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Combined introduction of Bmi-1 and hTERT immortalizes human adipose tissue-derived stromal cells with low risk of transformation

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ABSTRACT

Adipose tissue-derived stromal cells (ASCs) are increasingly being studied for their usefulness in regenerative medicine. However, limited life span and donor-dependent variation of primary cells such as ASCs present major hurdles to controlled and reproducible experiments. We therefore aimed to establish immortalized ASC cell lines that provide steady supply of homogeneous cells for *in vitro* work while retain essential features of primary cells. To this end, combinations of human telomerase reverse transcriptase (hTERT), murine Bmi-1, and SV40 large T antigen (SV40T) were introduced by lentiviral transduction into ASCs. The resulting cell lines ASC^{hTERT}, ASC^{Bmi-1}, $ASC^{Bmi-1+hTERT}$ and $ASC^{SV40T+hTERT}$ were tested for transgene expression, telomerase activity, surface immunomarkers, proliferation, osteogenic and adipogenic differentiation, karyotype, tumorigenicity, and cellular senescence. All cell lines have maintained expression of characteristic surface immunomarkers, and none was tumorigenic. However, ASC^{Bmi-1} had limited replicative potential, while the rapidly proliferating $ASC^{SV40T+hTERT}$ acquired chromosomal aberrations, departed from MSC phenotype, and lost differentiation capacity. ASC^{hTERT} and $ASC^{hTERT+Bmi-1}$, on the other hand, preserved all essential MSC features and did not senesce after 100 population doublings. Notably, a subpopulation of ASC^{hTERT} also acquired aberrant karyotype and showed signs of transformation after long-term culture. In conclusion, hTERT alone was sufficient to extend the life span of human ASC, but ASC^{hTERT} are prone to transformation during extensive subculturing. The combination of Bmi-1 and hTERT successfully immortalized human ASCs without significantly perturbing their phenotype or biological behavior.

1. Introduction

Mesenchymal stem cells (MSCs) are adult multipotent cells with immense therapeutic potential. MSCs were first discovered in the bone marrow stroma, but now it is widely accepted that cells with similar properties can be isolated from a variety of tissues [1]. While most experiments and clinical studies still focus on bone marrow-derived MSCs, adipose tissue is emerging as a promising alternative source of MSCs for the ease and relatively small discomfort of harvesting, and the high density of stem cells, specifically termed adipose-derived stromal cells or ASCs, in fat tissue [2].

Although the main therapeutic features of MSCs such as multilineage differentiation, regeneration-promoting trophic effect, and immunomodulation have been subject to intensive research in the last two decades, many of the underlying mechanisms remain to be clarified. Yet, controlled and reproducible *in vitro* work with MSC-like cell types is hampered by variation between cells isolated from different individuals and limited life span of primary cultures. Bone marrow MSCs from healthy donors exhibit striking heterogeneity in terms of both growth and differentiation, and the differences observed show no obvious correlation with age or gender [3]. Repeated use of cells from a single donor could eliminate inter-individual variation, but MSCs gradually lose multipotency and eventually become senescent as they undergo extensive proliferation *ex vivo* [4].

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Both heterogeneity and replicative senescence can be circumvented by the establishment of immortal cell lines from primary cell cultures. Since cellular senescence is associated with telomere shortening, immortalization strategies typically include forced expression of telomerase reverse transcriptase (hTERT). Successful immortalization of bone marrow MSCs from human, minipig, and rhesus monkey has been achieved by expressing exogenous hTERT [5–8]. However, several investigators have found that restoring telomerase activity is not sufficient for the immortalization of human primary cells [9,10]. Therefore, in other experiments hTERT was introduced into human bone marrow- or placenta-derived MSCs in combination with growth promoters such as human papillomavirus (HPV) E6/E7 oncoprotein or the $p16^{Ink4a}$ antagonist Bmi-1 [11,12]. In some instances, hTERT indeed proved to be insufficient for immortalization; moreover, observations made on bone marrow MSCs transduced with HPV E6/E7 [13] and embryonic stem cell-derived MSCs transduced with c-MYC [14] indicated that hTERT may even be dispensable under certain conditions.

Immortalization of ASCs of either human or animal origin has not been reported to date. Hence we set out to establish immortalized human ASC cell lines that retain essential features of primary cells including differentiation potential and growth capacity over large time scales, and thus provide steady supply of homogeneous cells for *in vitro* work. We used the lentiviral technique to introduce hTERT and murine Bmi-1, both separately and in combination, into human primary ASCs. For positive biological control of immortalization, hTERT was combined with SV40T, the prototypical and most potent immortalizing gene [15].

2. Materials and methods

2.1. Cell isolation and culture

Human ASCs were retrieved from a 30-year-old healthy female donor and processed with ethical permission from the Hungarian Medical Research Council. The liposuction specimen was treated with 0.1% w/v collagenase type IV (Sigma–Aldrich, St. Louis, USA) at 37 °C for 30 min. Digestion was stopped by adding basal medium (DMEM-F12 1:1 with 10% v/v fetal bovine serum, 2 mM L-glutamine, and 50 μg/mL gentamicin). Cells were pelleted, resuspended in basal medium, and plated at an approximate density of $10^5/cm^2$. After several passages, basal medium was replaced with growth medium (basal medium plus 1 ng/ml FGF-2). Media, cell culture reagents, and recombinant human FGF-2 were from Life Technologies (Carlsbad, USA).

2.2. Flow cytometry

Expression of cell surface immunomarkers was assessed by flow cytometry. Cells were washed with PBS containing 0.5% w/v bovine serum albumin (BSA), and incubated with fluorescently labeled primary antibodies (BD Biosciences, Erembodegem, Belgium) or the corresponding isotype controls at 37 °C for 30 min. Measurements were carried out in a 4-color FACSCalibur flow cytometer (BD Biosciences).

2.3. Lentiviral gene transfer

The lentiviral vectors pLOX-GFP-iresTK, pLOX-TERT-iresTK, pLOX-CWBmi1, and pLOX-TERT-iresTtag (abbreviated as LV-GFP, LV-hTERT, LV-Bmi-1, and LV-SV40T, respectively) were obtained from the laboratory of Didier Trono (Lausanne, Switzerland). Lentiviral particles were produced in HEK293T cells transfected by the calcium phosphate co-precipitation method. Viral titers of the supernatants were estimated from flow cytometric measurement of HEK cells infected with a dilution series of LV-GFP supernatant. ASCs were first transduced with either LV-Bmi-1 or LV-SV40T at a multiplicity of infection (MOI) of approx. 7. Subsequently, subpopulations of these cells, as well as previously unmodified ASCs, were infected with LV-hTERT at the MOI of cca. 2. Control cells were either left untransduced or infected with LV-GFP.

2.4. Quantitative PCR for the determination of proviral copy number

Total DNA was isolated from cell culture lysates, and a 59-bp region of the integrated provirus was quantified using TaqMan reagents (Life Technologies) along with RNase P as genomic internal reference. The average number of integrated proviral copies per genome was calculated by the comparative C_T method. Sequences of primers and probes used are listed in Table 1a.

2.5. Gene expression analysis

Total RNA was isolated from 2×10^5 to 1×10^6 cells with TRIzol (Life Technologies). cDNA was synthesized from 1 μg of RNA using random primers and the Reverse Transcription System from Promega (Madison, USA). hTERT, Bmi-1, and SV40T were quantified using Power SYBR Green or TaqMan reagents in StepOne or StepOne Plus qPCR instruments (Life Technologies) using the appropriate primer pairs/assays shown in Table 1a, and expression levels were calculated relative to beta-actin.

2.6. Immunofluorescence

Cells were fixed with 4% w/v paraformaldehyde, blocked against nonspecific staining with 1% w/v BSA and 4% v/v nonimmune serum, permeabilized with 0.1% v/v Triton X-100, and incubated with primary antibodies for hTERT (Epitomics, Burlingame, USA), human/murine Bmi-1 (Merck, Darmstadt, Germany), SV40 large T antigen (Santa Cruz, Santa Cruz, USA), RUNX2 (R&D Systems, Minneapolis, USA) or the appropriate isotype controls overnight at 4 °C. AlexaFluor (Life Technologies) or Northern Light (R&D Systems) secondary antibodies were applied for fluorescent detection, and nuclei were counterstained with DAPI.

2.7. Telomerase activity assay

The functional activity of telomerase enzyme in cell extracts was assessed using the TRAPeze XL Telomerase Detection Kit from Millipore according to the manufacturer's protocol. Telomerase activity was expressed in units of total product generated (TPG).

Table 1a
Primers and probes used in qPCR and RT-qPCR experiments.

Assay	Forward primer	Reverse primer	Probe (FAM-MGB)
Integrated provirus	CCG AAC AGG GAC TTG AAA GC	CGA GTC CTG CGT CGA GAG A	AAA GGG AAA CCA GAG GAG
RNase P (genomic)	AGC TGA GTG CGT CCT GTC ACT	TCT GGC CCT AGT CTC AGA CCT T	CAC TCC CAT GTC CCT T
hTERT (cDNA)	TaqMan assay # Hs00972650_m1 (applied biosystems)		
Mouse Bmi-1 (cDNA)	TTA ATG TGT GTC CTG TGT	GAA GAG TTT TAT CTG ACC	–
SV40 large T antigen	CAG GCA TAG AGT GTC TGC	CAA CAG CCT GTT GGC ATA TG	–
Beta-actin (cDNA)	CCA GGT CAT CAC CAT TGG C	TCC TTC TGC ATC CTG TCG G	–

2.8. Cell proliferation

For the quantification of live cells, resazurin (Sigma–Aldrich) was added to the growth medium at a final concentration of 0.1 mg/ml. After 1 h of incubation supernatants were transferred to an optical 96-well plate, and resorufin fluorescence was read at 540/579 nm. In other experiments, cells were plated at a density of $4 \times 10^3/cm^2$, allowed to grow for 1 week, then trypsinized and counted in a hemacytometer.

2.9. Osteogenic and adipogenic differentiation

For osteogenic differentiation, basal medium was supplemented with non-essential amino acids, 0.1% v/v 2-mercaptoethanol, 0.3 mM ascorbic acid, 10 mM β-glycerophosphate, and 100 nM dexamethasone. After 3 weeks, cells were fixed and immunostained for RUNX2, tested for alkaline phosphatase activity with 0.02% w/v 5-bromo-4-chloro-3-indolyl phosphate/0.03% w/v nitro blue tetrazolium dissolved in ALP buffer (100 mM TRIS, 100 mM NaCl, 5 mM MgCl2, 0.05% v/v Tween-20, pH 9.5), and stained with 2% w/v Alizarin red at pH 4.2. For adipogenic differentiation, cells were kept in basal medium supplemented with 100 nM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthine for 2 weeks, and stained with Oil Red O.

2.10. Cellular senescence staining

Senescence-associated beta-galactosidase staining was carried out using the Cellular Senescence Assay Kit from Millipore. Cells were cultured for 48 h in a 24-well multidish and stained according to the manufacturer's protocol.

2.11. Karyotyping

Mid-log cell cultures were arrested with 100 ng/ml colcemid, disrupted in 0.075 M KCl solution at 37 °C, fixed with 3:1 mixture of methanol and acetic acid, and allowed to air dry. Following brief trypsinization chromosomes were stained with Giemsa (Merck). Karyograms were assembled using LUCIA Cytogenetics software (Laboratory Imaging, Prague, Czech Republic).

2.12. In vivo tumorigenicity assay

Five 8-week old NOD/SCID gamma (NOD·Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice per group were injected subcutaneously with 2×10^6 control ASCs, immortalized ASCs, or HT1080 fibrosarcoma cells suspended in Matrigel (BD Biosciences). Animals inoculated with HT1080 carried large tumors and were terminated after 5 weeks. All other mice were sacrificed after 12 weeks of observation. Animal experimentation was carried out in accordance with the institutional ethical guidelines.

Table 1b
Oligonucleotides used in the ligation-mediated PCR (LM-PCR) method.

Name	Sequence
Blunt-end adaptor F	5′-phospho-CGTCCTAACTGCTGTGCCACT-3′
Blunt-end adaptor R	5′-GACCCGGGAGATCTGAATTCGAGTGGCACA-GCAGTTAGGACG-3′
Biotinylated primer	5′-biotin-AGACCAATGACTTACAAGGCAGCTG-3′
HIV-LTR primer 1	5′-AGTGCTTCAAGTAGTGTGTGCC -3′
HIV-LTR primer 2	5′-GTCTGTTGTGTGACTCTGGTAAC -3′
Adaptor primer 1	5′-GACCCGGGAGATCTGAATTCG-3′
Adaptor primer 2	5′- AGTGGCACAGCAGTTAGGACG-3′

2.13. Cell cycle analysis

Trypsinized cells were washed with PBS + 0.5% w/v BSA, and fixed with cold 70% v/v ethanol on ice for 1 h. After washing as above, cells were resuspended in PBS + 0.5% w/v BSA containing 40 μg/ml propidium iodide, and DNA content was measured in a FACSCalibur flow cytometer (BD Biosciences Europe). Cell cycle characteristics were evaluated using ModFit LT 3.0 software (Verity Software House, Topsham, ME, USA).

2.14. Ligation-mediated PCR (LM-PCR)

To determine the chromosomal location of lentiviral integration sites, genomic DNA isolated from the cell cultures was extensively digested with one of the frequent-cutting restriction endonucleases BtsCI, HpyCH4V, HaeIII, and TaqI, or the combination of NspI + AseI + BsmI (New England Biolabs, Ipswich, USA), and fragments carrying provirus-genome junctions were biotin-labeled by linear extension using a biotinylated primer specific for the 5′ end of the integrated provirus and Phusion high-fidelity polymerase (New England Biolabs). The biotin-labeled fragments were immobilized to streptavidin-agarose beads (Life Technologies), and blunt-end ligated to a universal adaptor. Provirus-genome junctions were amplified using provirus- and adaptor-specific primers. For each reaction, the mixture of PCR products was cloned into pCR-2.1 TOPO vector (Life Technologies), transformed into TOP10F' cells allowing blue/white selection, and several colonies carrying large inserts were picked. Inserts were PCR-amplified and subjected to direct sequencing. The sequences obtained were aligned to the human genome using the BLAST algorithm. All applied oligonucleotides are listed in Table 1b.

Table 2
Timeline of experiments.

Experiment type	PD level at the time of experiment			
	ASC^{hTERT}	ASC^{Bmi-1}	ASC$^{Bmi-1+hTERT}$	ASC$^{SV40T+hTERT}$
Immunofluorescence	83	33	107	108
RT-qPCR				
Time point #1	45	10	35	120
Time point #2	100	55	90	310
Proviral copy number				
Time point #1	–	2	–	10
Time point #2	23	25	23	87
Time point #3	103	56	90	312
Telomerase assay	84	26	104	350
Proliferation assay				
Time point #1	19	20	19	74
Time point #2	102	32	107	424
SA-βGal staining	100	57	103	357
Flow cytometry				
MSC markers	86	29	109	370
Hematopoietic markers	50	42	50	176
Osteogenic differentiation				
Alizarin red	50	42	50	176
RUNX2 & ALP	88	27	74	259
Adipogenic differentiation	98	27	74	280
Karyotyping	56	–	56	183
In vivo tumorigenicity	74	–	74	255
End of observation period	120	Entered senescence between 55 and 60	143	481

2.15. Statistical evaluation

Statistical differences were evaluated using Student's *t*-test where applicable. *P* values smaller than 0.05 were accepted as significant.

3. Results

3.1. Establishment of immortalized cell lines

Following lentiviral transduction, ASC populations were observed over a total period of 20 months. $ASC^{Bmi\text{-}1}$ entered senescence and ceased to proliferate between population doubling (PD) levels 55 and 60. All other cell lines were actively proliferating at the time of manuscript preparation. A timeline of experiments, with PD levels of different cell lines at the time of each assay, is provided in Table 2.

3.2. Expression of the immortalizing genes

Efficient introduction of the immortalizing genes was confirmed by immunofluorescence, RT-qPCR, and telomerase activity assay (Fig. 1A–C). Expression of hTERT mRNA, nuclear hTERT immunoreaction, and telomerase activity were observed in ASC^{hTERT}, $ASC^{Bmi\text{-}1+hTERT}$, and $ASC^{SV40T+hTERT}$, but not in wild-type ASC, $ASC^{Bmi\text{-}1}$, or ASC^{GFP}. Low basal levels of human BMI-1 were detected in the Bmi-1-untransduced cells using a non-species-specific antibody, in contrast with the robust overexpression of the murine protein seen in $ASC^{Bmi\text{-}1}$ and $ASC^{Bmi\text{-}1+hTERT}$. Expression of SV40T mRNA and protein was detected in $ASC^{SV40T+hTERT}$ only.

Expression of exogenous hTERT remained invariant over time in ASC^{hTERT} and $ASC^{SV40+hTERT}$ but decreased 4.1-fold ($P = 0.01$) in $ASC^{Bmi\text{-}1+hTERT}$ between PD35 and PD90. SV40T became mildly silenced over time in $ASC^{SV40T+hTERT}$, whereas the levels of Bmi-1 mRNA slightly tended to increase from early to late passages in both $ASC^{Bmi\text{-}1}$ and $ASC^{Bmi\text{-}1+hTERT}$; these differences, however, were not statistically significant.

3.3. Proviral copy number

Mean integrated proviral copy number values per genome were determined by qPCR (Fig. 1D). At the time of both early and late measurements, the majority of ASC^{hTERT} cells carried, on average, three copies of LV-hTERT, while $ASC^{SV40T+hTERT}$ cells probably harbored one copy of LV-SV40T and three copies of LV-hTERT. Thus, hTERT and SV40T proviral copy numbers quickly stabilized and

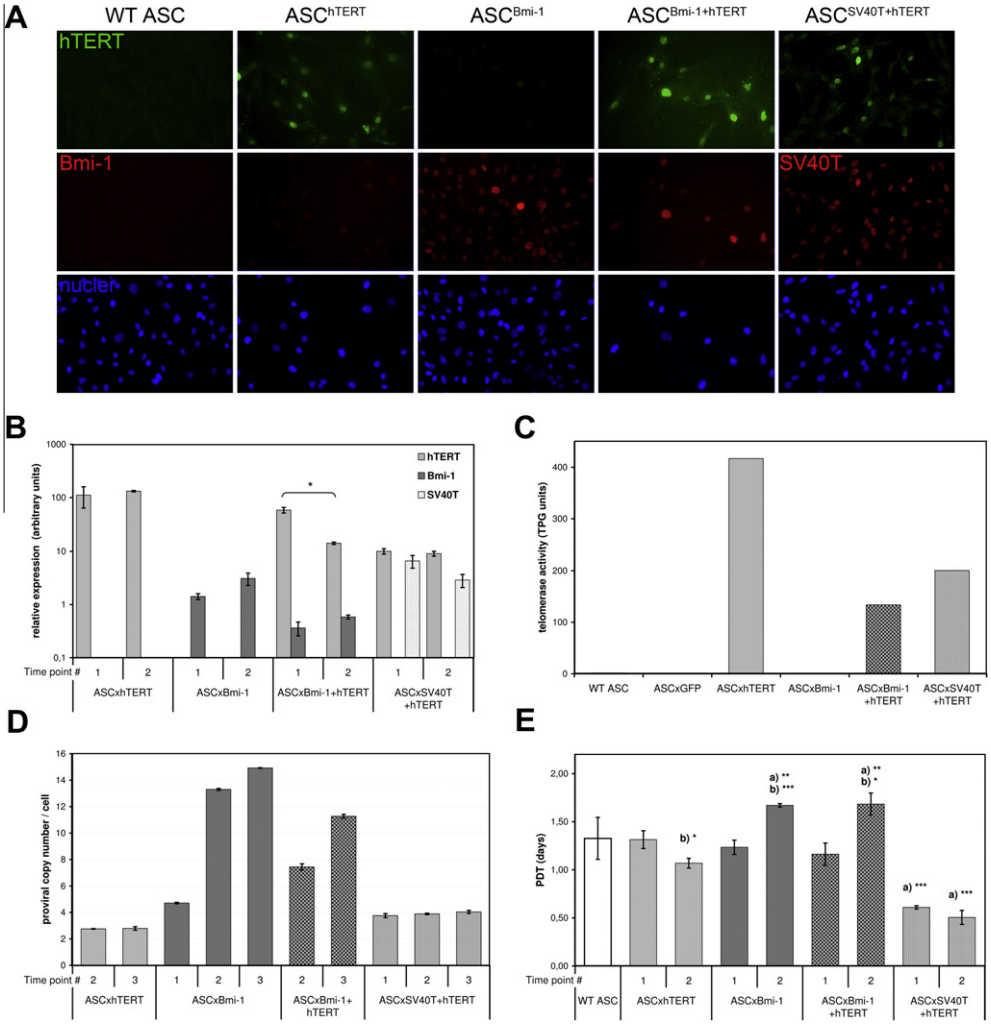

Fig. 1. Characterization of the immortalized cell lines by immunofluorescence, qPCR, telomerase assay, and proliferation assay. (A) Immunofluorescent detection of the immortalizing proteins. (B) mRNA expression levels of the immortalizing genes, measured at two different time points by RT-qPCR using β-actin as reference. $^{*}P = 0.01$. (C) Telomerase activity assessed by TRAPeze assay. (D) Average proviral copy number values per cell, measured at two or three different time points by qPCR using RNase P as reference. (E) Mean population doubling times (PDT), determined at two different time points by resazurin reduction assay. (a) Difference from wild-type ASC; (b) Difference between the two time points. $^{*}0.05 > P > 0.01$; $^{**}0.01 > P > 0.001$; $^{***}P < 0.001$. For PD level values at each time point, see Table 2.

remained unchanged over time. On the other hand, a gradual selection in favor of high copy number Bmi-1 clones was deducible from the increases seen in $MSC^{Bmi\text{-}1}$ (5 to 15) and $ASC^{Bmi\text{-}1+hTERT}$ (8 to 12).

3.4. Proliferation and cellular senescence

Population doubling time (PDT) values were calculated in mid-log phase populations at various PD levels (Fig. 1E). Initially, the PDT of ASC^{hTERT}, $ASC^{Bmi\text{-}1}$, and $ASC^{Bmi\text{-}1+hTERT}$ did not differ significantly from early-passage parental ASC. $ASC^{SV40T+hTERT}$, in contrast, was already twice as proliferative at the time of the early measurement. Decelerated growth of $ASC^{Bmi\text{-}1}$ was detected as soon as PD32, and the population arrived to complete arrest between PD55 and PD60. The doubling rate of $ASC^{Bmi\text{-}1+hTERT}$ also diminished over time, but the population kept on growing steadily. ASC^{hTERT}, on the other hand, exhibited a minor acceleration, and the growth rate of $ASC^{SV40T+hTERT}$ also tended to increase further with extensive subculturing.

Senescence-associated β-galactosidase (SA-βGal) staining (Fig. 2) was similar to early-passage wild-type ASC in ASC^{hTERT} at PD100 and $ASC^{Bmi\text{-}1+hTERT}$ at PD103. The majority of $ASC^{Bmi\text{-}1}$ cells were overtly senescent at PD57, whereas $ASC^{SV40T+hTERT}$ was completely negative for SA-βGal at PD357.

3.5. Surface immunomarkers and multipotency

All immortalized cell lines retained expression of surface MSC immunomarkers CD44, CD73, CD90, and CD105, and none expressed any of the hematopoietic lineage markers CD3, CD14, CD19, CD34, CD45, and HLA-DR (results not shown). Three weeks of osteogenic differentiation induced nuclear translocation of RUNX2, upregulation of alkaline phosphatase, and massive calcium

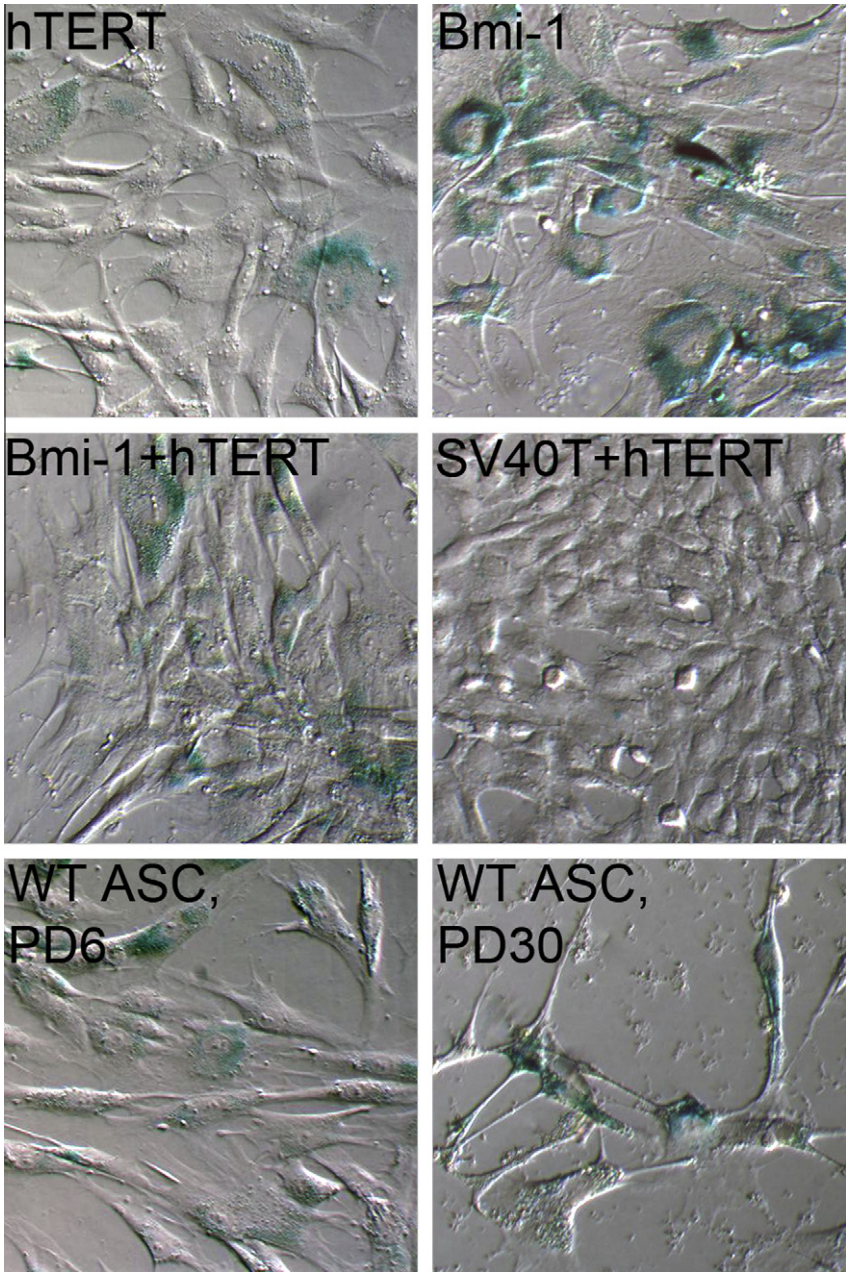

Fig. 2. Senescence-associated β-galactosidase staining.

deposition in ASC^{hTERT}, ASC^{Bmi-1}, and $ASC^{Bmi-1+hTERT}$ (Fig. 3A–C). The same cells also responded to adipogenic induction (Fig. 3D). $ASC^{SV40T+hTERT}$, on the other hand, showed aberrant nuclear localization of RUNX2 without osteogenic stimulus, and failed to respond to either differentiation protocol.

3.6. Karyotype and in vivo tumorigenicity

Cell lines that continued to proliferate after PD50 were subjected to cytogenetic analysis, and inoculated into immunodeficient mice to test tumor-forming ability. The karyotype of ASC^{hTERT} and $ASC^{Bmi-1+hTERT}$ was normal, whereas $ASC^{SV40T+hTERT}$ exhibited multiple aberrations including chromosomal losses and unbalanced translocations (see Supplementary Fig. 1). Despite the abnormal karyotype and aggressive *in vitro* behavior of $ASC^{SV40T+hTERT}$, none of the immortalized cell lines formed tumors in NOD/SCID gamma mice during a 12-week observation period (data not illustrated).

3.7. Potential transformation of ASC^{hTERT} at high passage numbers

After PD100, marked acceleration was noted in the proliferation of ASC^{hTERT}. This altered population was named $ASC^{hTERT}/2$ and henceforth treated separately. To replace ASC^{hTERT}, a cryopreserved aliquot archived at PD83 was reconstituted and kept under continuous observation.

ASC^{hTERT} between PD levels 84 and 110 and $ASC^{hTERT}/2$ at PD120 are compared in Fig. 4. Weekly population growth was 2.8 ± 1.5-fold vs. 6.0 ± 2.5-fold ($P = 0.04$) in ASC^{hTERT} and $ASC^{hTERT}/2$, respectively (Fig. 4A). ASC^{hTERT} was characterized by well-spread cellular morphology, and SA-βGal reaction similar to wild-type ASCs (few scattered, weakly positive cells), whereas many $ASC^{hTERT}/2$ cells

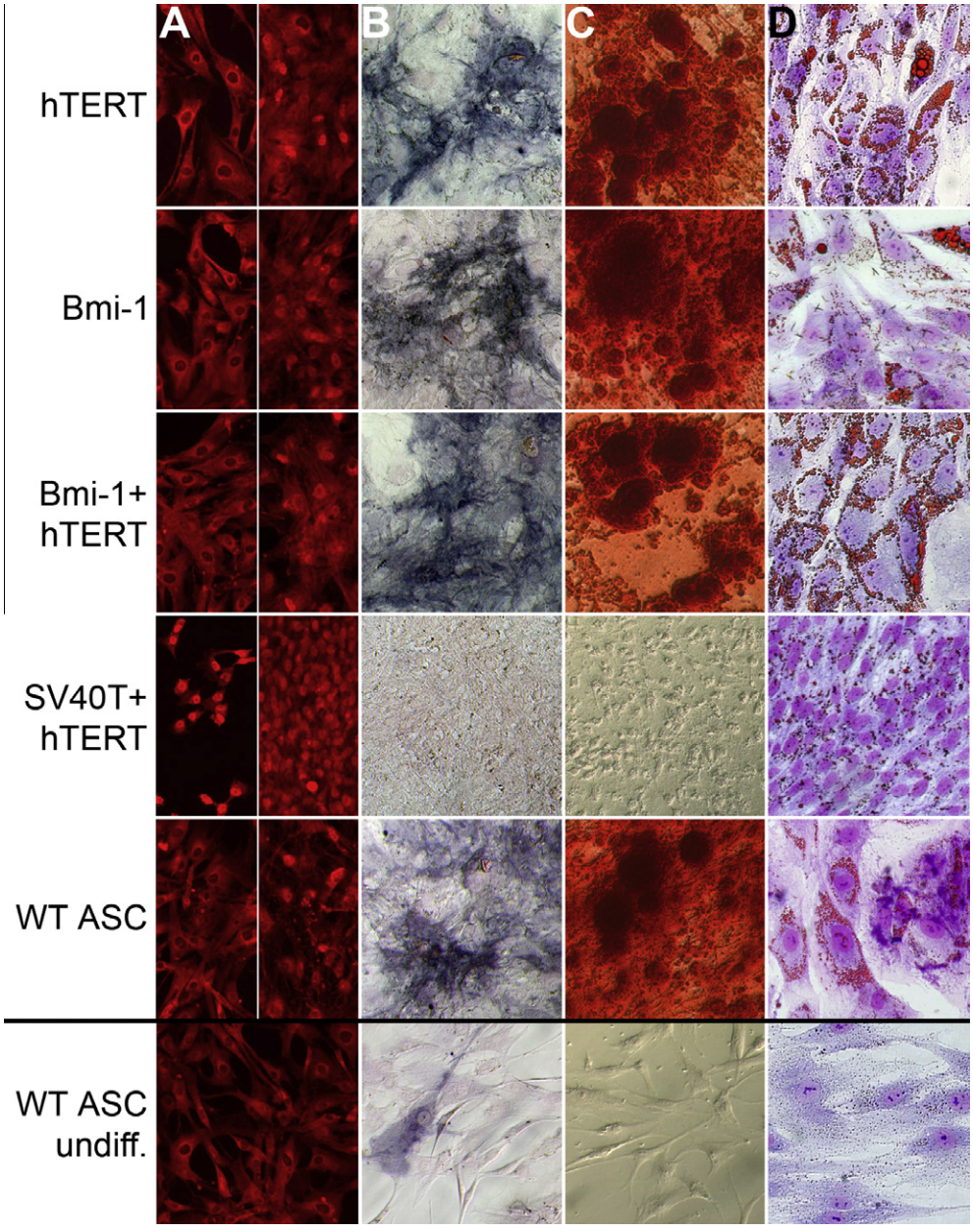

Fig. 3. Osteogenic and adipogenic differentiation. (A) Immunolocalization of RUNX2 on day 1 (left half image) and after 3 weeks (right half image). (B) Detection of alkaline phosphatase activity by addition of chromogenic substrate. (C) Detection of calcification by Alizarin red staining. (D) Assessment of adipogenic differentiation by Oil Red O staining. Abbreviation: undiff., undifferentiated.

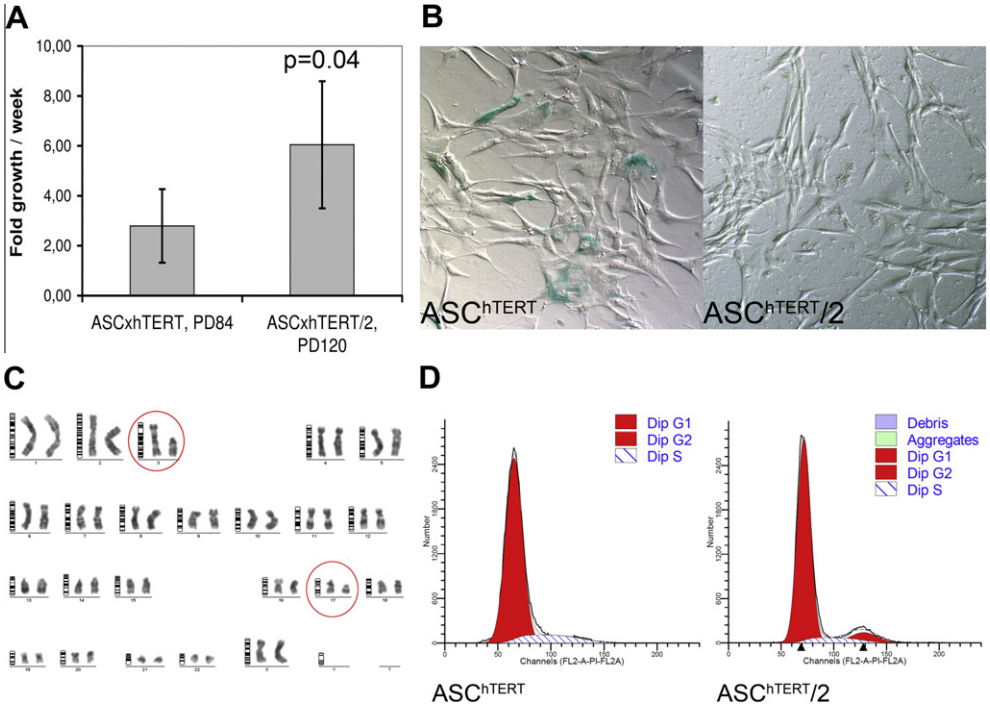

Fig. 4. Comparison of ASC^{hTERT} and ASC^{hTERT}/2 cells. (A) Weekly population growth estimated by cell counting. (B) SA-βGal staining. (C) A selected karyogram of ASC^{hTERT}/2 showing large deletions on 3p and 17p. (D) Cell cycle analysis of confluent cultures. G2/M: 0.28% vs. 8.57% in ASC^{hTERT} and ASC^{hTERT}/2, respectively.

assumed a more elongated shape and the population was completely negative for SA-βGal (Fig. 4B). As opposed ASC^{hTERT} where normal karyotype was retained, various nonclonal alterations were observed in ASC^{hTERT}/2 cells (an example is shown in Fig. 4C). Cell cycle analysis revealed no mitotic ASC^{hTERT} cells in confluent culture (G2/M: 0.28%); as a contrast, ASC^{hTERT}/2 lost contact inhibition and continued to proliferate after reaching confluence (G2/M: 8.57%) (Fig. 4D).

The possible transforming role of lentiviral insertion was investigated by ligation-mediated PCR. The same two integration sites were identified in both populations. One integrated proviral copy was localized on chromosome 5 within the gene of hypothetical protein LOC65250, while the other provirus was found to be inserted into the gene of TBC1 domain family member 5, isoform b, on chromosome 3.

4. Discussion

In this paper we report the establishment of immortalized cell lines from human primary ASCs. Immortalization was achieved by lentiviral transduction of hTERT in combination with the growth promoters Bmi-1 or SV40T. We also intended to clarify whether hTERT or Bmi-1 are able to immortalize ASCs on their own, or their combined application is effective only.

Some oncogenes such as HPV E6/E7 or c-MYC have been shown to immortalize MSCs without the addition of hTERT [13,14]. In such instances, telomerase induction by the oncogen is thought to obviate the need for exogenous hTERT. Both c-MYC and Bmi-1 have been demonstrated to activate telomerase in mammary epithelial cells [16,17], and Bmi-1 was proposed to suppress senescence by releasing telomerase inhibition in stem cells [18]. In our experience, the Bmi-1-only population was unable to evade senescence and eventually ceased to proliferate, although the increase of mean proviral copy number over time suggested that clones overexpressing Bmi-1 might have enjoyed selective advantage. Nevertheless, in repeated experiments several clones may emerge that escape replicative crisis and activate telomerase, as it was observed in the case of Bmi-1-transduced placental MSCs [12].

Unlike Zhang et al. [12] who found that hTERT-transduced cells stopped replicating after less than 20 PD, the group of Moustapha Kassem claimed that hTERT alone was sufficient to immortalize human bone marrow-derived MSCs [7]. Similarly, no blockage of the p16^{Ink4a}/Rb pathway was required for the hTERT-mediated immortalization of umbilical cord blood-derived MSCs [19]. These results put into question the mere necessity for any growth promoter. Doubtlessly, the avoidance of oncogenes in the immortalization process would be welcome as a more elegant and safer approach. It has turned out, however, that the limitless replicative potential ensured by hTERT is a 'fertile soil' for the acquisition of potentially transforming mutations. Several years later, the Kassem group reported on the transformation of hTERT-transduced human MSCs after extensive passaging, and identified a number of oncogenic alterations in the affected cell lines [20].

ASC^{hTERT}/2 is supposed to have undergone a similar transforming event which led to accelerated proliferation, complete absence of SA-βGal staining, and loss of contact inhibition. Since approx. 100 population doublings elapsed between lentiviral transduction and transformation, the proviral integration pattern of ASC^{hTERT}/2 was identical to its non-transformed counterpart, and neither of the two integration loci was suggestive of oncogene cis-activation or tumor suppressor inactivation, the causative role of lentiviral insertion seems unlikely. The expansive clone probably arose after PD83, as the transforming event did not reoccur until PD120 in a reconstituted lot of ASC^{hTERT} cryopreserved previously at PD83. Further investigation is underway to identify possible genetic alterations in ASC^{hTERT}/2, as well as to estimate the probability of transformation from repeated experiments. It is also unclear whether the chromosomal abnormalities seen were primary or subsequent to transformation. The possible contribution of FGF-2 added to the growth medium also needs to be considered, since spontaneous transformation of human bone marrow MSCs cultured in FGF-2-containing medium has been reported [21].

On the basis of our and others' observations, however, we can conclude that introduction of hTERT may predispose MSCs to the acquisition of transforming mutations [22,23], even though the actual occurrence of transformation is stochastic and may occasionally not happen up to very high PD levels [6].

In favor of the combined immortalization approach, i.e. concomitant transfer of hTERT along with a growth promoter, it can be speculated that forced expression of a specific oncogene may suppress the emergence of mutant clones that possess significant selective advantage. Thus, somewhat paradoxically, combination of hTERT with an appropriately chosen growth promoter, one which does not transform MSCs and permits the conservation of their phenotype and differentiation potential, may allow the generation of more stable cell lines. As expected, SV40 large T antigen, although very potent as an immortalizing agent, was not compatible with the preservation of MSC-like phenotype. Bmi-1 as a partner of hTERT, on the other hand, offered stable and continued growth without significantly perturbing cell behavior. In earlier studies, MSCs and related cell types such as cementoblast progenitors immortalized with hTERT and Bmi-1 were cultured up to 100 PD, and neither transformation nor loss of multipotency was reported [12,24]. In our hands, osteogenic and adipogenic induction of $ASC^{Bmi\text{-}1+hTERT}$ was successful at PD74, and no sign of transformation was noted even beyond PD120. Rather, and in spite of selection for high proviral copy number cells, the growth of $ASC^{Bmi\text{-}1+hTERT}$ slowed down at late passages; nevertheless, the population has not entered senescence.

In summary, combined application of hTERT and Bmi-1 resulted in the development of a stable ASC-derived immortal cell line with unaffected differentiation capacity and no observed occurrence of transformation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.04.088.

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