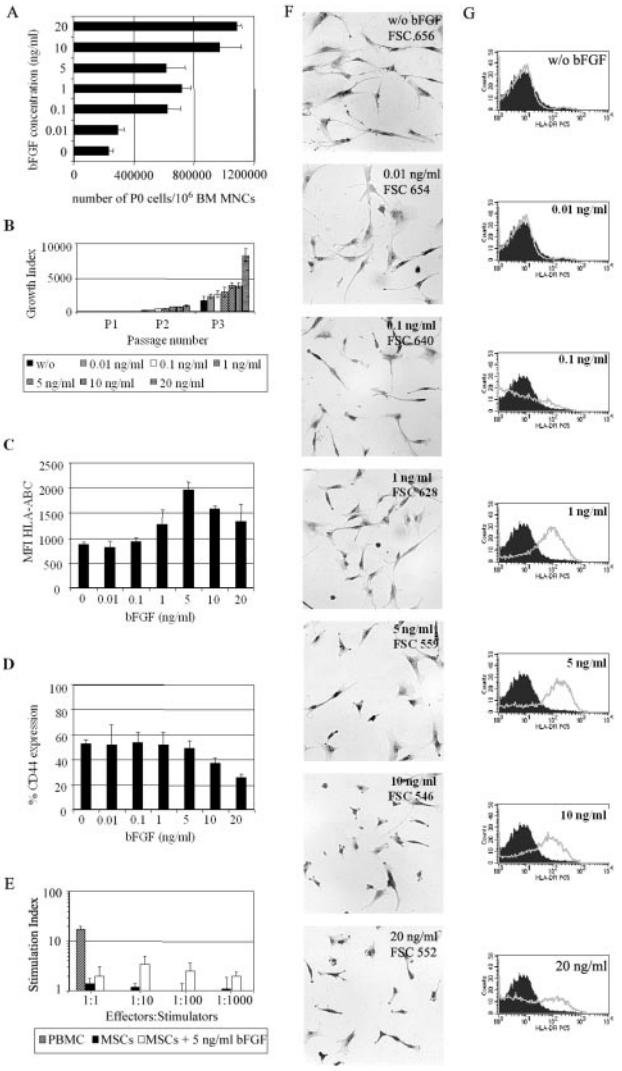

Figure 5. Effect of basic fibroblast growth factor (bFGF) supplementation of the culture media on human mesenchymal stem cells (MSCs). Passage-3 MSCs were cultured at 10,000/cm^2 in Nunc flasks, in aMEM with Glutamax with or with out the addition of bFGF at 0.01, 0.1, 1, 5, 10, and 20 ng/ml. **(A):** Number of adherent cells isolated from 10^6 bone marrow (BM) mononuclear cells (MNCs) plated. **(B):** Growth index of MSCs passaged at 100 cells/cm^2 at weekly intervals. **(C):** HLA-ABC mean fluorescence intensity (MFI) of MSCs derived from culture in the different concentrations of bFGF. **(D):** CD44 expression of MSCs derived from culture in the different concentrations of bFGF. **(E):** Irradiated (30-Gy) passage-3 MSCs, cultured in aMEM with Glutamax without (black bars) or in the presence of (white bars) bFGF at 5 ng/ml, as well as PBMCs from the same individuals as the MSCs (striped bar), were used as stimulators for PBMCs from an HLA-mismatched donor in a 5-day assay. Effectors were added at 100,000 cells/well, over the stimulators plated at the indicated ratios. **(F):** MSCs stained with Giemsa (FSC, forward scatter height). **(G):** Fluorescence-activated cell sorter analysis of HLA-DR expression. Filled histograms represent the matched isotype control, and open histograms show HLA-DR expression. **(A–E):** Data are expressed as mean ± standard deviation from four independently performed experiments **(F, G)**. Representative results from four independently performed experiments are shown.

dex, 2.5 ± 0.67, compared with 1.2 ± 0.17 elicited with MSCs cultured in the absence of bFGF, $p = .02$), but this proliferation induction is still very weak compared with the response obtained against PBMCs (stimulation index 17.8 ± 2.4) derived from the same donor as MSCs. When the potential of MSCs to prevent GvHD was tested, their immunosuppressive ability was found to be enhanced by the addition of bFGF at concentrations higher than 5 ng/ml (≥ 18.0 days of survival, compared with 16.4 ± 1.1 days with MSCs cultured in the absence of bFGF, $p < .05$). With respect to their ability to enhance tumor development, this remained unaffected by media bFGF supplementation. One could speculate that in such a model, even a small number of MSCs were capable of masking any potential differences in MSC suppression ability due to sufficient immunosuppression (Table 2).

Supplementation of culture media with bFGF limited the differentiation capacity of MSCs. As shown in Table 2, osteogenic, and, at a much lower extent, adipogenic potential were enhanced, whereas neurogenic differentiation ability was decreased. Enhancement of osteogenic potential was also reflected in in vivo bone formation, where osteogenesis was significantly augmented when MSCs were cultured in the presence bFGF at concentrations higher than 5 ng/ml.

Finally, bFGF supplementation of MSC media reduced the ability to support CD34 cell engraftment. When human $CD34^+$ cells were coinjected in SCID mice with MSCs cultured in the absence of bFGF, human $CD34^+$ cells constituted 3.60% ± 0.4% of BM MNCs and 3.22% ± 0.6% of spleen cells, compared with 2.12% ± 0.5% to 3.04% ± 0.4% of BM MNCs and 1.58% ± 0.3% to 2.75% ± 0.7% of spleen cells with MSCs cultured in different concentrations of bFGF ($p < .05$; Table 2).

DISCUSSION

MSC transplantation holds promise as cellular therapy for numerous human diseases, and clinical studies have already been initiated. Given the considerably low percentage of MSCs in adult BM as well as the vast numbers required for regenerative and immunotherapeutic approaches, in vitro expansion is a prerequisite for MSC transplantation. Furthermore, standardized culture conditions are required to facilitate comparison of clinical results involving MSCs.

Several methods have been proposed to test the quality of MSCs to be used for clinical protocols, varying from simple colony-forming assays [36] to more complex morphological characterizations [30, 38]. However, there is no uniform approach for MSC expansion equilibrating among extended proliferation and preservation of multilineage differentiation potential.

In this study, we compare numerous different culture conditions for MSC isolation and in vitro expansion, some of them already being used in experimental and clinical protocols. Evaluation of different culture media for MSCs revealed that those based on aMEM are more suitable for both isolation and expansion of multipotent MSCs. Low glucose concentration in DMEM-based media and Glutamax instead of L-glutamine in all different basal media consistently supported MSC growth. Greater proliferation in media with Glutamax has been observed in various cell types and has been attributed to the greater stability of the dipeptide L-alanyl-L-glutamine contained in media with Glutamax, in contrast to L-glutamine, which is chemically unstable, even at 4°C, and its chemical breakdown and cellular metabolism lead to ammonia formation and subsequent inhibition of cell growth [48]. Our results further confirm that human MSCs possess different culture requirements than murine MSCs, because the latter show high proliferation rates in IMDM [49], a type of medium that failed to support human MSC growth in our culture system.

Previous reports, evaluating critical parameters for MSC expansion, have proposed that plating MSCs at low density favors proliferation and stemness preservation [25, 26, 38, 44]. Our results, testing six different passage densities, confirm this finding. However, all of these reports have used high BM MNC plating density (always greater than 150,000 BM MNCs/cm^2). The results from our experiments, however, indicate that initial plating densities of 5,000 to 10,000 cells/cm^2 result in much higher numbers of the starting MSC-enriched adherent popula-

Table 2. Differentiation potential of P3 cells cultured in or without the addition of basic fibroblast growth factor (bFGF) at different concentrations

	Without bFGF	0.01 ng/ml bFGF	0.1 ng/ml bFGF	1 ng/ml bFGF	5 ng/ml bFGF	10 ng/ml bFGF	20 ng/ml bFGF
In vitro adipogenic differentiation[a]	51 ± 3	55 ± 6	55 ± 12	58 ± 8	59 ± 5	59 ± 8	55 ± 4
In vitro osteogenic differentiation[b]	57 ± 4	61 ± 5	69 ± 8	72 ± 5	89 ± 10	83 ± 7	87 ± 6
In vitro neurogenic differentiation[c]	49 ± 4	45 ± 5	40 ± 6	43 ± 4	32 ± 9	39 ± 8	33 ± 4
Human $CD45^+$ cells in bone marrow (%)	3.60 ± 0.4	3.66 ± 0.6	3.04 ± 0.4	3.14 ± 0.5	2.78 ± 0.2	2.06 ± 0.6	2.12 ± 0.5
Human $CD45^+$ cells in stem cells (%)	3.22 ± 0.6	3.04 ± 0.7	2.70 ± 0.6	2.30 ± 0.5	1.78 ± 0.2	1.58 ± 0.3	1.62 ± 0.4
In vivo bone formation[d]	3.0	3.1	3.4	3.3	3.9	3.9	3.9
GvHD survival (days)	16.4 ± 1.1	16.2 ± 2.6	15.6 ± 1.5	17.2 ± 1.3	18.0 ± 1.6	18.2 ± 1.6	18.0 ± 1.2
CT-26 lung infiltration[e]	2.3	2.4	2.9	3.0	2.3	2.4	2.2

[a]Percent Sudan Black IV^+ cells out of 500 total cells counted.
[b]Percent mineralized area.
[c]Percent of cells stained positive (of 500 total cells counted) for the lowest expressed neural-specific marker used.
[d]Mean score from seven animals, each one using MSCs from independent experiments. Scoring according to Kuznetsov et al. [41]: (4) bone spreads over more than 50% of the sections, (3) bone occupies less than 50% of the sections, (2) bone structures occupy a small part of each section or of some sections, (1) bone found only in a few sections, (0) no bone is formed.
[e]Mean score from 10 animals, each one using MSCs from independent experiments. Scores as described in Figure 1.

tion. Because the proliferative capacity of MSCs is very high, the starting population is crucial for final numbers of cells to be obtained.

Further factors affecting the expansion of human MSCs include the quality of plastic surface used for their adhesion. We tested culture flasks from four different companies and observed that after 4-week culture, greater numbers of MSCs were acquired in Falcon flasks. All flasks used are made from polystyrene permanently rendered hydrophilic with corona discharge, using high voltage to create a reactive gas plasma [50]. This process for Falcon flasks takes place in a closed chamber, thus creating a consistent treatment surface. On the contrary, during manufacturing of the rest of the flasks tested, the gas is exposed to ambient air and therefore subjected to day-to-day environmental changes. This difference may account for the better performance regarding MSC proliferation potential. However, the quality of cells produced did not differ among the different types of flasks used.

The most common growth factor used to induce proliferation of MSCs, while maintaining their multilineage capacity, is bFGF. Thus, we tested several bFGF concentrations and, in agreement with previous studies [31–34], found a dose-dependent increase of isolation and proliferative potential of MSCs. Unexpectedly, however, at least in our culture conditions, addition of bFGF in culture media results in significant alteration of MSC properties. Most importantly, bFGF causes HLA-DR induction, although in a low density on the cell surface, and also upregulates HLA class I expression. Still, this upregulation of HLA molecules results in weak allogeneic immune responses, compared with the responses elicited by allogeneic PBMCs. However, the in vivo immunosuppressive ability of MSCs is enhanced when cultured with bFGF, suggesting that the complex mechanisms used to suppress the cells of the immune system overcome their potential to induce alloreactivity. Another alteration in MSC phenotype by bFGF-supplemented media concerns CD44 expression. CD44 is involved in cell-cell and cell-matrix interactions, thus being responsible for extravasation and homing, also influencing cell growth and survival [51]. Consequently, bFGF-induced CD44 downregulation may possibly result in reduced effective engraftment of transplanted MSCs. Regarding MSC differentiation capacity in bFGF-supplemented cultures, osteogenic potential, both in vitro and in vivo, seems to be upregulated, adipogenesis is somewhat increased, whereas in vitro neurogenesis and CD34 engraftment support are slightly suppressed, thus probably restricting the use of bFGF-cultured MSCs to clinical approaches targeting bone and cartilage defects.

In conclusion, this is the first conclusive study providing the general guidelines for the establishment of a standardized protocol aiming at the production of vast numbers of high-quality MSCs to be used in preclinical studies and clinical protocols.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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