Human Bone Marrow–Derived Mesenchymal Stem Cells Do Not Undergo Transformation after Long-term *In vitro* Culture and Do Not Exhibit Telomere Maintenance Mechanisms

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Abstract

Significant improvement in the understanding of mesenchymal stem cell (MSC) biology has opened the way to their clinical use. However, concerns regarding the possibility that MSCs undergo malignant transformation have been raised. We investigated the susceptibility to transformation of human bone marrow (BM)–derived MSCs at different *in vitro* culture time points. MSCs were isolated from BM of 10 healthy donors and propagated *in vitro* until reaching either senescence or passage (P) 25. MSCs in the senescence phase were closely monitored for 8 to 12 weeks before interrupting the cultures. The genetic characterization of MSCs was investigated through array-comparative genomic hybridization (array-CGH), conventional karyotyping, and subtelomeric fluorescent *in situ* hybridization analysis both before and after prolonged culture. MSCs were tested for the expression of telomerase activity, human telomerase reverse transcriptase (hTERT) transcripts, and alternative lengthening of telomere (ALT) mechanism at different passages. A huge variability in terms of proliferative capacity and MSCs life span was noted between donors. In eight of 10 donors, MSCs displayed a progressive decrease in proliferative capacity until reaching senescence. In the remaining two MSC samples, the cultures were interrupted at P25 to pursue data analysis. Array-CGH and cytogenetic analyses showed that MSCs expanded *in vitro* did not show chromosomal abnormalities. Telomerase activity and hTERT transcripts were not expressed in any of the examined cultures and telomeres shortened during the culture period. ALT was not evidenced in the MSCs tested. BM-derived MSCs can be safely expanded *in vitro* and are not susceptible to malignant transformation, thus rendering these cells suitable for cell therapy approaches. [Cancer Res 2007;67(19):9142–9]

Introduction

In recent years, a significant improvement in the understanding of multipotent mesenchymal stem cell (MSC) biology (1) has opened the way to their clinical use. MSCs have been used in several approaches for reparative/regenerative cell therapy, as well as in the perspective of modulating immune response against alloantigens (2–13). MSCs have the ability to differentiate into multiple lineages, such as osteoblasts, tenocytes, adipocytes, and chondrocytes (14–16) and may be identified by both their capacity to adhere to plastic and their phenotypic characterization through a panel of cell surface molecules, including CD90, CD105, and CD13. However, a unique and specific MSC marker, allowing their exclusive identification, has not yet been found. The large interest in MSC applicability for clinical approaches relies on the ease of their isolation from several human tissues, such as bone marrow (BM), adipose tissue, placenta, and amniotic fluid (17–19), on their extensive capacity for *in vitro* expansion and on their functional plasticity.

Concerns that adult human MSCs may be prone to malignant transformation have been recently raised. In fact, human adipose tissue–derived MSCs have been shown to undergo spontaneous transformation after long-term *in vitro* culture (20). The same phenomenon was also noted in murine BM-derived MSCs (21), which, after numerous passages in culture, increased telomerase activity and proceeded to malignant transformation. A previously published study (22) has also documented that murine gastric epithelial cancer originates from BM-derived cells, presumably MSCs, after recruitment of these cells to the chronically injured mucosal site. Thus, use of MSCs for clinical approaches in many fields of medicine requires that the biosafety of these cells be carefully investigated through appropriate and sensitive tests. Indeed, the absence of transformation potential in cultured MSC has to be documented before considering infusion of these cells into patients, particularly into immunocompromised subjects where failure of immune surveillance mechanisms might further favor the development of tumors *in vivo*.

The aim of this study was to investigate the potential susceptibility of human BM-derived MSC to malignant transformation at different *in vitro* culture time points and to ascertain whether the biological properties of these cells after *ex vivo* expansion remain appropriate for cell therapy approaches.

Materials and Methods

BM donors. BM cells were harvested, under local or general anesthesia, from 10 healthy hematopoietic stem cell donors (median age 18 years), after obtaining written informed consent. Twenty to 30 milliliters of heparinized BM from each donor were used for MSC generation and expansion. The institutional review board of Pediatric Hematology-Oncology approved the design of this study.

Isolation and long-term culture of BM-derived MSCs. Mononuclear cells were isolated from BM aspirates by density gradient centrifugation

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(Ficoll 1.077 g/mL; Lymphoprep, Nycomed Pharma) and plated in noncoated 75 to 175 cm^2 polystyrene culture flasks (Corning Costar) at a density of 160,000/cm^2 in complete culture medium: Mesencult (StemCell Technologies) supplemented with 10% FCS (Mesenchymal Stem Cell Stimulatory Supplements, StemCell Technologies), 2 mmol/L L-glutamine, and 50 μg/mL gentamicin (Life Technologies). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. After 48 h adhesion, nonadherent cells were removed and culture medium was replaced twice a week. MSCs were harvested after reaching ≥80% confluence, using trypsin (Sigma-Aldrich), and propagated at 4,000 cells/cm^2 continuously until reaching a senescence phase or passage (P) 25. The senescence phase was defined as a decrease in MSC proliferative capacity, finally leading to cell cycle arrest. MSCs in the senescence phase were closely monitored for an additional 8 to 12 weeks before interrupting the cultures, to look for cells that had escaped from senescence and recommenced proliferation. Postsenescence clones were isolated by limiting dilution: To obtain single cell–derived clones, MSCs were seeded at 1 cell per well in a 96-well culture plate (Corning Costar) and cultured as described above. The cells were observed daily for 4 to 6 weeks to examine colony formation.

The population doubling of cultured MSCs was calculated at every passage according to the equation, $\log_2$ (the number of harvested cells / the number of seeded cells). The finite population doublings were determined by cumulative addition of total numbers generated from each passage until the cells ceased dividing.

Flow cytometry. To phenotypically characterize MSCs and to define their purity, FITC or phycoerythrin-conjugated monoclonal antibodies specific for CD45, CD14, CD34, CD13, CD80, CD31, HLA A-B-C, HLA-DR, CD90 (BD PharMingen); CD73, CD105 (Serotec); CD133 (Miltenyi Biotec S.r.l.); and vascular endothelial growth factor receptor 2 (VEGFR2; Sigma-Aldrich) were used. MSCs expanded from the 10 BM donors were analyzed every three passages, starting from P3 (P3, P6, P9, P12, etc.) Appropriate, isotype-matched, nonreactive fluorochrome-conjugated antibodies were used as controls. Analysis of cell populations was done by means of direct immunofluorescence with a FACSCalibur flow cytometer (BD PharMingen) and data were calculated using CellQuest software (BD PharMingen).

Multilineage differentiation potential of MSCs. To assess their differentiation capacity, MSCs cultured from all BM donors were induced into adipocytes and osteoblasts at P3, P6, and at later passages whenever possible, using a method previously described (23, 24).

Telomerase activity detection assay. Telomerase activity was measured by the PCR-based telomeric-repeat amplification protocol (TRAP; ref. 25). Samples containing 0.1, 0.5, and 1 μg of protein were analyzed by the TRAPeze kit (Intergen Company). Protein extract (0.5 μg) from the telomerase-positive tumor cell line (JR8) was used as a positive control sample in each TRAP assay. Each reaction product was amplified in the presence of a 36-bp internal TRAP assay standard. A sample was scored as telomerase activity–positive when positive TRAP results were obtained from at least one protein concentration.

RNA extraction and reverse transcription-PCR analysis of the human telomerase reverse transcriptase. Total cellular RNA was extracted from frozen samples with RNeasy micro kit (Qiagen GmbH). Total RNA (0.5 μg) from each sample was reverse-transcribed by using the reverse transcription-PCR (RT-PCR) Core kit (Applied Biosystems) with random hexamers, and the resultant cDNA was then amplified with the same kit. Amplification of full-length and alternatively spliced human telomerase reverse transcriptase (hTERT) cDNA was obtained as previously described (26).

Detection of alternative lengthening of telomere–associated promyelocytic leukemia bodies. Cells were fixed in 1:1 methanol/acetone and processed to detect associated promyelocytic leukemia (PML) bodies by combined PML immunofluorescence and telomere fluorescent *in situ* hybridization (FISH) according to Henson et al. (27). Images were captured on a Nikon Eclipse E600 fluorescence microscope using ACT-1 (Nikon) image analysis software and processed using Adobe Photoshop Image Reader 7.0 software. Associated PML body status was determined according to previously defined criteria (27). Samples from alternative lengthening of telomere (ALT)–positive (IIICF/c-EJ-*ras*) or telomerase-positive (JR8) tumor cell lines were used as positive and negative controls.

Telomere length analysis. Total DNA was isolated using QuicKpicK genomic DNA kit (BioNobile, Medi Diagnostici), digested with the *Hin*fI restriction enzyme, electrophoresed using CHEF-DR II Pulsed Field system (Bio-Rad), transferred to a nylon membrane, and hybridized with a 5′-end [γ-^{32}P]dATP-labeled telomeric oligonucleotide probe $(TTAGGG)_4$ as previously reported (28). Autoradiographs were scanned (ScanJet IIcx/T, Hewlett

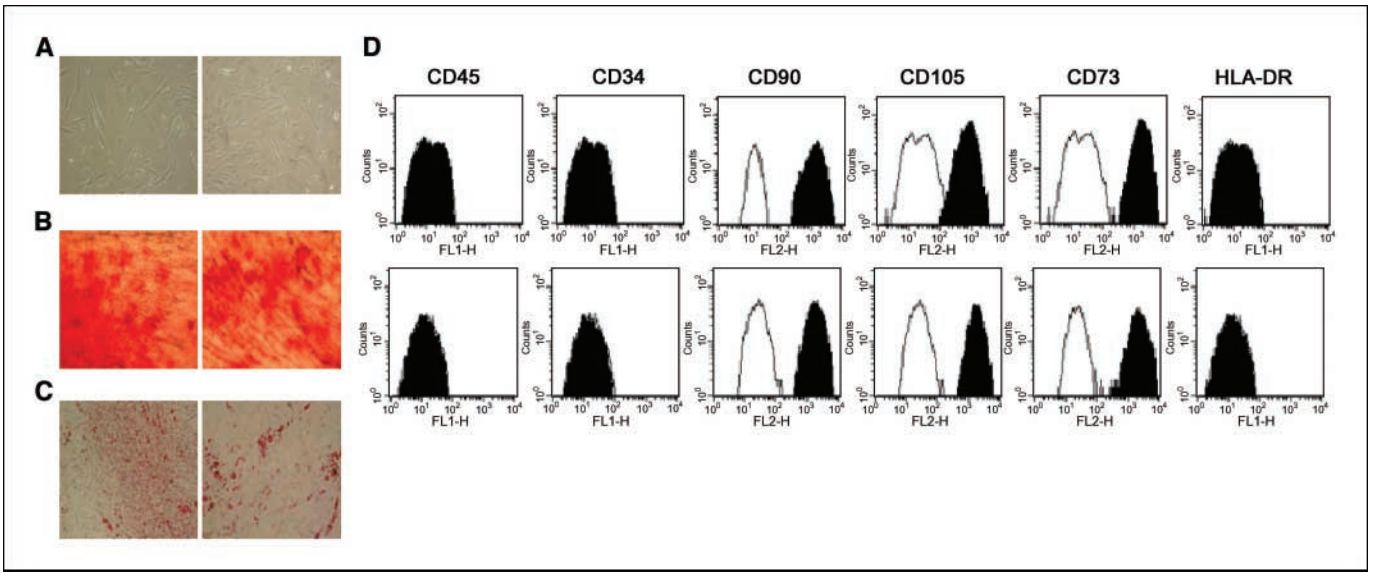

Figure 1. Characterization of human BM-derived MSCs during long-term *in vitro* culture. *A,* morphologic appearance of BM-derived MSCs from donor 7 at P3 (*left*) and P15 (*right*). MSCs at P15 are smaller in size compared with the same cells at P3, although they maintained the typical spindle shape. Magnification, ×10. *B,* osteogenic differentiation capacity of MSCs from donor 5 at P3 (*left*) and P15 (*right*). The differentiation into osteoblasts is shown by the histologic detection of alkaline phosphatase activity (*purple reaction*) and calcium deposition stained with Alzarin red. Magnification, ×20. *C,* adipogenic differentiation capacity of MSCs from donor 5 at P3 (*left*) and P15 (*right*). The differentiation into adipocytes is revealed by the formation of lipid droplets stained with Oil Red O. Magnification, ×20. *D,* immunophenotypic characterization of MSCs from donor 3 at P3 (*top row*) and P15 (*bottom row*).

Packard) and digitalized by Image Quant (Molecular Dynamics). Each gel was standardized by inclusion of DNA from GM847 (ALT-positive) and HeLa (telomerase-positive) cell lines. ALT status was determined as previously reported (27). Statistical analysis of telomere restriction fragment (TRF) length distributions was done using the Telometric software (29).

Direct *P53* sequencing. Cultured MSC DNA obtained from presenescence cultures of donors 1 and 2 was used as a template for PCR amplification of the exon and intronic boundaries of the *p53* gene. Primers were designed to intron sequences flanking exon of the *p53* gene (exons 2–11) as previously reported (30). Direct sequencing of DNA amplicons was done on an Applied Biosystems 373 automated DNA sequencer. Sequences were analyzed using Chromas Lite software (Technelysium) and verified against the sequence deposited in Genbank (National Center for Biotechnology Information).

Molecular karyotyping. Molecular karyotyping was done through array comparative genomic hybridization (array-CGH) with the Agilent kit (Human Genome CGH Microarray, Agilent Technologies). The array-CGH platform is a 60-mer oligonucleotide-based microarray that allows a genome-wide survey and molecular profiling of genomic aberrations with a resolution of ~75 kb (kit 44B). The genetic situation of the 10 BM donors was tested before culture (defined as time 0 or T_0), using either BM mononuclear cells or peripheral blood lymphocytes (PBL), and after *in vitro* culture on MSCs at P3 (the passage at which MSCs are usually harvested for clinical use or T_1). Six MSC samples were also evaluated at later passages, between P11 and P15 (T_2), after prolonged *in vitro* culture. The method for array-CGH analysis of MSCs has been reported in detail elsewhere (24).

Cytogenetic analysis (karyotyping and subtelomeric FISH). Before harvest, the cultures of all MSC BM donors at various passages (P2–P11) were incubated at 37°C with colcemid (Irvine Scientific) at 1 μg/mL final concentration for 2 h. The cells were fixed and spread according to standard procedures. Metaphases of cells were Q-banded and karyotyped in accordance with the International System for Human Cytogenetic Nomenclature recommendations (1995). FISH with chromosome subtelomeric-specific probes (ToTelVysion, Vysis) was done on fixed metaphase chromosomes obtained from 3 of the 10 karyotyped MSC donors, according to manufacturer's instructions and protocol. In total, the kit makes it possible to analyze 36 short- and long-arm subtelomeres and the 5 long-arm subtelomeres of the acrocentric chromosomes.

Results

Characterization of human BM-derived MSCs during long-term *in vitro* culture. MSCs from the 10 healthy donors were characterized by morphology, differentiation capacity, and immune phenotype at different culture time points, namely P3, P6, P9, P12, P15, and later, whenever possible, every two to three passages (Fig. 1*A–D*).

A wide variability between donors was noted in terms of proliferative capacity and *in vitro* life span of their cultured MSCs (see Fig. 2). The first two donors (donors 1 and 2) showed an early arrest of MSC growth; these two samples entered the senescence phase after 44 and 56 days culture at P4 [cumulative population doublings (CPD) = 2.58; mean population doublings per passage (MPD/P) = 0.86] and P5 (CPD = 3.70; MPD/P = 0.93), respectively. Thereafter, MSCs from both donors were monitored, during their senescence phase, daily for 12 weeks. A crisis phase did not occur in the two samples, even after repeated cryopreservation and thawing procedures. In fact, the MSCs progressively died during the senescence period and detached spontaneously from the flasks. In the case of donor 1, after 8 weeks in the senescence phase, we observed the appearance of a few spindle-shaped cells growing in clones, at a very low rate (postsenescence phase). These cells were analyzed by flow cytometry and showed the typical MSC markers (CD90, CD105, CD73, CD13: >95% positive cells; HLA-DR: <5% positive cells), whereas they were negative for hematopoietic

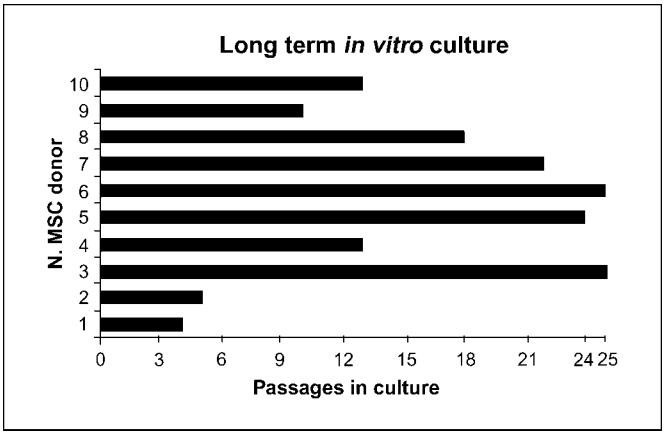

Figure 2. *In vitro* life span of MSC cultures, defined as number of passages before observation of senescence, derived from 8 of the 10 different donors (1, 2, 4, 5, and 7–10). MSC cultures from donors 3 and 6 were interrupted at P25 to allow data analysis. A large variability between the donors is observed.

markers (CD45, CD34) and for both CD133 and VEGFR2 (data not shown). Postsenescence MSCs could not be further propagated; repeated attempts to obtain single cell–derived clones by plating 1 cell per well in a 96-well plate failed. Three BM donors (donors 9, 10, and 4) showed an intermediate arrest in MSC growth. These cells displayed a progressive decrease in their proliferative capacity until they reached a senescence phase at P10 (CPD = 9.32; MPD/P = 1.04), P13 (CPD = 12.44; MPD/P = 1.13) and P13 (CPD = 24.67; MPD/P = 2.06), respectively. MSCs from these three donors maintained their typical spindle-shaped morphology, differentiation capacity to form osteoblasts and adipocytes, and their surface markers throughout the culture period. Donors 8, 7, and 5 showed a late MSC growth arrest, respectively, at P18 (CPD = 25.8; MPD/P = 1.57), P22 (CPD = 20; MPD/P = 1), and P24 (CPD = 22.48; MPD/P = 1.12). Also, in this group, MSCs were regularly characterized every three passages and did not display any relevant abnormality (Fig. 1*B* and *C, right*; Fig. 1*D, bottom*). Moreover, when exceeding P10, MSCs were routinely screened for the expression of CD133 and VEGFR2; these markers, which have been found to be expressed on a transformed MSC subpopulation derived from human BM (31), resulted negative (data not shown). However, MSCs from donors 7 and 5, when cultured after P13, became smaller in size compared with the same cells at P3 (Fig. 1*A, right*), although maintaining the typical spindle shape and a constant growth rate. MSCs reached the senescence phase at 26 and 32 weeks, respectively, for donors 7 and 5, and required a rather uniform amount of time to reach confluence at 8 to 10 days at every passage. In the case of donor 7, MSCs spontaneously differentiated into adipocyte-like cells when approaching senescence at P22 and could not be further propagated. MSCs expanded from donors 3 and 6 were trypsinized and replated 25 times continuously with a total culture period of 33 weeks (corresponding to CPD = 25.05; MPD/P = 1.19) and 44 weeks (corresponding to CPD = 23.16; MPD/P = 1.01), respectively. Thereafter, their long-term cultures were interrupted to allow data analysis. The behavior of MSCs expanded from the last two donors was very similar to that of MSCs from the previous couple of donors. With the exception of the acquisition of a smaller cell size, all other phenotypic and functional characterization variables, including telomerase activity and hTERT expression, were in agreement with the definition of *in vitro* expanded MSCs (1, 32).

Lack of expression of telomere maintenance mechanisms in human BM-derived MSCs during long-term *in vitro* culture and analysis of *p53* gene. MSC cultures obtained from all 10 donors were tested at different *in vitro* passages (from two to six passages for each culture) for the expression of telomerase catalytic activity (Fig. 3*A*) by TRAP assay. Specifically, in all cultures, an early passage (P1–P3) and later passages (P6–P24) were studied. TRAP results failed to evidence the presence of enzyme catalytic activity in all tested samples, including a postsenescence culture obtained from donor 1. To gain insights into the molecular mechanisms responsible for the repression of telomerase activity in MSCs, we assessed the expression of the *hTERT* gene, which codes for the catalytic component of human telomerase (33), in the same cultures screened for telomerase activity. Because it has been shown that alternative splicing of hTERT is involved in the regulation of telomerase activity (34), we analyzed the expression of the different hTERT transcripts (including not only the hTERT full-length transcript, but also three additional splice variants, α^-, β^-, and $\alpha^-\beta^-$) through the use of a specific primer set for the reverse transcriptase domain of hTERT. RT-PCR results failed to evidence the expression of any hTERT transcript in all cultures examined (Fig. 3*B*), thus indicating that the absence of telomerase activity in cultured MSCs was ascribable to a lack of *hTERT* gene transcription.

Consistent with the lack of telomerase activity, when we analyzed telomere length in cultures obtained from four donors (3, 5, 6, and 7) at early (P1–P3) and late (P14–P24) passages, we found evidence of telomere shortening as indicated by a progressive reduction in the mean TRF length (from 12 to 9.3 kb in the cultures obtained from donor 6) or appearance of shorter TRFs (<4 kb in late cultures from donors 3, 5, 6, and 7; Fig. 4).

To address the possibility that alternative mechanisms of telomere maintenance, ALT (35), are operating in telomerase-negative MSCs, we screened them for the expression of associated PML bodies, which are subnuclear structures containing telomeric DNA, telomere-specific binding proteins, and proteins involved in DNA recombination and replication, and represent a peculiar characteristic of ALT cells (36). However, the results we obtained through a combined immunostaining/FISH approach in the same cultures assayed for telomere length failed to evidence the presence

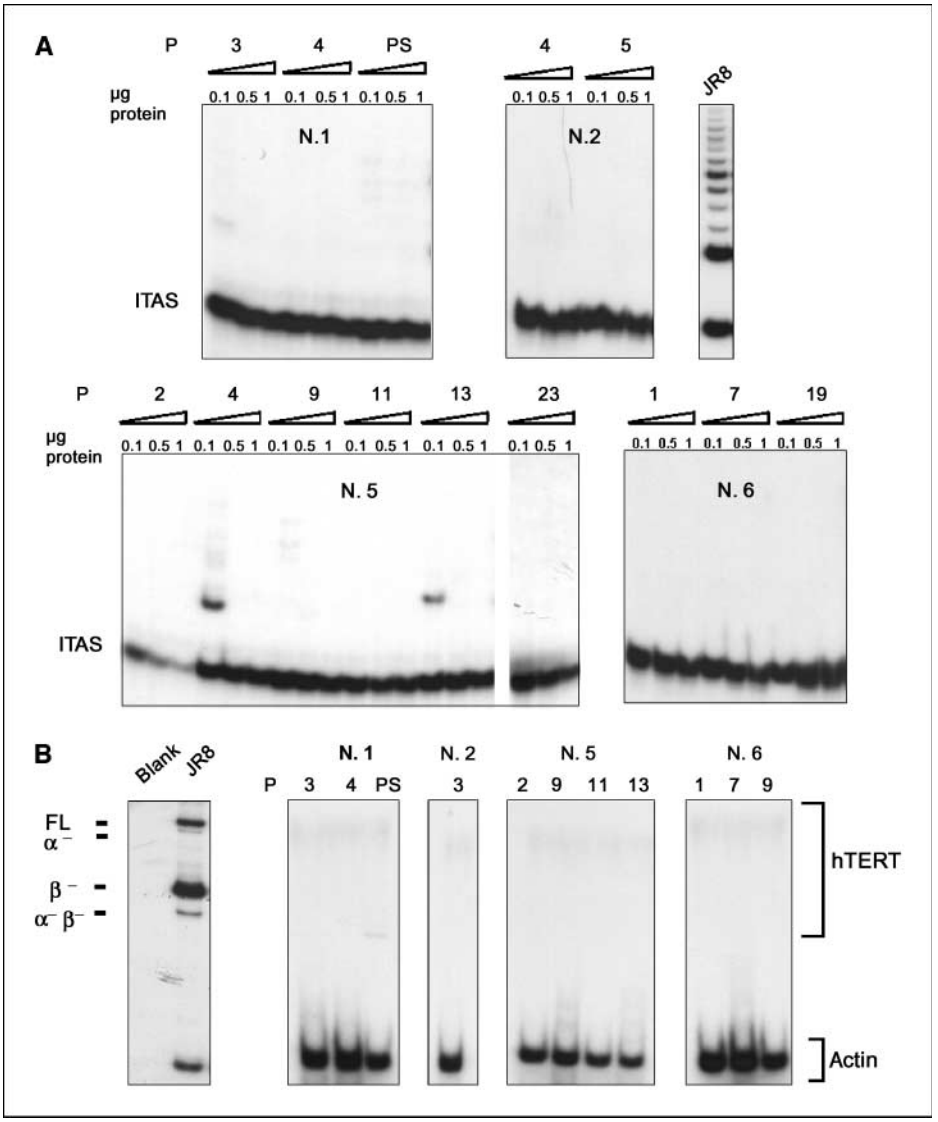

Figure 3. *A,* telomerase activity of MSC cultures derived from donors 1, 2, 5, and 6 at different passages (*P*). Telomerase activity was detected by the TRAP assay using different protein concentrations. *PS,* postsenescence culture. The telomerase-positive cell line JR8 was used as a positive control. The location of the internal amplification standard (*ITAS*) is reported. *B,* expression of hTERT mRNA transcripts, including the full-length (*FL*) and alternative splicing variants α^-, β^-, and $\alpha^-\beta^-$, as detected by RT-PCR in MSC cultures derived from donors 1, 2, 5, and 6 at different passages. Telomerase subunits were coamplified with β-actin as the internal standard. The telomerase-positive cell line JR8 was used as a positive control. The blank represents a negative control to which no RNA was added.

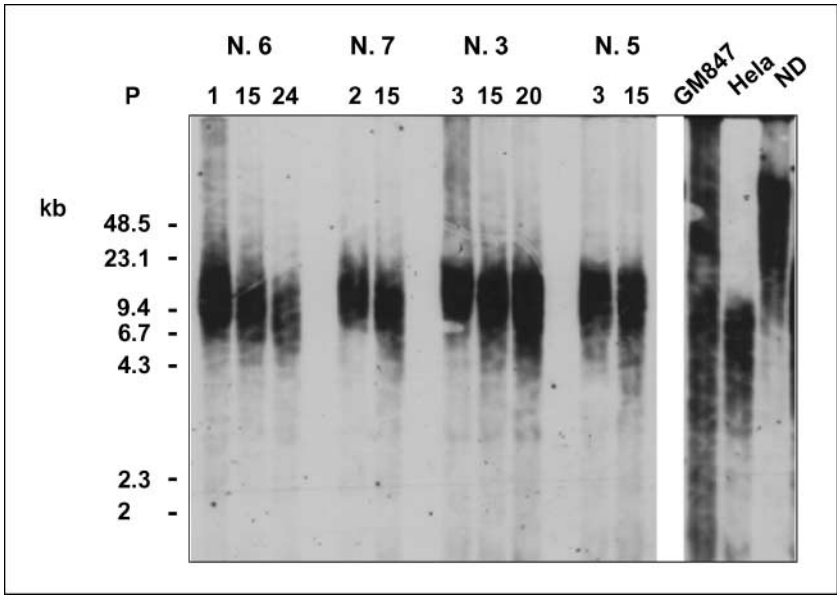

Figure 4. Telomere length distribution of MSC cultures derived from donors 3, 5, 6, and 7 at different passages. GM847 and HeLa cell lines were used as ALT-positive and telomerase-positive controls, respectively. *ND,* nondigested DNA.

of associated PML bodies (data not shown). The absence of an ALT phenotype in these cultures was further confirmed by the pattern of TRF length distribution. In fact, ALT-type telomeres, which are extremely long and heterogeneous (36), were not observed in MSCs (Fig. 4).

As further confirmation of the absence of carcinogenic potential of *ex vivo* cultured MSCs, direct DNA sequencing of exons 2 to 11 in presenescence cultures from donors 1 and 2 did not reveal the presence of mutation in the *p53* gene (data not shown).

Karyotype and subtelomeric FISH analysis. BM mononuclear cells/PBLs (T_0) and MSCs at P3 (T_1) derived from all donors were tested for their genetic situation; MSCs at P11 to P21 (T_2) from 6 of the 10 donors were also studied. In all cases, molecular karyotyping was analyzed by means of array-CGH. The comparison of results from two or three experiments for each donor (T_0, T_1, and T_2) allowed us to distinguish between large copy number variations (LCV; ref. 37), constitutionally present in donor's genome, and true chromosomal imbalances. Results of the array-CGH analysis showed that BM-derived MSCs expanded *in vitro* did not show unbalanced chromosomal abnormalities, as well as submicroscopic rearrangements, considering that the resolution of our approach is ~75 kb (Fig. 5). In fact, the array-CGH profiles of the repeated experiments from the same donor were perfectly overlapping and no deletions or duplications were present, besides the LCVs reported in the available databases.[4] Because array-CGH is not able to unravel balanced chromosomal rearrangements, classic cytogenetics with conventional QFQ banding and FISH analysis with chromosome subtelomeric-specific probes were done on chromosome metaphases obtained from MSCs at variable passages in culture (P2–11). Karyotyping was done in all donors: Only 1 of the 10 MSC samples was characterized by a pericentric inversion of chromosome 9, which represents a well-known variant without any phenotypic effect. Therefore, all MSC donors were characterized by a normal karyotype (Fig. 6*A*). FISH analysis with chromosome subtelomeric-specific probes was done on metaphase chromosomes of three donors (two at T_1 and one at T_2; Fig. 6*B*) and the results were normal in all cases.

Discussion

In the present study, we have generated, propagated in long-term *in vitro* culture, and monitored human MSCs derived from 10 BM donors. BM represents the source of MSCs most commonly used in cell therapy approaches; the biosafety features of these cells need to be carefully investigated to exclude the occurrence of functional or genetic alterations before their release for clinical use.

Our results show that human BM-derived MSCs can be cultured long-term *in vitro*, without losing their peculiar morphologic, phenotypical, and functional characteristics. Moreover, MSCs propagated in culture continuously for up to 44 weeks maintained a normal karyotype, without showing expression of telomere maintenance mechanisms. Consistent with these findings, a progressive reduction in the mean MSC TRF length, or appearance of shorter TRFs, was observed.

In contrast to what has been recently described by Rubio et al. (20) for human, adipose tissue–derived MSCs, none of our MSC samples bypassed the senescence period by developing a crisis phase characterized by a cell cycle rate accelerated compared with presenescence MSCs. On the contrary, all BM-derived MSCs showed a progressive decline in their proliferative/expansion capacity mainly resulting into the development of a senescence phase after variable *in vitro* culture times (6–44 weeks; Fig. 2). These observations on the proliferative life span of MSCs are in agreement with previously published studies on cultured MSCs (19, 20). Only in one case (donor 1), after 8 weeks in the senescence phase, the appearance of a few spindle-shaped cells growing in clones at a very low rate was observed (postsenescence phase). However, these postsenescence MSCs could not be propagated further and never developed a crisis phase. Repeated attempts to obtain single cell–derived clones by plating 1 cell per well in a 96-well plate failed; this cell behavior is very different from that described by Wang et al. (31) on a subpopulation of human MSCs derived from one single BM sample (named "huBM020") that

[4] http://projects.tcag.ca/variation/

showed very rapid population doubling and could be easily cloned in a single-cell assay. Postsenescence MSCs were analyzed by flow cytometry and showed the typical MSC markers at high levels (including CD90 and CD105), whereas they were negative for hematopoietic markers (CD45, CD34) and for CD133 and VEGFR2. On the contrary, the transformed cells described by Henson et al. (36) were shown to express the endothelial markers CD133 and VEGFR2, as well as low levels of CD90, and they were CD105 negative. The same down-regulation of the MSC membrane markers CD90 and CD105 was noted in the postcrisis cells described by Rubio et al. (20), which were derived from adipose tissue. Furthermore, the TRAP assay done on postsenescence MSCs derived from donor 1 failed to exhibit the expression of telomerase catalytic activity. All these findings show that our postsenescence cells are not different from normal MSCs.

Regarding morphology, we noticed that MSCs from four donors (3, 5, 6, and 7) became smaller in size when cultured over P13 to P15, although they maintained the typical spindle shape and a constant growth rate (Fig. 1*A, right*). On the contrary, the cells described by both Wang and Rubio (20, 31) resulted in cells morphologically distinct from typical MSCs; they were round or cuboidal, and, in the article published by Wang et al. (31), also exhibited contact-independent growth and formed foci with cells released into suspension. In our study, MSCs from all 10 donors, tested both at early (P3, infusion passage) and late passages (P9–P25), did not show immunophenotypic abnormalities and maintained a high level of purity throughout the culture period (Fig. 1*D*). Moreover, all cultures exceeding P10 were analyzed for the expression of CD133 and VEGFR2 and resulted negative. Also, the ability to differentiate into osteoblasts and adipocytes was preserved throughout the culture period in all MSC donors (Fig. 1*B* and *C*).

In agreement with previous reports (38), all our MSC samples lack expression of telomerase activity both at early passages and after long-term *in vitro* culture (up to P24). This finding was confirmed by the absence of hTERT transcript expression and reflected by a progressive telomere shortening in cultured MSCs. On the contrary, the transformed mesenchymal cells described by other authors (20, 31) exhibited telomerase activity. This phenomenon was also observed by Miura et al. (21) in murine BM-derived MSCs that, after numerous passages in culture, gradually increased telomerase activity and proceeded to a malignant state, resulting in fibrosarcoma formation *in vivo*. Some human tumors, mainly those of mesenchymal origin, including soft tissue and osteogenic sarcomas and glioblastomas (27), maintain their telomeres by the ALT mechanism. Telomere dynamics in ALT cells are consistent

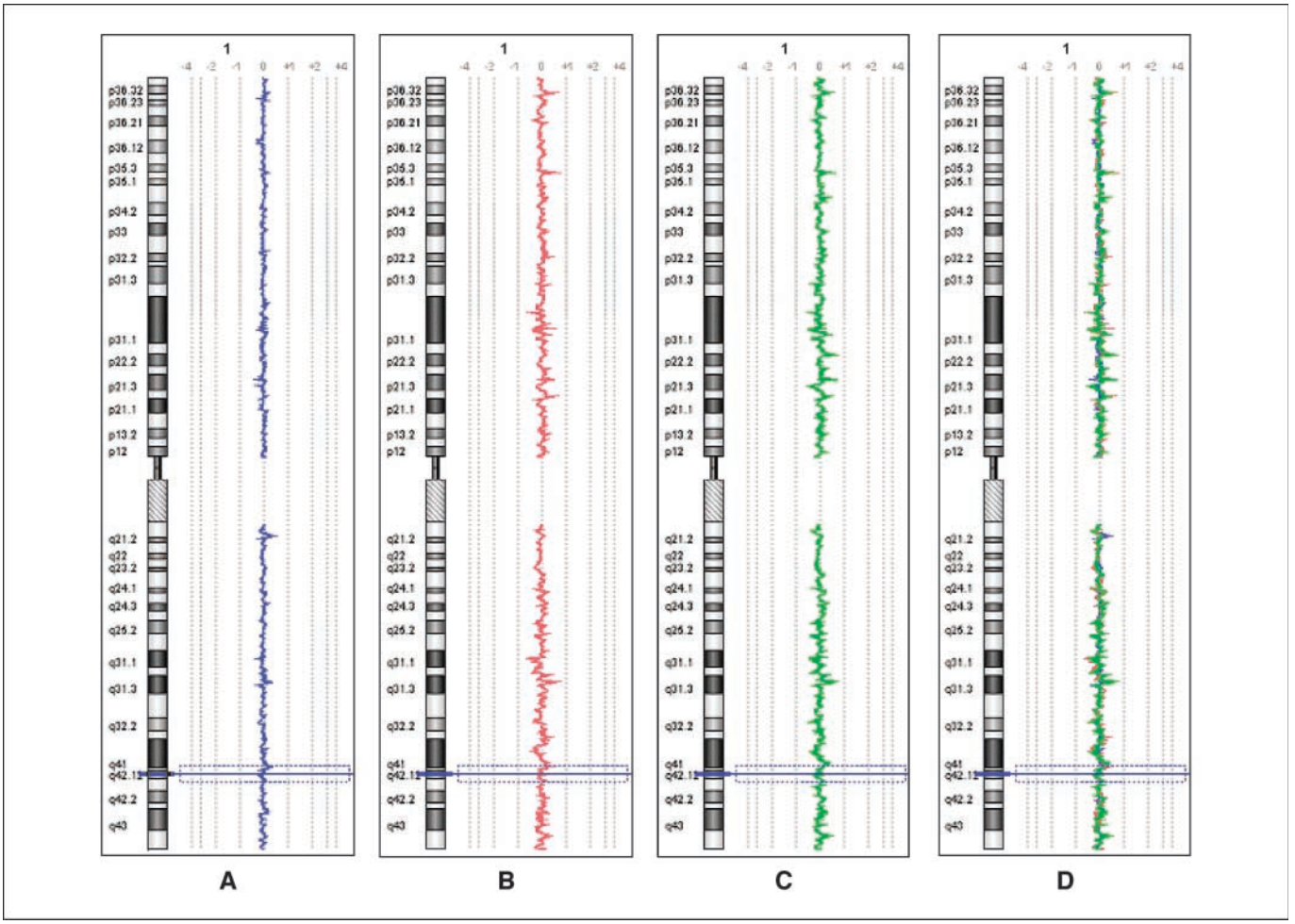

Figure 5. Representative MSC array-CGH profiles of chromosome 1 from donor 6 at (*A*) T_0: PBLs; (*B*) T_1: MSCs at P3; (*C*) T_2: MSCs at P14. *D,* three overlapping experiments: *blue line,* PBLs; *red line,* P3; *green line,* P14. The array-CGH profiles of MSCs are linear and perfectly overlapped, even when there are duplications or deletions caused by LCVs. This shows that *in vitro* expanded MSCs do not show unbalanced chromosomal rearrangements.

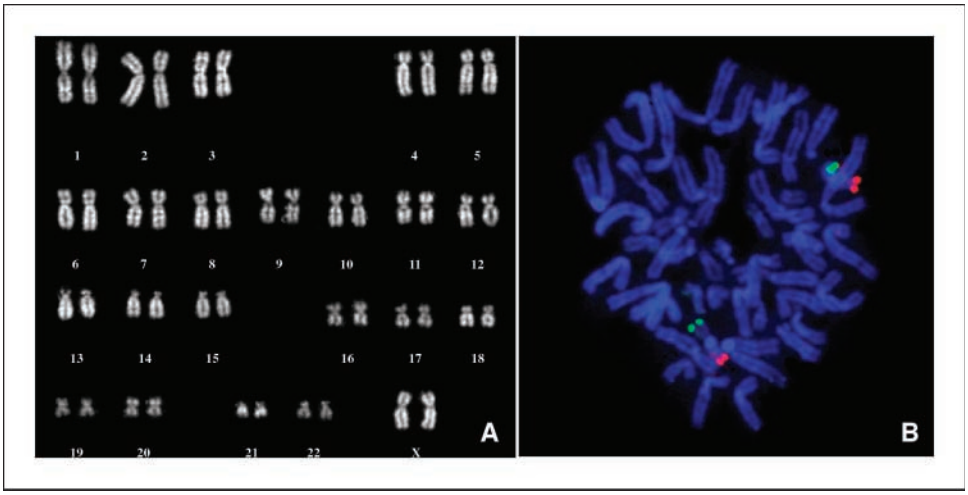

Figure 6. *A,* normal Q-banding karyotype (360–400 band) of MSCs from donor 2 at P2. *B,* FISH analysis with chromosome 16 subtelomeric-specific probes on a metaphase from donor 5 at P18. *Green signals,* subtelomeric regions of the short arm of chromosome 16; *red signals,* subtelomeric regions of the long arm. The location of signals on chromosome 16 only shows that no translocation involving this chromosome is present.

with a recombination-based mechanism, and characteristics of ALT cells include unusually long and heterogeneous telomeres, as well as the presence of peculiar subnuclear structures termed associated PML bodies. In all MSC samples tested, the presence of associated PML bodies failed to be evidenced. Moreover, the pattern of TRF distribution that we observed in the cultures was not consistent with the ALT phenotype.

The biosafety of BM-derived MSCs was further investigated by molecular karyotyping done by array-CGH, classic cytogenetics. and subtelomeric FISH analysis. For its high-resolution capacity and in view of the difficulty in obtaining cultured MSC metaphases, array-CGH may be considered the method of choice for characterizing the genomic situation of MSCs expanded *in vitro* (Fig. 5; ref. 24). However, this technique is unable to detect balanced chromosomal rearrangements that have been excluded in our MSC samples by performing in parallel classic karyotype and subtelomeric FISH analysis. Altogether, the karyotype analysis experiments, done before culture, as well as at early and late passages, showed that extensively *in vitro* expanded human BM-derived MSCs are devoid of chromosomal abnormalities, as well as of unbalanced submicroscopic rearrangements. Of course, we cannot completely exclude that point mutations or other subtle molecular events affecting oncogenes or tumor-suppressor genes might have occurred in cultured cells, predisposing them to transformation. Likewise, mechanisms of oncogenesis facilitated by infusion into an immunocompromised host are not explored by our approach.

In conclusion, our data indicate that human BM-derived MSCs do not display an aptitude for spontaneous transformation and can be safely expanded *in vitro* without any sign of immortalization or development of chromosomal abnormalities. The susceptibility to malignant transformation described in murine BM-derived MSCs by Miura et al. (21) might be related to the species of origin of the cells, which display a high degree of chromosome instability, characterized by the development of both structural and numerical aberrations even at early culture passages. The same authors could not show a similar behavior in human BM-derived MSCs, which were propagated *in vitro* under similar culture conditions. Also, in the case of human adipose tissue–derived MSCs (20), the susceptibility to malignant transformation might be strictly connected with the origin of the tissue; indeed, in comparison with BM, which is very rich in stem cells, fat tissue contains mainly differentiated cells, and it generates MSCs that are immunophenotypically slightly different from those derived from BM. Finally, in the study by Wang et al. (31), it is very likely that the *in vitro* culture of BM-derived MSCs caused the transformation of a subpopulation of cells capable to express endothelial markers. Our results provide support to the concept that the biological properties of human BM-derived MSCs after *ex vivo* expansion remain suitable for use in cell therapy approaches; however, considering the relevant interest in the utilization of MSCs in several fields of medicine and the potential risk of developing alterations during the expansion period, it is strongly recommended that phenotypic, functional, and genetic characteristics of MSCs after *in vitro* culture are tested to further guarantee safety for the patient.

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