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ORIGINAL PAPER

Differentiation and Neuro-Protective Properties of Immortalized Human Tooth Germ Stem Cells

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Abstract Stem cells are considered to be promising therapeutic options in many neuro-degenerative diseases and injuries to the central nervous system, including brain ischemia and spinal cord trauma. Apart from the gold standard embryonic and mesenchymal origin, human tooth germ stem cells (hTGSCs) have also been shown to enjoy the characteristics of mesenchymal stem cells (MSCs) and the ability to differentiate into adipo-, chondro-, osteo- and neuro-genic cells, suggesting that they might serve as potential alternatives in the cellular therapy of various maladies. Immortalization of stem cells may be useful to avoid senescence of stem cells and to increase their proliferation potential without altering their natural characteristics. This study evaluated the expression of stem cell markers, surface antigens, differentiation capacity, and karyotype of hTGSCs that have been immortalized by human telomerase reverse transcriptase (hTERT) or simian vacuolating virus 40 (SV40) large T antigen. These undying cells were also evaluated for their neuro-protective potential using an in vitro SH-SY5Y neuro-blastoma model treated with hydrogen-peroxide or doxo-rubicin. Although hTGSC-SV40 showed abnormal karyotypes, our results suggest that hTGSC-hTERT preserve their MSC characteristics, differentiation capacity and normal karyotype, and they also possess high proliferation rate and neuro-protective effects even at great passage numbers. These peculiars indicate that hTGSC-hTERT could be used as a viable model for studying adipo-, osteo-, odonto- and neuro-genesis, as well as neuro-protection of MSCs, which may serve as a springboard for potentially utilizing dental waste material in cellular therapy.

Keywords Human tooth germ stem cell · Neuro-protection · Immortalization · Differentiation

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Introduction

Stem cell transplantation is an innovative approach to the treatment of various diseases characterized by cellular depletion. Replacement of necrotized tissue in cerebral or cardiac ischemia, regenerating lost neurons in various neuro-degenerative diseases, rehabilitating traumatized organs such as in spinal cord injury, renewing depleted insulin-producing β-cells of the islets of Langerhans in type-1 diabetes, revitalizing the sensory apparatus in blindness, and restoring structure and regaining functions in several other disorders by viable new cells is a promising alternative to current conventional strategies. Despite promising results in animal models, exact therapeutic effects remain largely unknown, leading to yet less impressive outcomes in clinical trials [1, 2]. One of the main hypotheses behind the beneficial roles of stem cell transplantation explains that grafted MSCs migrate to the damaged area and differentiate into new brain cells such as neurons and glias. Another theory is that transplanted MSCs exert their therapeutic effect through paracrine stimulation of neuro-genesis, as well as providing neuro-protective and neuro-trophic support [3]. In addition, MSCs were shown to induce angio-genesis and synapse formation, known to reduce apoptosis and cyto-toxicity, and have also been demonstrated to regulate inflammation [4]. Considering the complex patho-physiology of neuro-degenerative diseases and the lack of evidence that MSCs differentiate into functional neurons in vivo, indirect paracrine effects of MSCs are more likely [5].

Adult bone marrow-derived stem cells (BMSCs), also referred to as mesenchymal stem cells (MSCs) or mesenchymal stromal cells, are considered to be the primary sources for many cellular therapy and tissue engineering applications. Their isolation, however, involves invasive and painful procedure associated with various medical and surgical risks. The recently emerged embryonic stem cells (ESCs) have excellent self renewal and differentiation capacity, however they impose risk of teratoma formation and also exert ethical controversies. Search for minimally invasive alternative adult stem cells sources has focused on, among others, dental stem cells (DSCs) which are mostly derived from waste materials obtained after routine orthodontic treatments or maxillo-facial surgery in the adults [6]. Among them dental pulp stem cells (DPSCs) and dental follicle stem cells (DFSCs) originate from neural crest and contain both mesenchymal and neuro-genic cells [7]. DPSCs were shown to increase survivability of dopaminergic neurons treated with neuro-toxic molecules [8] and also increase regeneration of injured spinal cord [9]. DPSCs isolated from 3rd molar (wisdom) teeth can also differentiate into cells of all three germ layers (endo-, meso- and ecto-derm) [10]. DFSCs have the capacity to transform into neuro-genic cells and might be used for biomedical and tissue engineering applications [11, 12]. DSCs also have the capacity to induce angio- [13] and neuro-genesis [9], as well as regulate inflammatory/immune response [14], all of which are essential for efficient regeneration of injured neural tissues.

Although all structures of the body develop pre-natally, the organo-genesis of human 3rd molars starts only at around age 6: tooth germs (tooth buds) give rise to wisdom teeth later post-embryonically, which became a target of investigation [7]. Recently we have isolated human tooth germ stem cells (hTGSCs) from developing teeth of young adults. They originate from neural crest and have mesenchymal and neuro-ectodermal components. This cell population differs from DPSCs and DFSCs that are obtained from fully formed adult teeth. Having extensively characterized hTGSCs, we have demonstrated that these cells are multi-potent and easily accessible after routine orthodontic treatments without any ethical concerns [15].

The use of stem cells in therapeutic applications is limited due to abnormal chromosomal changes and aging (i.e. lost differentiation capacity and reduced proliferation rate) leading to diminished functionality after successive passaging to reach the adequate number of cells for transplantation. Apart from their ready availability and lack of ethical concerns, hTGSCs can be immortalized as well in order to preserve their differentiation capacity and proliferation [16, 17]: eternal hTGSCs keep their ability to transform into odonto-, adipo- and osteo-genic cells [18], and exert neuro-protective effects [19] even at high passage numbers.

This study evaluates select immortalization methods and characterizes undying hTGSCs. Of the main alternative techniques, human telomerase reverse transcriptase (hTERT)-mediated immortalization approach has proved to keep cells in un-differentiated state and preserve their high transformational capacity, suggesting that it might be a suitable model for studying the molecular and biochemical background of stem cell differentiation and neuro-protection.

Materials and Methods

Isolation of hTGSCs

Human impacted 3rd molar tooth germs were surgically removed from 3 healthy patients (age: 13–15) as part of a prophylactic treatment for orthodontic reasons. Written informed consent was obtained from each patient and their parents following approval by the Institutional Ethics Committee of Yeditepe University, Turkey. Enucleated tooth germs were placed in sterile physiological saline

solution. Isolation of hTGSCs was performed according to the protocol described previously by our group [15]. Cell-lines were maintained in growth medium containing Dulbecco's modified essential medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM of L-glutamine (Invitrogen, Carlsbad, CA, USA) and 1% of penicillin–streptomycin-fungizone (PSF) solution (Invitrogen, Carlsbad, CA, USA), and incubated at 37°C in a humidified atmosphere of 5% CO_2. Established hTGSC lines were sub-cultured using trypsin–EDTA solution (1X) (Invitrogen, Carlsbad, CA, USA).

Generation of Recombinant Lenti-Virus and Infection of hTGSCs

Packaging plasmids pCMV-VSV-G (Addgene plasmid 8454) and psPAX2 (Addgene plasmid 12260), as well as vector plasmids pBABE-hygro-hTERT (Addgene plasmid 1773) or pBABE-puro-SV40 LT (Addgene plasmid 13970) were obtained from Addgene (Cambridge, MA, USA). Production of recombinant hTERT and simian vacuolating virus 40 (SV40) large T antigen (LT) lenti-viruses was achieved by transfecting human embryonic kidney 293T (HEK293T) cells (cat#: CRL-11268, American Type Culture Collection (ATCC), Manassas, VA, USA) with pCMV-VSV-G + psPAX2 + pBABE-hygro-hTERT or pCMV-VSV-G + psPAX2 + pBABE-puro-SV40 LT plasmids, respectively, using standard calcium phosphate method. Supernatant containing viral particles were collected at 24–48 h after transfection.

For infection of stem cells, hTGSCs were seeded in tissue culture flasks. At 60% confluency, fresh medium containing recombinant lenti-viruses was added. Selection of virally transduced cells was performed by 150 μg/mL of hygro-mycin and 1 mg/mL of puro-mycin for hTERT and SV40 immortalized cells, respectively. At 90% confluency, hTGSC-hTERT and hTGSC-SV40 were maintained in regular hTGSCs growth medium using standard cell culture technique.

In order to compare growth rates of normal and immortalized cells, hTGSCs were seeded into 12-well plates in triplicates. Lines were cultured, trypsinized and counted using hemo-cytometer.

Chromosomal Analysis

hTGSC-hTERT and hTGSC-SV40 were cultured in DMEM + Glutamax™-1 (1X) medium containing 10% FBS and 1% of PSF solution. Cyto-genetic analysis was performed at 40–50% confluency of cells. Incubation with ProCell chromosome resolution reagent (Genial Genetic Solutions, Wirral, UK) to prevent chemical contraction and to encourage chromosome elongation was followed 2.45 h later by treatment with metaphase arresting solution for 75 min. Subsequently, cells were collected by 0.25% trypsinization (Gibco-Invitrogen, Carlsbad, CA, USA), incubated in pre-warmed hypotonic solution (0.075 M of KCl), fixed with Carnoy's fixative (3:1 mixture of methanol and acetic acid) and spread onto clean glass slides. After air-drying, samples were incubated at 65°C overnight, and were stained with 5% Giemsa solution, prepared by mixing Giemsa's azur-eosin-methylene-blue solution (Merck, Darmstadt, Germany) with Gurr solution (Gibco-Invitrogen, Carlsbad, CA, USA) for 4.5 min. Images of the captured metaphase chromosomes were obtained through 100X objective lens of light microscope (Olympus America, Melville, NY, USA). Scanned chromosome pics were split from each other and karyotyped using the Cytovision ChromoScan software (Applied Imaging, Santa Clara, CA, USA). hTGSC-hTERT and hTGSC-SV40 cell lines with choromosomal abnormalities were not used in further analysis.

Flow Cytometry Analysis

Surface antigen expression profile of immortalized and non-immortalized hTGSCs was analyzed by flow cytometry as described previously [20]. Cells were trypsinized and incubated in PBS for 45 min with primary anti-bodies against CD14 (cat #SC-7328), CD29 (cat #BD556049), CD34 (cat #SC-51540), CD45 (cat #SC-70686), CD90 (cat #SC-53456), CD105 (cat #SC-71043), CD133 (cat #SC-65278), CD166 (cat #SC-53551) (all from SantaCruz Biotechnology Inc., Santa Cruz, CA, USA) and CD73 (cat #550256) (Zymed, San Francisco, CA, USA). After washing, cells were incubated with fluorescein-iso-thio-cyanate (FITC)-conjugated secondary anti-bodies (cat #SC-2989, SantaCruz Biotechnology Inc., Santa Cruz, CA, USA) at 4°C for 45 min, except for CD29 against which a monoclonal anti-body conjugated to chromophore-containing phyco-erythrin protein (PE) was used (for budgetary reasons only). The flow cytometry analysis of the cells was carried out using Becton–Dickinson FACSCalibur flow cytometry system (Becton–Dickinson, San Jose, CA, USA).

Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA from hTGSC-hTERT and normal hTGSCs was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. ESC mRNA from human embryonic stem cell line Moscow 01 (hESM01) was kindly provided by dr. Sergey L. Kiselev (Vavilov Institute of General Genetics, Russian Academy of Sciences, Moscow) [21]. cDNA synthesis was performed using random hexamer primers and moloney murine leukemia virus reverse-transcriptase (MMLV-RT)

(Promega, Madison, WI, USA) at 37°C for 1 h. TaqMan primers and probes were designed as described previously [15], and were synthesized by Syntol (Moscow, Russia). Pre-mix (2.5X) for TaqMan RT-PCR was purchased commercially (Syntol, Moscow, Russia) and was used according to the manufacturer's instructions. The amount of RNA was normalized by using β-actin. Serial dilution of ESC cDNA was used for relative quantification of the expression of *oct4*, *klf4* and *sox2* genes. Relative expression of *htert* mRNA was analyzed using SYBRgreen RT-PCR method. The PCR primers were as follows: GAPDH (sense: TAT CGT GGA AGG ACT CA, anti-sense: GCA GGG ATG ATG TTC TGG A) [22], hTERT (sense: CTG AGG AGT AGA GGA AGT G, anti-sense: TGG TTT CTG TGT GGT GTC). cDNAs were mixed with primers and SYBR Premix Ex Taq (including TaKaRa Ex Taq HS, dNTP Mixture, Mg^{2+}, SYBRgreen-I) in a final volume of 20 μL. GAPDH (glycer-aldehyde-3-phosphate-de-hydrogenase) gene was used as the reference house-keeping gene for normalization of the data. mRNA expression level in hTGSCs was considered as 100%. All RT-PCR experiments were done using iCycler RT-PCR detection system (Bio-Rad, Hercules, CA, USA).

Differentiation of hTGSCs

To confirm MSC characteristics, immortalized hTGSCs were differentiated into osteo-, odonto- and adipo-genic cells as described previously [15, 17, 23], using hTGSC-hTERT at passage #35. For osteo-genic transformation, cells were counted and cultured in 6-well plates at a concentration of 3,000 cells/cm^2 in growth medium. After 48 h, growth medium was replaced with osteo-genic medium: DMEM supplemented with 10% FBS, 0.1 mM/L dexa-methasone, 10 mM/L β-glycerol-phosphate, 50 mM/L ascorbate (Sigma Chemical Co., StLouis, MO, USA). Medium was changed every other day. On day 14th, vonKossa staining and immuno-cytochemistry were performed to confirm osteo-genic differentiation. For adipogenic transformation, cells were counted and cultured in 6-well plates at a concentration of 3,000 cells/cm^2 in growth medium. After 24 h, the medium was replaced with DMEM supplemented with 10% FBS, 1 mM/L dexamethasone, 5 μg/mL insulin, 0.5 mM/L iso-butyl-methyl-xanthine and 60 mM/L indomethacin (Sigma Chemical Co., StLouis, MO, USA). After 2 weeks of incubation, intra-cellular lipid vesicles were observed under light microscope (Nikon TS100, Minnesota, MN, USA). Cells were induced to differentiate into odonto-genic cells by incubating them with odonto-genic differentiation medium consisting of DMEM supplemented with 10% FBS, PSF, 2 mM L-glutamine, 50 μg/mL ascorbic-acid and 2 mM of 2-glycerol-phosphate (Sigma Chemical Co., StLouis, MO, USA) for 14 days with medium changed every other day.

Immuno-Cyto-Chemical Analysis

hTGSC-hTERT grown on glass cover-slips were fixed with 2% of *p*-formaldehyde and permeabilized by incubating cells with 0.1% Triton-X100/PBS for 5 min. Non-specific binding of anti-bodies was blocked by adding 2% goat serum (diluted in PBS) for 20 min. Samples were incubated with primary anti-bodies (IgG): anti-DSPP (dentin sialo-phospho-protein, Cat# sc-33586), anti-DMP-1 (dentin matrix protein-1, Cat# sc-73633), anti-collagen type-I (Cat# sc-80565) and anti-osteocalcin (Cat# sc-30044) (SantaCruz Biotechnology Inc., Santa Cruz, CA, USA) overnight at 4°C. After washing twice for 5 min with PBS to remove unbound primary anti-bodies, goat polyclonal anti-rabbit and anti-mouse secondary IgG anti-bodies conjugated to Alexa-Fluor 488 fluorescent dye (Invitrogen, Carlsbad, CA, USA) were added and incubated for 1 h. DAPI (6′-di-amidino-2-phenyl-indole) (Sigma Chemical Co., StLouis, MO, USA) was used as nuclear counter-stain. Cover-slips were mounted on clean glass slides using Mowiol mounting medium (Calbiochem, La Jolla, CA, USA). Prepared slides were observed under Leica TCS SP2 SE confocal microscope (Leica, Bensheim, Germany) immediately after preparation.

vonKossa Staining

After 14 days of incubation with osteo-genic medium in 6-well plates, cells were fixed with 2% of *p*-formaldehyde at 4°C for 30 min. Subsequently, cells were stained using vonKossa technique (Bio-optica, Milano, Italy) and calcium depositions were observed with a light microscope (Nikon TS100, Minnesota, MN, USA).

Preparation of Conditioned Medium of hTGSC-hTERT and in vitro Neuro-Protection Assay

hTGSC-hTERT at passage #5 were cultured in T-75 tissue culture flasks in growth medium. At 70% confluency, conditioned medium was collected, passed through 0.22 μm filters to avoid any cell debris, and divided into 2 mL aliquots. Tubes were kept at −20°C until use. SH-SY5Y neuro-blastoma cells (cat#: CRL-2266, American Type Culture Collection (ATCC), Manassas, VA, USA) were plated in 12-well plates at a concentration of 40,000 cells/well with either growth medium only or growth medium supplemented with 20% hTGSC-hTERT conditioned medium. Cells were exposed to 100 μM of H_2O_2 (Riedel-de-Haen, Hanover, Germany) or 5 μM of doxo-rubicin for 24 h. After treatment, cell viability was assessed by the colori-metric 3-(4,5-dimethyl-thiazol-2-yl)-

2,5-di-phenyl-tetrazolium-bromide (MTT)-assay (CellTiter96 Aqueous One Solution, Promega, Madison, WI, USA) according to the manufacturer's instructions by measuring the activity of enzymes in living cells that reduce MTT to purple formazan dyes as detected by spectro-fluorimeter.

Data Analysis

Results are expressed as mean ± standard deviation (SD). Mann–Whitney *U*-test was used for statistical calculations, utilizing Sigma Stat Sofware (Jandel Scientific, San Rafael, CA, USA). Values of $P < 0.05$ were accepted as statistically significant.

Results

Using recombinant lenti-viral vector, immortalized hTGSC-hTERT and hTGSC-SV40 cell-lines were obtained (n = 3 each), encoding either hTERT or SV40 genes.

At least 20 metaphases were karyotyped and analyzed for numerical and structural chromosomal abnormalities. Anomalies on both domains were detected in 1 of the hTGSC-hTERT cell-lines, including trisomy of chromosome 7 and apparently balanced translocation between chromosomes 1q and 16q, formulated as 47XY + 7t(1;16)(q32;q24) (data not shown). The remaining hTGSC-hTERTs displayed normal 46 XY karyotype (Fig. 1a). On the other hand, all hTGSC-SV40 cell-lines showed highly abnormal chromosomal characteristics: the most common aberrations included telomeric associations, deletions and duplications (Fig. 1b). Cell-lines with abnormal karyotype were excluded from the study.

The chromosomally intact hTGSC-hTERT cell-lines were free from any morphological changes even at high passage numbers (Fig. 2), and their proliferation rate had increased significantly when compared to their non-transfected counterparts (Fig. 3). Flow cytometry demonstrated that these cells expressed MSC markers CD29, CD73, CD90, CD105 and CD166, but not hematopoietic markers such as CD14, CD34, CD45 and CD133 (Fig. 4).

RT-PCR analysis showed the level of expression of hTERT mRNA in hTGSC-hTERT being 3-times higher than in non-modified cells (Fig. 5a). In order to assess the state of differentiation before and after hTERT transfection, expression of genes associated with pluri-potency was analyzed. An increased expression of *oct4* and *sox2*, but decreased *klf4* production was observed (Fig. 5b). Induction of hTGSC-hTERT to transform into adipo-, osteo- and odonto-genic cells has confirmed their ability to differentiate into all of these cell types. Immuno-cyto-chemistry analysis revealed that hTGSCs were stained positive for odonto-genic markers DSPP (Fig. 6a) and DMP-1 (Fig. 6b), as well as osteo-genic markers collagen type-I (Fig. 6c) and osteo-calcin (Fig. 6d).

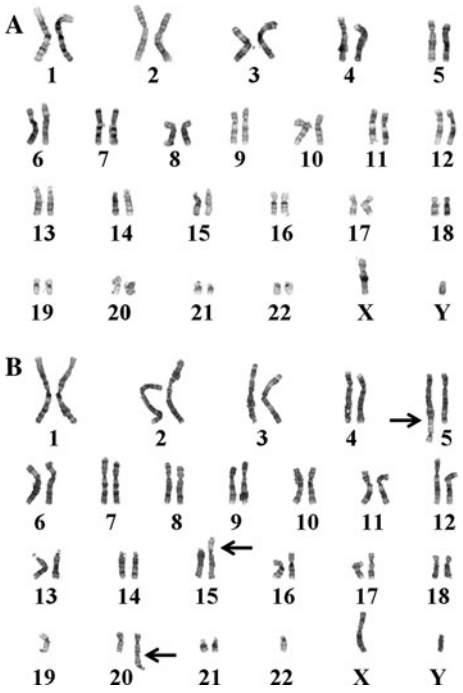

Fig. 1 Cyto-genetic examination of hTGSC-hTERT and hTGSC-SV40. Karyotyping was performed at passage #15 of the smears stained with 5% Giemsa and observed under light microscope (magnification: 100X). hTGSC-hTERT exhibited normal karyotype (**a**), but hTGSC-SV40 (**b**) showed chromosomal abnormalities such as telomeric associations (e.g. between chromosomes 5–19 and 20–22), deletions and duplications (e.g. that of the *p*-arm of chromosome 15) (indicated by *arrows*)

Osteo-genic differentiation was also confirmed by visualizing calcium depositions using vonKossa technique (Fig. 6e). After adipo-genic differentiation, cells had formed classical lipid vesicles which were observed under light microscope (Fig. 6f).

The in vitro neuro-protection assay showed a marked decrease in stressor-induced neuro-toxicity: in the presence of hTGSC-hTERT conditioned medium (20%), cell viability of SH-SY5Y increased by 12 and 16% following treatment with 100 μM of H_2O_2, or 5 μM of doxo-rubicin, respectively (Fig. 7) at 24 h. Selection of a short (1-day) treatment only was based on the fact that over half of the cells exposed to such a strong noxa died in 24 h without conditioned medium.

Discussion

Despite vast clinical trials, routine and wide-spread application of stem cell therapy is marred by several factors. Among them, aging of cells is a limiting technical problem: although stem cells have the ability to self-renew and give rise to subsequent generations with variable degrees of

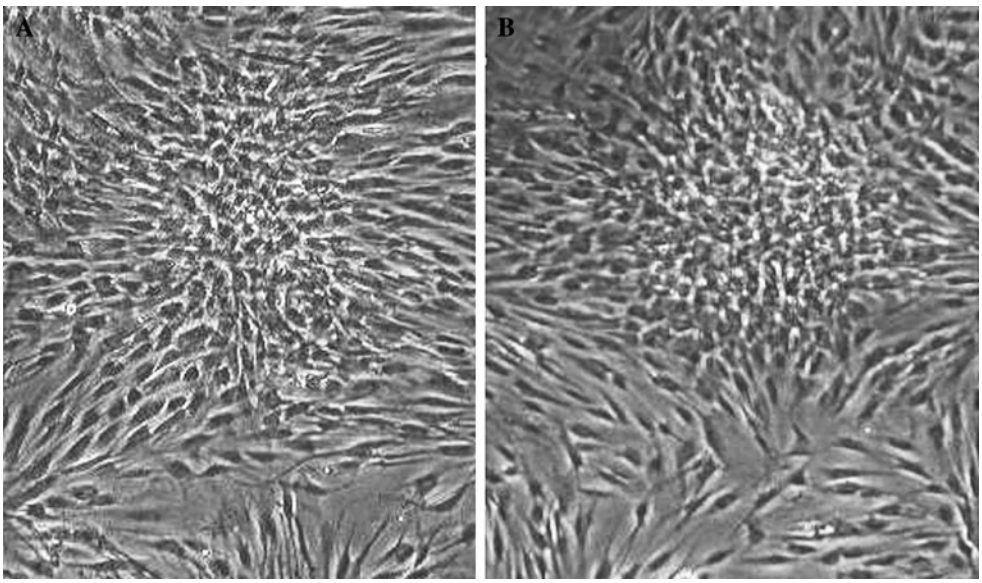

Fig. 2 Morphology of stem cells. Cultured in growth medium, hTGSCs (**a**) and hTGSC-hTERT (**b**) have shown classical MSC morphology

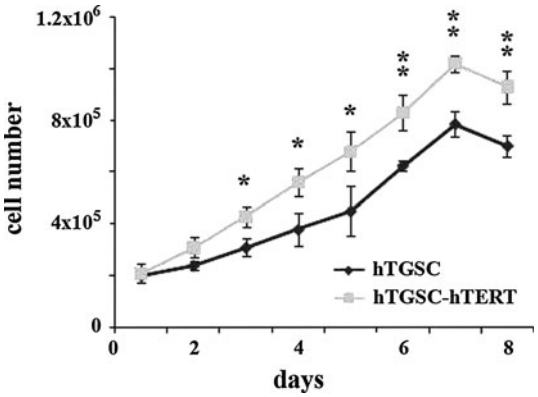

Fig. 3 Comparison of proliferation rate of hTGSC and hTGSC-hTERT. Late passage hTGSC-hTERT (n = 2) has higher proliferation rate than non-transfected cells (n = 3). Values are expressed as mean ± SD, $*P < 0.05$, $**P < 0.001$

differentiation capacities, these progenitor cells are prone to relatively fast senescence with decreased proliferation activity, which hampers their use in biomedical and tissue engineering applications [24]. Immortalization is a readily available mode to bypass the replicative senescence (non-division/proliferation) and aging of stem cells.

Telomeres are non-coding guanine-rich repetitive DNA sequences at the end of the chromosomes that undergo progressive shortening. They prevent constant loss of genetic material from chromosome ends, and their gradual truncation is therefore responsible for aging on the cellular level and sets a limit on life-span. As human telomeres grow shorter, cells reach the limit of their replicative capacity and progress into senescence. hTERT is a catalytic subunit of telomerase that elongates telomeres. By adding short guanine-rich repeats, it prevents cells from gradually losing telomeric sequences. Telomerase activity is repressed in post-natal somatic cells, resulting in progressive shortening of telomeres and therefore in natural aging, but is expressed by cancer and immortalized cells maintaining constant (non-truncated) telomere-length during replication [25]. hTERT has been successfully used to immortalize stem cells resulting in expanding their life-span without changing their unique characteristics [16].

This study, along with previous ones, shows that hTERT-induced immortalization yields less karyotypic alterations or DNA damage, and results in technically no change in normal differentiation of cells [24] when compared to other techniques, such as SV40. We have successfully transformed hTGSC-hTERT into adipo-, osteo- and odonto-genic cells, demonstrating that these cell-lines are multi-potent, and preserve their MSC characteristics and differentiation capacity. The expression of ESC markers *oct4* and *sox2*, which are associated with pluri-potency, has increased after transfection with hTERT. On the other hand, marked repression of *klf4*, which plays a role in chromosome remodeling, was observed. These might indicate that undying cells have modified gene expression without altered DNA sequence (i.e. epi-genetic changes).

All cell-lines demonstrated normal karyotypes before immortalization, however after transfection each hTGSC-SV40 displayed aberrant chromosomal appearances. The most common anomalies included telomeric associations of choromosomes 19 and 20, where the ends of these chromosomes were fused. Duplications and deletions were also observed. This is in line with previous studies

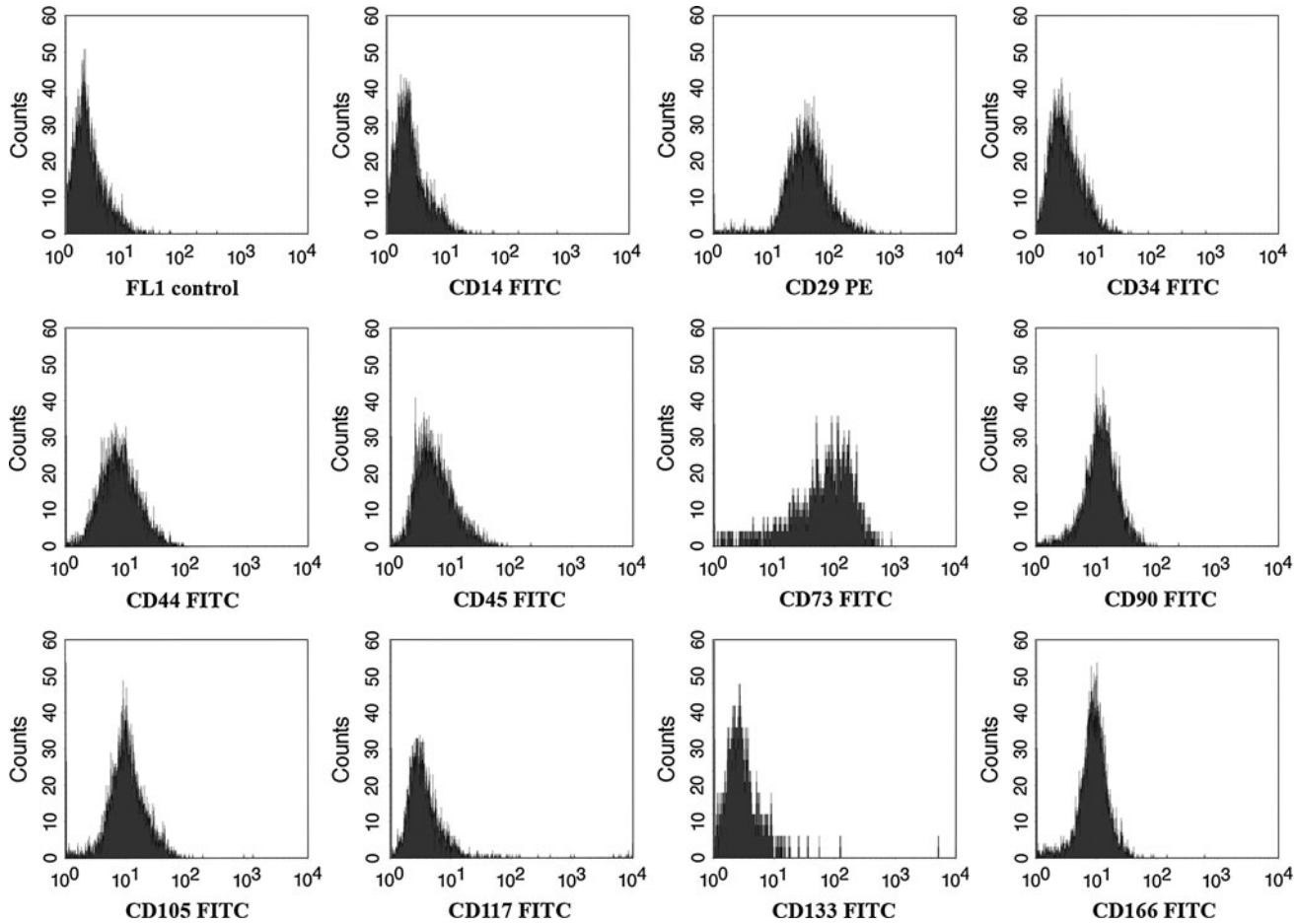

Fig. 4 Analysis of MSC surface antigens. Flow cytometry revealed hTGSC-hTERT as being positive for cell surface antigens CD29, CD73, CD90, CD105 and CD166, but negative for hemato-poietic markers CD14, CD34, CD45 and CD133. *FITC* fluorescein-iso-thio-cyanate, *PE* phyco-erythrin

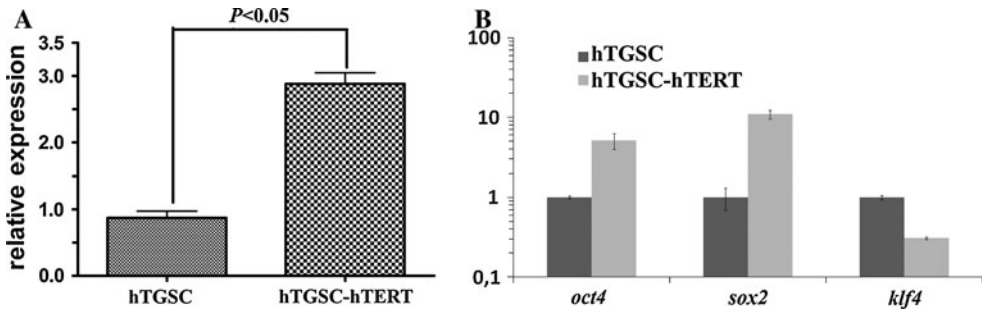

Fig. 5 Analysis of mRNA expression using quantitative PCR. After transfection of cells, hTERT expression level has increased by threefold, as confirmed by RT-PCR (**a**). Analysis of pluri-potency associated genes by RT-PCR demonstrated elevated expression of *oct4* and *sox2*, and repression of *klf4* in hTGSC-hTERT when compared to non-transfected stem cells. Values are expressed as mean ± SD

demonstrating loss of differentiation capacity, DNA damage and karyotypic abnormalities following SV40-induced immortalization [26].

In contrast, only 1 hTGSC-hTERT has undergone chromosomal rearrangement, which has occurred only after reaching high number of population doubling (90 pd), and presumably caused by accumulation of mutations during cell divisions and exposure to stress conditions associated with cell passaging rather than by direct effect of increased intra-cellular hTERT levels. These data suggest that

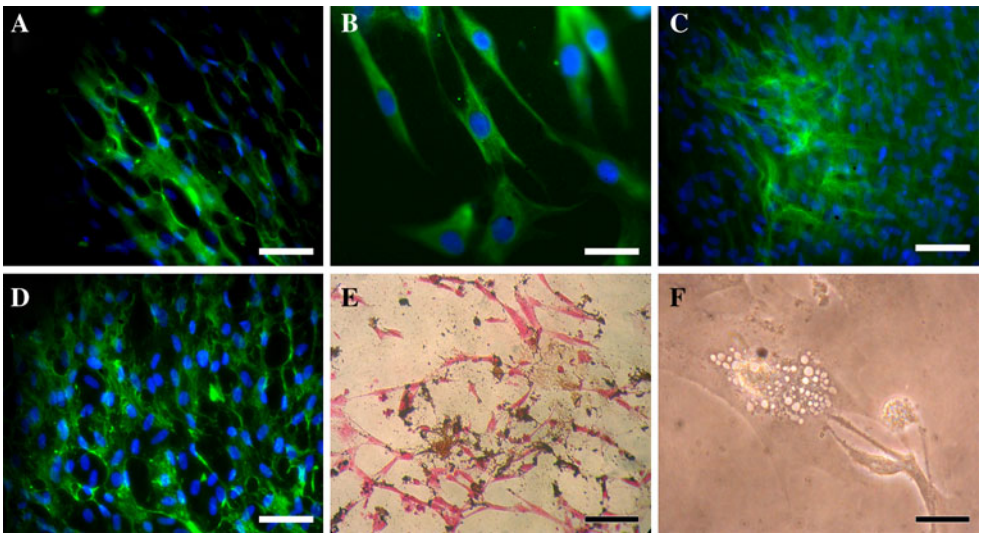

Fig. 6 Differentiation of hTGSC-hTERT. Immuno-cyto-chemistry analysis using anti-bodies against odonto-genic markers DSPP (**a**) and DMP-1 (**b**), and against osteo-genic markers collagen type-I (**c**) and osteo-calcin (**d**), as well as positive vonKossa staining (**e**) confirm transformation of hTGSC-hTERT into these lineages. Adipo-genic differentiation of immortalized stem cells has resulted in classical lipid vesicle formation as observed under light microscope (**f**). *Scale bars*: **a**: 25 μm, **b**: 10 μm, **c**: 25 μm, **d**: 20 μm, **e**: 100 μm and **f**: 10 μm

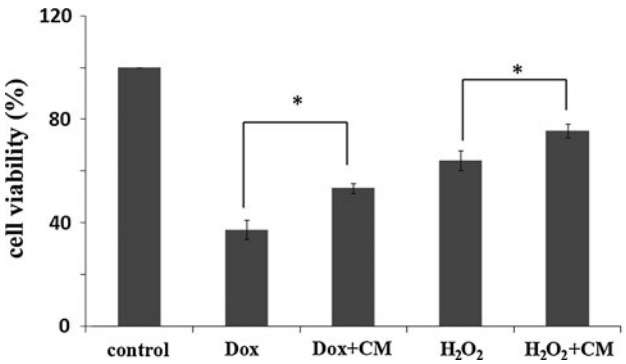

Fig. 7 Viability of cells after 24 h-incubation with H_2O_2 or doxo-rubicin (Dox). Viability of SH-SY5Y neuro-blastoma cells was significantly increased by 20% of hTGSC-hTERT conditioned medium (CM), $*P < 0.05$

hTERT-induced immortalization of hTGSCs is a safer alternative to SV40. Moreover, SV40 LT is a proto-oncogene: it inactivates cellular tumor suppressor proteins (e.g. p53), causing cells to enter into S phase thereby promoting DNA replication and cell proliferation. Possible tumoro-genicity, therefore, also limits interest in transfection of cells with SV40.

In addition to intact differentiation capacity and karyotype, hTGSC-hTERT showed neuro-protective effects on SH-SY5Y cells by reducing neuro-toxicity induced by either oxidative stress (H_2O_2) or the cyto-toxic/static antibiotic (anti-cancer drug) doxo-rubicin in this study. Perhaps similarly to MSCs [27, 28], hTGSC-hTERT may also secrete growth factors, such as vascular endothelial growth-factor (VEGF), brain-derived neuro-trophic factor (BDNF), as well as fibroblast growth-factor 2 (FGF_2), and might also release anti-oxidative enzymes (e.g. super-oxide dismutase, catalase and glutathione-*s*-transferase) into the culture medium which, when applied on SH-SY5Y cell-lines, could efficiently exert neuro-protection. Further studies, however, are warranted to characterize this effect.

These data suggest that hTGSC-hTERT might be used as a model system to understand the cross-talk between MSCs and neurons in many neuro-degenerative diseases where neuro-toxicity is an underlying pathological event. The advantage of using hTGSC-hTERT over intact MSCs in this regard is their long-term preservation of functional properties which is crucial during lengthy experiments. These results also serve as springboard for future studies evaluating the potential therapeutic role of dental waste materials in neuro-degenerative disorders.

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