British Journal of Haematology, 2003, **123**, 702–711

Murine marrow-derived mesenchymal stem cell: isolation, *in vitro* expansion, and characterization

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Received 8 July 2003; accepted for publication 20 August 2003

Summary. In spite of the attention given to the study of mesenchymal stem cells (MSCs) derived from the bone marrow (BM) of humans and other species, there is a lack of information about murine MSCs. We describe the establishment of conditions for the *in vitro* expansion of plastic-adherent cells from murine BM for over 50 passages, and provide their characterization regarding morphology, surface marker profile and growth kinetics. These cells were shown to differentiate along osteogenic and adipogenic pathways, and to support the growth and differentiation of haematopoietic stem cells, and were thus operationally defined as murine mesenchymal stem cells (mMSCs). mMSCs were positive for the surface markers CD44, CD49e, CD29 and Sca-1, and exhibited a homogeneous, distinctive morphology. Their frequency in the BM of adult BALB/c and C57Bl/6 mice, normal or knockout for the α-L-iduronidase (*IDUA*) gene, was preliminarily estimated to be 1 per 11 300–27 000 nucleated cells. The emergence of a defined methodology for the culture of mMSCs, as well as a comprehensive understanding of their biology, will make the development of cellular and genetic therapy protocols in murine models possible, and provide new perspectives in the field of adult stem cells research.

Keywords: mesenchymal stem cell, fibroblast colony-forming unit, bone marrow, mouse, *in vitro* cultivation.

Postnatal bone marrow (BM) contains cells that can differentiate along mesenchymal pathways during *in vitro* cultivation, given adequate conditions. Culture conditions for the maintenance of these cells were initially described in the 1970s (Prockop, 1997). Following the initial observations, the nomenclature regarding this cell type varied from the early designation of fibroblast colony-forming unit (CFU-F), through mesenchymal progenitor cells, marrow stromal cells and mesenchymal stem cells (MSCs) (Phinney, 2002). MSCs have been isolated from a number of species, including human (Pittenger *et al*, 1999), mouse (Pereira *et al*, 1995), rat (Wakitani *et al*, 1995), dog (Kadiyala *et al*, 1997), baboon, pig, sheep, goat, rabbit (Mosca *et al*, 2000) and cat (Martin *et al*, 2002). In these studies, MSCs were isolated by adherence to plastic substrates, and they were shown to be capable of osteogenic, chondrogenic, adipogenic and myogenic differentiation. Another important characteristic of MSCs is their ability to produce cytokines and growth factors that support and regulate haematopoiesis (Haynesworth *et al*, 1996; Majumdar *et al*, 1998). Neuronal differentiation of these cells (Woodbury *et al*, 2000; Sanchez-Ramos *et al*, 2000) is an important finding, as it denotes transcendence of the mesodermal to the ectodermal developmental pathway. In fact, it may be that the term 'mesenchymal' is not the best one to describe this particular stem cell. Recently, a related cell type, termed multipotent adult progenitor cell (MAPC), was obtained from humans (Reyes *et al*, 2001), rat and mouse (Jiang *et al*, 2002a). Similar cells can be isolated from postnatal murine BM, muscle and brain (Jiang *et al*, 2002b). The methodology described for the culture of MAPCs includes growth on a fibronectin-coated surface, and murine MAPCs were reported to be leukaemia inhibitory factor dependent. It is not clear how close MSCs and MAPCs are, and further studies are necessary to verify whether or not they represent two faces of the same coin.

Expansion of MSCs during culture is still difficult to achieve for they tend to loose the capacity to proliferate and differentiate under standard culture conditions (Banfi *et al*, 2000; Sekiya *et al*, 2002). The isolation of murine mesenchymal stem cells (mMSCs) from BM using standard methods usually results in a heterogeneous cell population with a high degree of haematopoietic contaminants (Phinney *et al*, 1999), which hinders the study of the embedded genuine MSCs. The *in vitro* cultivation and expansion of homogeneous mMSC cultures may help to circumvent the interference of such contaminants on the authentic mMSC. Furthermore, the expansion of homogeneous,

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undifferentiated mMSCs may provide raw matter for *in vivo* studies concerning cell and genetic therapy using the ease of the murine models, and subsequent development of preclinical protocols to treat a wide range of diseases. MSC transplantation, however, may imply immunoregulatory effects, as direct contact between MSCs and memory antigen-specific T cells has been shown to inhibit the response to their cognate peptide in a mouse model (Krampera *et al*, 2003).

We have established simple culture conditions for murine BM that result in the establishment of a homogeneous cell population capable of differentiating along osteogenic and adipogenic pathways and supporting haematopoiesis. This cell population could be expanded for over 50 passages without loosing its differentiation potential, and for this reason is operationally referred to as mMSCs. Flow cytometry analysis using 14 surface markers showed that mMSCs were positive for CD29, CD49e, CD44 and Sca-1. They shared morphologic characteristics with cells in colonies of CFU-F assays, which enabled the evaluation of their frequency in BM by this method.

MATERIALS AND METHODS

Animals. C57Bl/6 and BALB/c adult mice were purchased from LACEN (Porto Alegre RS, Brazil). C57Bl/6 knockout mice, deficient for α-L-iduronidase (*IDUA*-KO), represent a murine model of human mucopolysaccharidosis type I (MPS I). The analysis of the mesenchymal stem cell compartment in this animal model is pertinent as the disease involves pathological alterations of mesenchymal tissues. Furthermore, our group is currently interested in the development of an MSC-based genetic therapy for MPS I. *IDUA*-KO were derived from animals provided by Dr Elizabeth Neufeld (UCLA School of Medicine, CA, USA). The mice were produced by targeted disruption of the murine *IDUA* gene (Ohmi *et al*, 2003). Homozygous mutants were identified at birth by polymerase chain reaction (Clarke *et al*, 1997). The animals were kept in standard conditions, and used in the experiments at 2 to 10 months of age.

mMSC primary culture. Normal culture medium (NM) consisted of Dulbecco's modified Eagle's medium (DMEM; catalogue no. D5523; Sigma Chemical Co., St Louis, MO, USA) containing 10% (v/v) fetal bovine serum (FBS; Cultilab, São Paulo, Brazil) and 0–5·96 g/l HEPES buffer (Gibco BRL, Grand Island, NY, USA). No antibiotics were added to the NM.

Bone marrow was obtained from 8–39-week-old mice. The animals were killed by cervical dislocation and BM was flushed out of tibias and femurs. After washing by centrifugation at $400 \times g$ for 10 min and counting of viable cells with trypan blue in a Neubauer chamber, the cells were resuspended in NM to a final concentration of 5×10^6 viable cells per millilitre. To initiate an mMSC culture, cells were plated in six-well tissue culture dishes (TPP, Trasadingen, Switzerland), at 3·5 ml/well ($1{\cdot}94 \times 10^6$ cells/cm^2). The culture was kept in a humidified 5% CO_2 incubator at 37°C for 72 h, when non-adherent cells were removed by changing the medium.

mMSC subculture. Confluent primary cultures were washed once with Ca^{2+} Mg^{2+}-free Hank's balanced salt solution (Sigma). A 0·25% trypsin solution containing 0·01% ethylenediaminetetraacetic acid (Sigma) was laid onto the monolayer and incubated for 10 min at 37°C. After thorough detachment of the adherent cells using a pipette, cells were resuspended in NM to a final volume of 10·5 ml, and the resulting suspension was split into three new wells. Subsequent passages were performed similarly, but incubation was for 5 min at room temperature (RT) and split ratios were 1:2. By passage 4 or 5, cultures were transferred to 75 cm^2 flasks (TPP). Subculture was performed when cultures reached a minimum of 90% confluence. The split ratio was set to 1:6 at passage 5 or 6, 1:9 at around passage 11 and reached a maximum of 1:24 from passage 28 onwards. Those values were defined empirically to permit subculture to be performed twice a week. Culture medium was changed every 3–4 days.

Morphological analysis and photographs. The mMSC cultures were routinely observed on an inverted phase-contrast microscope (Zeiss, Jena, Germany). For detailed observation, cells were rinsed with phosphate-buffered saline (PBS), fixed with ethanol for 5 min at RT in some cases, and stained for 2·5 min with Giemsa. Photomicrographs were taken with a digital camera (AxioCam; Zeiss) coupled to an inverted microscope (AxioVert; Zeiss), using AxioVision 3.1 software (Zeiss).

Differentiation assays. Osteogenic differentiation was induced by culturing mMSCs for up to 4 weeks in NM supplemented with 10^{-8} mol/l dexamethasone, 5 μg/ml ascorbic acid 2-phosphate and 10 mmol/l β-glycerophosphate (Phinney *et al*, 1999). To observe calcium deposition, cultures were washed once with PBS, and stained for 5 min at RT with Alizarin Red S stain (Nuclear, São Paulo SP, Brazil), pH 4·2. Excess stain was removed by several washes with distilled water.

To induce adipogenic differentiation, mMSCs were cultured for up to 4 weeks in NM supplemented with 10^{-8} mol/l dexamethasone and 5 μg/ml insulin, a slight modification of a previously described protocol (Phinney *et al*, 1999). Adipocytes were easily discerned from the undifferentiated cells by phase-contrast microscopy. To further confirm their identity, cells were fixed with 4% paraformaldehyde in PBS for 1 h at RT, and stained with either Oil Red O (Sigma) solution (three volumes of 3·75% Oil Red O in isopropanol plus two volumes of distilled water) or Sudan Black B (Sigma) solution (three volumes of 2% Sudan Black B in isopropanol plus two volumes of distilled water) for 5 min at RT.

*Haematopoietic support assay.*To determine the capacity of mMSCs to support haematopoiesis, long-term cultures (LTCs) established from C57Bl/6 BM were analysed. Cells were transferred to 24-well plates (TPP) and used on day 10, when they were confluent. Cells were irradiated with 1500 rad using a linear accelerator with 6 MV photons at the Hospital de Clínicas de Porto Alegre (RS, Brazil). One $\times 10^5$ haematopoietic stem cells (HSCs), isolated from 8-week-old C57Bl/6 mice by positive magnetic-activated cell sorting using anti-Sca-1 antibody (Sca-1 Multisort kit;

Miltenyi Biotec, Auburn, CA, USA), were added to four wells of each of the irradiated LTCs and also to four wells containing no adherent layer. The haematopoietic maintenance medium used was Iscove's modified Dulbecco's medium (IMDM; Sigma) containing 2% FBS and 1% antibiotic-antimycotic solution (Gibco BRL). After 7 days of culture with no change in medium, the non-adherent cells were collected and the presence of haematopoietic precursors was determined by the haematopoietic colony-forming unit (CFU-H) assay. For the CFU-H assay, cells were transferred to 0·5 ml semisolid medium which included stem cell factor, interleukin 3 (IL-3), IL-6 and erythropoietin (Methocult GF M3434; StemCell Technologies, Vancouver, Canada). The cells were plated in 24-well plates on a well-to-well basis, and cultivated in a humidified 5% CO_2 incubator at 37°C. Colonies were scored by observation under an inverted microscope, after 6–14 days of culture.

Flow cytometry. Cells were trypsinized, collected and incubated for 30 min at 4°C with phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated antibodies against murine Sca-1, Gr-1, CD11b, CD13, CD18, CD19, CD29, CD31, CD44, CD45, CD49d, CD49e, CD90·2, CD117 and IgG (Pharmingen, San Diego, CA, USA). Excess antibody was removed by washing. Detection of PE and FITC labelling was accomplished on a FACScalibur cytometer equipped with 488 nm argon laser (Becton Dickinson, San Diego, CA, USA) using CellQuest software. At least 10 000 events were collected. WinMDI 2.8 software was used to create the histograms.

CFU-F assay. The CFU-F assay was performed using a modification of a previously described protocol (Castro-Malaspina *et al*, 1980). Cells obtained from fresh BM were resuspended in NM to a concentration of $2{\cdot}25 \times 10^5$ viable cells/ml. Two millilitre of this cell suspension were dispensed into each well of a six-well plate ($4{\cdot}98 \times 10^4$ cells/cm^2). Medium was changed on days 3 and 8 of culture.

On the 13th day, cultures were fixed and stained with Giemsa. The number of colonies displaying five or more cells was scored under an inverted microscope. Colonies with four cells were counted when one of them presented two nuclei. Colonies whose morphology clearly differed from the mMSC morphology were excluded from the results.

RESULTS

mMSC long-term culture

Pilot CFU-F assays using different cell concentrations ranging from 1×10^3 to 5×10^5 cells per 2 ml (approximately $1{\cdot}11 \times 10^2$ to $5{\cdot}54 \times 10^4$ cells/cm^2) demonstrated that a maximal number of non-overlapping colonies was reached using $4{\cdot}5 \times 10^5$ BM cells in 2 ml of NM per 9·03 cm^2 (approximately 5×10^4 cells/cm^2). In higher cell densities colonies overlapped and haematopoietic contamination was higher, while a lower cell density rendered proportionally lower CFU-F counts (data not shown), an indication that the initial cell density had striking effects on the growth of CFU-F.

While still determining the conditions for the establishment of LTCs, the media used were α-minimal essential medium (α-MEM; Sigma), IMDM (Sigma), MesenCult (StemCell Technologies) and DMEM containing 10% FBS, which was established as the most appropriate for mMSC growth. Alkalinization observed during the handling of DMEM seemed to be a disadvantage for the mMSC, so that NM pH was adjusted by the addition of HEPES buffer to a maximum of 5·96 g/l. The concentration of choice was 3·7 g/l, which rendered a final pH of around 7·3.

The initial conditions tested included incubation times of 1, 4 and 72 h before the removal of non-adherent cells, and the latter was chosen for providing more confluent or near-to-confluence primary cultures in a shorter period of time. Non-adherent cells were usually transferred to new plates. Such cultures consisted mainly of haematopoietic cells, although colonies exhibiting the characteristic mMSC morphology were also present at a low frequency (not shown). As the work focused on the BM plastic-adherent cells, the non-adherent fraction was not studied in further detail.

One of the most important factors to determine the establishment of the LTC of MSCs was the density of the starting culture. We initially used 2·5 ml of a 1×10^6/ml cell suspension per well ($2{\cdot}77 \times 10^5$ cells/cm^2), which resulted in primary cultures that failed to reach confluence, and could not be expanded through subculture. The cell concentration was then raised to 5×10^6/ml using the same plating volume (i.e. $1{\cdot}38 \times 10^6$ cells/cm^2), providing confluent primary cultures with low proliferative capacity. When cultures were initiated with 3·5 ml of a 5×10^6/ml cell suspension per well ($1{\cdot}94 \times 10^6$ cells/cm^2), LTCs could be established. Lower plating volumes with the same cell concentration (i.e. lower plating densities) did not provide the same result, rendering primary cultures which generally did not reach confluence, as depicted in Fig 1. Once a confluent primary culture was obtained, the split ratios exerted great influence on the LTC. When diluted at a ratio greater than 1:3 during the first passage, the establishment of the LTC was impaired, and hence after the first passage the split ratios were set to 1:2 until the culture kinetics indicated that higher ratios could be used, which occurred by the fourth or fifth passage.

Under the conditions described above, cultures usually remained morphologically highly heterogeneous until passage 3 or 4, presenting round, spindle-shaped and flattened cells (Fig 1). The proportion of flattened cells in relation to the other distinct cell morphologies observed increased gradually with time, and usually some degree of morphological heterogeneity could still be observed up to passages 8–9. From then on, the flattened cell type was predominantly observed and cultures could be split at higher ratios. The absence of haematopoietic precursors was shown by the fact that mMSCs from two of the cultures (LTC1 and LTC2) at passage 22 and 16, respectively, did not produce any observable haematopoietic colonies when subjected to CFU-H assay (see below).

Five LTCs were analysed in greater detail. The morphology of the MSCs was very similar among all cultures (Fig 2). mMSCs are flat, plastic-adherent cells, with a size of around 90 μm. They present a nucleus of approximately 30 μm

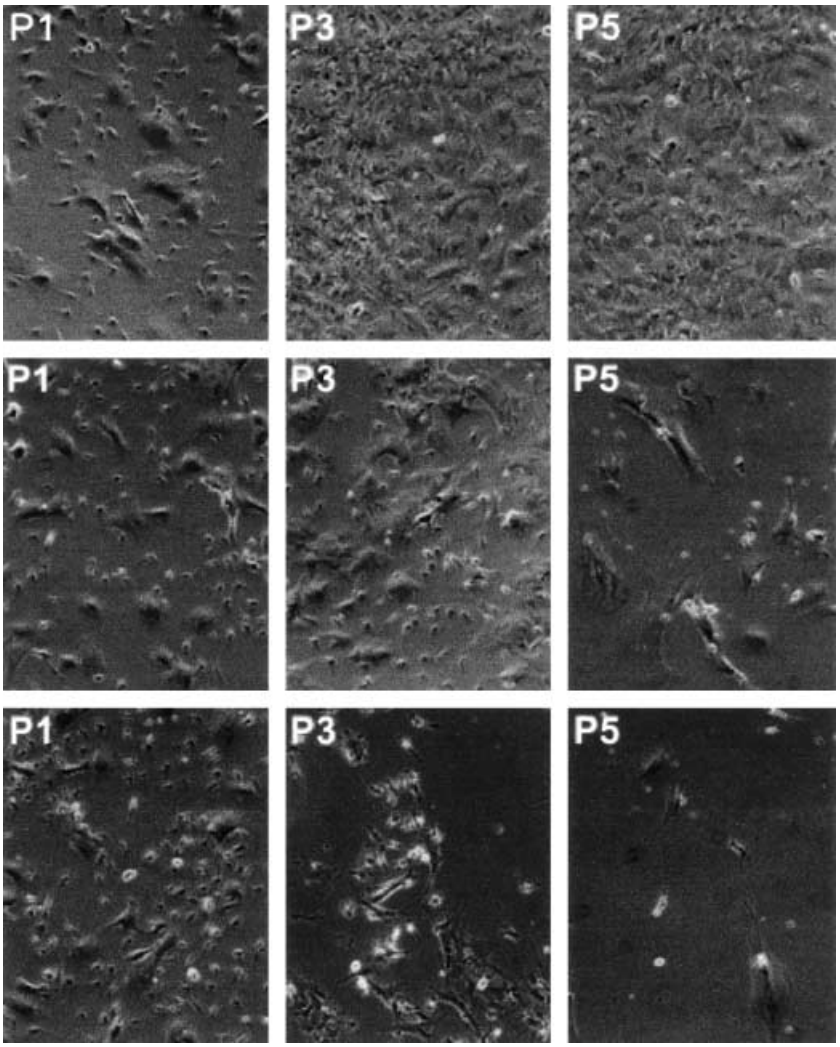

Fig 1. Phase contrast images of three different cultures established from the bone marrow of a C57Bl/6 mouse, at passages 1, 3 and 5. The cultures were initiated from 3·5 ml (top row), 2 ml (middle row) and 0·8 ml (bottom row) of a 5×10^6 nucleated marrow cell/ml suspension, and kept in 3·5 ml of normal medium from the first medium change onwards. Passages of the three cultures were performed at the same time, at dilution rates of 1:3 on the first passage and 1:2 from then onwards. Cells in the cultures shown on the middle and bottom rows failed to thrive at passage 4, while those shown on the upper row established a LTC. Original magnification: ×100 for all images.

diameter, with characteristic scattered granules. Cryopreservation of cells from one of the cultures (LTC2) at passage 4, followed by thawing and cultivation, resulted in cultures with the same morphological and functional characteristics of cells being grown continually.

mMSC growth kinetics

Confluence was usually reached 6 or 7 days after beginning of the cultures, under the conditions defined in this work. The interval between passages varied greatly until passage 10, and from then on was established at around 4 days. Cell growth was continuous for all cultures (Fig 3). LTC1 showed an increasing growth rate that reached a stable value after about 50 days in culture, while the growth rate of LTC2 seemed to become stable about 70 days after thawing. LTC3 reached the maximal growth rate among the cultures, after about 110 days of culture, and LTC4 exhibited a stable growth rate after passage 5, by the 35th day of culture. LTC5 showed a somewhat extended initial lag phase, entering a log phase by 90 days from the onset of the culture.

Differentiation of mMSCs

Three of the cultures were tested for their ability to differentiate into other cell types, at different time points starting at passage 8. When subjected to osteogenic and adipogenic media, mMSCs differentiated into osteoblasts (Fig 4A) and adipocytes (Fig 4B). mMSC LTC1 differed from the other LTCs in that osteogenic differentiation was accompanied by adipogenic differentiation. In addition, spontaneous adipogenic differentiation was observed when mMSCs were kept confluent for extended periods.

mMSCs haematopoietic support

The ability of mMSCs to support haematopoiesis was assessed through co-culture with Sca-1^+ HSCs for 1 week. Despite the short period of co-culture, non-adherent horizontal haematopoietic clusters were present in direct contact with the mMSCs (not shown). The results are summarized in Table I. A small number of mMSCs cultured for 2 days immediately after passage 29, which exhibited 30% confluence, were shown to nurse as many HSCs as a stromal monolayer derived from BM primary culture.

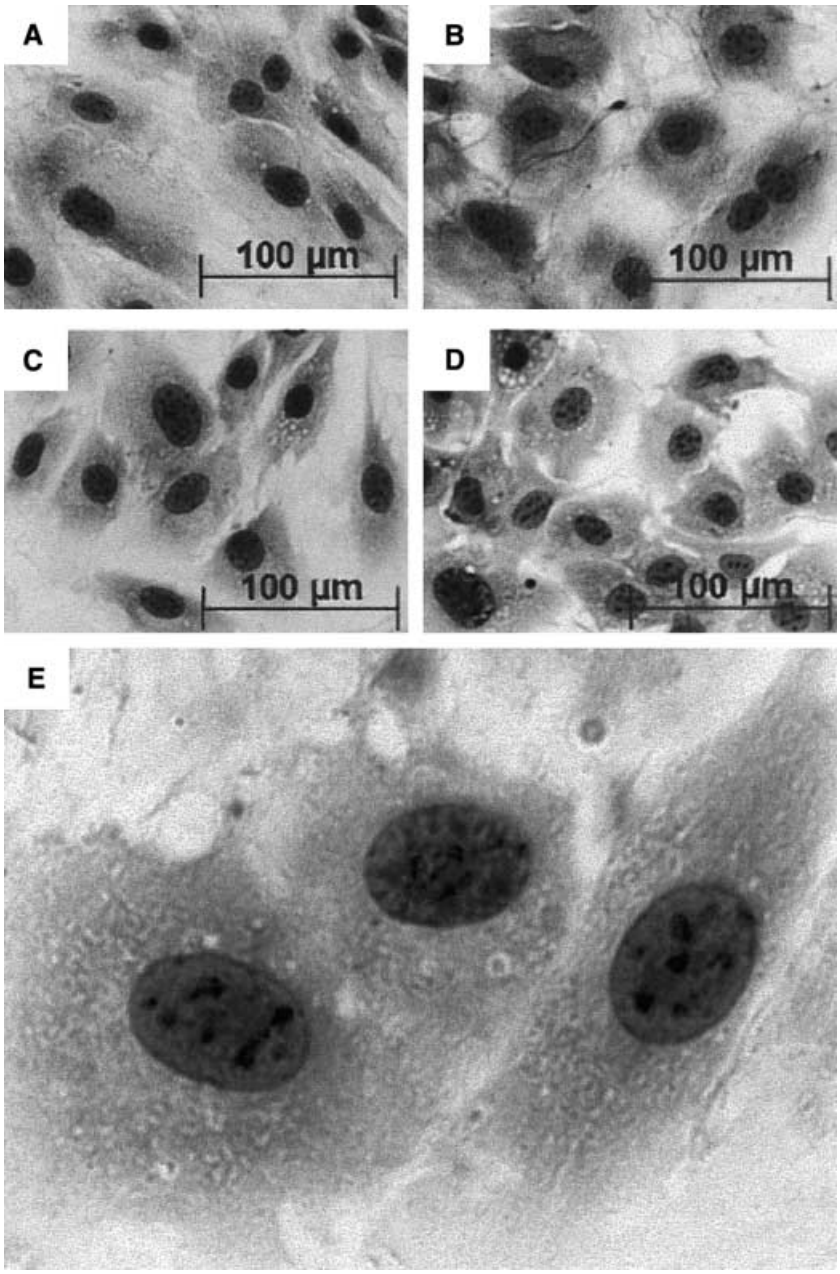

Fig 2. mMSC Morphology. LTCs were generated from BM of two normal (A, C and E) and two *IDUA*-KO C57Bl/6 animals (B and D). mMSCs are shown after passage 19 (A), 8 (B), 39 (C, E) and 14 (D), stained with Giemsa. Original magnification: (A–D)s ×100; (E) ×400.

Confluent monolayers, derived from LTC2 at passage 9 and LTC1 at passage 26, were much more efficient at keeping HSCs alive, so that CFU-H assays presented too many colonies to be precisely counted. The colonies observed included CFU granulocyte–macrophage, CFU granulocyte, CFU granulocyte–erythrocyte–monocyte–megakaryocyte and burst-forming-unit erythroid.

Flow cytometry

The morphological homogeneity of the cultures after around 10 passages was also apparent on the flow cytometric analyses. After several passages in culture, mMSCs exhibited a typical forward scatter (FSC-H) × side scatter (SSC-H) plot (Fig 5A).

The expression of LTC3 was checked at passages 6, 10 and 30 (Fig 5B). Cells that expressed CD11b at the sixth passage did not persist in the culture, as they could not be detected in passages 10 and 30. These data indicate that after 10 passages, mMSC cultures can be described as free from terminally differentiated haematopoietic cells. This is in agreement with the results concerning the morphological development of LTCs, which indicate that homogeneity of the cell population is achieved around passages 8–9.

The reproducibility of the methodology was also apparent when surface markers were analysed at different time points. From passage 10 onwards, different cultures exhibited similar surface marker and SSC × FSC profiles.

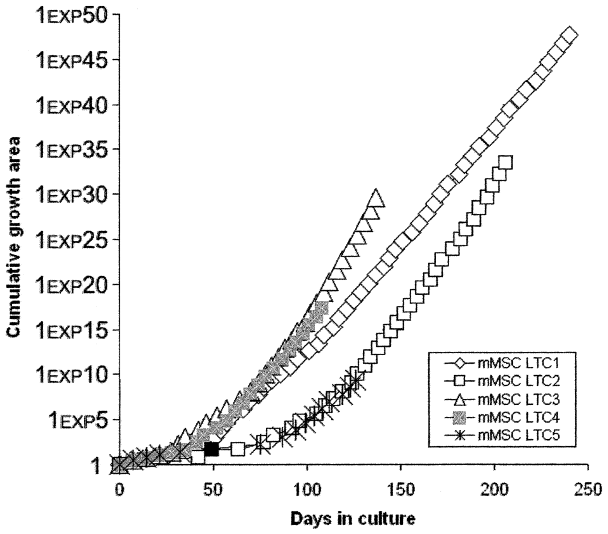

Fig 3. Growth kinetics of mMSC LTCs. The value 1 was substituted for the growth area (GA) occupied by a primary mMSC culture, which corresponded to 9·03 cm^2, as a matter of simplification. When the second passage took place, the split ratio at passage 1 (1:3) was multiplied by that value, meaning that at the end of passage 1 the cumulative GA was 3 (i.e. three times the growth area occupied by a primary culture). At the end of the second passage, the split ratio at passage 2 (1:2) was multiplied by the cumulative GA at passage 1 (3), giving the cumulative GA a value of 6 at passage 2. This procedure was repeated for each passage, providing a theoretical growth curve that is directly proportional to the cell number. The black box in the LTC2 curve indicates the fourth passage, which involved cryopreservation of the cells for a week, thawing and transfer of the whole cell number to a new flask of same size. LTC1 and LTC2: C57Bl/6, normal, male, 10 and 8 weeks old respectively; LTC3 and LTC4: C57Bl/6, *IDUA*-KO, female 39 weeks old and male 21 weeks old respectively; LTC5: BALB/c, normal, female, 8 weeks old.

Fig 5C shows representative surface profiles, for mMSC LTCs 1, 2 and 3. LTCs were homogeneously positive for CD29, CD44 and CD49e, and negative for CD11b, CD13, CD18, CD31, CD45, CD49d, CD19, surface immunoglobulin and Gr-1 (not shown). mMSC LTC2 differed from the others in that it expressed a low level of CD117, which became lower as the culture progressed (not shown). In turn, LTC1 was the only culture to express CD90·2 (Fig 5D). Sca-1 expression varied among the LTCs, exhibiting a tendency to increase with higher number of passages in culture. LTC2 presented two subpopulations regarding the expression of Sca-1. A more detailed observation of LTC2 showed that among the population observed in the characteristic mMSC FSC × SSC plot, cells with larger size and higher granularity expressed higher Sca-1 levels. These data suggest that the expression of some molecules such as Sca-1 by mMSCs may be influenced by cell cycle. On the contrary, observation of cell clusters by fluorescence microscopy after detachment by trypsin digestion showed that Sca-1 was preferentially located at cell–cell contact zones (not shown), suggesting that this molecule was trypsin sensitive.

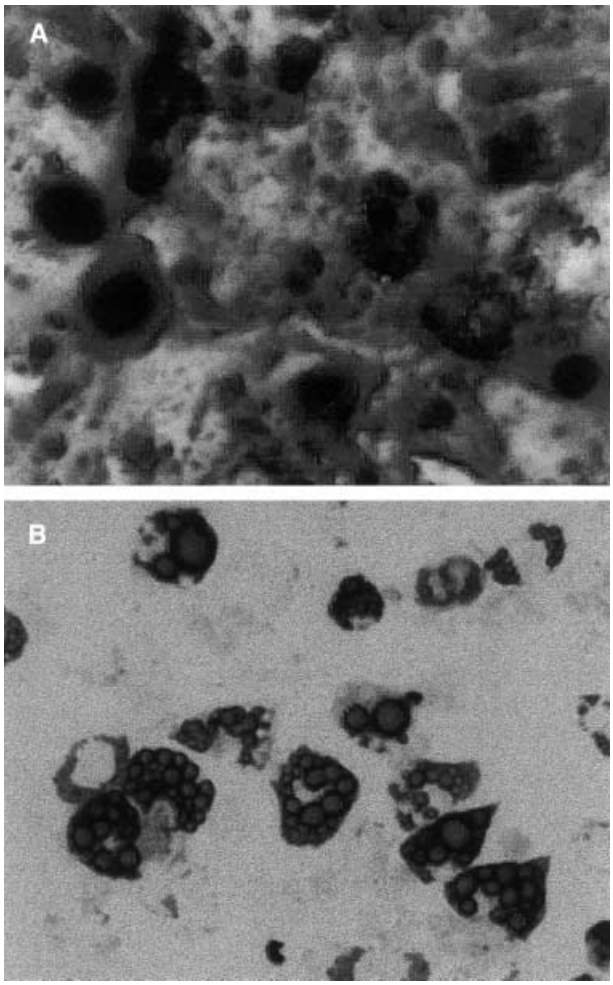

Fig 4. Differentiation of mMSCs. Eighth passage cells from mMSC LTC3 subjected to osteogenic differentiation, stained with Alizarin Red S (A). Induced adipogenic differentiation of 33rd passage cells from LTC1 is evidenced by Oil Red O-stained fat vacuoles (B). Original magnification: ×200.

Estimation of the mMSC frequency in BM

The morphological similarity among mMSC LTC cells and colonies observed in CFU-F assays suggested the use of this assay to evaluate the frequency of the mMSC in fresh BM. Counting of colonies was performed after a period of 13 days, and excluded isolated adherent cells that exhibited a typical mMSC morphology, as one of the criteria to define such a cell type is the capacity to proliferate. Hence, colonies with at least five mMSC-like cells, and occasionally colonies with four cells in which one was binucleated, were considered.

Thirteen individuals were analysed, and there was great variation in the number of colonies detected in CFU-F assays (Table II). By the time the CFU-F assays were performed, only two *IDUA*-KO mice were available. As the data did not fit the requirements for the use of parametric tests, comparison among groups was performed using Kruskal–Wallis independent samples test, which showed that no

Table I. Haematopoietic support provided by mMSCs. 1×10^5 Sca-1^+ cells were cultured for 7 days in the presence or absence of an adherent layer formed by cells from LTC1, LTC2 or BM stroma cultured for 5 days. After co-culture, cells were transferred to semi-solid medium containing haematopoietic growth factors, and haematopoietic colonies were enumerated 6–14 days later. CFU-H assay revealed that the number of CFU-H per 1×10^3 Sca-1^+-enriched cells was 6·5 ± 0·75 (mean ± SEM).

Adherent layer	Number of haematopoietic colonies after 7 days of co-culture (mean ± SEM)
None	0
Confluent monolayer formed by BM cultured in standard conditions for 5 days	9·0 ± 1·68*
30% Confluent LTC1 cells at passage 29	14·5 ± 2·18*
Confluent monolayer of LTC2 cells at passage 9	>20
Confluent monolayer of LTC1 cells at passage 26	>25

*Not statistically significant. Student's *t*-test, $P = 0{\cdot}1843$.

noticeable differences were present. Nevertheless, we observed a tendency for the groups to exhibit differences related to age, for multivariate analysis showed that the highest values were generally accompanied with higher ages. Future studies using larger sample sizes are necessary to elucidate the factors that influence the number of CFU-Fs in BM. As a preliminary estimate for the frequency of MSC in adult murine BM, all mean values were grouped, resulting in a value of 28·218 ± 11·589 MSCs per $4{\cdot}5 \times 10^5$ nucleated cells (95% confidence interval), which corresponded to one mMSC per 11 300–27 000 BM cells.

DISCUSSION

The cell populations described in this work presented characteristics that enabled their classification as MSCs in an operational way. They sustain prolonged self-renewal, maintaining homogeneous characteristics for over 50 passages (over 8 months), can differentiate along osteogenic and adipogenic pathways, and give support to HSCs. Enhanced haematopoietic support by human MSC with the addition of growth factors has been previously demonstrated by Kadereit *et al* (2002) using a 12-day protocol that resulted in the expansion of $CD34^+$ $CD38^-$ HLA-DR^- progenitors with maintenance of high levels of p21 (a cell

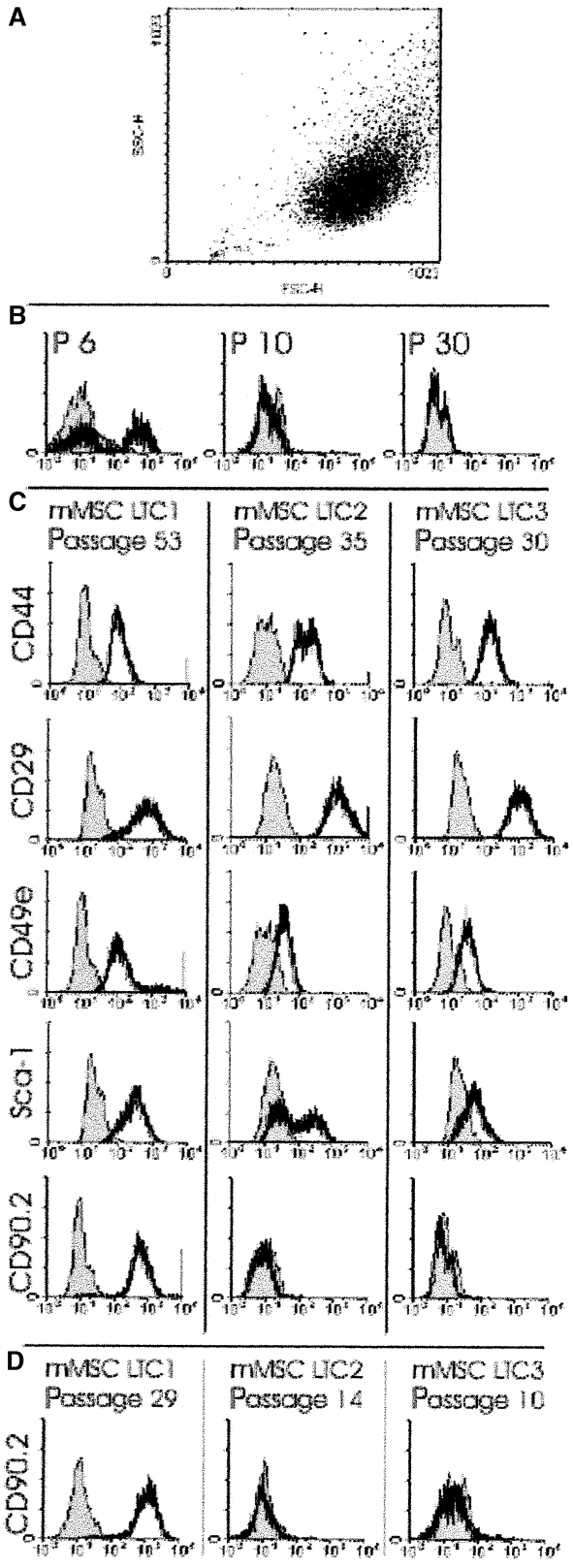

Fig 5. Expression of surface markers by mMSCs. Characteristic homogeneous FSC-H × SSC-H plot exhibited by mMSC, as exemplified by 53rd passage LTC1 cells (A). Cells expressing CD11b at passage 6 were not observed from passage 10 onwards (B). Cells from LTCs 1–3 were analysed during passages 53, 35 and 30, respectively. Histograms demonstrating the expression of surface molecules were plotted against controls (anti-CD19 and anti-IgG, shaded) (C). Expression of CD90·2 on LTC1, but not on LTC2 and LTC3 cells, was not dependent on the number of passages (D).

Table II. Number of colonies observed in CFU-F assays per $4{\cdot}5 \times 10^5$ viable BM cells from 13 individuals.

Age (weeks)	Genotype	Gender	Strain	CFU-F number (mean ± SEM)
21	*IDUA*-KO	Male	C57Bl/6	53·50 ± 2·45
21	*IDUA*-KO	Male	C57Bl/6	23·17 ± 1·30
21	Normal	Male	C57Bl/6	47·50 ± 2·17
12	Normal	Male	C57Bl/6	18·83 ± 1·33
12	Normal	Male	C57Bl/6	13·00 ± 1·00
8	Normal	Male	C57Bl/6	63·00 ± 2·77
8	Normal	Male	C57Bl/6	54·83 ± 0·79
8	Normal	Female	C57Bl/6	23·33 ± 2·26
8	Normal	Female	C57Bl/6	8·67 ± 0·99
8	Normal	Female	C57Bl/6	14·33 ± 1·76
8	Normal	Male	BALB/c	21·67 ± 1·17
8	Normal	Male	BALB/c	13·83 ± 1·47
8	Normal	Male	BALB/c	11·17 ± 1·17

cycle inhibitor) and BCL-2 (an antiapoptotic protein). Haematopoietic support provided by the MSC in the absence of exogenous growth factors observed in the present study and in others (Dormandy *et al*, 2001; Majumdar *et al*, 1998), evidence the role played by such a cell type in the control and regulation of haematopoiesis. Adipogenic differentiation after so many passages is noteworthy, as other groups have reported the loss of MSC adipogenic differentiation capacity after extended subculture (Quirici *et al*, 2002; Muraglia *et al*, 2000). Although not formally tested, the possibility that prolonged maintenance of the cells in the present study is the result of viral transformation is unlikely, as the results were reproducible even using animals derived from different stocks. When these cells were transferred to siliconized glass flasks at routine subculture dilutions, they failed to proliferate (not shown), which indicates anchorage-dependent growth and suggests that no transformation processes took place during the LTC.

This is, to our knowledge, the first report of prolonged *in vitro* expansion of murine MSCs from total BM using standard tissue culture techniques. The postulate that, given the appropriate conditions, a stem cell will remain and proliferate in culture while more differentiated cells will be lost directed the experiments. The results showed that some of the adherent cells from BM could win the competition against terminally differentiated cells in LTC. In addition, we found that the initial culture conditions were critical for the establishment of the LTC. In particular, the starting cell density and the initial split ratios were shown to have a dramatic effect on the expansion capability of mMSCs.

The growth kinetics differences observed among the LTCs were a consequence of intrinsic and extrinsic factors. The former relates to the inherent characteristics of each mMSC LTC population, while the latter includes slight differences regarding the culture conditions among the different LTCs (e.g. medium pH or trypsinization effectiveness) and the human factor, which determined when and how cultures would be passaged. Hence, the extended time for reaching the maximal growth rate required by LTC3 in relation to the other LTCs may be attributed to the great resistance to increase split ratios for the first *IDUA-KO* mMSC LTC, as only a few *IDUA-KO* animals were available by the time the study was performed, and that LTC should not be lost.

Immunophenotyping of the five LTCs showed that the surface marker profile of mMSCs is compatible with that of murine BM stromal cells (Wieczorek *et al*, 2003), murine stromal cell lineages (Charbord *et al*, 2002) and human MSCs (reviewed by Deans & Moseley, 2000). Little difference was observed among the cultures. In particular, LTC2 presented two subpopulations regarding Sca-1 expression, suggesting that the expression of that marker is influenced by cell cycle, or that Sca-1 is trypsin-sensitive. Other molecules used in this work (CD44 and CD49d) have been show to be sensitive to treatment with trypsin (Nolte *et al*, 2002), and we attributed the CD44 apparent low fluorescence intensity, shown in Fig 5, to this condition. The other dissimilarity found was the expression of CD90·2 by mMSC LTC1. Expression of CD90·2 by one of the five mMSC LTCs analysed, which in other aspects (culture kinetics, differentiation patterns) did not differ from the other cultures, probably reflects the existence of phenotypic (and functional?) heterogeneity within the BM MSC compartment, as has been shown for HSCs (Pranke *et al*, 2001). Consequently, the expression of CD90 as an mMSC subpopulation determinant remains to be studied in detail. Recently, murine MSCs isolated with a different methodology were reported, which also presented a similar surface profile (Baddoo *et al*, 2003).

The finding that mMSCs had a distinctive morphology was surprising and useful to drive the search for the best conditions for their expansion, after the first LTC was established. Cells with this morphology have already been described in the murine BM (Friedenstein *et al*, 1976), but not in strict association with the MSC phenotype. One direct consequence of that was the comparison with colonies observed in CFU-F assays. The results of CFU-F

assays (Table II) were used to estimate that one mMSC can be found in a range of 11 300–27 000 BM cells from adult mice, consistent with other data from the literature (Wu *et al*, 2000). No differences between C57Bl/6 and BALB/c mice CFU-F frequency could be detected. This is in contrast to the results obtained by Phinney *et al* (1999), which reported great CFU-F frequency variation among different mouse strains. In fact, the culture conditions and the criteria for defining CFU-F colonies used in the present study are different. Phinney *et al* (1999) used high plating densities in α-MEM to obtain CFU-Fs, which were defined by size (larger than 2 mm in diameter) and positive staining by Giemsa, while the CFU-F assay used in the present work included low plating densities in DMEM in addition to morphological analysis by Giemsa staining. Under these conditions, haematopoietic contamination and colony overlapping were greatly reduced. Thus, we believe that the differences in CFU-F frequency among different mouse strains found previously (Phinney *et al*, 1999) are because of the low resolution of the CFU-F assay used and the haematopoietic contamination resulting from the high plating density, which may exert negative effects on the mMSC and can vary among mouse strains.

Mucopolysaccharidosis type I (MPS I) is an autosomal recessive disease resulting from deficiency of the lysosomal enzyme IDUA, and *IDUA*-KO animals have been reported to show phenotypic features similar to severe MPS I in humans (Russell *et al*, 1998). No obvious differences could be observed among mMSCs generated from both normal and *IDUA*-KO animals. BM from normal and *IDUA*-KO mice also does not seem to present differences in MSC frequency. This indicates that the murine model of MPS I would be adequate for future experiments aiming to correct IDUA levels through cellular or genetic therapy mediated by mMSCs, as the pathology is not expected to affect the normal conditions of these cells *in vivo*.

The *in vitro* expansion and the characterization of mMSCs have important implications for the development of basic research and therapeutic strategies such as cellular and genetic therapy using murine models. Procedures that require large amounts of defined mMSCs such as *in vivo* administration shall be performed in the near future, and experiments that require a highly purified mMSC population, such as the assessment of the growth factors and surface molecules involved. The factors influencing the prolonged self-renewal and the differentiation of mMSCs remain to be investigated, and further studies are necessary to explore the full potential of these cells.

ACKNOWLEDGMENTS

We would like to thank Lizandra Nied, MD, for irradiating the adherent cell layers, Dr Sidia Callegari-Jacques for providing assistance with statistical analyses, and Dr Katia Kvitko and Daniel Garcia dos Santos for identifying the *IDUA*-KO mice by PCR. This work was supported by FAPERGS, CAPES and CNPq.

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