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# Lack of telomerase activity in human mesenchymal stem cells

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**Telomerase activity and telomere maintenance have been associated with immortality in tumor and embryonic stem cells. Whereas most normal somatic cells are telomerase negative, low levels of this enzyme have been found in adult stem cells from the skin, gut and the hematopoietic system. Here, we show that telomerase activity is not detectable in human mesenchymal stem cells (hMSCs), which have the phenotype SH2$^+$, SH3$^+$, SH4$^+$, CD29$^+$, CD44$^+$, CD14$^-$, CD34$^-$ and CD45$^-$, and have the capacity to differentiate into adipocytes, chondrocytes and osteoblasts. These data suggest that hMSCs have a different telomere biology compared to other adult stem cells. Alternatively, true mesenchymal stem cells might be a very rare subpopulation that have a detection level that is below the sensitivity of the TRAP assay.**
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## Introduction

Human mesenchymal stem cells (hMSCs) have attracted much attention because of their multipotential properties with regard to differentiation, and their possible use for cell and gene therapy.[1] hMSCs from bone marrow are referred to as stroma-derived stem cells which give rise to multiple nonhematopoietic cell lineages, such as osteoblasts, chondrocytes, adipocytes and hematopoiesis-supporting stroma.[2–4] hMSCs have been described to be negative for CD14, CD34, CD45, and positive for SH2, SH3, CD29, CD44, CD71, CD90, CD106, CD120a and CD124.[4,5] Furthermore, hMSCs have the ability to proliferate extensively *in vitro,* and maintain the capacity to differentiate into multiple cell types upon culture, which has been associated with their stem cell nature.[4,6,7] Recently, a more primitive multipotent adult progenitor cell population (MAPCs) was identified, which differentiates not only into mesenchymal cells, but also into cells with characteristics of visceral mesoderm, neuroectoderm and endoderm.[7,8]

It has been established that the proliferative capacity of human cells is causally linked to the functional maintenance of their telomeres, which constitute the final ends of chromosomes. Telomeres consist of TTAGGG repeats and associated proteins in human cells.[9] They serve to protect the ends of chromosomes from end-to-end fusion, recombination and degradation. In most normal somatic cells, telomere sequences are lost during replication *in vitro* and *in vivo,* limiting their proliferative capacity.[10–13] In contrast, immortal cells such as tumor and embryonic stem cells are capable of maintaining telomere function, in general, by activation of the reverse transcriptase telomerase.[14–16] Unlike most normal somatic cells that are telomerase negative, low to moderate levels of the enzyme have been described in adult stem cells from skin,[17] gut,[18] and from the hematopoietic system.[19,20] Furthermore, telomerase activity in hematopoietic cells has been associated with a capacity for self-renewal.[21] In order to define the telomerase status in mesenchymal stem cells, we determined the activity of the enzyme in several human specimens, which surprisingly revealed that these cells are telomerase negative using the TRAP assay.

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## Materials and methods

### *Isolation, characterization and culture of hMSCs*

hMSCs (MSC nos. 1–3) with the capacity for adipogenic, chondrogenic and osteogenic differentiation were obtained from Osiris Therapeutics Inc., Baltimore (via CellSystems, St. Katharinen, Germany). The cryopreserved cells (HMSC 6830) showed the following flow-cytometric expression of surface antigens after the second passage, according to the certificate of analysis: positive for SH2, SH3, SH4, CD29, CD44 and negative for CD14, CD34 and CD45. Cells were plated at a density of 3000–6000 cells/cm$^2$ in 0.2–0.4 ml MSCGM Medium (Osiris Therapeutics Inc.) per cm$^2$ growth area and were supplemented with 10% mesenchymal cell growth supplement (Osiris Therapeutics Inc.), 2% L-glutamin, 0.05 U/ml penicillin, 0.05 $\mu$g/ml streptomycin (MSCGM SingleQuots®, Osiris Therapeutics Inc.). The medium was replenished every 3–4 days and cells were passaged upon reaching a confluency of 80–90%.

MSC nos. 4–8 were derived from fresh bone marrow samples of healthy individuals according to the guidelines of the University of Freiburg. Mononuclear cells (MNCs) were separated by density gradient centrifugation and plated at a density of 100 000–150 000 MNCs/cm$^2$ in MSCGM medium. Nonattached cells were washed away the following day. Once adherent cells were more than 50% confluent, they were detached with 1 × trypsin–EDTA (Invitrogen GmbH, Karlsruhe, Germany) and replated at a density of 1000 cells/cm$^2$ under the same culture conditions. This method produced a continuous culture density between 1000 and 4000 cells/cm$^2$. Adipogenic and osteogenic differentiation potential was verified using the same RT-PCR and staining methods previously shown by others.[4] Flow-cytometric analysis revealed that these cells were positive for SH2, SH3, CD90, and negative for CD34, CD45 and AC133 using FITC-labeled monoclonal antibodies (Becton Dickinson, Heidelberg, Germany).

### *Telomerase activity*

Telomerase activity was determined using the *Telo*TAGGG PCR ELISA$^{PLUS}$ kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. Briefly, $2 \times 10^5$ cells per sample were lysed in 200 $\mu$l Chaps buffer, and 0.5 $\mu$g of protein supernatant was used for PCR reaction in a total volume of 50 $\mu$l. Telomerase activity was described in a semiquantitative manner as 'relative telomerase activity' (RTA), relating the ELISA signal of the

sample to the one obtained by a control template with a known number of telomeric repeats. Frozen aliquots of Phoenix ampho cells, which form a second-generation retrovirus producer line based on the 293 T cell line, were analyzed with each test as an additional positive control. Telomerase activity was expressed as a percentage of the RTA of hMSCs relative to the RTA of phoenix ampho cells.

In addition, 20 $\mu$l of PCR product was resolved by a 12% polyacrylamide gel, and visualized by a Biotin Luminescent Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol.

## Results and discussion

Telomerase activity was measured in previously frozen hMSCs with the phenotype $SH2^+$, $SH3^+$, $SH4^+$, $CD29^+$, $CD44^+$, $CD14^-$, $CD34^-$ and $CD45^-$ [4] from three different donors (MSC nos. 1–3). Interestingly, we did not detect telomerase activity using the TRAP-ELISA assay at the beginning of culture (Table 1), nor at later stages during cell expansion (Table 2). To exclude that this finding was because of prior cryopreservation of hMSCs, we also analyzed telomerase activity in freshly isolated hMSCs (MSC nos. 4–8), which were positive for SH2, SH3, CD90 and negative for CD34, CD45 and AC133. Again, we found that these MSCs had no detectable levels of telomerase. We also measured telomerase activity when hMSCs were plated at a lower density, which has been reported to support the growth of recycling stem cells with higher proliferative capacity.[22] However, even under these conditions we were unable to detect significant levels of telomerase (data not shown) indicating that hMSCs are either truly telomerase negative or the activity is below the level of detection using the TRAP-ELISA assay.

In order to evaluate the sensitivity of the TRAP-ELISA assay, we diluted hMSCs with telomerase-positive phoenix cells at different concentrations (Figure 1a). Similar to a previous report, telomerase activity was detected in the range of 1:1000 to

**Table 1** Absence of telomerase activity in hMSCs

| *Donor* | *Telomerase activity (%)* |
|---|---|
| MSC 1 | 1 |
| MSC 2 | 0 |
| MSC 3 | 1 |
| MSC 4 | 1 |
| MSC 5 | 0 |
| MSC 6 | 0 |
| MSC 7 | 0 |
| MSC 8 | 0 |
| CB (*n*=7) | Median: 51 (range: 29–82) |
| Phoenix | 100 |

Telomerase activity was measured in eight samples of human mesenchymal stem cells (hMSCs). MSC nos. 1–3 were previously frozen; MSC nos. 4–8 were derived from fresh bone marrow samples. Telomerase activity was expressed as a percentage relative to the activity of an immortal control cell line (Phoenix ampho cells). As additional positive control, the activity in seven cord blood (CB) samples with the phenotype $CD34^+$ was measured. Telomerase activity is considered to be positive if the difference in absorbance of hMSCs is higher than the two-fold background activity which was not observed in any of the investigated MSC samples.

**Table 2** Absence of telomerase activity in hMSCs during cell expansion

| *CPDL* | *Telomerase activity (%)* |
|---|---|
| 2.2 | 1 |
| 4.7 | 1 |
| 6.6 | 0 |
| 10.1 | 1 |
| 12.2 | 1 |
| 13.7 | 1 |
| 14.6 | 0 |
| 15.3 | 0 |

Sequential analysis of telomerase activity during *in vitro* expansion of MSC 1. Telomerase activity was expressed as a percentage relative to the activity of an immortal control cell line (see Figure 1).
CPDL: cumulative population doubling levels.

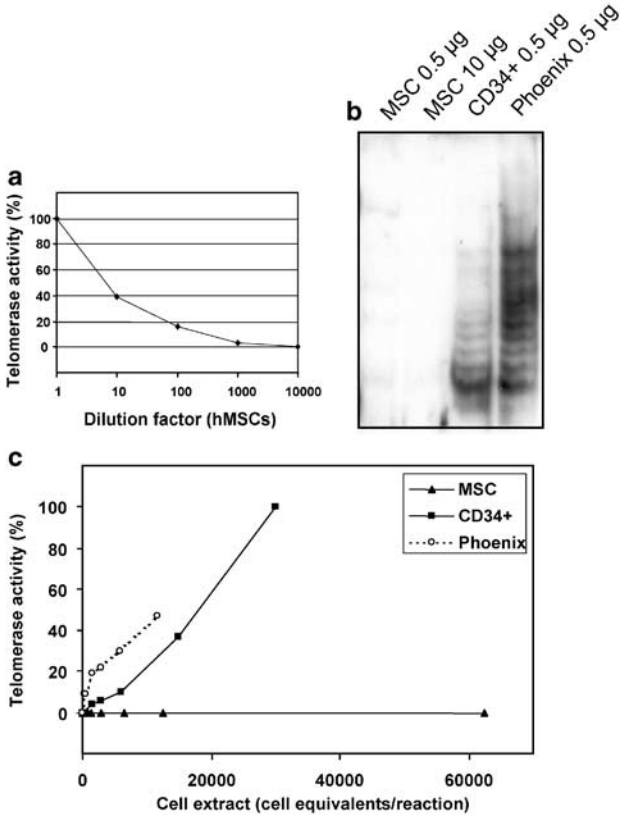


**Figure 1** Telomerase activity in hMSCs. (a) Detection of telomerase-positive cells in the presence of telomerase-negative cells in order to determine the sensitivity of the TRAP-ELISA assay. Telomerase activity is expressed as a relative percentage of the RTA values. Telomerase-positive Phoenix cells were mixed with telomerase-negative hMSCs at indicated ratios. (b) Representative Southern blot from a TRAP assay experiment demonstrating the typical telomerase-mediated six-nucleotide ladder in a telomerase-positive Phoenix cell line. hMSCs and $CD34^+$ cells were derived from the same bone marrow donor (no. 8) using different amounts of protein for hMSCs. (c) Telomerase activity was analyzed using the TRAP-ELISA assay at different concentrations of cell extracts. Telomerase activity is expressed as a relative percentage of the RTA values. Cell equivalents of hMSCs (triangles) and $CD34^+$ cells (squares), which were derived from the same bone marrow donor (no. 8) were assayed at indicated numbers per reaction. Phoenix ampho cells (open circles) were used as a positive control. Note that more than 60 000 cell equivalents of hMSCs ($=10\,\mu$g protein, see b) assayed in a single reaction demonstrated no telomerase activity.

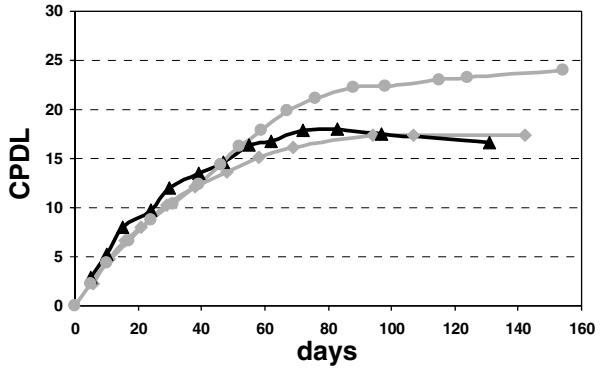


**Figure 2** Replicative senescence of hMSCs upon cell expansion. hMSCs with the phenotype $SH2^+$, $SH3^+$, $SH4^+$, $CD29^+$, $CD44^+$, $CD14^-$, $CD34^-$ and $CD45^-$ [4] from three different donors (MSC nos. 1–3) were cultured after plating at a density of 3000–6000 cells/cm$^2$. CPDL: cumulative population doubling levels. MSC 1 ◆, MSC 2 ▲, MSC 3 ●.

1:10 000 cells.[23] Finally, the activity of telomerase was determined in $CD34^+$ cells and hMSCs from the same individual assaying different numbers of cell equivalents. Increasing telomerase activity was measured in the Phoenix control as well as in hematopoietic progenitor cells. However, no detectable telomerase activity was observed in hMSCs even when a protein concentration of 10 $\mu$g was used, which corresponds to more than 60 000 cell equivalents (Figure 1B and C).

In order to determine the *in vitro* lifespan, cells from MSC nos. 1–3 were cultured at a density of 3000–6000 cells/cm$^2$ over multiple passages until proliferation stopped. The number of population doublings per day decreased continuously and the maximum was determined as $18.7 \pm 2.5$ (Figure 2a). This time, the cells showed typical features of senescence with a broad and flattened shape. Thus, the proliferative *in vitro* lifespan of hMSC appears to be limited similar to normal somatic cells, which is in agreement with a previous report.[24]

Our findings are in contrast to the observation by Pittenger *et al*[4] who detected high telomerase activity in hMSCs of the same phenotype. However, in their studies it was surprising that a typical negative control (human diploid fibroblasts) was also telomerase positive as indicated in the supplementary data. In general, telomerase activity is absent in normal somatic cells, but has been detected in cells with self-renewal capacity from skin, gut and the hematopoietic system.[17–19] In contrast to embryonic stem cells, which are capable of maintaining telomeres by telomerase, adult stem cells undergo progressive telomere shortening and appear to be mortal, although the activity of telomerase might extend their proliferative capacity. Similarly, the proliferative capacity of hMSCs has been reported to be limited,[24] which was also observed in our expansion cultures. Furthermore, preliminary data indicate that hMSCs are also subject to continuous telomere erosion (data not shown).

Our observation that hMSCs are telomerase-negative implicates that these cells are different from other adult stem cells, at least with respect to their telomerase status. Thus, it raises the question whether cells with the described phenotype and with the capability to differentiate into multiple cell types should be referred to as mesenchymal stem cells instead of fibroblast-like stromal cells. In contrast, more primitive MAPCs have recently been reported to maintain telomere length upon cell expansion. This appears to be associated with extended growth potential, although the telomerase status has yet to be defined in human MAPCs.[25] Therefore, we cannot exclude that a rare subpopulation of bone marrow stromal cells is indeed telomerase positive, but which might be below the level of sensitivity of the TRAP assay. The speculation that the frequency of such a cell is in the range of one in $10^7$ or one in $10^8$ is in agreement with this possibility.[7]

Assuming that telomerase activity is associated with self-renewal and is a feature of stem cells, it could be useful to identify bone marrow stromal cells on the basis of their telomerase status. Alternatively, ectopic transfer of the catalytic component hTERT could circumvent the critical technical barriers that result from induction of replicative senescence upon large-scale expansion of stromal cells.[26,27]

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