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P16/p53 expression and telomerase activity in immortalized human dental pulp cells

Obi Egbuniwe,[1] Bernadine D. Idowu,[1] Juan M. Funes,[2] Andrew D. Grant,[3] Tara Renton[4] and Lucy Di Silvio[1,*]

[1]Biomaterials and Biomimetics; [3]Wolfson Centre for Age-Related Diseases; [4]Department of Oral Surgery; Dental Institute; King's College London; [2]University College London Cancer Institute; London, UK

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Introduction: Residing within human dental pulp are cells of an ectomesenchymal origin that have the potential to differentiate into odontoblast-like cells. These cells have a limited growth potential owing to the effects of cell senescence. This study examines the effects of immortalizing odontoblast-like cells on cell proliferation and mineralization by comparing transformed dental pulp stem cells (tDPSCs) and non-transformed dental pulp stem cells (nDPSCs).

Results: With the exogenous expression of hTERT, tDPSCs maintained a continued expression of odontogenic markers for cell proliferation and mineralization (ALP, COL-1, DMP-1, DSPP, OCN and OPN), as did nDPSCs. Oncoprotein expression was seen in both groups except for a noted absence of p16 in the tDPSCs. nDPSCs also showed lower levels of total ALP and DNA activity in comparison to tDPSCs when assayed as well as low telomerase activity readings.

Methods: Using a retroviral vector, exogenous human telomerase reverse transcriptase (hTERT) was expressed in tDPSCs. Both cell groups were cultured and their telomerase activities were determined using a telomerase quantification assay. Also examined were the expression of genes involved in proliferation and mineralization such as human alkaline phosphatase (ALP), β-actin, collagen I (col-1), core binding factor (cbfa)-1, dentin matrix protein (DMP-1), dentin sialophosphoprotein (DSPP), GAPDH, hTERT, osteocalcin (OCN), osteopontin (OPN), as well as oncoproteins involved in senescence (p16, p21 and p53) using RT-PCR. DNA and alkaline phosphate activity was also assayed in both cell groups.

Conclusion: These results indicate maintenance of odontoblast-like differentiation characteristics after retroviral transformation with hTERT and suggest a possible link with a reduced p16 expression.

Introduction

Human pulp tissue is populated by a group of multipotent cells of mesenchymal origin known as dental pulp stem cells (DPSCs). These cells are heterogeneous in nature and have a potential for pluripotency.[1-3] During the Bell Stage of tooth development, the pulp is formed by the differentiation of the dental papilla.[4] Separating the pulp from tooth dentine are mature odontoblasts that form the outer layer of the dental pulp and are responsible for the secretion of the collagenous and non-collagenous proteins[5] needed to produce reparative (secondary) dentine.[6-8] These odontoblasts have been referred to as the regenerative unit of the dentine-pulp complex.[2,9] Lying beneath this layer of odontoblasts is a mixed population of undifferentiated cells that are able to differentiate under appropriate stimuli to yield different cell types.[10] The process of dentinogenesis is influenced by the presence of an enabling environment controlled by several growth factors and signaling pathways.[4,11] In appropriate culture media, isolated DPSCs have the potential to differentiate into adipocytes, neural-like cells and odontoblasts.[1,2,12,13] The subject of a considerable amount of research interest are the odontoblasts and primary cell cultures of these cells will provide better understanding of their roles in dentin matrix formation. As is common with most human cell cultures, odontoblasts in vitro exhibit limited proliferation and senescence within several generations owing to a loss of telomeres.[14]

The in vitro study of primary cultures of human cells is dependent either on a constant supply of fresh tissue or the ability to maintain the cells in a steady proliferative state. Many cell/tissue types are only rarely available, depending on the frequency of biopsy. This difficulty is not associated with teeth, which are routinely extracted during many dental procedures, providing a ready supply of tissue. However, extraction of the pulp and expansion of the pulp cells is time-consuming, and cultures are often contaminated by bacteria from infected teeth, so the availability of immortalized pulp cells would be advantageous. Attempts to obtain cultures of human cells have been successful in the main, although often they only have limited proliferative ability.[15] The death of these cells is mostly due to oncogenic stress as a result of an increased amount of cell divisions.[14-16] As cell division progresses, there is a shortening of telomeres, which causes cellular instability and activates an arrest of cell division. This cell cycle arrest is mediated by growth suppressing proteins p16 and p53.[16,17] The protein p53 controls the senescence of damaged or mutated cells and its activity is dependent on the presence of p16 [18] and p21,[19] which are both damage response proteins.

*Correspondence to: Lucy Di Silvio; Email: lucy.di_silvio@kcl.ac.uk
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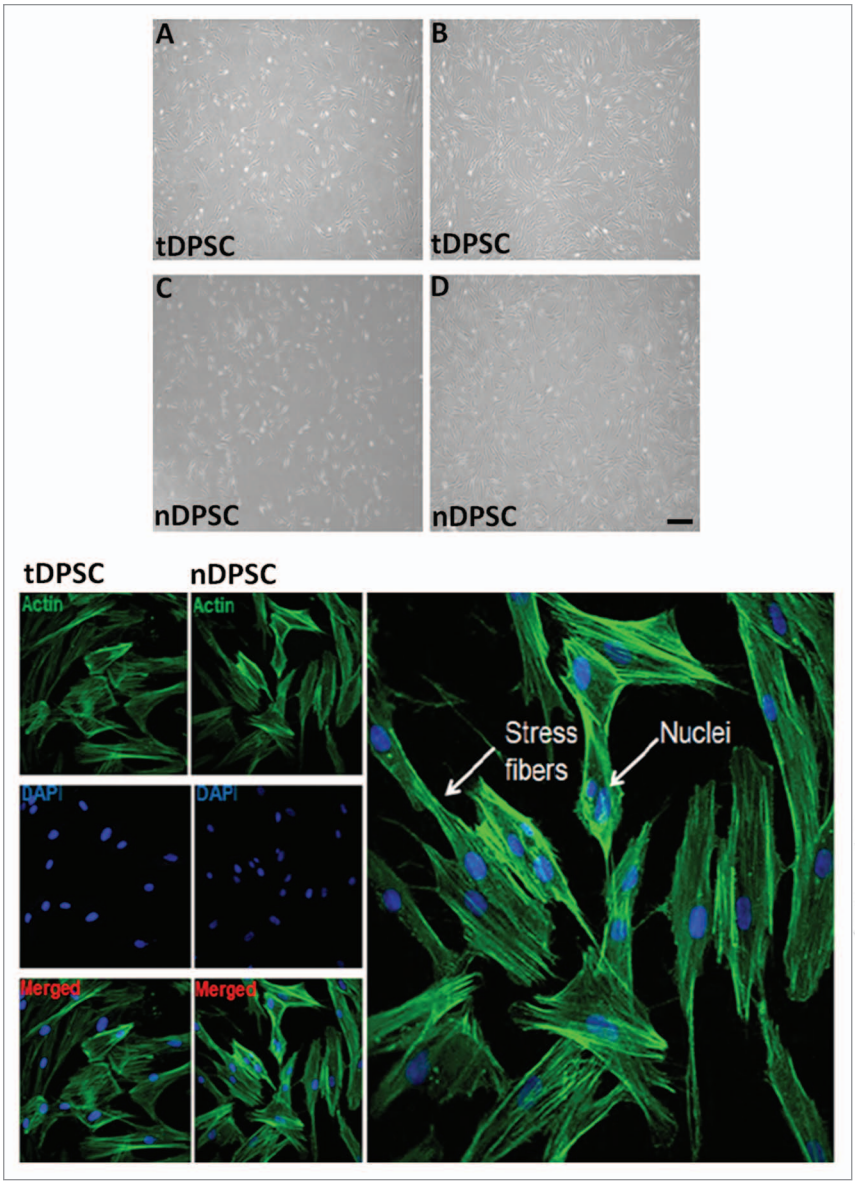

Figure 1. Optical phase contrast microphotographs showing the morphology tDPSCs and nDPSCs under phase contrast microscopy (x400) during the growth phase, day 3 (A and C) and at confluence, day 7 (B and D). Also shown is some immunopositive staining of tDPSCs and nDPSCs.

The maintenance of telomere length of cells in culture is dependent on the expression of human telomerase reverse transcriptase (hTERT).[20,21] This gene encodes the catalytic subunit of a protein complex known as telomerase that permits the addition of telomeric DNA repeat sequences during cell division.[22] Uninhibited cell division has been demonstrated by research groups who have used a combination of viral oncogenes that allow for the ectopic expression of the telomerase enzyme to successfully establish human cell lines with increased periods of cell growth. Protein products of Simian Virus 40 (SV40) or retroviral vectors have been used to transduce the hTERT gene into human cell lines.[23-25] This deactivates the p53 pathway and immortalizes the cells.[26,27] A disadvantage in some human cell lines is the possibility of development of tumor cells.[28] There is a suggestion that the expression of p16 may still prevent further cell division as its function is not dependent on telomerase expression.[29]

To further understand the effect of ectopic telomerase expression and senescence, we have used a retroviral vector to transfect human DPSCs with odontoblastic differentiation. While this method of transduction has been reported in reference 24 and 30, this study examines the effects of p16, p21 and p53 levels on the proliferative and mineralising activities of odontoblast-like cells following ectopic telomerase expression.

Results

Cell morphology, phase contrast microscopy. The morphology of tDPSCs and nDPSCs was analyzed microscopically at different time periods (**Fig. 1**). Both cells, as depicted by the optical phase contrast microphotograph reported in **Figure 1A**, spread along the surface of the culture plates, showing a rapid phase of growth within the first day. By Day 7, both tDPSCs and nDPSCs had assumed a heterogeneous morphology typified by the appearance of spindle-like shaped cells with extensive cellular processes and this phenotype was seen as the cells (tDPSCs and nDPSCs) grew to confluence (**Fig. 1B**).

The tDPSCs maintained this spindle-like phenotype beyond day 7, through further culture passages, in keeping with the normal growth morphology as observed by other groups.[3,11,30,31]

Cytoskeletal structure analysis by fluorescence microscopy. Using a fluorescent-labeled phalloidin, the actin filaments of tDPSCs and nDPSCs were observed. In both cell groups, there was an identical pattern of parallel fiber orientation (**Fig. 1**). This was mainly in the cytoplasmic areas of the visualized cells, transversing the nuclei of the spindle-like cells.

TRAP (telomere repeat amplification protocol) assay. Cell extracts of tDPSCs, nDPSCs, telomerase-positive and telomerase-negative samples were made using equivalents of 500 and 1,000 cells (**Fig. 2**). The fluorescence was measured and showed a significant change in the ΔFl/ΔR values for tDPSCs at 500 and 1,000 cell equivalents. The telomerase-positive extracts showed ΔFl/ΔR values indicative of telomerase activity at 500 cell equivalents. ΔFl/ΔR values for nDPSCs at 500 and 1,000 cell equivalents were zero (data not shown) and less than 1 per cent of the telomerase positive cells respectively, indicating a low level of telomerase activity. This would suggest background levels of telomerase activity in nDPSCs in keeping with the faint expression bands detected on mRNA levels by PCR (**Fig. 4**).

Telomerase-negative samples showed a zero ΔFl/ΔR at 500 cell equivalents (data not shown).

β-galactosidase staining. DPSCs undergoing senescence showed a blue peri-nuclear stain indicating positivity for β-galactosidase (at a pH of 6.5) activity in these cells (**Fig. 3**). The early passage tDPSCs (passage 6–9) did not stain for β-galactosidase. A few late passage tDPSCs (passage 30) and a significant number of nDPSCs (passage 9) showed β-galactosidase activity. The cells were re-plated after the experiment and there was a failure of the positively staining nDPSCs to proliferate.

RT-PCR analysis. Initially, the presence of hTERT mRNA was confirmed using RT-PCR. There was expression in both nDPSCs and tDPSCs, with a fainter hTERT band appearing in nDPSCs (**Fig. 4**). The expression of mRNA for the growth regulating factors p16, p21 and p53 was also investigated in both cell groups. There was no evidence of downregulation of p16, p21 and p53. However, the p16 bands were fainter in both cell groups, even more so in the nDPSCs (**Fig. 4**). To confirm the presence of odontoblastic differentiation markers we examined the presence of cbfa-1, DMP-1, DSPP and osteopontin in both cell groups. Also performed was the detection of mRNA expression of markers of mineralization such as ALP, COL1 and osteocalcin (**Fig. 4**).

Colorimetric assays. *DNA assay.* To analyze the proliferative activities of tDPSCs and nDPSCs, both cell groups (40,000 cells/ml) were cultured in DMEM/S growth medium for a week. Lysates were taken from both groups and a DNA assay was run. The optical absorbance values were obtained and represented graphically (**Fig. 5A**). There is a steady increase in the total proliferative activity in the tDPSCs compared with the nDPSCs over the 7-d culture period.

ALP assay. The mineralising activity of both tDPSCs and nDPSCs was evaluated using lysates from cells from each group (40,000 cells/ml) cultured in DMEM/S growth media over a 7 d period. The data obtained showed a steady-state of increase in total ALP activity in the tDPSCs compared with the nDPSCs, rising to significant levels ($p \leq 0.001$) from day 1 to day 7 (**Fig. 5B**).

Discussion

The immortalization of human dental cells is not entirely novel. A literature review demonstrated that several authors have successfully transformed and immortalized these cells, having obtained them from a variety of oral sources. Kamata and colleagues in 2004 performed a series of transformation studies using cells from human dental papilla, dental pulp, periodontal ligament and gingival fibroblasts.[24] In the same year, Kitagawa and coworkers also successfully

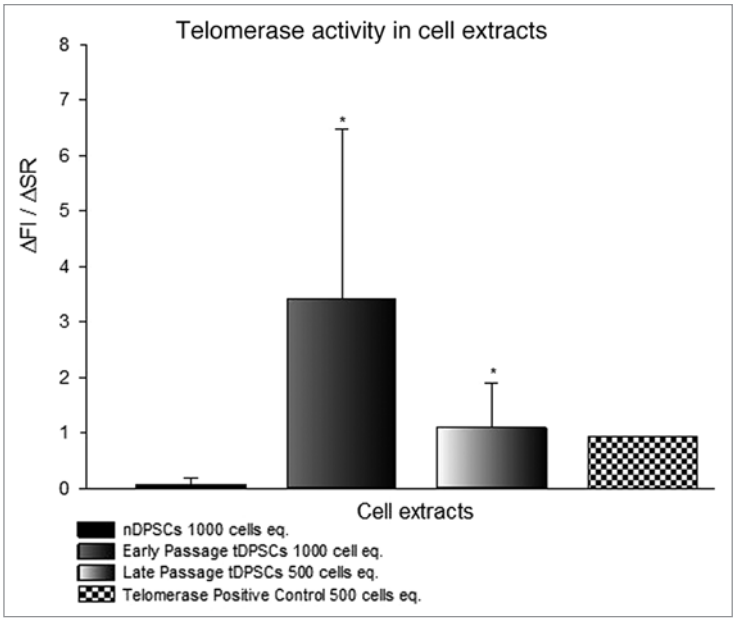

Figure 2. Relative fluorescent readings used to assess telomerase activity in tDPSCs and nDPSCs after the introduction of exogenous HTERT. The results are expressed as the means ± SD (n = 4, *p < 0.001).

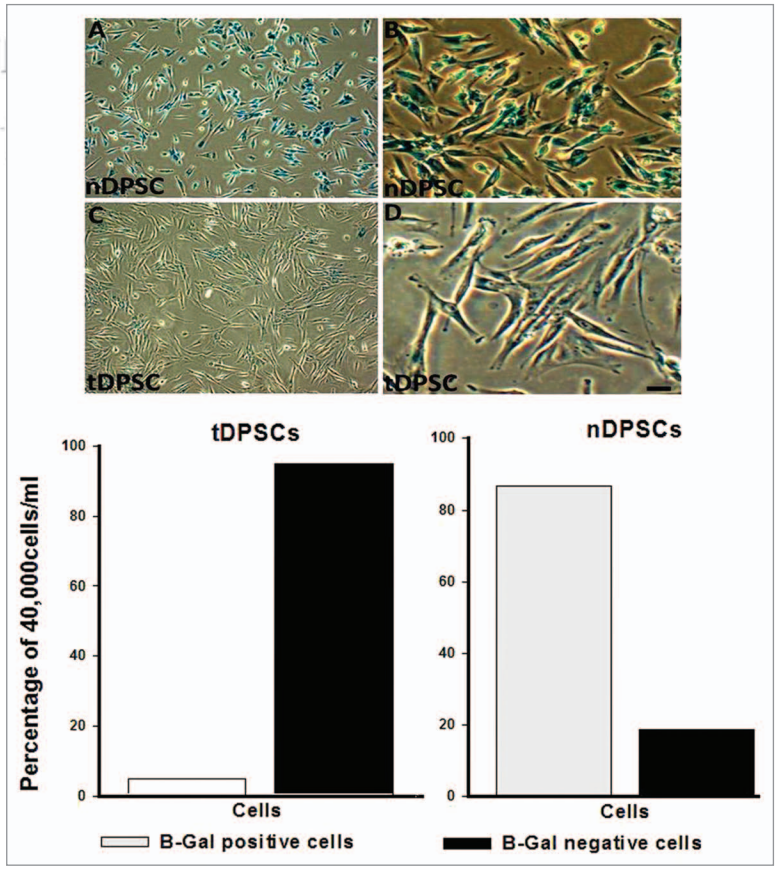

Figure 3. β-Galactosidase staining in cultured human dental pulp stem cells. Cells were stained and photographed at 100x (A and C) or 200x (B and D) magnification. (A and B) Early passage nDPSC showing >80% positivity and (C and D) Late passage tDPSCs showing <10% positivity for β-Galactosidase staining both depicted in the graph above. Positive staining disappeared after re plating tDPSCs.

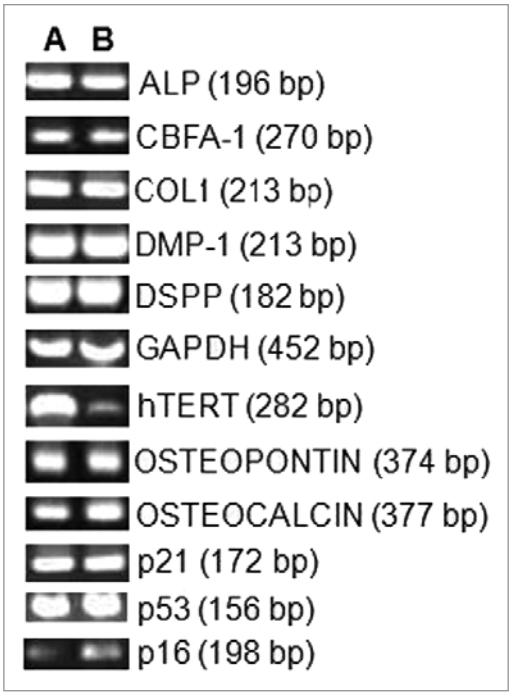

Figure 4. RT-PCR results for odontoblastic differentiation genes (ALP, cbfa-1, Col-1, DMP-1, DSPP, Osteopontin and Osteocalcin) and growth regulating factors p16, p21 and p53. Lane A, tDPSCs and Lane B, nDPSCs. GAPDH was run as an internal control and water as the negative control, for both groups.

immortalized dental pulp cells.[30] In 2005 Fujita and colleagues immortalized periodontal ligament cells.[14] Previous studies have demonstrated that transfected cells are able to avoid the normal senescence program and hence extend the life span of the cells.[32]

In this study we used the HIV retroviral packaging system intending to explore the possibility of establishing a hDPSC cell line that would circumvent normal programmed senescence and maintain its usefulness as a tool for further study of cellular activity. The detection of hTERT expression at mRNA levels following induction in a variety of cell lines has been widely reported in reference 24, 30 and 33. The presence of hTERT mRNA was successfully demonstrated in this study following transfection of the tDPSCs with the hTERT gene. Also, the telomerase activity in tDPSCs was 3 times greater than in nDPSCs (**Fig. 2**). We have demonstrated perpetual cell growth over 30 population doublings while maintaining characteristics of differentiation and phenotypic cellular identity, in line with literature-defining parameters for a successful immortalization of dental pulp stem cells. The evidence to support continued odontoblastic differentiation is demonstrated by the expression of mRNA for cbfa-1, DMP-1, DSPP and osteopontin (**Fig. 4**). This corroborates the typical features of odontoblasts in culture.[1-3,30] The genetic expression of ALP, COL1 and osteocalcin,[1-3] in addition the mineralization activities of the tDPSCs is further evidence to show a lack of change in cellular characteristics as demonstrated in the morphology and cytoskeletal organization of the cell (see **Fig. 1**).

In addition, the non transduced DPSCs (nDPSCs), demonstrated a very high degree of β-galactosidase associated senescence activity (**Fig. 3**), a feature absent from a vast majority of the transduced DPSCs (tDPSCs) owing to the introduction of hTERT into these cells. With the prolongation of the life span of the DPSCs, we considered the possible effects of transfection on the silencing of senescence associated factors (oncoproteins). hTERT has long been associated with oncoproteins such as p16, p21 and p53 owing to the link between these proteins and telomerase activity.[18,34-36] To the knowledge of the authors, this is the first study in dental pulp stem cells showing a possible link between the introduction of exogenous hTERT and mRNA expression of p16. The presence of p16 has been attributed to unfavorable culture conditions.[37] Also noteworthy is its inverse relationship with the aging and proliferation of some cell types.[38,39] It is uncertain whether the alteration of the pathways controlled by any of the three oncoproteins examined is solely dependent on the functional presence of hTERT.[29] It is our opinion that this "downregulation" of p16 activity maybe promoted by the arresting of senescence of DPSCs owing to the introduction of exogenous hTERT, which promotes p53 activity in certain human cell types.[18,28,29,34,40,41] The results from this study suggest an ability of DPSCs to maintain phenotypic characteristics of odontoblasts and circumvent senescence in the presence of ectopic hTERT, which may alter p16 expression.

Materials and Methods

Cell culture. The dental pulp cells were prepared from human third molars extracted from four adult patients at the Department of Oral Surgery, Dental Institute, King's College London (with ethical approval (KCH REC ref. 08/H0808/104) and patient consent). After collection, the teeth were washed with 70% ethanol and then with Hank's balanced saline solution (HBSS) (Invitrogen), pH 7.4. The crowns and roots of the teeth were separated aseptically by making horizontal indentations along the cervical margin using a low speed circular diamond saw (Agar Scientific Ltd.,) and the pulp tissue retrieved from the pulp chamber using sterile forceps. After pooling the pulp cell samples, disaggregation was effected through enzymatic digestion in a solution of 3 mg/ml collagenase type I and 4 mg/ml dispase (Sigma-Aldrich®) for 30 min at 37°C after initially mincing the pulp tissue into small pieces measuring 1–2 mm by 3–4 mm with a scalpel blade under sterile conditions.

The cells were cultured following modification of the method described by Gronthos and coworkers (Gronthos et al. 2000). Following digestion, the pulpal tissue explants was grown in 25 cm^3 culture flasks, T25 (Cell Star®), using Dulbecco's modified Eagle's medium DMEM (Pharmakine®) supplemented with 10% fetal bovine serum, FBS, 2.5 x 1,000 U/ml penicillin and 2.5 mg/ml streptomycin. The cultures were incubated at 37°C in an atmosphere of 95% O_2 and 5% CO_2. The media in the culture flasks was changed every 4 d to remove non adherent cells and cellular debris. After 10–14 d, outgrowths from the minced pulp tissue explants were observed. At confluence, the cells were trypsinized and passaged in T25 flasks. These cells were designated Passage 2. The cells were maintained for 3 passages before differentiation was induced by using a supplemented media. This

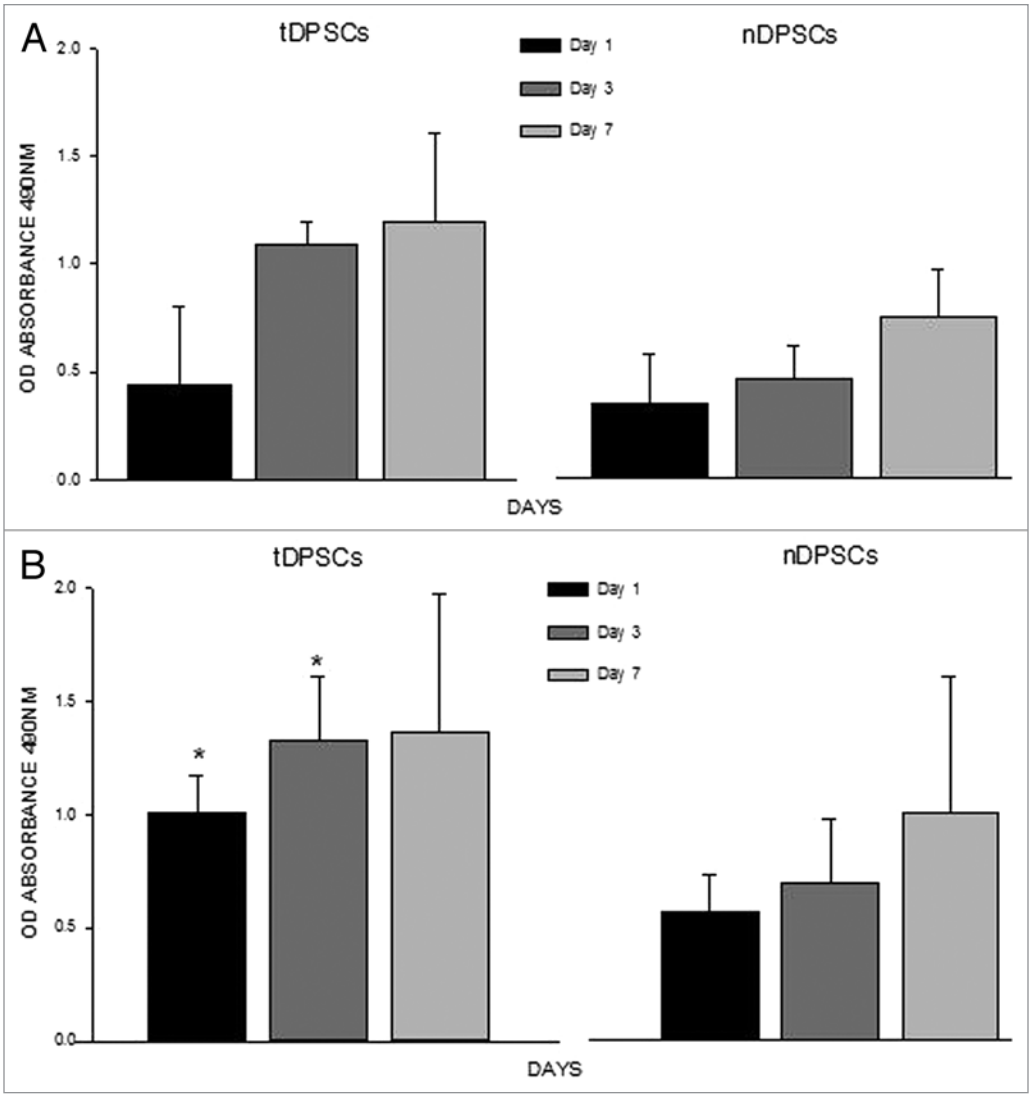

Figure 5. Analyses of cell proliferation using a DNA assay (A) and cell mineralization by way of an ALP assay (B) in tDPSC and nDPSC cell groups. The experiments were performed in triplicate. The values are means of the absorbance readings at 490 nm ± SD (n = 4, *p < 0.001).

supplementation was effected by conditioning the culture media with 50 μg/ml ascorbic acid, 10 mM β-glycerol phosphate and 10^{-7} M dexamethasone. A batch of the "non-differentiated" cells was harvested, frozen and stored in liquid nitrogen. The culture media was changed to a high glucose DMEM supplemented with 10% FCS, 5 x 1,000 U/ml Penicillin, 5 mg/ml Streptomycin and 0.11 g/L Sodium pyruvate (Sigma Aldrich®), DMEM/S. All other culture conditions were kept constant. Periodic monitoring of the cells was continued and sub culturing at confluence was done at a ratio of 1:4 to new culture 25 cm^3 flasks.

Transduction of DPSCs with hTERT. The retroviral vector containing the cloned hTERT gene, pBABE-puro-hTERT (**Fig. 1**),[42] was a kind gift from Dr. J.M. Funes, University College London Cancer Institute. One x 10^4 DPSCs were plated in each well of a 24-well plate. This was followed by exposure to the hTERT retrovirus that was added to DMEM/S growth media, for 24 h. After 24 h the virus was removed and replaced with DMEM/S growth media for a further 72–90 h period at 37°C in a humidified incubator. This was followed by selection using 1 μg/ml puromycin (Sigma) for between 7–14 d. The cells were then expanded. The DPSCs that survived were used for the rest of this study and are referred to as tDPSCs.

Cell extract TRAP (Telomere repeat amplification protocol) assay. Telomerase activity was determined using a TRAPeze®XL Telomerase Detection Kit (Millipore™). The cells were pelleted, washed in PBS and dissolved in 200 μL of 1x CHAPS lysis buffer. After a 30 min incubation period on ice, the cell samples were centrifuged at 12,000 g for 20 min at 4°C. 160 ul from each of the sample supernatants were collected for protein content analysis using a standard by Bicinchoninic acid protein assay. Two μL of the cell-extract (an equivalent of 2.5 μg protein/sample supernatant) was added to a 48 μL reaction solution consisting of 5x TRAPeze®XL reaction mix, a cloned and unmodified Taq polymerase (Sigma-Aldrich®) and distilled water. A 1,000 cell equivalents of telomerase positive cell extract (provided with the kit) was used as a positive control. Heat inactivated samples,

Table 1. Primer sequences and cycling conditions for the genes analyzed

Primer	Sequence Product	Length (bp)
ALP	F: 5'-CCA CGT CTT CAC ATT TGG TG-3' R: 5'-AGA CTG CGC CTG GTA GTT GT-3'	196 bp
β-ACTIN	F: 5'-ATG AGG ATG CTC ACG GAG CGC GGC TAC AGC-3' R: 5'-ACA CCA CTG TGT TGG CGT ACA GGT CTT TGC-3'	331 bp
CBFA-1	F: 5'-TGA GAG CCG CTT CTC CAA CC-3' R: 5'-GCG GAA GCA TTC TGG AAG GA-3'	270 bp
COL-1	F: 5'-CCA AAT CTG TCT CCC CAG A-3' R: 5'-TCA AAA ACG AAG GGG AGA T-3'	213 bp
DSPP	F: 5'-TCA CAA GGG AGA AGG GAA TG-3' R: 5'-TGC CAT TTG CTG TGA TGT TT-3'	182 bp
DMP-1	F: 5'-CAG GAG CAC AGG AAA AGG AG-3' R: 5'-CTG GTG GTA TCT TGG GCA CT-3'	213 bp
GAPDH	F: 5'-ACC ACA GTC CAT GCC ATC AC-3' R: 5'-TCC ACC ACC CTG TTG CTG TA-3'	452 bp
hTERT	F: 5'-CCT CTG TGC TGG GCC TGG ACG ATA-3' R: 5'-ACG GCT GGA GGT CTG TCA AGG TAG-3'	282 bp
OCN	F: 5'-ATG AGA GCC CTC ACA CTC CT-3' R: 5'-CAA GGG GAA GAG GAA AGA AG-3'	377bp
OPN	F: 5'-CAG TGA CCA GTT CAT CAG ATT CAT C-3' R: 5'-CTA GGC ATC ACC TGT GCC ATA CC-3'	374bp
p16	F: 5'-GAC ATC CCC GAT TGA AAG AA-3' R: 5'-TTT ACG GTA GTG GGG GAA GG-3'	198 bp
p21	F: 5'-GAC ACC ACT GGA GGG TGA CT-3' R: 5'-CAG GTC CAC ATG GTC TTC CT-3'	172 bp
p53	F: 5'-GGC CCA CTT CAC CGT ACT AA-3' R: 5'-GTG GTT TCA AGG CCA GAT GT-3'	156 bp

a minus Taq polymerase and a minus telomerase sample were used as negative controls. The tubes containing the mixture were incubated at 30°C for 30 min in thermocycler block. This was followed by a 4-step PCR at 94°C for 30 sec, 59°C for 30 sec, 72°C for 1 min (36 cycles), a 72°C for 3 min extension step, 55°C for 25 min and a 4°C incubation phase. The 50 μl PCR reaction product for each sample was transferred into a black walled 96-well plate containing 150 μl of 10 nM TRIS-HCl/0.15 M NaCl/2 mM $MgCl_2$ buffer (pH 7.4). Using a fluorescent plate reader, fluorescence was measured for each sample using excitation/emission parameters (in line with the manufacturers' protocol) for fluorescein, F (485 nm/534 nm) and sulforhodamine, R (584 nm/610 nm). The net change in fluorescence (ΔFl/ΔR) was determined for each sample.

β-galactosidase staining. The expression of β galactosidase by the nDPSCs and tDPSCs was assessed using a Senescence Cells Histochemical Kit (Sigma-Aldrich®). The cells were washed in PBS, fixed for 6–7 min (room temperature) in a 1 x 20% formaldehyde/2% glutaraldehyde buffer. After three washes, the cells were incubated at 37°C in a non-CO_2 oven with a β-galactosidase senescence associated stain solution. The staining of cells was evident (under a microscope) after 4 h and was at a maximum at 12 hrs.

RT-PCR analysis. Using an RNA extraction kit (Qiagen), the total cellular RNA was isolated from the cells as per the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of total RNA using a High Capacity RNA-to-cDNA Master Mix (Applied Biosystems). Products were amplified by PCR using primers specific for human alkaline phosphatase (ALP), β-actin, collagen I (col-1), core binding factor (cbfa)-1, dentin matrix protein (DMP-1), dentin sialophosphoprotein (DSPP), GAPDH, hTERT, osteocalcin (OCN), osteopontin (OPN), p16, p21 and p53 (see **Table 1**). PCR products were separated by electrophoresis (1.5% agarose gel) and detected with ethidium bromide under UV light.

Colorimetric assays. *Alkaline phosphatase (ALP) assay.* tDPSCs and nDPSCs were seeded in 24-well plates at a density of 1 x 10^4 cells/well. ALP activity was measured after 1, 3 and 7 d cultures in DMEM/S using a modified assay that employs p-nitro phenyl phosphate as a substrate for measuring the enzyme protein present.[43] The alkaline phosphatase enzyme cleaves the phosphate group from p-nitro phenyl phosphate to yield p-nitro phenol, which is yellow at an alkaline pH (10.3). The absorbance was then measured at 405 nm with a spectrophotometer (Opsys MR Microplate reader).

DNA assay. Following cell seeding at 1 x 10^4 cells/well in 24-well plates, the DNA content of tDPSCs and nDPSCs was examined using the method of Rao and colleagues:[44] three cycles of freezing—thawing at -80°C and 37°C respectively; 100 μl aliquots of each cell group was then transferred to a 96 well-plate; 100 μl Hoechst 33,258 (Sigma®) fluorimetric dye was added to each well; florescence was measured at an excitation of 355 nm and emission wavelength of 450 nm on a plate reader (Chameleon™, Hidex, Finland). DNA standards (Sigma®) were prepared and the DNA assay was performed in parallel for standardization. The cell lysates used for the experiment were taken at days 1, 3 and 7 of the cell culture and the experiment was performed using triplicate samples.

Immunocytochemistry. To analyze the actin microfilament cytoskeletal protein, visualization was performed using FITC-conjugated phalloidin (1/1,000). The cells were cultured to 70% confluence in 6-well plates and fixed in 4% paraformaldehyde supplemented with 1% (w/v) sucrose at 37°C for 15 min. After washing in PBS, the cells were permeabilized at room temperature with 0.1% Triton-X 100 for 5 min and incubated with 1% bovine serum albumin at 37°C for 10 min to saturate non specific binding sites. This was followed by incubation with phalloidin at 37°C for 1 h. Control wells were incubated without phalloidin. The nuclei were counterstained with DAPI for 10 min. The cells were washed before viewing. The plates were visualized using a Leica DMRE upright confocal microscope (Leica Microsystems Heidelberg GmbH).

Statistical analyses. The analyses of the data were performed using independent t-test with Sigma Plot 11.0 software (©Systat Software, Inc.). Statistical significance of the data was

determined at $p \leq 0.001$ and the values were expressed as mean ± standard deviation (SD).

Conclusions

We have isolated hTERT immortalized DPSCs that exhibit and maintain phenotypic characteristics of odontoblastic differentiation. The "downregulation" of p16 activity is an important feature in the characterization of these cells as it may have an important role in the understanding of the mechanisms that control cellular senescence. This may provide a greater understanding of cellular mechanisms with respect to tissue regeneration. As a more readily available source of stem cells, involving a less invasive procedure compared with bone marrow aspiration for example, it may provide a viable method for cell-seeded scaffold in situ and lead to the development of new clinical treatment modalities.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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