CHAPTER 4

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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Summary

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 8-12 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 (FISH) analysis both before and after prolonged culture. MSCs were tested for the expression of telomerase activity, 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 ten 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 demonstrated that MSCs expanded in vitro did not document 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.
Introduction

In recent years, a significant improvement in the understanding of multipotent mesenchymal stromal cell (MSC) biology\(^1\) has opened the way to the clinical use of these adult stem cells. MSCs have been employed 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, which would allow 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 \textit{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 \textit{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.

The use of MSCs for clinical approaches in many fields of medicine first requires that the bio-safety 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 immune-compromised 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

Bone marrow 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-thirty ml of heparinized BM from each donor were employed 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 (Ficoll 1.077 g/ml; Lymphoprep, Nycomed Pharma, Oslo, Norway) and plated in non-coated 75-175 cm² polystyrene culture flasks (Corning Costar, Celbio, Milan, Italy) at a density of 160,000/cm² in complete culture medium: Mesencult (StemCell Technologies, Vancouver, Canada) supplemented with 10 % FCS (Mesenchymal Stem Cell Stimulatory Supplements, StemCell Technologies), 2 mM L-glutamine and 50 µg/ml gentamycin (Gibco-BRL, Life Technologies, Paisely, UK). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. After 48 hour adhesion, non-adherent cells were removed and culture medium was replaced twice a week. MSCs were harvested after reaching ≥ 80% confluence, using Trypsin (Sigma-Aldrich, Milano, Italy), and propagated at 4,000 cells/cm².
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 8-12 weeks before interrupting the cultures, in order to look for the appearance of a crisis phase defined as uncontrolled cell proliferation. Post-senescence clones were isolated by limiting dilution: to obtain single cell-derived clones, MSCs were seeded at 1 cell/well in a 96-well culture plate (Corning Costar) and cultured as described above. The cells were observed daily for 4-6 weeks to examine colony formation.

Flow cytometry
To phenotypically characterize MSCs and to define their purity, fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated monoclonal antibodies specific for CD45, CD14, CD34, CD13, CD80, CD31, HLA A-B-C, HLA-DR, CD90 (BD PharMingen, San Diego, CA), CD73, CD105 (Serotec, Kidlington, Oxford, UK), CD133 (Miltenyi Biotec S.r.l., Bologna, Italy), 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, non-reactive fluorochrome-conjugated antibodies were employed as controls. Analysis of cell populations was performed by means of direct immunofluorescence with a FACScalibur flow cytometer (BD BD PharMingen) and data 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, employing a method previously described. To detect osteogenic differentiation, cells were stained for alkaline phosphatase (AP) activity using Fast Blue (Sigma-Aldrich) and for calcium deposition with
Alzarin Red (Sigma-Aldrich). The adipogenic differentiation was evaluated through the morphological staining of fat droplets with Oil Red O (Sigma-Aldrich).

Telomerase activity detection assay
Telomerase activity was measured by the polymerase chain reaction (PCR)-based telomeric-repeat amplification protocol (TRAP).\textsuperscript{25} Samples containing 0.1, 0.5 and 1 \(\mu\)g of protein were analyzed by the TRAPeze kit (Intergen Company, Oxford, UK) according to the manufacturer’s protocol. After extension of the substrate TS (5’-AATCCGTCGAGCAGAGTT-3’) oligonucleotide by telomerase, the telomerase products were amplified by PCR in the presence of a 5\textsuperscript{[32P]}-end-labeled TS primer for 28 cycles and resolved in 10% polyacrylamide gels. Protein extract (0.5 \(\mu\)g) 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 (ITAS). A sample was scored as telomerase activity-positive when positive TRAP results were obtained from at least one protein concentration.

RNA extraction and reverse transcriptase (RT)-PCR analysis of the human telomerase reverse transcriptase (hTERT)
Total cellular RNA was extracted from frozen samples with RNeasy micro kit (QUIAGEN GmbH, Hilden, Germany) according to the manufacturer’s protocol. Total RNA (0.5 \(\mu\)g) from each sample was reverse-transcribed by using the RT-PCR Core kit (Applied Biosystems, Branchburg, NJ) with random hexamers, and the resultant cDNA was then amplified with the same kit. Amplification of full length and alternatively spliced hTERT cDNA was obtained using TERT-2164S (5’-GCCTGAGCTGTACTTTGTCAA-3’) and TERT-2620AS(5’-CGCAAAACAGCTTGTTCTCCATGTC-3’) oligonucleotides.
with initial heating at 95°C for 3 min, followed by 35 cycles at 95°C for 30 s, 62°C for 50 s, 72°C for 50 s, and 72°C for 5 min. The amplification was performed in a mixture containing 0.3 μCi [α-32p]deoxycytidinetriphosphate (300 Ci/mmol; Amersham Pharmacia Biotech, Cologno Monzese, Milan, Italy). Primers for the internal β-actin control were added during cycle 15 at 72°C. Amplified products were electrophoresed on a 5% non-denaturing polyacrylamide gel in 1X Tris-borate EDTA buffer. The gel was dried and autoradiographed.

Detection of alternative lengthening of telomere (ALT)-associated promyelocytic leukemia (PML) bodies (APBs)

Cells were fixed in 1:1 methanol:acetone and processed to detect APBs by combined PML immunofluorescence and telomere FISH according to Henson et al. Images were captured on a Nikon Eclipse E600 fluorescence microscope using ACT-1 (Nikon, Tokyo, Japan) image analysis software and processed using Adobe Photoshop Image Reader 7.0 software. APB status was determined according to previously defined criteria. The presence of an APB was defined by the localization of a telomeric DNA focus within a nuclear PML body; samples were scored as APB-positive if they contained APBs in ≥0.5% of cells. To avoid false positives, an APB was considered to be present only when the telomeric DNA fluorescence within a PML body was more intense than that of telomeres, and a cell was not considered to contain APBs if >25% of the co-localized foci occurred outside the nucleus. To avoid false negatives, at least 2,000 nuclei were examined, and the assay was repeated in the presence of negative results. Samples from ALT-positive (IIICF/c-EJ-ras) or telomerase-positive (JR8) tumor cell lines were used as positive and negative controls for the APB assay.
Telomere length analysis

Total DNA was isolated using QuicKpicK genomic DNA kit (BioNobile, Medi Diagnostici, Milan, Italy), digested with the *Hinf*I restriction enzyme, electrophoresed using CHEF-DR II Pulsed Field system (BioRad, Hercules, CA), transferred to a nylon membrane, and hybridized with a 5'-end [γ-32P]dATP-labeled telomeric oligonucleotide probe (TTAGGG)₄ as previously reported. Autoradiographs were scanned (ScanJet IIcx/T, Hewlett Packard, Milan, Italy) and digitalized by Image Quant (Molecular Dynamics, Sunnyvale, CA). Each gel was standardized by inclusion of DNA from GM847 (ALT-positive) and HeLa (telomerase-positive) cell lines. ALT status was determined by calculating whether the mean, variance and semi-interquartile range of the terminal restriction fragment (TRF) length distribution was greater than 16 kb, 1,000 kb, and 4 kb, respectively. Samples were classified as ALT-positive when 2/3 or 3/3 of these criteria were met for unimodal or bimodal TRF length distributions, respectively. Statistical analysis of TRF length distributions was performed using the Telometric software.

Molecular karyotyping

Molecular karyotyping was performed through array comparative genomic hybridization (array-CGH) with the Agilent kit (Human Genome CGH Microarray, Agilent Technologies, Santa Clara, CA). 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 about 75 kb (kit 44B). The genetic situation of the 10 BM donors was tested before culture (defined as time 0 or T₀), using either BM mononuclear cells (BMMNCs) or peripheral blood lymphocytes (PBLs), and after *in vitro* culture on MSCs at P3 (the passage at which MSCs are usually harvested for clinical use or T₁). Six
MSCs samples were also evaluated at later passages, between P11 and P15 (T2), 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)
Prior to harvest, the cultures of all MSC BM donors at various passages (P2-P11) were incubated at 37°C with colcemid (IrvineScientific, Santa Ana, CA) at 1 μg/ml final concentration for 2 hours. 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 (ISCN, 1995). Fluorescent in situ hybridization (FISH) with chromosome subtelomeric-specific probes (ToTelVysion, Vysis, Downer’s Grove, IL) was performed on fixed metaphase chromosomes obtained from three of the ten karyotyped MSC donors, according to manufacturer’s instructions and protocol. In total, the kit makes it possible to analyze 36 short and long arms subtelomeres and the 5 long arms subtelomeres of the acrocentric chromosomes.

Results
Characterization of human BM-derived MSCs during long term in vitro culture MSCs derived from BM of the 10 healthy donors were isolated and propagated in long term in vitro culture. MSCs from all 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 2-3 passages (Figure 1A, 1B, 1C and 1D).
Figure 1. Characterization of human BM-derived MSCs during long term in vitro culture. A) Morphological appearance of BM-derived MSCs from DONOR #7 at P3 (left) and P15 (right). MSCs at P15 are smaller in size as compared to the same cells at P3, although they maintained the typical spindle shape. Magnification x10. B) Osteogenic differentiation capacity of MSCs from DONOR N. 5 at P3 (left) and P15 (right). The differentiation into osteoblasts is demonstrated by the histological detection of AP activity (purple reaction) and calcium deposition stained with Alizarin Red. Magnification x 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 x 20. D) Immunophenotypic characterization of MSCs from DONOR #3 at P3 (superior row) and P15 (inferior row). E) Expression of CD133 and VEGFR2 molecules on the surface of MSC from DONOR #3 at P15.
A wide variability between donors was noted in terms of proliferative capacity and \textit{in vitro} life span of their cultured MSCs (see Figure 2). The first two donors (donor #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 and P5, respectively. Thereafter, MSCs from both donors were monitored, during their senescence phase, daily for twelve 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 (characterized by appearance of picnotic bodies within the cells) 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 (post-senescence 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 markers (CD45, CD34) and for both CD133 and VEGFR2 (data not shown). Post-senescence MSCs could not be further propagated; repeated attempts to obtain single cell-derived clones by plating 1 cell/well in a 96-well plate failed.

![Figure 2. In vitro life span of MSC cultures, defined as number of passages before observation of senescence, derived from 10 different donors (#1-10). A large variability between the donors is observed.](image-url)
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, P13 and P13, 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, P22 and P24. Also in this group, MSCs were regularly characterized by morphology, differentiation potential and immune phenotypic analysis every 3 passages and did not display any relevant abnormality (Figure 1B and 1C, pictures on the right; Figure 1D, lower panel). 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 BM35, resulted negative (Figure 1E). However, MSCs from donor #7 and #5, when cultured after P13, became smaller in size as compared to the same cells at P3 (Figure 1A, picture on the right), although maintaining the typical spindle shape and a constant growth rate. MSCs reached the senescence phase at 26 and 32 weeks respectively for donor #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 donor #3 and #6 were trypsinized and replated 25 times continuously with a total culture period of 33 and 44 weeks 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 parameters, including telomerase activity and hTERT expression, were in
agreement with the definition of in vitro expanded MSCs.\textsuperscript{1,29} In particular, we did not observe any acceleration in cell growth rates.

Lack of expression of telomere maintenance mechanisms in human BM-derived MSCs during long term in vitro culture

MSC cultures obtained from all 10 donors were tested at different in vitro passages (from 2 to 6 passages for each culture) for the expression of telomerase catalytic activity (Figure 3A) 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 post-senesceence 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\textsuperscript{30}, in the same cultures screened for telomerase activity. Since it has been demonstrated that alternative splicing of hTERT is involved in the regulation of telomerase activity\textsuperscript{31}, we analyzed the expression of the different hTERT transcripts (including not only the hTERT full-length transcript, but also three additional splice variants, $\alpha^\prime$, $\beta^\prime$ and $\alpha\beta^\prime$) 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 (Figure 3B), thus indicating that the absence of telomerase activity in cultured MSCs was ascribable to a lack of hTERT gene transcription.
Figure 3. A) Telomerase activity of MSC cultures derived from donors #1,2,5,6 at different passages (P). Telomerase activity was detected by the TRAP assay using different protein concentrations. PS, post-senescence 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 h-TERT mRNA transcripts, including the full length (FL) and alternative splicing variants $\alpha$, $\beta$, and $\alpha\beta$, as detected by RT-PCR in MSC cultures derived from donors #1,2,5,6 at different passages (P). Telomerase subunits were coamplified with $\beta$-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.

Consistent with the lack of telomerase activity, when we analyzed telomere length in cultures obtained from 4 donors (#3-5-6-7) 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 Kb to 9.3 Kb in the cultures obtained from donor #6) or appearance of shorter TRFs (<4 Kb in late cultures from donors #3-5-6-7) (Figure 4).

To address the possibility that alternative mechanisms of telomere maintenance referred to as ALT33 are operating in telomerase-negative MSCs, we screened them for the expression of APBs, 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 cells33. However, the results we obtained through a combined immunostaining/FISH approach in the same cultures assayed for telomere length failed to evidence the presence of APBs (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 heterogeneous33, were not observed in MSCs (Figure 4).

Figure 4. Telomere length distribution of MSC cultures derived from donors #3,5,6,7 at different passages (P). GM847 and HeLa cell lines were used as ALT-positive and telomerase-positive controls, respectively. ND, non-digested DNA.
Karyotype and subtelomeric FISH analysis

BMMNCs/PBLs (T₀) and MSCs at P3 (T₁) derived from all donors were tested for their genetic situation; MSCs at P11-21 (T₂) from six of the ten 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 (T₀, T₁, and T₂), allowed us to distinguish between large copy number variations (LCVs)³⁴, constitutionally present in donor’s genome, and true chromosomal imbalances. Results of the array-CGH analysis demonstrated that, even after long term culture, 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 about 75 kb (Figure 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 databases¹.

Figure 5. Representative MSC array-CGH profiles of chromosome 1 from donor #6 at: A) T₀: PBLs; B) T₁: MSCs at P3; C) T₂: MSCs at P14. D) Three overlapping experiments: blue line applies to PBLs, red line to P3 and green line to P14. The array-CGH profiles of MSCs are linear and perfectly overlapped, even when there are duplications or deletions caused by LCVs. This demonstrates that in vitro expanded MSCs do not show unbalanced chromosomal rearrangements.

¹ http://projects.tcag.ca/variation/

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Since array-CGH is not able to unravel balanced chromosomal rearrangements, classical cytogenetics with conventional QFQ banding and FISH analysis with chromosome subtelomeric-specific probes were performed on chromosome metaphases obtained from MSCs at variable passages in culture (P2-11), in order to detect reciprocal translocations. Karyotyping was performed in all donors: only one out of the ten 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 (Figure 6A). FISH analysis with chromosome subtelomeric-specific probes was performed on chromosome metaphase of three donors (two at T1 and one at T2, Figure 6B) and resulted normal in all cases.

![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 represent subtelomeric regions of the short arm of chromosome 16; red signals those of the long arm. The location of signals on chromosome 16 only demonstrates that no translocation involving this chromosome is present.](image-url)
Discussion

In the present study, we have generated, propagated in long term \textit{in vitro} culture and monitored human MSCs derived from 10 BM donors. Importantly, BM represents the source of MSCs most commonly used in cell-therapy approaches. MSCs from all donors were characterized, at several culture time points, on the basis of the typical parameters for MSC definition (such as morphology, immune phenotype and differentiation capacity), from a genetic point of view and by the expression of telomerase activity and alternative mechanisms of telomere maintenance.

Considering the wide interest in MSCs, in particular for those derived from BM, for clinical approaches, the bio-safety features of these cells need to be carefully investigated, in order to exclude the occurrence of functional or genetic alterations before releasing these cells for clinical use.

Our results demonstrate that human BM-derived MSCs can be cultured long term \textit{in vitro}, without losing their peculiar morphological, 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.\textsuperscript{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 to pre-senescence MSCs. On the contrary, all BM-derived MSCs demonstrated a progressive decline in their proliferative/expansion capacity mainly resulting into the development of a senescence phase after variable \textit{in vitro} culture times (6-44 weeks; Figure 2). These observations on the proliferative life-span of MSCs are in agreement with previously published studies on cultured MSCs.\textsuperscript{19,20} Only in one case (donor #1), after 8 weeks in the

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senescence phase, the appearance of a few spindle-shaped cells growing in clones at a very low rate was observed (post-senescence phase). However, these post-senescence MSCs could not be propagated further and never developed a crisis phase. Repeated attempts to obtain single cell-derived clones by plating 1 cell/well in a 96-well plate failed; this cell behavior is very different from that described by Wang et al.\textsuperscript{35} on a subpopulation of human MSCs derived from one single BM sample (named ‘huBM020’) that showed very rapid population doubling and could be easily cloned in a single-cell assay. Post-senescence 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 Wang and colleagues\textsuperscript{35} 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 post-crisis cells described by Rubio et al\textsuperscript{20}, which were derived from adipose tissue. Furthermore, the TRAP assay performed on post-senescence MSCs derived from donor N.1 failed to exhibit the expression of telomerase catalytic activity. All these findings demonstrate that our post-senescence cells are not different from normal MSCs, since they display the same particular MSC characterization parameters.

Regarding morphology, we noticed that MSCs from four donors (#3-5-6-7) became smaller in size when cultured over P13-15, although they maintained the typical spindle shape and a constant, growth rate (Figure 1A, right). On the contrary, the cells described by both Wang and Rubio\textsuperscript{35,20} resulted in cells morphologically distinct from typical MSCs; they were round or cuboidal, and, in the paper published by Wang and colleagues\textsuperscript{35}, also exhibited contact-independent growth and formed foci with cells released into suspension. In our study MSCs from all ten donors, tested both at early (P3, infusion passage) and
late passages (P9-25) did not show immune phenotypic abnormalities and maintained a high level of purity throughout the culture period (Figure 1D). 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 (Figure 1B and 1C).

In agreement with previous reports, 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 exhibited telomerase activity. This phenomenon was also observed by Miura *et al.* 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, maintain their telomeres by the ALT mechanism. Telomere dynamics in ALT cells are consistent 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 APBs. In all MSC samples tested, the presence of APBs failed to be evidenced. Moreover, the pattern of TRF distribution that we observed in the cultures was not consistent with the ALT phenotype.

The bio-safety of BM-derived MSCs was further investigated by molecular karyotyping performed by array-CGH, classical cytogenetics and subtelomeric FISH analysis. Array-CGH is a rapid and high-resolution technique useful for the detection of both benign and disease-causing genomic copy-number variations in tumors and genetic disorders, and also for testing cultured embryonic stem cells. 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 \emph{in vitro} (Figure 5).\textsuperscript{24} However, this technique is unable to detect balanced chromosomal rearrangements, that have been excluded in our MSC samples by performing in parallel classical karyotype and subtelomeric FISH analysis. Altogether, the karyotype analysis experiments, performed before culture, as well as at early and late passages, demonstrated that extensively \emph{in vitro} expanded human BM-derived MSCs are devoid of chromosomal abnormalities, as well as of unbalanced submicroscopic rearrangements. These findings are different from what is observed in embryonic stem cells maintained \emph{in vitro} for a high number of passages.\textsuperscript{39} 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 a immune compromised 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 \emph{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 and colleagues\textsuperscript{21} might be related to the animal 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 demonstrate a similar behavior in human BM-derived MSCs which were propagated \emph{in vitro} under similar culture conditions. Also in the case of human adipose tissue-derived MSCs\textsuperscript{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 immune phenotypically slightly different from those derived from BM. Finally, in the study by Wang and colleagues\textsuperscript{36}, it is very likely that the \textit{in vitro} culture of BM-derived MSCs caused the transformation of a subpopulation of cells capable to express endothelial markers. This is further demonstrated by the fact that the same cells, thawed a few months later, propagated normally in culture and did not give rise to any transformed population.

Our results provide support to the concept that the biological properties of human BM-derived MSCs after \textit{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 phenotype, functional and genetic characteristics of MSCs after \textit{in vitro} culture are tested, to further guarantee safety for the patient.

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