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Mesenchymal Stromal Cell Irradiation Interferes with the Adipogenic/Osteogenic Differentiation Balance and Improves Their Hematopoietic-Supporting Ability

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Bone marrow mesenchymal stromal cells (MSCs) are precursors of adipocytes and osteoblasts and key regulators of hematopoiesis. Irradiation is widely used in conditioning regimens. Although MSCs are radioresistant, the effects of low-dose irradiation on their behavior have not been extensively explored. Our aim was to evaluate the effect of 2.5 Gy on MSCs. Cells from 25 healthy donors were either irradiated or not (the latter were used as controls). Cells were characterized following International Society for Cellular Therapy criteria, including in vitro differentiation assays. Apoptosis was evaluated by annexin V/7-amino-actinomycin staining. Gene expression profiling and reverse transcriptase (RT)-PCR of relevant genes was also performed. Finally, long-term bone marrow cultures were performed to test the hematopoietic-supporting ability. Our results showed that immunophenotypic characterization and viability of irradiated cells was comparable with that of control cells. Gene expression profiling showed 50 genes differentially expressed. By RT-PCR, SDF-1 and ANGPT were overexpressed, whereas COL1A1 was downregulated in irradiated cells ($P = .015$, $P = .007$, and $P = .031$, respectively). Interestingly, differentiation of irradiated cells was skewed toward osteogenesis, whereas adipogenesis was impaired. Higher expression of genes involved in osteogenesis as SPP1 ($P = .039$) and lower of genes involved in adipogenesis, CEBPA and PPARG ($P = .003$ and $P = .019$), together with an increase in the mineralization capacity (Alizarin Red) was observed in irradiated cells. After differentiation, adipocyte counts were decreased in irradiated cells at days 7, 14, and 21 ($P = .018$ $P = .046$, and $P = .018$, respectively). Also, colony-forming unit granulocyte macrophage number in long-term bone marrow cultures was significantly higher in irradiated cells after 4 and 5 weeks ($P = .046$ and $P = .007$). In summary, the irradiation of MSCs with 2.5 Gy improves their hematopoietic-supporting ability by increasing osteogenic differentiation and decreasing adipogenesis.

INTRODUCTION

The bone marrow (BM) microenvironment controls growth and differentiation of hematopoietic stem cells (HSCs) and is composed of several cell types, including osteoblastic-lineage cells. They have a critical role in the regulation of primitive HSCs, stimulating growth and maintaining the clonogenic potential of $CD34^+$ cells [1-3]. *CXCL12*-abundant

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reticular cells and a subset of Nestin$^+$ cells are also key regulators contributing to HSC maintenance in an undifferentiated state [4]. Adult BM also contains adipocytes, which exert an inhibitory effect on hematopoiesis and preventing hematopoietic progenitor cell expansion. The number of correlates inversely with the hematopoietic activity in the BM [5]. Adipocytes block granulopoiesis and inhibit the release of granulocyte colony-stimulating factor production by macrophages via neuropilin-1 (*NRP1*) expression [6]. Adipocytes also secrete transforming growth factor β1, a well-known inhibitor of hematopoiesis [7].

Many cells of the BM microenvironment (including osteoblasts and adipocytes) derive from mesenchymal stromal cells (MSCs) [8]. MSCs are a key component of the hematopoietic microenvironment despite the fact that they constitute a small proportion of BM mononuclear cells [9,10]. Communications between marrow stromal cells and hematopoietic cells regulate homing, cell survival, proliferation, and mobilization of blood cells [11]. MSCs are not only able to differentiate into osteoblast and adipocytes but also exert a potent immunomodulatory activity [12,13]. Thus, they represent an attractive therapeutic tool, being currently evaluated in multiple cell therapy programs. Most beneficial effects of MSCs are related to paracrine actions rather than to direct differentiation of MSCs [14].

On the other hand, the use of hematopoietic cells in hematopoietic cell transplantation remains as the most extended cell therapeutic approach for a variety of hematopoietic diseases. Preparative or conditioning regimens are needed before cell infusion to reduce the amount of malignant cells and to produce an adequate immunosuppression to allow engraftment. One of the components of classical conditioning regimens has been total body irradiation at high doses (12 Gy) [15]. Nevertheless, the use of a low dose of irradiation as a unique conditioning regimen for elderly or frail patients was successfully introduced in 2001 by the Seattle group [16,17]. Low-dose irradiation allows engraftment with significant reduced toxicity and has broadened the ability to undergo HSC transplantation for unfit or elderly patients.

When the hematopoietic system is exposed to ionizing radiation, hematopoietic homeostasis is altered. Hematopoietic stem and progenitor cells are extremely radiosensitive [18]. The abolition of the host hematopoietic system creates a new space in the BM niche where donor hematopoietic cells can engraft.

Research in this field has focused mostly on BM hematopoietic recovery after irradiation and transplantation, whereas less attention has been paid to the changes induced by irradiation into the microenvironment. It has been shown that after total body irradiation followed by BM transplantation, hematopoietic cells are from the donor and MSCs remain of host origin [19]. These results indicate that MSCs are able to survive doses of irradiation that are lethal to hematopoietic cells.

Although MSCs are resistant to radiation in terms of viability, cellular function can be altered. MSCs do not loss their differentiation capacity, but this capacity can be altered even at low irradiation doses [20]. In this regard the expression of adipogenic markers is significantly lower in murine cells exposed to irradiation [21], but it is controversial if the differentiation ability to osteoblastic cells is induced or not [22-25]. There is scarce information on the effects of irradiation on human MSCs, especially the effects of low-dose irradiation. Therefore, in the current study we assessed if a reduced dose (2.5 Gy) of irradiation modifies the differentiation ability of human MSCs and also the functional changes induced in terms of their hematopoietic-supporting ability.

METHODS

Cell Isolation and Expansion

BM human MSCs were isolated from 25 healthy donors (18 men and 7 women) with a median age of 38 years (range, 21 to 65). In all cases written informed consent was previously obtained according to institutional guidelines and the Declaration of Helsinki. All experimental procedures were also approved by Ethics Committee of the Hospital Universitario de Salamanca (70/07/2015).

Ten to 20 mL BM was obtained from the iliac crest under local anesthesia. BM mononuclear cells were isolated and separated by Ficoll-Paque density-gradient centrifugation (Ficoll-Paque density, 1.077 g/mL; GE Healthcare BioSciences, AB, Uppsala, Sweden). Cells were counted and seeded at a density of 1×10^6 cells/cm^2, and expansion was carried out as previously described [26] in DMEM (GIBCO, Life Technologies, Carlsbad, CA) supplemented with FBS (GIBCO, Life Technologies) and 1% penicillin/streptomycin.

MSC Irradiation

Two aliquots of each MSC sample from passage 3 were cultured in parallel. One aliquot was irradiated with a single doses of 2.5 Gy, delivered at a rate of 2 to 3 Gy/min, using a cesium source (GammaCell 1000; Nordion International, Ottawa, Ontario, Canada), whereas the non-irradiated aliquot was used as control. Cells from both groups were subjected to the subsequent assays.

Characterization of MSCs by Flow Cytometry

For immunophenotypic characterization MSCs from passage 3 were incubated with the following monoclonal antibodies conjugated with either FITC, PE, peridinin chlorophyll protein, or allophycocyanin: anti-CD34 FITC (eBioscience Inc., San Diego, CA); anti-CD105 (RD Systems, Minneapolis, MN); anti-CD14 (Cytognos, Salamanca, Spain); and anti-CD73, -CD45, -CD44, -CD19, -CD90, -CD166, and HLA-DR (BD Biosciences, San Jose, CA). Unstained MSCs were used as control. Samples were acquired on a FACS Calibur flow cytometer using Cellquest Pro software (Becton Dickinson, San Jose, CA). Data were analyzed using the Infinicyt software (Cytognos).

Apoptosis Assays

One hour and 72 hours after irradiation, cells were harvested, washed, and incubated with Annexin V, 7-AAD (7-amino-actinomycin) using the BD Pharmigen PE Annexin V Apoptosis Detection Kit I (BD Biosciences). FITC-conjugated CD90 (eBioscience) was added before the sample was acquired on a FACS Calibur flow cytometer using Cellquest Pro software (Becton Dickinson). At least 5×10^4 events per sample were recorded. Data were analyzed using Infinicyt (Cytognos). Cells were considered to be in an early apoptotic state, late apoptosis, or dead if they were Annexin V$^+$/7-AAD$^-$, Annexin V$^+$/7-AAD$^+$, or Annexin V$^-$/7-AAD$^+$, respectively. Five pairs of MSC samples were used for this analysis.

Gene Chip Human Gene ST Arrays

Because of cost constraints only 5 pairs of MSC samples (non-irradiated and irradiated) were used for this analysis. Total RNA was isolated and purified using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The RNA integrity was assessed using the 2100 Bioanalyzer (Agilent, Palo Alto, CA). Labeling and hybridizations were performed according to protocols from Affymetrix (Santa Clara, CA). Briefly, 100 ng of total RNA were amplified and labeled using the WT Plus reagent kit (Affymetrix) and then hybridized to Human Gene 2.0 ST Array (Affymetrix). Washing and scanning were performed using the GeneChip System (GeneChip Hybridization Oven 645, GeneChip Fluidics Station 450, and GeneChip Scanner 7G; Affymetrix).

Raw data were extracted from unprocessed cell intensity files and normalized using the robust multi-array average algorithm [27], implemented in the Affymetrix expression console (version 1.4.1.46). The significance analysis of microarrays technique [28] was used for the identification of differentially expressed genes between paired samples. Genes with a q value $< .05$ were considered to be significantly over- or infra-expressed.

Reverse Transcriptase PCR of Genes Involved in Hematopoiesis

Total RNA was extracted from 10 pairs of non-irradiated and irradiated MSCs. cDNA was prepared by reverse transcription using the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA), and the converted cDNA was analyzed for genes involved in hematopoietic maintenance, including stromal-derived factor 1 (*SDF1*), angiopoietin 1 (*ANGPT*), thrombopoietin (*THPO*), collagen type I alpha 1 chain (*COL1A1*), nerve growth factor cd271 (*NGF*), C-X-C motif chemokine receptor 4 (*CXCR4*), CD44 molecule (Indian blood group) (*CD44*), and integrin subunit alpha 4

(*ITGA4*), to specific gene expression of osteogenic lineage, including alkaline phosphatase (*ALP*), runt related transcription factor 2 (*RUNX2*), and osteopontin (*SPP1*), and for adipogenic specific gene expression, including peroxisome proliferator activated receptor gamma (*PPARG*) and enhancer binding protein alpha (*CEBPA*). *GADPH* was used as a control gene for genes implicated in differentiation, and *GUS* was used as a control for genes implicated in hematopoietic maintenance.

Genes were quantified using TaqMan gene expression assays and the Step One Plus Real-Time PCR System (Applied Biosystems). Relative quantification was calculated from the $2^{-\Delta Ct}$ values where $\Delta C_t = C_t Gene - C_t Control$.

MSC Differentiation Analysis

BM-MSCs on third passage were seeded in 9-cm^2 tissue culture dishes (Corning, Ithaca, NY) and irradiated on the following day. For osteogenic differentiation cells were seeded at a density of 3.5×10^3 cells/cm^2, and for adipogenic differentiation control cells were seeded at a density of 22.5×10^3. One hour after irradiation, differentiation medium was added to each well, and cells were grown for 21 days. Osteogenic differentiation was induced using NH Osteodiff Medium (Miltenyi Biotec, Bergisch Gladbach, Germany) supplemented with 1% penicillin/streptomycin. Adipocytic differentiation was induced with human MSC Adipogenic Induction SingleQuots (Lonza, Basel, Switzerland) and human MSC Adipogenic Maintenance SingleQuots (Lonza). Differentiation medium was replaced twice a week. To evaluate the osteoblastic differentiation cells were stained with alkaline phosphatase using an NBT/BCIP solution kit (Nitroblue tetrazolium chloride/5-bromo-4.chloro-3-indolyl-phosphate; Roche, Basel, Switzerland). Calcium deposits were quantified by Alizarin Red S staining in which the absorbance was proportional to calcium deposits in the sample and with the mineralization level of the cells (Sigma-Aldrich, St. Louis, MO). Ten pairs of MSC samples were used for this analysis. Oil-red-O staining (Merck KGaA, Darmstadt, Germany) was used for the assessment of adipogenic differentiation in 7 pairs of MSC samples.

CD34$^+$ Cell Isolation

Mobilized CD34$^+$ progenitor cells were isolated from leukapheresis products of 7 healthy donors (6 men and 1 woman) with a median age of 37 years (range, 26 to 52) by immunomagnetic sorting in an AutoMACS (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) after labeling cells with the human CD34 MicroBead Kit (Miltenyi Biotec), according to the manufacturer's recommendations. Purity of cells was confirmed by flow cytometry using FITC-CD34 (eBioscience), and the viability was evaluated by labeling cells with 7-AAD.

Long-Term BM Culture and Clonogenic Assays

To establish the long-term BM culture (LT-BMC), the stromal layer was first induced by seeding 1.5×10^5 MSCs onto 9-cm^2 tissue culture dishes (Corning) in DMEM until confluence was obtained. Eight MSC samples were used in this experiment. Then, cells were irradiated, and 1 hour later medium was replaced by LT-BMC medium, containing 62.4% Iscove (GIBCO, Invitrogen, Grand Island NY), 10% horse serum (PAA Laboratories GmbH, Pasching, Austria), 10% FBS, and 1% hydrocortisone 5×10^{-5} M. Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 for 1 week to establish the stromal layer. At day 7, 3×10^5 CD34$^+$ progenitor cells were added to the stromal layer. Cultures were fed weekly by replacing half of the growth medium and maintained for 5 weeks.

Clonal growth of progenitor cell population was assayed weekly culturing 1.25×10^3 CD34$^+$ progenitor cells isolated each week from the removed medium of LT-BMC into methylcellulose MACS Media Stem MACS HSC-CFU complete w/o Epo human (Miltenyi Biotec) during 2 weeks at 37°C in a fully humidified atmosphere of 5% CO_2. After these 2 weeks the colonies formed were scored using an inverted microscope.

Statistical Analysis

Values were summarized as median and range. The Wilcoxon signed ranks test was used to compare the differences between paired results (irradiated versus non-irradiated samples). Differences were considered to be statistically significant for values of $P < .05$. All statistical analyses were performed on the GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Characterization of Irradiated and Non-Irradiated MSCs

Both non-irradiated and irradiated MSCs displayed the characteristic fibroblastic-like morphology and were able to adhere to plastic surfaces. Immunophenotypic analysis by flow cytometry showed that all samples expressed CD73, CD90, CD105, CD44, and CD166, whereas they were negative for CD34, CD45, CD14, CD19, and HLA-DR, demonstrating a characteristic immunophenotypic profile of MSCs without differences between non-irradiated and irradiated cells (Figure 1).

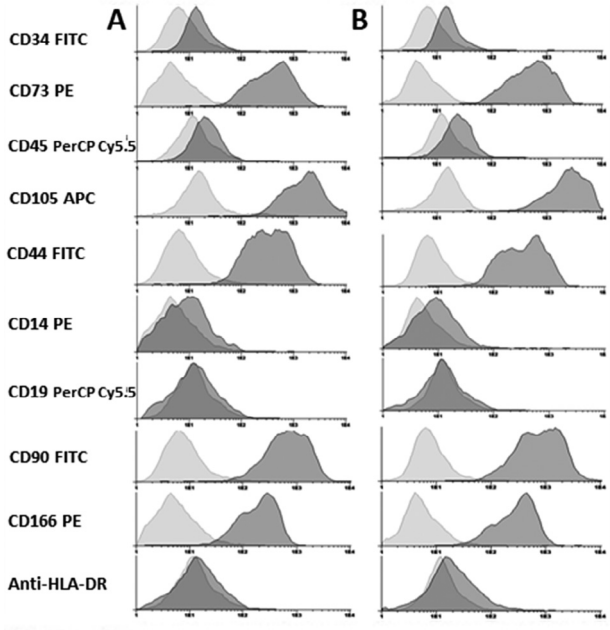

Figure 1. Phenotypic analysis of non-irradiated (A) and irradiated (B) BM MSCs. MSCs were isolated from human BM and expanded to passage 3. Then, expression of different cell surface markers was analyzed by flow cytometry. Data are representative of 3 MSC samples.

MSC Viability

To evaluate if a 2.5-Gy irradiation dose induced apoptosis in MSCs, cell viability assay was performed both 1 hour and 72 hours after irradiation (n = 5). There were no differences in the percentage of viable cells (Annexin V$^-$/7AAD$^-$), early apoptotic cells (Annexin V$^+$/7AAD$^-$), late apoptotic cells (Annexin V$^+$/7AAD$^+$), and dead cells (Annexin V$^-$/7AAD$^+$) between both groups neither at 1 hour nor at 72 hours after irradiation (Figure 2).

Gene Expression Profiling of Irradiated MSC

Applying significance analysis of microarrays analysis, statistically significant differences were found in 50 of 6661 tested genes by applying the established filtering criteria. From these 50 genes, 5 were overexpressed and 45 down-expressed in irradiated compared with non-irradiated MSCs (Figure 3 and Supplementary Table S1). The most downregulated gene was pyruvate dehydrogenase kinase 1 (*PDK1*), which is involved in the regulation of adipogenesis.

Expression of Genes Involved in Hematopoiesis

The expression of some important genes involved in homing or in the maintenance of hematopoiesis was analyzed by reverse transcriptase (RT)-PCR (n = 10). *NGF*, *THPO*, *CD44*, *CXCR4*, and *ITGA4* were expressed in both non-irradiated and irradiated MSCs without statistically significant differences between both groups. *COL1A1* showed lower expression in irradiated cells ($P = .031$). On the contrary, *SDF1* and *ANGPT* were significantly overexpressed in irradiated compared with control MSCs ($P = .015$ and $P = .007$, respectively; Figure 4).

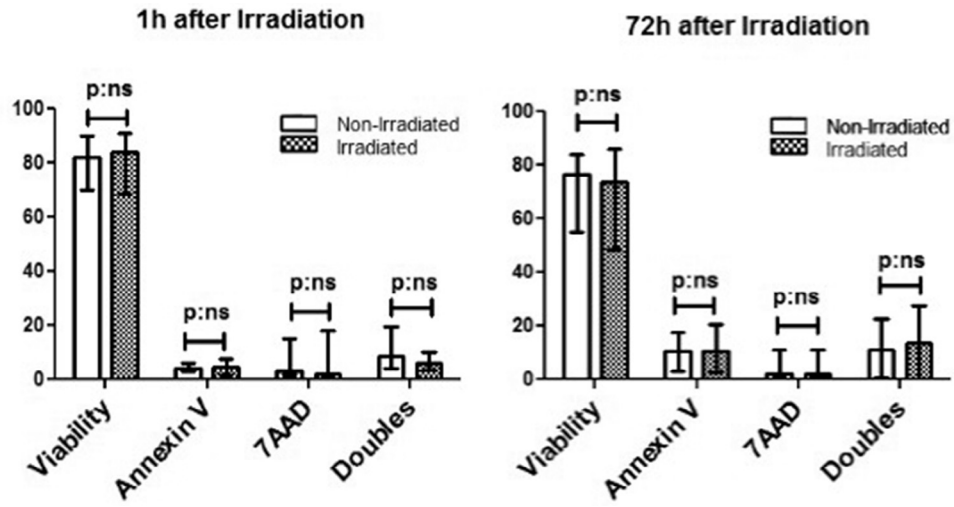

Figure 2. Apoptosis assays in non-irradiated and irradiated MSCs 1 hour and 72 hours after irradiation. MSCs were incubated with Annexin V, 7-AAD, and CD90. Then, the expression of different cell surface markers was analyzed by flow cytometry. Cells were considered to be viable (Annexin V^-/7-AAD^-), in an early apoptotic state (Annexin V^+/7AAD^-), late apoptosis (Annexin V^+/7-AAD^+), or dead (Annexin V^-/7-AAD^+).

Differentiation Ability

Both non-irradiated and irradiated MSCs were positive for alkaline phosphatase staining and contained lipid droplets stained with Oil-red-O after culture with specific osteogenic and adipogenic differentiation media, respectively (Figure 5A-D). To quantify the adipogenic capacity of MSCs, the number of adipocytes growing in adipogenic differentiation medium were counted at days 7, 14, and 21 of the differentiation process (n = 7). In all cases the number of adipocytes was significantly reduced in irradiated MSCs

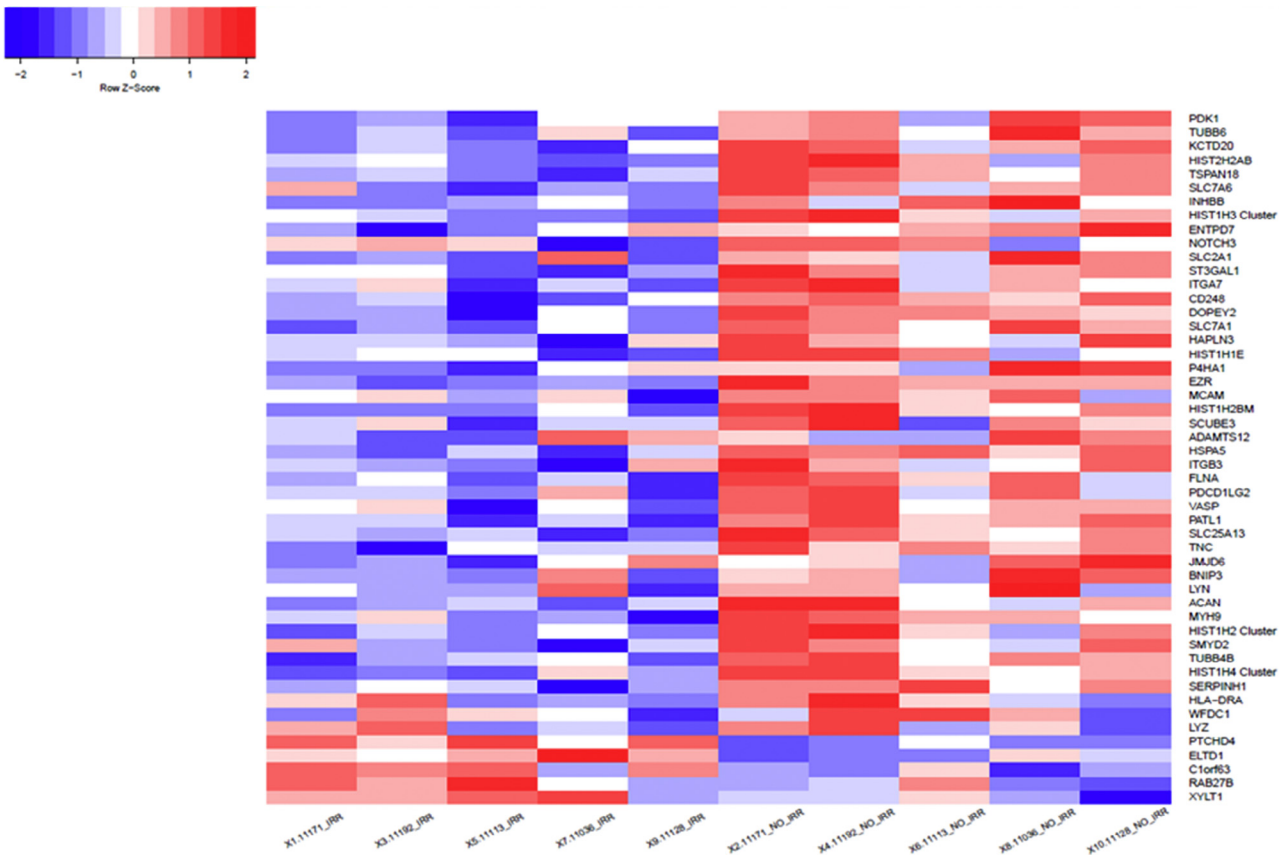

Figure 3. Heat map showing the top 50 genes found either downregulated or upregulated upon irradiation with 2.5 Gy. Purified RNA from 5 pairs of non-irradiated and irradiated MSC samples was hybridized in Gene Expression Arrays (Affymetrix). The significance analysis of microarrays technique was used for the identification of differentially expressed genes between paired samples. Gene downregulation is represented in *blue* and gene upregulation in *red*.

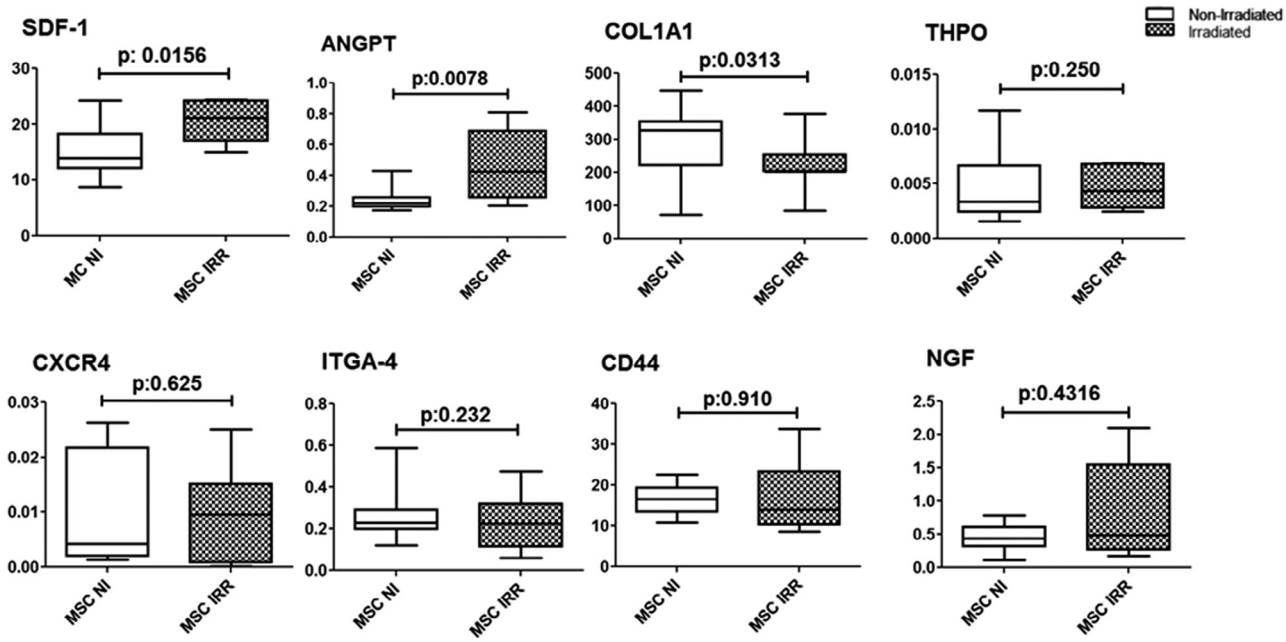

Figure 4. Expression of genes involved in hematopoiesis. Expression of *SDF1*, *ANGPT*, *COL1A1*, *THPO*, *CXCR4*, *ITGA4*, *CD44*, and *NGF* in non-irradiated (*white*) and irradiated (*black*) MSCs was performed by RT-PCR 3 days after irradiation. β-glucuronidase was used as a control.

compared with non-irradiated cells in all 3 time points ($P = .018$, $P = .046$, and $P = .018$, respectively; Figure 5E).

In addition, the expression of adipogenic differentiation genes, *CEBPA* and *PPARG*, was significantly lower in irradiated MSCs compared with non-irradiated cells ($P = .003$ and $P = .019$, respectively). Regarding genes involved in early osteogenesis, *RUNX2* was less expressed in irradiated cells ($P = .003$) compared with non-irradiated cells, whereas there were no differences in the expression of *ALP* between both groups. *SPP1*, involved in late osteogenesis and mineralization, was overexpressed in irradiated MSCs ($P = .039$; Figure 5F). Moreover, the capacity of mineralization quantified by Alizarin Red staining (n = 10) showed the presence of a slightly higher amount of calcium deposits in irradiated cells, although differences did not reach statistically significant differences ($P = .084$; Figure 5G).

Hematopoietic-Supportive Capacity by LT-BMCs

The ability of non-irradiated and irradiated MSCs to support hematopoiesis in vitro was assessed in LT-BMC (n = 8). Purity of $CD34^+$ progenitor cells isolated for these experiments was superior to 90%.

$CD34^+$ cultured with both types of stromal layers produced similar number of colonies in the first week. From the second week on more colony-forming unit (CFU)-granulocyte macrophage colonies were scored in $CD34^+$ cells grown on stromal layers from irradiated MSCs, with significant differences at the fourth and fifth weeks ($P = .046$ and $P = .007$, respectively; Figure 6).

During the 5 weeks of LT-BMC the predifferentiation status of the stromal layer coming from non-irradiated or irradiated MSCs was assessed (n = 5). From the first to the fifth week of culture the number of adipocytes per field in the irradiated stromal layer was significantly lower compared with the non-irradiated stromal layer (Figure 7A-C). In addition, stromal layers at the third and fifth week (n = 6) of LT-BMC were stained by Alizarin Red. At both time points the mineralization level of cells was higher in stromal layers from irradiated MSCs, with significant differences at the third week ($P = .046$; Figure 7D).

DISCUSSION

After irradiation, a depletion of hematopoietic cells in the BM and subsequently in peripheral blood takes place. This depletion creates an "empty space" that favors hematopoietic engraftment after transplantation [29]. However, these effects of irradiation are generally dose dependent. After low-dose irradiation, peripheral blood mobilization and hematopoietic reconstitution are stimulated [30,31]. In this regard, Li et al. [32] reported in a murine model that transplantation of HSCs from a whole body irradiated donor led to a better engraftment than HSCs from non-irradiated mice.

The overall effects of irradiation on MSCs are only beginning to be disclosed. Despite the fact that MSCs maintain their viability, their global gene expression [33] and their capacity of differentiation can be altered even at low doses of irradiation [20].

The originality of our study is that we studied changes occurring after low-dose irradiation on human MSCs, addressing together MSC differentiation capacity and its impact on hematopoietic supportive function. The interest in low doses of irradiation is based on the fact that many groups are using low doses of total body irradiation as the sole conditioning regimen in patients with advanced age and/or comorbidities, allowing them to benefit from transplantation [16,17].

Regarding the radio-resistance of MSCs, we report that human BM MSCs irradiated with 2.5 Gy neither lose their viability nor modify their immunophenotypic profile. It is known that MSCs are less susceptible to radiation than HSCs. Sugrue et al. found that mouse MSC lines express high levels of key DNA damage response (DDR) and antiapoptotic proteins but low levels of proapoptotic proteins. Consequently, after 10 Gy of irradiation a delay of cell cycle progression was

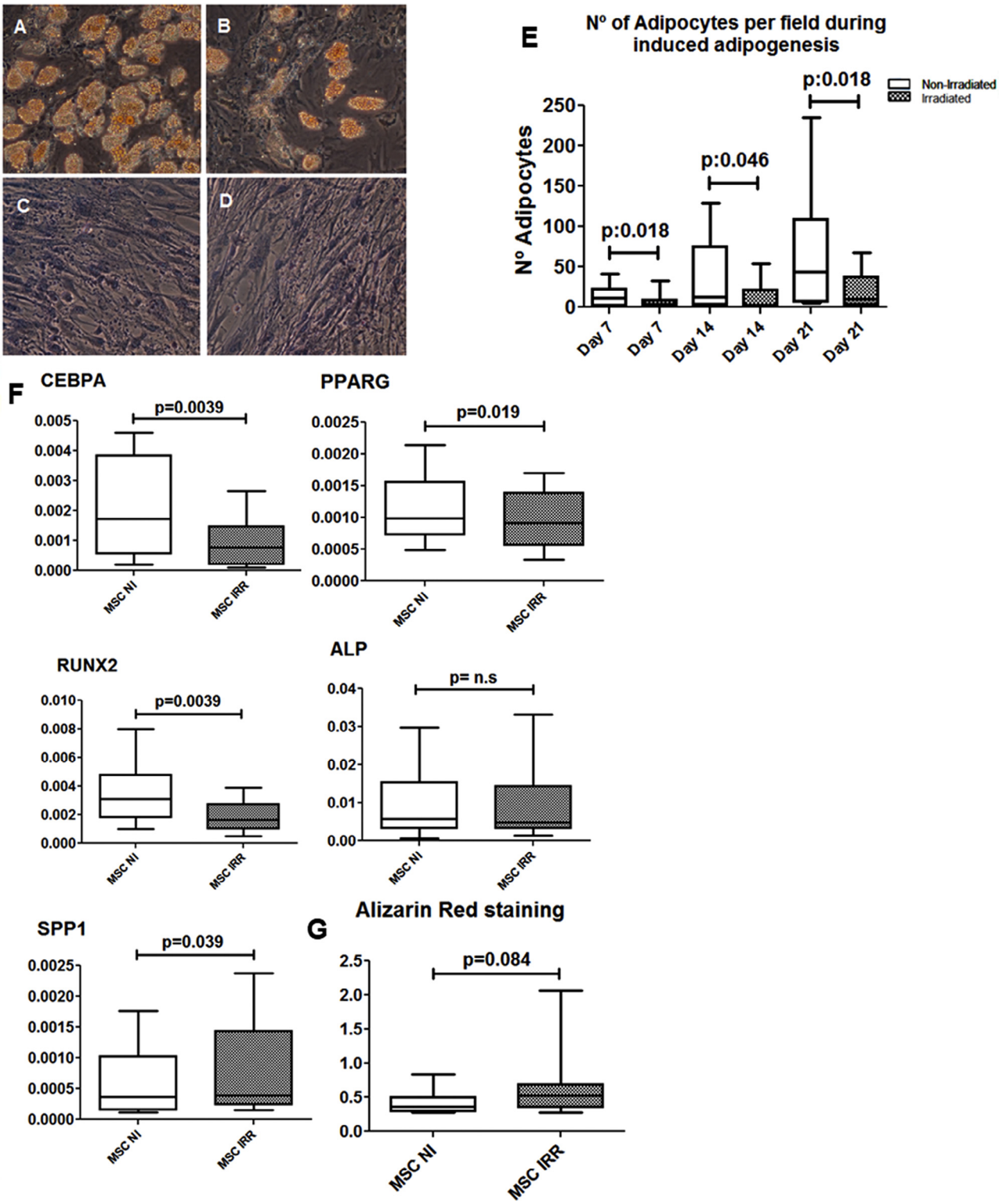

Figure 5. Differentiation capacity of irradiated MSCs. After exposure to 2.5 Gy, MSCs were cultivated in differentiation-inducing media during 21 days. Adipogenic differentiation of non-irradiated (A) and irradiated MSCs (B) was visualized by Oil-Red-O staining. Osteogenic differentiation of non-irradiated (C) and irradiated MSCs (D) was detected by alkaline phosphatase staining. The number of adipocytes growing in adipogenic differentiation medium was counted in non-irradiated (*white*) and irradiated MSCs (*black*) at days 7, 14, and 21 of the differentiation process (E). Expression of *CEBPA*, *PPARG* (adipogenesis), *RUNX2*, *ALP* (osteogenesis), and *SPP1* (mineralization) in non-irradiated (*white*) and irradiated MSCs (*black*) was performed by RT-PCR 3 days after irradiation. β-glucuronidase was used as a reference gene (F). Calcium deposition was quantified at day 21 after irradiation in non-irradiated (*white*) and irradiated MSCs (*black*) by Alizarin Red S staining in which the absorbance was proportional to calcium deposits in the sample and with the mineralization level of the cells (G).

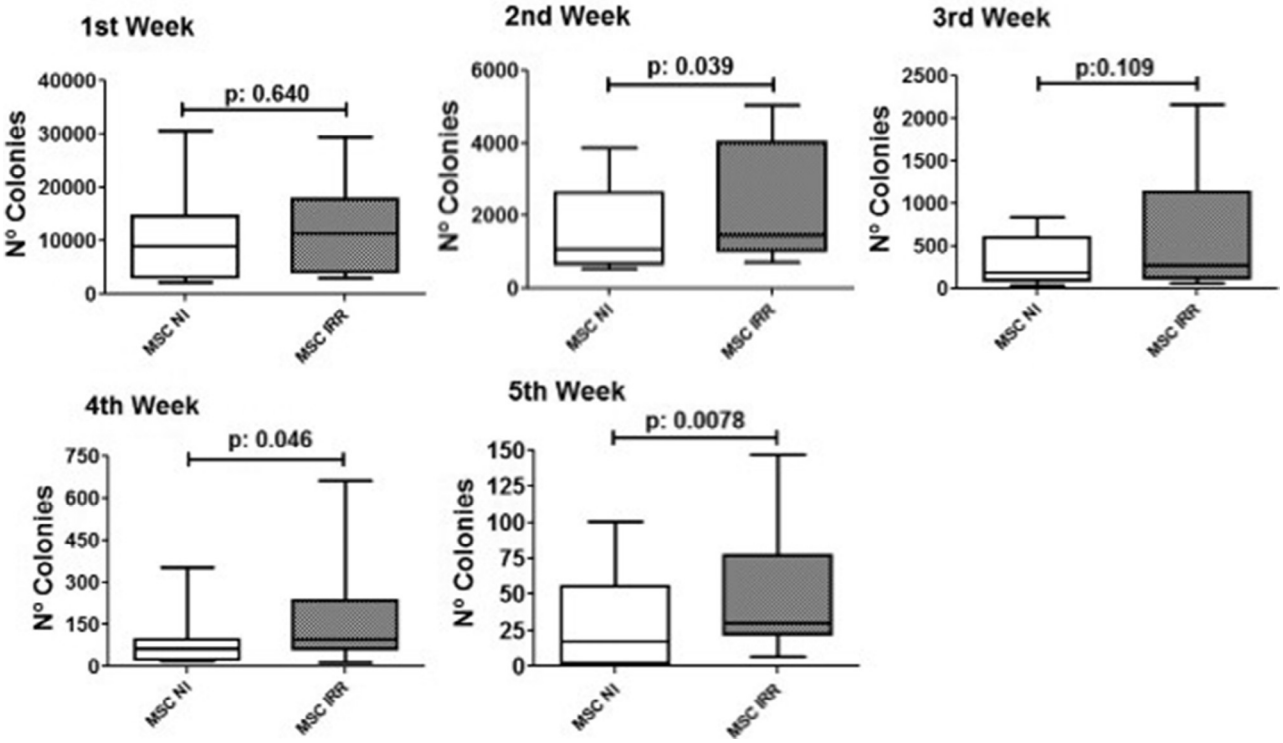

Figure 6. Colonies scored in LT-BMC. Mobilized CD34⁺ progenitor cells were isolated from leukapheresis and added to stromal layers from non-irradiated or irradiated MSCs. Clonal growth of progenitor cell population from non-irradiated (*white*) and irradiated (*black*) MSCs was assayed weekly.

observed in these cells to facilitate DNA repair before entering the next phase [34,35]. It has been also described that human MSCs display variable anatomic site-dependent response and recovery from irradiation. After exposition of 2.5 to 10 Gy of γ-irradiation, maxilla and mandible MSCs recover more quickly than iliac crest [36]. The effects on MSC as previously indicated are dose-dependent. In fact, proliferation of irradiated MSCs seems to be suppressed in a dose-dependent manner with 4 Gy of irradiation [37], whereas exposure of MSCs to low-dose irradiation was reported to result in increased proliferation of cultured rat MSCs [38,39].

We have detected an alteration in MSC global gene expression after irradiation, which is in accordance to previously reported data [33]. Genes involved in cellular cycle, protein dimerization, and vesicles formation were upregulated, whereas more than 40 genes were downregulated after irradiation. Interestingly, *PDK1*, the most downregulated gene in our analysis, is involved in adipogenesis through the activation of *CEBPA* via *GSK3* [40]. The downregulation of *PDK1* in irradiated MSCs would finally result in a downregulation of *CEBPA* (thus decreasing adipogenesis), as confirmed by RT-PCR.

We have also shown a significant overexpression of *ANGPT* and *SDF1* in MSCs after irradiation. The proper maintenance of normal HSCs within the niche is heavily dependent on the expression of these hematopoietic factors. In accordance with these data, it has been shown that low doses of irradiation could induce changes in the secretion of stem cell factor, granulocyte macrophage colony-stimulating factor, and *IL-11*, regulating the BM microenvironment [39].

In this regard, after irradiation treatment we have shown that MSCs retained their differentiation capacity as already described in human MSCs treated with different doses of irradiation 2 to 10 Gy [41] or even 30 to 60 Gy [42]. Although MSCs are able to differentiate after being irradiated, we have shown here that the balance osteogenesis/adipogenesis was modified by low-dose irradiated MSCs. In the present study the expression levels of *CEBPA* and *PPARG*, key factors driving the adipogenic differentiation of BM MSCs, were decreased after irradiation. A clear reduction in the amount of adipocytes after the specific differentiation culture was also observed. This is concordant with a number of studies that describe the decrease of the adipogenic differentiation in irradiated MSCs [20,21,24]. What is more subtle are the changes in the osteogenic capacity of MSC induced by low-dose irradiation. We have seen by RT-PCR that the expression of *RUNX2* and *ALP* involved in early osteogenesis are decreased, whereas the expression of *SPP1*, involved in late osteogenesis or mineralization, increases after irradiation. In concordance with the expression of *SPP1*, the quantification of Alizarin Red–positive staining mineralization deposits are also increased in irradiated MSCs. These results are also supported by some previous reports. In this respect, Abramovitch-Gottlib et al. [22] saw that low level laser irradiation stimulates osteogenic differentiation of MSC seeded on a 3-dimensional biomatrix, and Soleimani et al. [23] showed that low-level laser irradiation increased the BM MSC differentiation to osteoblast.

It has been shown that an altered differentiation capacity may impact the hematopoietic supportive ability of MSCs. Previous studies in murine models where mice were irradiated to induce BM aplasia have shown a significant increase in osteogenic and a decrease in adipogenic markers. Colony forming units–fibroblast from these mice favors HSC proliferation in vitro compared with colony forming units–fibroblast obtained from control mice [43]. Accordingly, we report from LT-BMC experiments that stromal layers from irradiated MSCs had a reduced adipogenic and increased osteogenic capacity

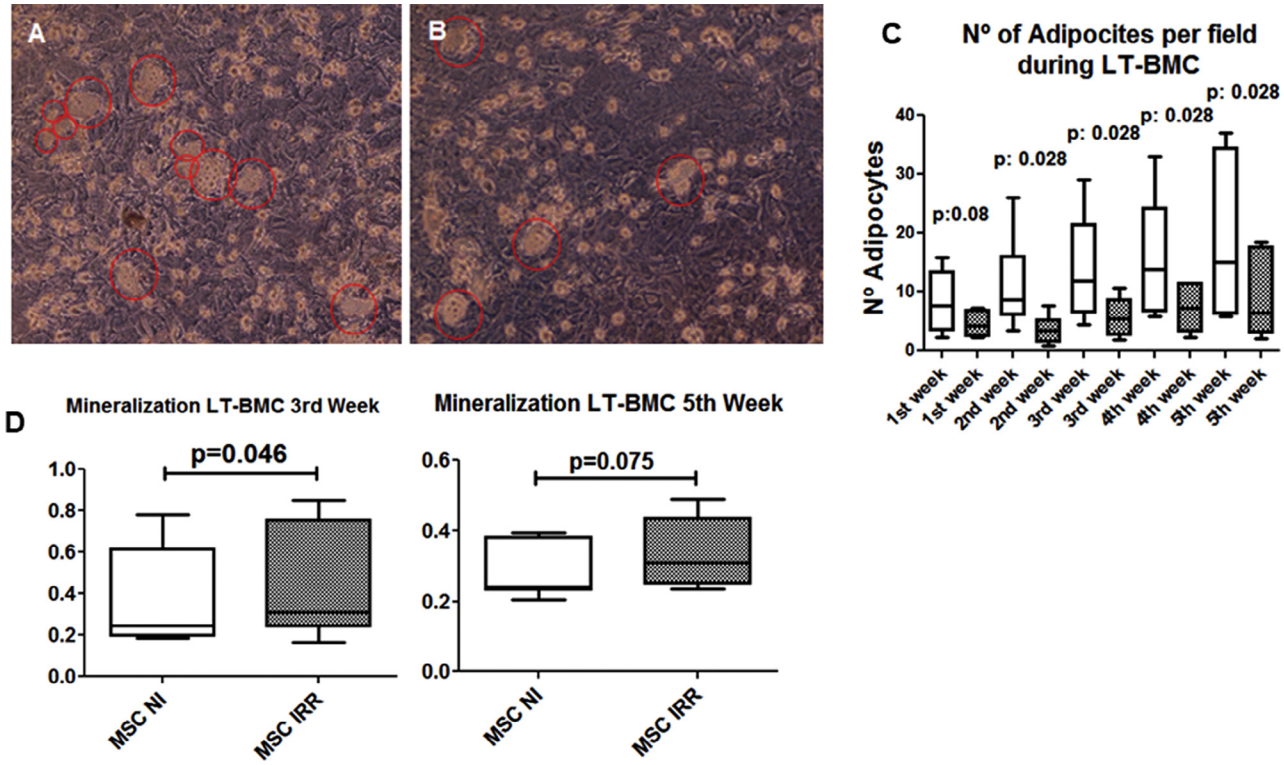

Figure 7. Differentiation status of LT-BMC stromal layer. During the 5 weeks of LT-BMC, the predifferentiation status of the stromal layer coming from non-irradiated or irradiated MSCs was assessed. Adipogenic differentiation of non-irradiated (A) and irradiated stromal layer (B) was visualized and the number of adipocytes growing were counted in non-irradiated (*white*) and irradiated (*black*) MSCs at days 7, 14 and 21 of the differentiation process (C). Calcium deposition was quantified at the third and fifth week of the LT-BMC in non-irradiated (*white*) and irradiated MSCs (*black*) by Alizarin Red S staining in which the absorbance was proportional to calcium deposits in the sample and with the mineralization level of the cells (D).

of differentiation, resulting in an increased hematopoietic colony-forming capacity. Our results are also in agreement with Naveiras et al. [5], who demonstrated that adipocyte-rich BM regions had a reduced frequency of HSCs and that adipocyte ablation in murine models enhanced hematopoietic recovery after irradiation, showing a negative regulation of hematopoiesis by BM adipocytes. There are also in vitro studies in which human MSCs induced to adipogenic differentiation attenuated the hematopoietic colony-forming capacity of $CD34^+$ cells [44]. Nevertheless, some of our results showed some variability. Thus, further confirmatory studies performed on a paired sample with higher numbers of donors are warranted to assess the real impact of our results.

In summary, our results suggest that the modifications induced by low doses of γ-irradiation on the capacity of differentiation of MSCs improves their hematopoietic-supporting ability. Nevertheless, future studies are needed, especially in murine models to prove this effect in vivo. In addition, because some of the results obtained showed several variations, further studies with a higher number of paired samples are warranted. In addition, different doses of irradiation should be compared in the future to totally understand the effect of the irradiation dose on these effects.

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Authorship statement: M.C.C. and F.S.-G. designed the study, reviewed data, and interpreted results. S.P. and S.M. performed sample preparation, experiments, and statistical analysis. M.D.C. provided BM samples for most patients and obtained informed consent. C.R., L.I.S.A., and A.R. performed and analyzed flow cytometry studies. L.A.C. performed overall bioinformatic analysis. J.B. and L.A.P.R. irradiated the cells. T.L.R. and R.O. collaborated with LT-BMC. M.C.C., F.S.-G., S.P., and S.M. wrote the manuscript. All authors reviewed the manuscript during its preparation and approved the final version submitted.

SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at doi:10.1016/j.bbmt.2017.11.007.

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