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Mesenchymal stem cells are present in peripheral blood and can engraft after allogeneic hematopoietic stem cell transplantation

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A B S T R A C T

Background and Objectives. Whether human mesenchymal stem cells (MSC) can be transplanted is controversial and their presence in peripheral blood is not fully accepted. In the present study we have analyzed whether, within the allogeneic transplantation setting, MSC are of host or donor origin.

Design and Methods. Bone marrow MSC from 19 patients who had undergone allogeneic transplantation were expanded and identified using immunophenotypic markers. After that, chimerism studies were performed using reverse transcription polymerase chain reaction of short tandem repeat (STR) loci. Analyses were carried out at different time-points after transplantation, with a total of 44 samples studied. Bone marrow was used as the source of stem cells for transplantation in 4 cases and peripheral blood in 15 cases. The conditioning regimen was standard in 9 patients and non-myeloablative in 10 patients.

Results. Our results show that in the great majority of cases analyzed (17 out 19), MSC were of host origin. However, in 2 patients with multiple myeloma who had received a reduced intensity transplantation using peripheral blood stem cells, MSC were partially of donor origin (60.17% and 26.13% of total MSC).

Interpretation and Conclusions. These findings indicate that after allogeneic transplantation MSC from the donor can engraft in bone marrow. Moreover, since the stem cells were obtained from peripheral blood, it can be concluded that MSC circulate among mobilized peripheral blood stem cells and can engraft in bone marrow after allogeneic transplantation.

Key words: mesenchymal cells, chimerism, allogeneic hematopoietic stem cell transplantation.

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The bone marrow stroma is a heterogeneous mixture of cells and extracellular matrix which provide both physical support and growth factors, as well as cytokines or chemokines for the regulation of hematopoiesis. These cells come from an immature progenitor cell termed the mesenchymal stem cell (MSC).¹⁻³ Under specific *in vitro* conditions, MSC can give rise to bone,⁴ cartilage,^{5,6} adipose tissue⁷ or skeletal muscle.^{8,9}

Although in some animal models it has been shown that MSC can be transferred within the allogeneic transplantation setting,¹⁰⁻¹³ the transplantability of MSC remains controversial in humans. Moreover, while it has been claimed that there are mesenchymal precursors in the peripheral blood (PB) of normal individuals,¹⁴⁻¹⁶ these results have not been confirmed by other authors¹⁷ and the transplantability of cir-

culating MSC has not been previously investigated. The main goal of the present study was to investigate whether the MSC are of donor or host origin in the context of hematopoietic allogeneic transplantation. Concomitantly, we wanted to analyze the presence of MSC in the peripheral blood progenitor stem cell graft. For this purpose, bone marrow MSC from patients who had been allo-transplanted were isolated, cultured and characterized, and chimerism studies were performed.

Design and Methods

Isolation and ex vivo MSC culture

MSC were isolated, expanded and immunophenotypically characterized from bone marrow (BM) cells of 5 healthy donors and 19 patients who had previously received a

hematopoietic progenitor cell allogeneic transplantation from an HLA-identical sibling. Patients had the following diagnoses: chronic myeloid leukemia (n=4), Hodgkin's disease (n=2), acute lymphoblastic leukemia (n=5), chronic lymphocytic leukemia (n=1), acute myeloid leukemia (n=3), multiple myeloma (n=3) and aplastic anemia (n=1). Conditioning regimens were standard (cyclophosphamide + total body irradiation or cyclophosphamide + busulphan) in 9 cases, and reduced intensity (RIC) (fludarabine + busulphan or fludarabine + melphalan) in the remaining 10 patients.¹⁸ The source of stem cells was PB and BM in 16 and 3 patients, respectively. The patients' characteristics are shown in Table 1. A total of 45 BM samples were analyzed at different time-points after transplantation (from day +28 until day +910). The BM grafts were collected from the posterior iliac crest under local anesthesia according to standard institutional procedures. In all cases BM samples were obtained after informed consent, according to the Ethical Committee of the University Hospital of Salamanca (Spain).

MSC were obtained using the method previously reported by Minguell *et al.*¹ Briefly, low density mononuclear cells (MNC) from BM were separated with a Ficoll-Paque (Seromed® Biochrom KG) gradient and plated for adherence to a plastic surface (3–5 days) in culture medium (DMEM; Gibco) containing 10% fetal calf serum (Biowhittaker). Twice a week, adherent cells were fed by complete replacement of the medium. When the layer was confluent, the culture was trypsinized and cells were then seeded in a 25 cm² flask until confluency. The process was repeated at least once in order to obtain a sufficient number of cells for analysis. In order to avoid any hematopoietic contamination, CD45 depletion was performed prior to immunophenotypic or chimerism studies. After trypsinization, the CD45 cells were removed using MACS MicroBeads (CD45 MicroBeads; Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's recommendations. Briefly, MSC were incubated for 15 minutes at 6°C with CD45 antibody (mouse anti-human CD45). After washing with phosphate-buffered saline (PBS)-0.5% bovine serum albumin, labeled cells were loaded onto a column installed within a magnetic field using an immunomagnetic separator (AutoMACS, Miltenyi Biotec, Bergisch Gladbach, Germany).

Differentiation and phenotype of MSC

Adipogenic and osteogenic differentiation was induced as previously described.¹⁹ Adipogenesis was measured by the accumulation of neutral lipids in fat vacuoles, stained with oil-red-O. For osteogenic characterization, specimens were stained for alkaline phosphatase for morphological examination. For flow cytometry, detached cells were washed and re-suspended in

Table 1. Characteristics of the patients and their transplantations.

Diagnosis	n	Conditioning regimen	Cell source	MSC Chim
		Stand./RIC	BM/PB	H/mix/donor
CML	4	3/1	3/1	4/0
AL	8	4/4	0/8	7/0
HD	2	0/2	0/2	2/0
CLL	1	0/1	0/1	1/0
AA	1	1/0	1/0	1/0
MM	3	0/3	0/3	1/2

CML: chronic myeloid leukemia; AL: acute leukemia; CLL: chronic lymphoid leukemia; AA: aplastic anemia; MM: multiple myeloma; Conditioning Stand: standard conditioning; RIC: reduced intensity conditioning; MSCChim: mesenchymal stem cells chimerism Mix: mixed chimerism. H: host cells. Data expressed as number of patients.

PBS. Cells were incubated for 15 minutes with conjugated monoclonal antibodies against CD105, CD34, CD45, CD56, HLA-DR, CD54, CD62L, CD106, CD104, CD90, CD49b and CD133 (Miltenyi Biotec). Cells were then washed and resuspended in PBS.

Phenotypic characterization was performed using the following monoclonal antibody combinations (FITC/PE/PerCP/APC): CD105/CD56/CD45/CD34; CD90/CD133/CD45/CD34; CD90/CD104/CD45/-; CD90/CD106/CD45/CD34; CD54/CD62L/CD45/-; CD49b/CD106/CD45/CD34; CD90/-/HLA-DR/CD34. Data acquisition was performed in a FACScalibur flow cytometer (Becton Dickinson Biosciences; BDB) and the analysis performed with the Paint-A-Gate program (BDB) as previously described.²⁰

Chimerism studies

Chimerism was analyzed both in BM and PB samples. For investigation of chimerism in BM MSC, the samples had been depleted of CD45⁺ cells as described above. The chimerism status of unfractionated hematopoietic BM cells was also analyzed. In those patients receiving RIC transplants, chimerism studies were also carried out on separated CD15⁺ and CD3⁺ cells from PB. The CD3 and CD15 cells were selected using MACS Microbeads (CD3 MicroBeads and CD15 MicroBeads; Miltenyi Biotec) following the manufacturer's recommendations, and using the AUTOMACS device (Miltenyi Biotec). Chimerism studies were performed with a commercially available automated kit (PowerPlex® 16 System; Promega Corporation; USA) with semiautomatic electrophoresis (ABI Prism 377 DNA Sequencer, Applied Biosystems, Foster City, CA, USA). The kit uses multiplex amplification of fifteen short tandem repeat (STR) loci (Penta E, D18S51, D21S11, TH01, D3S1358, FGA, TPOX, D8S1179, vWA, Penta D, CSF1PO, D16S539, D7S820, D13S317 and D5S818) and Amelogenin.

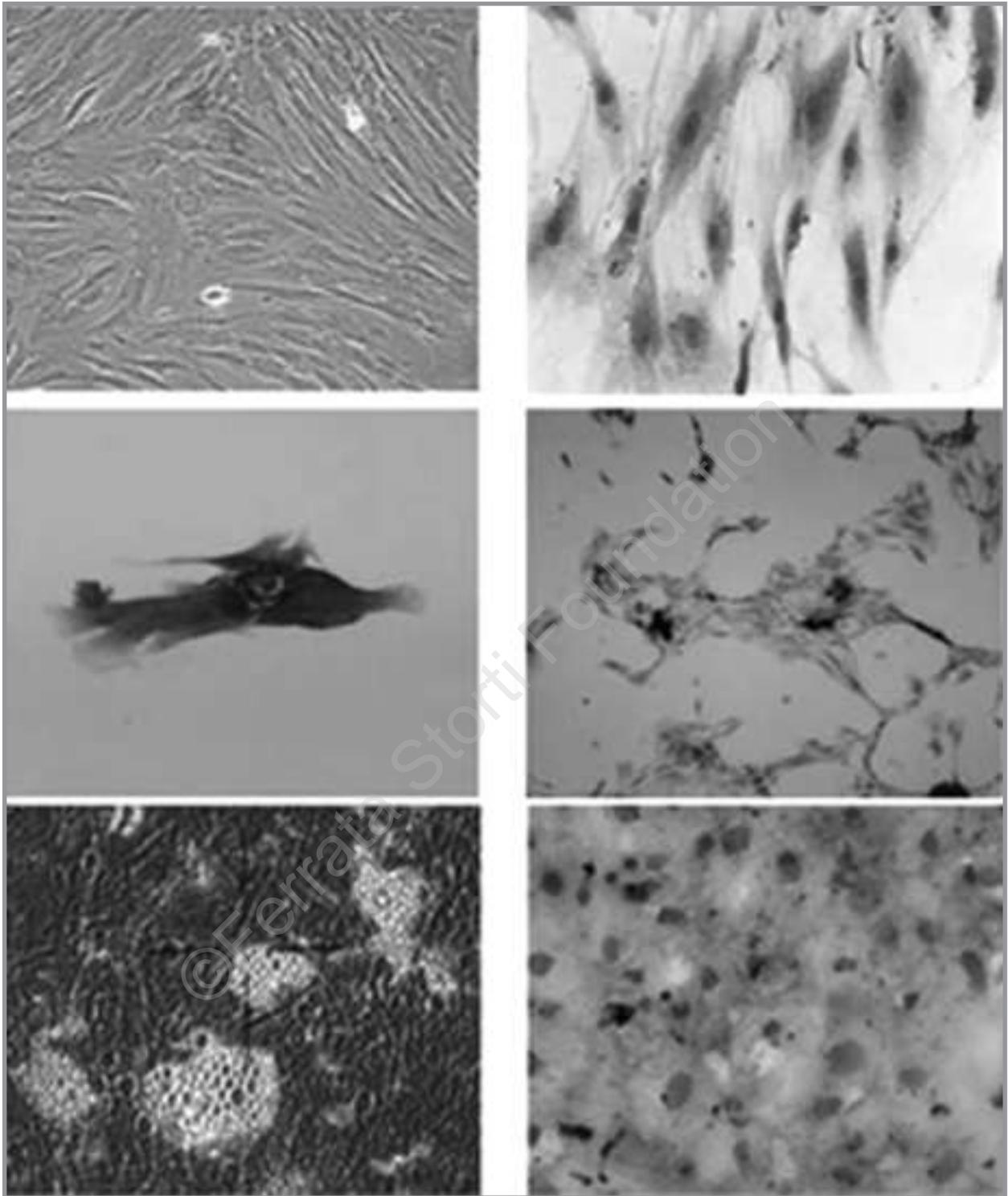


Figure 1. Morphology and differentiation potential of MSC *in vitro*. **A.** A typical light microscopic view of MSC in culture. **B.** Light microscopic view of MSC counterstained with May-Grünwald Giemsa. **C and D.** Immunochemical staining showing cells expressing alkaline phosphatase under osteoinductive conditions. **E.** Light microscopic view of adipocytes in MSCs culture. **F.** Lipid-containing adipocytes were also detected by Oil-red-O staining under adipogenic induction.

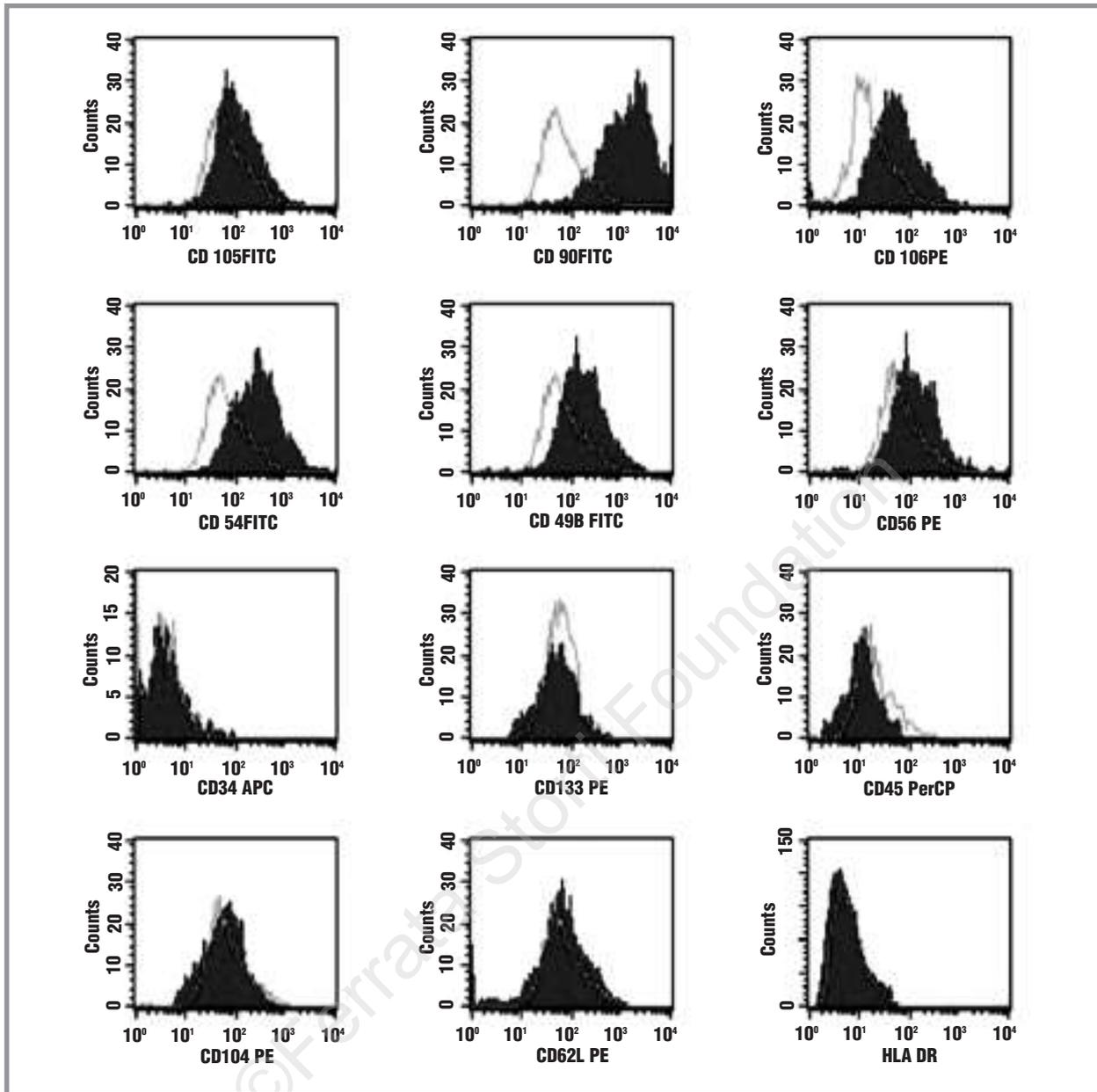


Figure 2. Representative immunophenotype of *in vitro* expanded undifferentiated MSC. Gray line: control. Black line: specific antibody.

Results

Isolation and *ex vivo* culture of MSC

Adherent MSC could be expanded in BM cultures from all but two of the patients analyzed, who were subsequently excluded from the present analysis. After 3-4 passages, cells with fibroblastic appearance reached confluence (Figure 1).

Phenotype and differentiation of MSC

Flow cytometry analyses demonstrated that MSC expressed CD90, CD49^{low}, CD106, CD54^{low}, CD105

(endoglin) and CD56^{low} in all cases, but were negative for CD34, CD45, CD133, CD62L, CD104 and HLA-DR antigens (Figure 2). To establish the pluripotency of bone marrow-derived MSC, these cells were induced to differentiate into osteoblasts and adipocytes. In all cases studied, positive results were obtained for alkaline phosphatase and adipocytes (Figure 1).

Chimerism studies

When chimerism was analyzed we observed that the great majority of MSC were of host origin whether the stem cell source had been BM or PB (Table 1). Neither

Table 2. Chimerism studies of patients with mixed chimerism in MSC.

	Day +28				Day +180				Day +280				Day +400			
	BM	CD3	CD15	MSC	BM	CD3	CD15	MSC	BM	CD3	CD15	MSC	BM	CD3	CD15	MSC
1	C	C	C	ND	C	C	C	ND	C	C	C	H	C	C	C	M (26.13%)
2	C	C	C	H	C	C	C	M (60.17%)	C	C	C	ND	ND	ND	ND	ND

Day: day after transplantation. CD3: CD3⁺ purified cells; CD15: CD15⁺ purified cells; MSCs: mesenchymal stem cells; BM: bone marrow; C: complete chimerism; H: host origin of MSC; M: mixed chimerism; (%): percentage of MSC from donor origin.

Table 3. Clinical characteristics of patients with mesenchymal mixed chimerism.

	Diagnosis	CR	Disease status at study	Chimerism analysis	Complete chimerism reached	Acute GVHD	Chronic GVHD	On treatment at study for chronic GVHD	Evolution +18 months
Case #1	MM IgG λ	Fludarabine + Melphalan	Day +400 BM: 0.35% PC CD3: C	BM: C CD15: C CD3: C	+28	No	Extensive +133	YES	+ 18 months : CR
Case #2	MM IgG κ	Fludarabine + Melphalan	Day +180 BM: 1.4% PC	BM: C CD15: C CD3: C	+28	No	Limited +180	NO	+ 18 months: CR

Case #1: patient showed mixed chimerism of MSC with 26.13% of MSC from donor origin on day +400; Case #2: patient showed mixed chimerism of MSC with 60.17% of MSC from donor origin on day +180; CR: conditioning regimen; BM: bone marrow; CD15: peripheral blood CD15⁺ cells; CD3: peripheral blood CD3⁺ cells; C: complete. GVHD: graft-versus-host disease; CR: complete remission.

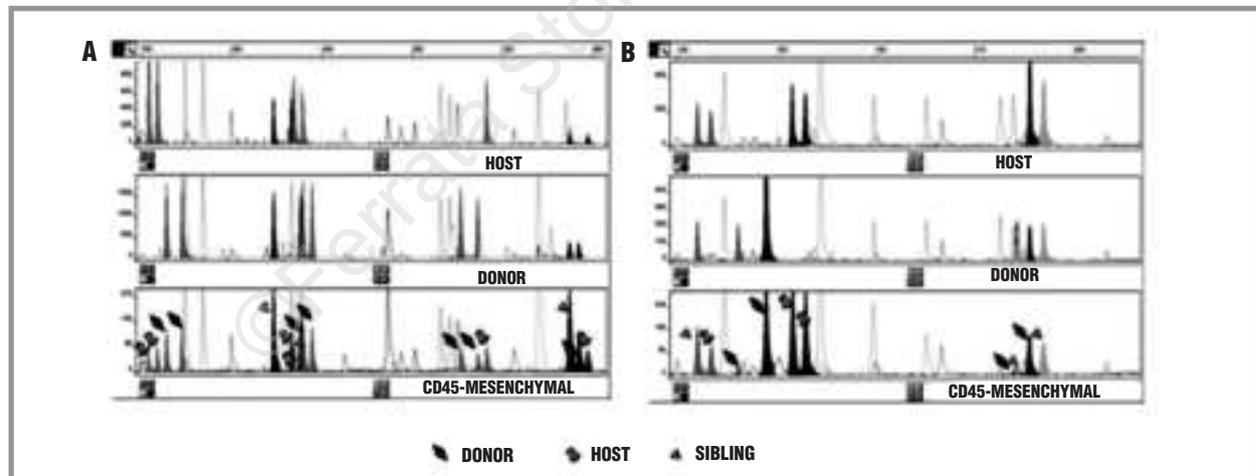


Figure 3. Chimerism studies of patients with mixed chimerism in MSC. A. Patient showing a mixed chimerism: 26.13% on day +400 post-transplantation. B. Patient with mixed chimerism: 60.17% on day + 180.

conditioning regimen had any influence on MSC origin and these results applied to all times after transplantation between day +28 and +910. Only two patients showed mixed chimerism of MSC, with 26.13% and 60.17% MSC of donor origin on day +400 and +180 respectively, after transplantation (Figure 3). Chimerism

studies on MSC from these two patients had previously shown cells originating from the patients (Table 2). The clinical characteristics of these two cases are shown in Table 3. When hematopoietic chimerism was studied in these cases, complete chimerism in both BM and PB CD15⁺ cells and CD3⁺ cells was observed (Table 2).

Discussion

Despite considerable interest in the potential therapeutic applications of the MSC population, there is currently no well-defined isolation or characterization protocol. To date, the acquisition and identification of MSC is based on the capacity of these cells to adhere to plastic surfaces and posterior immunophenotypic analysis.¹⁻³ Previously reported immunophenotypic studies have shown that there are no specific marker for MSC, but they express antigens and receptors for cell adhesion molecules, as well as being devoid of typical hematopoietic antigens.¹⁻³ Flow cytometry analyses performed in this study demonstrated that, in accordance with previously published data, MSC showed high expression of CD90 and an intermediate/low expression of the following antigens: CD49b, CD106, CD54, CD105, and CD56. They were negative for CD34, CD45, CD133, HLA-DR, CD62-L and CD104 antigens. In order to confirm their mesenchymal origin, cells were induced to differentiate toward bone and adipose tissues as done by other authors.¹⁹

Several *in vitro* and *in vivo* studies in non-human models have shown that MSC can migrate and incorporate into various tissues after syngeneic or xenogeneic transplantation.¹⁰⁻¹³ However, in humans, the transplantability of BM stromal cells remains controversial. Some studies, using sex-mismatched allografts, showed that some BM stromal cells are from donor origin.²¹⁻²² When MSC chimerism was analyzed in the present study, we observed that MSC were from host origin in the great majority of cases – 17 out of 19 (89%) – whether BM or PB had been used as the source of cells for transplantation. The conditioning regimen did not have any influence on MSC origin at any time point analyzed after transplantation (from day +28 to +910). However, two patients did show mixed chimerism of MSC with 26.13% and 60.17% of MSC of donor origin on day +400 and +180 after transplantation, respectively. Previous chimerism studies on MSC from these two patients had shown complete chimerism according to both patient origin and hematopoietic chimerism in BM and PB (CD15⁺ and CD3⁺) cells. Our results show that at least some allogeneic MSC of donor origin can graft into the a recipient's bone marrow. Why this feature occurs later in the post-transplant period is not easy to explain, but our data concur with those

reported by Devine *et al.*¹¹ on non-hematopoietic tissues. These authors observed mesenchymal cells⁹⁻²¹ months after transplantation but failed to show them in an earlier period. What is more striking is that the two patients in whom donor MSC were identified were two of the three cases with multiple myeloma. It is well known that bone marrow stroma is heavily impaired in multiple myeloma.²³ Data from our laboratory corroborate the hypothesis that MSC from MM patients have impaired *in vitro* growth capacity (*data not shown*). Perhaps their *in vitro* expansion capacity could be overcome by the normal/healthy MSC. This altered behavior could justify an easier graft of primary non-expanded MSC. Recent data demonstrating that expanded MSC can help children with osteogenesis imperfecta^{24,25} and other diseases corroborate our hypothesis, suggesting that MSC can graft better in damaged tissues.²⁶⁻²⁸

The second relevant piece of information from our study is that MSC may be derived not only from BM but also from mobilized PB stem cells.²⁹ Some authors showed that MSC can be obtained and expanded from mobilized PB,^{14,15,30} but other studies failed to confirm this¹⁷ and isolation of these cells is clearly difficult.

The results of the present analysis show that MSC not only circulate among mobilized PB stem cells from healthy donors but also that they can engraft into the BM of allogeneic hematopoietic stem cell recipients. Surprisingly, we only detected MSC from donors in PB transplanted patients; donor MSC in patients who had undergone allogeneic BM transplantation were not detected. These observations could be due to the low number of BM recipients analyzed (n=4), and the fact that none of them was a MM patient.

In summary, the present study shows the capacity of MSC to circulate among mobilized PB stem cells and to graft into BM after allogeneic hematopoietic stem cell transplantation. The fact that this feature was observed in MM patients warrants further study.

EMV: performed the majority of cell cultures and immunophenotypic analyses, and she wrote the manuscript; JA: supervised the phenotypic analysis and MSCs identification; MAIco: carried out the chimerism studies; NL, MAIbe and LIS-A: contributed to all the laboratory studies; JAP-S and FMS-G: oversaw patient care and their evolution; JFSM: supervised and critically revised the manuscript; MCDc: designed and controlled the experiments and revised the manuscript. The authors reported no potential conflicts of interest.

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