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Dental Stem Cells SV40, a new cell line developed *in vitro* from human stem cells of the apical papilla

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Abstract

Aim: To establish and fully characterize a new cell line from human stem cells of the apical papilla (SCAPs) through immortalization with an SV40 large T antigen.

Methodology: Human SCAPs were isolated and transfected with an SV40 large T antigen and treated with puromycin to select the infected population. Expression of human mesenchymal surface markers CD73, CD90 and CD105 was assessed in the new cell line named Dental Stem Cells SV40 (DSCS) by flow cytometry at early and late passages. Cell contact inhibition and proliferation were also analysed. To evaluate trilineage differentiation, quantitative polymerase chain reaction and histological staining were performed.

Results: DSCS cell flow cytometry confirmed the expression of mesenchymal surface markers even in late passages [100% positive for CD73 and CD90 and 98.9% for CD105 at passage (P) 25]. Fewer than 0.5% were positive for haematopoietic cell markers (CD45 and CD34). DSCS cells also showed increased proliferation when compared to the primary culture after 48 h, with a doubling time of 23.46 h for DSCS cells and 40.31 h for SCAPs, and retained the capacity to grow for >45 passages (150 population doubling) and their spindle-shaped morphology. Trilineage differentiation potential was confirmed through histochemical staining and gene expression of the chondrogenic markers *SOX9* and *COL2A1*, adipogenic markers *CEBPA* and *LPL*, and osteogenic markers *COL1A1* and *ALPL*.

Conclusions: The new cell line derived from human SCAPs has multipotency, retains its morphology and expression of mesenchymal surface markers and shows higher proliferative capacity even at late passages (P45). DSCS cells can be used for *in vitro* study of root development and to achieve a better understanding of the regenerative mechanisms.

KEYWORDS

mesenchymal stromal cell, plasmid, regenerative endodontics, senescence, sv40, transformed cell

INTRODUCTION

Multipotent mesenchymal stromal cells (MSCs) are an adult cell population with self-renewal potential and the ability to differentiate into diverse specialized cell types (Mushahary et al., 2018). MSCs were originally isolated from rat bone marrow (Friedenstein et al., 1968) and have subsequently been identified in almost all postnatal tissues (da Silva et al., 2006). Significant differences in their characteristics have been described depending on the source tissue (Heo et al., 2016).

Several types of dental MSCs have been isolated from the pulp of human-impacted third molars (Gronthos et al., 2000), exfoliated deciduous teeth (Miura et al., 2003), periodontal ligaments (Seo et al., 2004), apical papilla (Sonoyama et al., 2006), dental follicle cells (Miura et al., 2003), gingiva (Zhang et al., 2009) and bone chips of dental implant sites (Park et al., 2012). Dental MSCs, like those from other sources, meet the minimum criteria proposed in 2006 by the International Society for Cellular Therapy (Dominici et al., 2006) to establish a set of standards to define human MSCs for laboratory-based scientific investigations and preclinical studies. These characteristics include adherence to plastic in standard cultures; specific surface antigen expression (95% positive for CD105, CD73 and CD90, and ≤2% positive for CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II); and that cells must differentiate into osteoblasts, adipocytes and chondroblasts under standard *in vitro* differentiation conditions.

Stem cells from the apical papilla (SCAPs) are a dental MSC population derived from the ectomesenchyme that can be demonstrated in the apical papilla attached to the apex of the developing tooth root in erupting permanent teeth (Nagata et al., 2021). SCAPs are also the source of primary odontoblasts responsible for the formation of root dentine (Huang et al., 2009). Recent studies have shown the potential of SCAPs for dental regenerative procedures (Arslan et al., 2019; El-Kateb et al., 2020) because they can survive infection and advanced apical periodontitis (Diogenes & Hargreaves, 2017; Lin et al., 2018). Furthermore, their superior neural stem cell properties mean that they are a promising source for the therapy of spinal cord injury (De Berdt et al., 2015). This important role highlights the ongoing requirement for models of SCAP *in vitro*, but the long-term culture of MSCs leads to their senescence, including morphological changes and decreased potential for differentiation (García-Bernal et al., 2021; Yang et al., 2018). To address this problem, several methods are available to immortalize cell lines from primary cultures. The most common ones include transduction with simian virus 40 large T antigen (SV40LT) to repress p53 and retinoblastoma tumour supressor (Rb)-mediated pathways or human telomerase reverse transcriptase to prevent telomere shortening, either alone or in combination (Piñeiro-Ramil et al., 2020).

Some authors have shown that simultaneous transduction with SV40LT and human telomerase reverse transcriptase is needed to achieve stable immortalization (Darimont et al., 2002; Liu et al., 2013); however, SV40 LT not only transforms cells allowing them to enter S phase and inhibiting the cell cycle suppressor action of p53 (Ahuja et al., 2005), furthermore, cell transformation through SV40 LT could secondarily alter telomerase and cause maintenance of telomere length (Li et al., 2021; Toouli et al., 2002), therefore the generation of cell lines through overexpression of SV40LT alone, could be an effective method to obtain immortalized cell lines (Li et al., 2020).

To date, no human SCAP cell lines have been developed through immortalization with SV40LT. Thus, the aim of the present study was to establish and characterize a new dental stem cell line from human SCAPs through immortalization with SV40LT named Dental Stem Cells SV40 (DSCS). We also aimed to characterize the new cell line by analysing the differences in proliferation, differentiation and morphology between the primary culture and the new cell line.

MATERIALS AND METHODS

The report of this laboratory study has been written according to Preferred Reporting Items for Laboratory studies in Endodontology 2021 guidelines (Nagendrababu et al., 2021 doi: 10.1111/iej.13542).

SCAP isolation and culture

An immature mandibular third molar at stage 7 of Nolla was extracted for orthodontic purposes from a healthy 12-year-old boy, after obtaining informed consent from his legal guardian. The apical papilla of the molar was minced under sterile conditions into 1-mm^3 fragments and digested by incubation with a solution of 3 mg/ml collagenase type I (Merck) and 4 mg/ml dispase (Merck) for 1 h, as described previously (Trevino et al., 2011). The digested cells were filtered through a 70-μm cell strainer (BD Falcon). Cells in suspension were centrifuged at 126 *g* for 2 min and resuspended in Dulbecco's minimum essential medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2 mmol/L L-glutamine, and 100 U/ml penicillin and streptomycin (P/S; Biological Industries). Cells were plated (Corning) and allowed to expand in culture until 70%–80% confluency. Cells from the first

passage (P) were used for immortalization. All the procedures received the approval of the Ethical Committee of the Hospital Odontologic of the Universitat de Barcelona (Ceim HOUB protocol number 2018-021-1).

Cell culture

SCAPs were cultured in DMEM supplemented with 10% FBS, 2 mmol/L L-glutamine and 100 U/ml P/S and incubated at 37°C with 5% CO_2. For osteogenic differentiation, cells were cultured in α-MEM with 10% FBS, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 50 μmol/L ascorbic acid, 10 mmol/L β-glycerophosphate and 100 U/ml P/S for 14 days. For adipogenic differentiation, cells were cultured in α-MEM with 10% FBS, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 1 μmol/L dexamethasone, 1 μg/ml insulin, 0.5 mmol/L 3-isobutyl-1-methylxanthine and 100 U/ml P/S for 14 days. For chondrogenic differentiation, cells were cultured in high-glucose DMEM with 10% FBS, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 50 μmol/L ascorbic acid, 10 mmol/L β-glycerophosphate, 100 nmol/L dexamethasone, 40 μg/ml L-proline, 10 ng/ml recombinant human transforming growth factor-β3, 50 mg/ml ITS-premix stock (BD Biosciences) and 100 U/ml P/S for 21 days.

Establishment of an immortalized human SCAP cell line

Retroviral particles were generated in Platinum-E cells from pBABE-puro *SV40 LT* plasmid (plasmid# 13970; Addgene) using Lipofectamine LTX (Thermo Fisher) as a transfection reagent, as described previously (Sánchez-de-Diego et al., 2019). The Platinum-E cells were left for 72 h in the transfection medium and the retroviral supernatants were harvested and filtered with a 0.45-μm cellulose acetate filter (Corning). SCAPs at P1 were seeded at 7×10^4 per well in a six-well plate (Corning), which resulted in 40%–50% confluence after 24 h. Cells were infected with 300 μl virus-containing medium, 5 μg/ml Polybrene (Sigma–Aldrich) and DMEM complete medium up to 1 ml. Cells were incubated for 24 h, and then, the virus-containing medium was replaced with a fresh culture medium and incubated for 24–48 h to allow the incorporation of the virus genetic material in the cells and obtain a higher expression of ectopic proteins. After this, cells were treated for 48 h with 5 μg/ml puromycin to select the infected population.

Lentiviral infection was confirmed by assessing mRNA expression of *puroR* gene by quantitative real-time polymerase chain reaction (qRT-PCR). Forward and reverse primers were:

5′-ATGACCGAGTACAAGCCCAC-3′ and 5′-GTTCT TGCAGCTCGGTGAC-3′.

Flow cytometry

Dental MSCs from the apical papilla infected with SV40 (DSCS cells from now on) were characterized at P4 and P25 using the Human MSC Analysis Kit (BD Bioscience). Cells were detached using BD™ Accutase™ Cell Detachment Solution, washed and resuspended at 10^7 cells/ml in BD Pharmingen Stain Buffer. One hundred microlitre of the cell suspension was added to each tube and stained with the following antibodies: FITC mouse anti-human CD90, PE mouse anti-human CD44, PerCP-Cy 5.5 mouse anti-human CD105, APC mouse anti-human CD73, hMSC-positive isotype control Cocktail, PE hMSC-negative isotype control cocktail, hMSC-positive cocktail or PE hMSC-negative cocktail. Tubes were incubated in the dark for 30 min on ice. Prior to flow cytometry analysis, cell suspensions were filtered through a 0.70-μm nylon mesh to remove aggregates (Corning). Flow cytometry assay was performed in the BD FACSCanto II Flow Cytometer System (BD Biosciences). Results were analysed using FlowJo Software.

Multilineage differentiation qRT-PCR analysis

Total RNA was isolated from primary SCAPs or DSCS cells using TRIsure reagent (Bioline). Purified RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCRs were carried out using the ABI Prism 7900 HT Fast Real-Time PCR System and Taqman 5′-nuclease probes (Applied Biosystems). Designed Taqman assays (Applied Biosystems) were used to quantify the expression of CCAAT Enhancer Binding Protein Alpha (*CEBPA*; Hs00269972_s1), Lipoprotein Lipase (*LPL*; Hs00173425_m1), Alkaline Phosphatase (*ALPL*; Hs01029144_m1), Collagen type I, Alpha 1 (*COL1A1*; Hs00164004_m1), SRY-Box Transcription Factor 9 (*SOX9*; Hs00165814_m1), Collagen Type II, Alpha 1 (*COL2A1*; Hs00264051_m1) and TATA-box binding Protein (*TBP*; Hs00427620_m1) genes. All transcripts were normalized to *TBP* expression.

Multilineage differentiation histochemical analysis

SCAPs at P6, DSCS cells at P15 and DSCS cells at P45 were seeded in 24-well plates (2×10^4 cells/well) and distributed

in control and differentiated groups. After 14 days in osteogenic and adipogenic differentiation media and 21 days in chondrogenic differentiation media, cells were rinsed twice with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (PFA) for 20 min.

To evaluate calcified tissue in the osteogenic group, fixed cells were incubated with Alizarin Red staining solution (Sigma–Aldrich) at room temperature in the dark for 45 min. Cells were rinsed twice with PBS and images of the wells were taken with a Canon 90D camera (Canon) and Tokina macro 100 F 2.8 D AT-X PRO objective (Kenko Tokina). For quantification of tissue mineralization, Alizarin Red was extracted following the protocol of Gregory et al. (2004). Briefly, 400 µl 10% acetic acid was added to each well. Wells were incubated for 30 min, followed by neutralization of the acid with 10% ammonium hydroxide. Absorbance was read at 405 nm with a Tecan Sunrise Microplate Reader (Tecan Trading AG). Absorbance measure was plotted and labelled as arbitrary absorbance units.

For quantification of glycosaminoglycans in the chondrogenic group, each well was incubated with Alcian Blue 8-GX (Sigma–Aldrich) at room temperature overnight. Bound Alcian Blue was extracted using guanidine HCl and the absorbance was read at 650 nm with a Tecan Sunrise Microplate Reader, as described previously (Prosser et al., 2019). Absorbance measure was plotted and labelled as arbitrary absorbance units.

For staining of lipid droplets in the adipogenic group, each well was incubated with 60% isopropanol for 2 min, followed by incubation with freshly prepared Oil Red O working solution (Sigma–Aldrich) for 30 min. The wells were rinsed four times with PBS. For colorimetric quantification, Oil Red O was extracted from lipid droplet deposits using 100% isopropanol for 1 min (Yu et al., 2021). Absorbance was read at 510 nm with a Tecan Sunrise Microplate Reader. Absorbance measure was plotted and labelled as arbitrary absorbance units.

Confocal fluorescence microscopy

The actin cytoskeleton was stained with phalloidin to compare the differences in morphology between primary culture SCAPs (P6) and DCSC cell line at early (P15) and late (P45) passages. Cells (1.5×10^4) of each group were seeded in an eight-well chambered coverslip (µ-Slide 8 Well Grid 500; Ibidi). After 24 h, cells were fixed with 4% PFA for 5 min and rinsed twice with PBS. Cells were permeabilized with 1% Triton X-100 (Sigma–Aldrich) for 20 min and blocked in 3% bovine serum albumin (Sigma–Aldrich) for 2 h. Double staining with 1: 5000 Hoechst 33342 (Invitrogen) and 1:500 AlexaFluor 633 conjugated phalloidin (Thermo Fisher) was performed. Cell morphology was analysed under confocal laser scanning microscope (Zeiss LSM880).

Cell proliferation and contact inhibition assays

SCAPs at P6 and DSCSs at P15 were seeded in 12-well plates at 3×10^4 cells/well and allowed to proliferate for 24, 48 and 72 h. At 0 (t0), 24, 48 and 72 h, cells were fixed with 4% PFA and nuclei were stained with 1 µg/ml 4,6-diamidino-2-phenylindole dihydrochloride (D9542; Merck) for 15 min. For each well, images of four microscopic fields were captured using a fluorescence microscope (Leica DM-IRB; Leica). The number of cell nuclei was quantified using ImageJ (National Institutes of Health). All results were expressed as a percentage of t0. In parallel, to determine the cell monolayer formation and arrest of cell growth once the cells reached confluence, SCAPs at P6 and DSCS at P15 and P45 were seeded in 24-well plates at 1.5×10^4 cells/well and allowed to proliferate for 1, 3, 5 and 7 days. Cells were fixed with 4% PFA and nuclei were stained with 1: 5000 Hoechst 33342 (Invitrogen). For each well, images of four microscopic fields were captured using a fluorescence microscope (Leica DM-IRB). The number of cell nuclei was quantified using ImageJ (National Institutes of Health). All results were expressed as a percentage of t0.

Statistical analysis

Statistical analysis was performed using GraphPad Prism. First, Shapiro–Wilk normality test was run per each study and Q–Q plots were represented to assess the normality of the samples. Then, statistical analysis was performed using the Student's *t*-test (for Gaussian distributions), nonparametric test (for no normal or lognormal distributions) or two-way analysis of variance. Quantitative data are presented as the mean ± standard error of the mean. Differences were considered significant at $*p<.05$, $**p<.01$ and $***p<.001$. All experiments were performed twice and in technical triplicates.

RESULTS

Establishment of the DSCS cell line

SCAPs were transduced with the retroviral vector for expression of SV40LT (Figure 1a) or green fluorescent

protein (GFP) as a control. After 3 days of cell culture, GFP expression was assessed as a control of infection. Due to the presence of a puromycin resistance gene downstream of SV40LT, we performed the selection using 5 mg/ml puromycin. After 48 h, almost 100% of the GFP control cells were dead, while around 40% of SV40-infected cells remained alive. DSCS cells infected with SV40 maintained their characteristic spindle-shaped morphology and no increase in death was observed.

To confirm the integration of the SV40 plasmid into the DSCS genome, we cultured DSCS cells for 15 passages and analysed the expression of *PuroR*, included in the pBabe vector. RT-PCR showed a significant increase in *PuroR* gene expression in DSCS cells compared with the primary cells (SCAPs; Figure 1b; Table S1). These data suggest that *PuroR* and SV40LT were stably inserted into the DSCS genome.

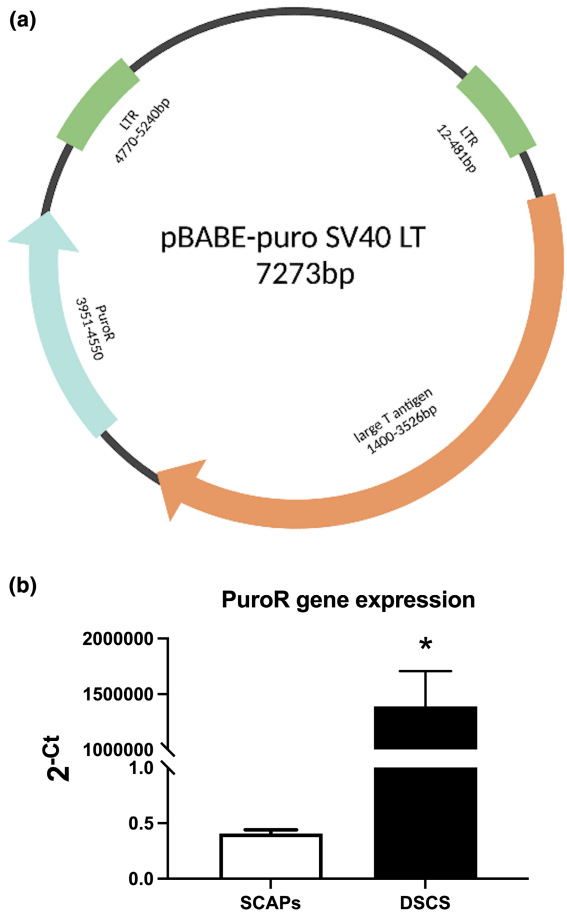

FIGURE 1 Generation of DSCS cells by SV40 infection. (a) Map of principal sequences of pBABE-puro SV40 LT (Addgene #13970). Plasmid image created with BioRender. (b) Expression analysis of *PuroR* gene in SCAPs and DSCS cells using RT-PCR assay. *$p < .05$.

DSCS cells express MSC surface markers

To determine whether immortalization affected the stemness of DSCS throughout passaging, we assessed the expression of stem cell surface markers at P4 and P25. Flow cytometry at P4 showed that <0.9% of cells presented with haematopoietic cell markers (CD45 and CD34) or immune cell markers (CD11b, CD19 and HLA-DR; Figure 2). By contrast, >98% of cells expressed MSC surface markers (99.7% positive for CD73, 98.5% for CD90 and 99.6% for CD105). Similarly, flow cytometry at P25 showed that <0.5% of cells presented with haematopoietic cell markers or for immune cell markers, while >98.9% of the cells at P25 expressed MSC surface markers (100% positive for CD73, 100% for CD90 and 98.9% for CD105). These data indicate that DSCS cells maintained their original cell surface markers even after multiple passages.

DSCS differentiate into osteoblasts, adipocytes and chondroblasts under standard *in vitro* conditions

To confirm that the new DSCS cell line expressed and retained its multilineage differentiation potential after multiple passages, histological staining and colorimetric quantification were performed with Alizarin Red, Oil Red O and Alcian Blue stains in P6 SCAPs, P15 DSCS cells and P45 DSCS cells. In SCAPs and P15 and P45 DSCS cells, Alizarin Red, Alcian Blue and Oil Red O staining were significantly higher in all the differentiated groups compared with the controls, confirming the multilineage differentiation potential. Alcian Blue staining was significantly higher in differentiated DSCS cells than in differentiated SCAPs. By contrast, Oil Red O staining showed an increase higher of adipogenic phenotype in differentiated SCAPs than in differentiated DSCS cells (Figures 3a–c, S1; Table S1).

Multilineage differentiation potential of DSCS cells (P6) was also assessed through the expression of the chondrogenic markers *SOX9* and *COL2A1*, adipogenic markers *CEBPA* and *LPL*, and osteogenic markers *COL1A1* and *ALPL* (Figure 3d; Table S3). Undifferentiated DSCS cells showed higher basal expression of chondrogenic and osteogenic gene markers compared with undifferentiated SCAPs. In both cell types, chondrogenic or osteogenic media induced differentiation and upregulation of chondrogenic or osteogenic genes, respectively. In both cases, gene induction was greater in SCAPs. Undifferentiated SCAPs showed higher basal expression of adipogenic gene marker *CEBPA* compared with undifferentiated DSCS cells. In both cell

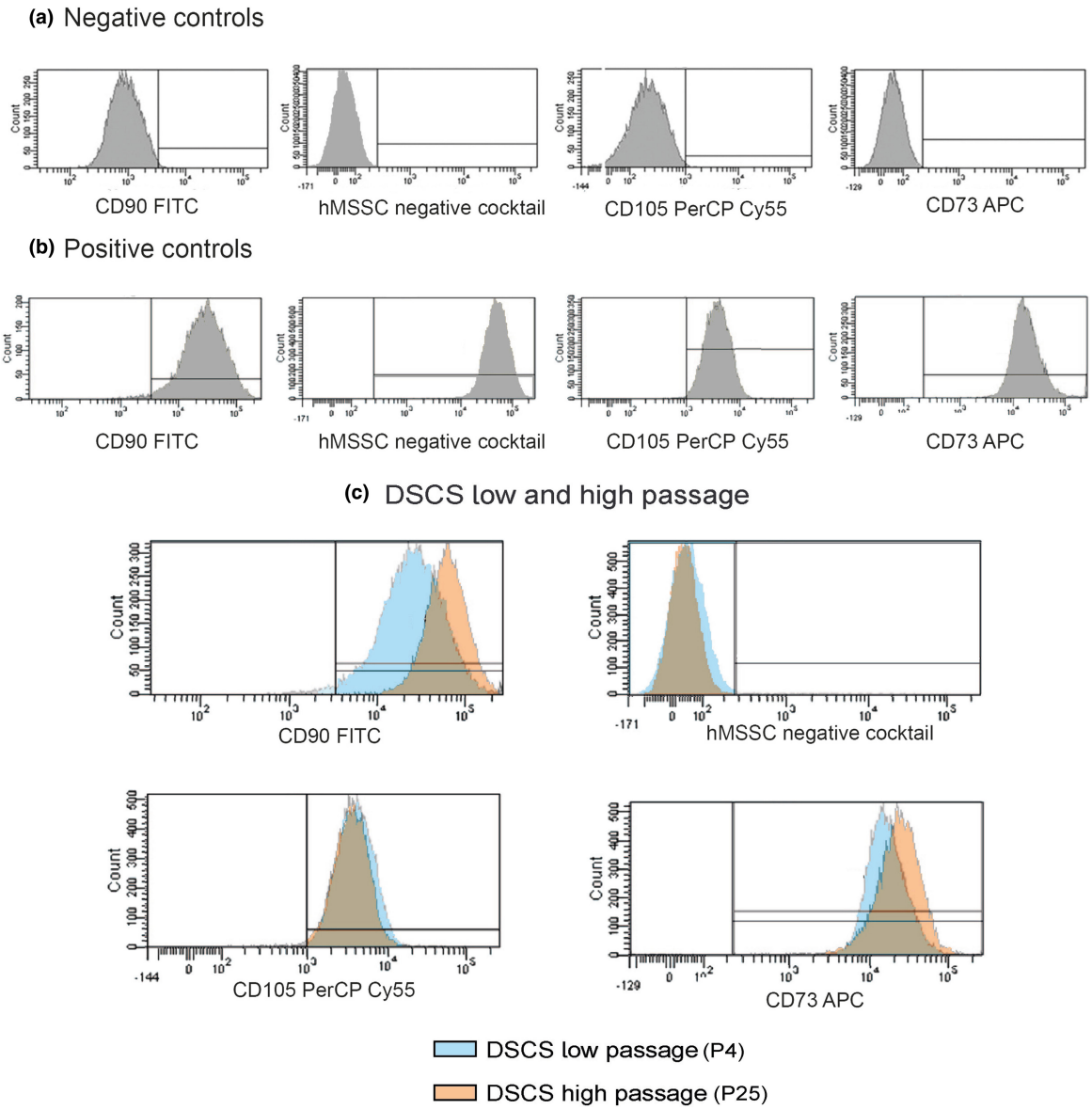

FIGURE 2 Flow cytometry of representative MSC-positive (CD90, CD105 and CD73) and -negative (CD45, CD34, CD11b, CD19 and HLA-DR) cell surface markers in DSCS cells at early [passage 4 (P4)] and late [passage 25 (P25)].

types, adipogenic medium induced differentiation and up-regulation of both adipose gene markers. In this case, gene induction was significant in the case of DSCS cells.

Cell morphology

Morphology of SCAPs P6, P15 DSCS cells and P45 DSCS cells was compared under phase contrast and confocal laser scanning microscopy (Figure 4b). DSCS cells exhibited the typical spindle shape of SCAPs after immortalization and were maintained through passaging (Figure 4).

SV40 induces increased cell proliferation and maintains contact inhibition

Proliferation of SCAPs P6 and DSCS P15 cells was compared at 24, 48 and 72 h. DSCS cells showed significantly increased proliferation at 48 and 72 h, with a doubling time of 23.46 h for DSCS cells and 40.31 h for SCAPs (Figure 5; Table S4). These results indicate that *SV40 LT* enhanced the proliferation capacity of SCAPs. After day 4, proliferation was inhibited in all cell groups in the culture. P6 SCAPs, P15 DSCS cells and P45 DSCS cells grew in a monolayer exhibiting contact inhibition (Figures 5 and S2; Table S5).

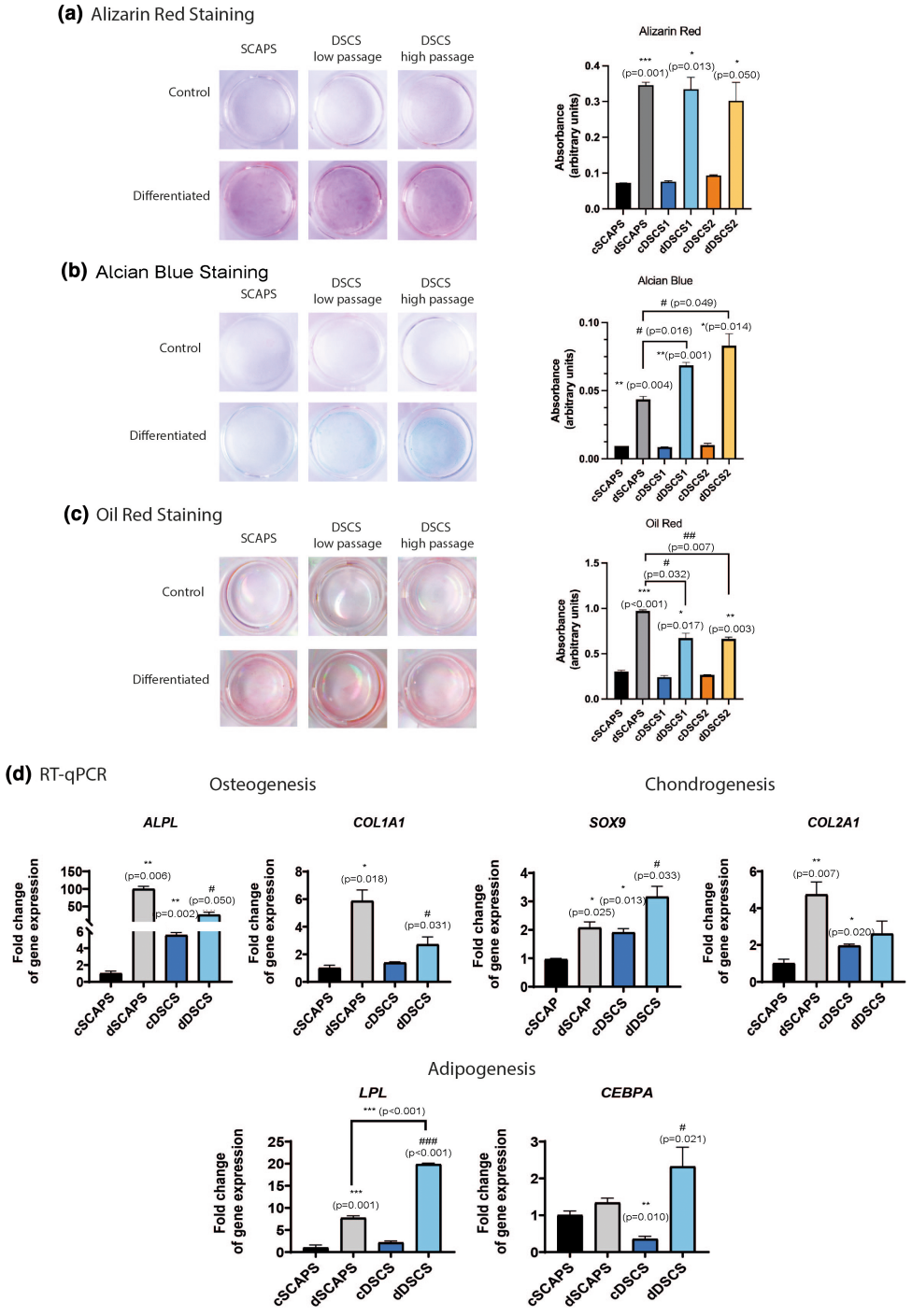

FIGURE 3 (a–c) Histochemical staining and absorbance measurements showed significantly higher values in the SCAPs (dSCAPS) at passage 6 (P6), DSCS at early passage [passage 15 (P15)] (dDSCS1) and DSCS at late passage [passage 45 (P45)] (dDSCS2) differentiated cells when compared to the undifferentiated cells (cSCAPS, cDSCS1 and cDSCS2). (d) Trilineage differentiation potential of SCAPs and DSCS cells by RT-PCR and normalized to *TBP* expression. Differentiated dSCAPs and dDSCS cells showed high levels of adipogenic marker *LPL* with a significant difference between dSCAPs and dDSCS cells, and a higher level of *CEBPA* expression in the dDSCS group. After 3 weeks of chondrogenic culture, significantly higher levels of *SOX9* were found in dSCAPs and dDSCS cells and significant upregulation of *COL2A1* in the dSCAPs. After 2 weeks of osteogenic culture, dSCAPs and dDSCS showed higher *ALP* and *COL1A1* activity than the undifferentiated group. * or $^{\#}p < .05$, ** or $^{\#\#}p < .01$, *** or $^{\#\#\#}p < .001$ using the Student's *t*-test. *Significance for comparison with undifferentiated SCAP (control) group; $^{\#}$significance for comparison with undifferentiated DSCS (control) group.

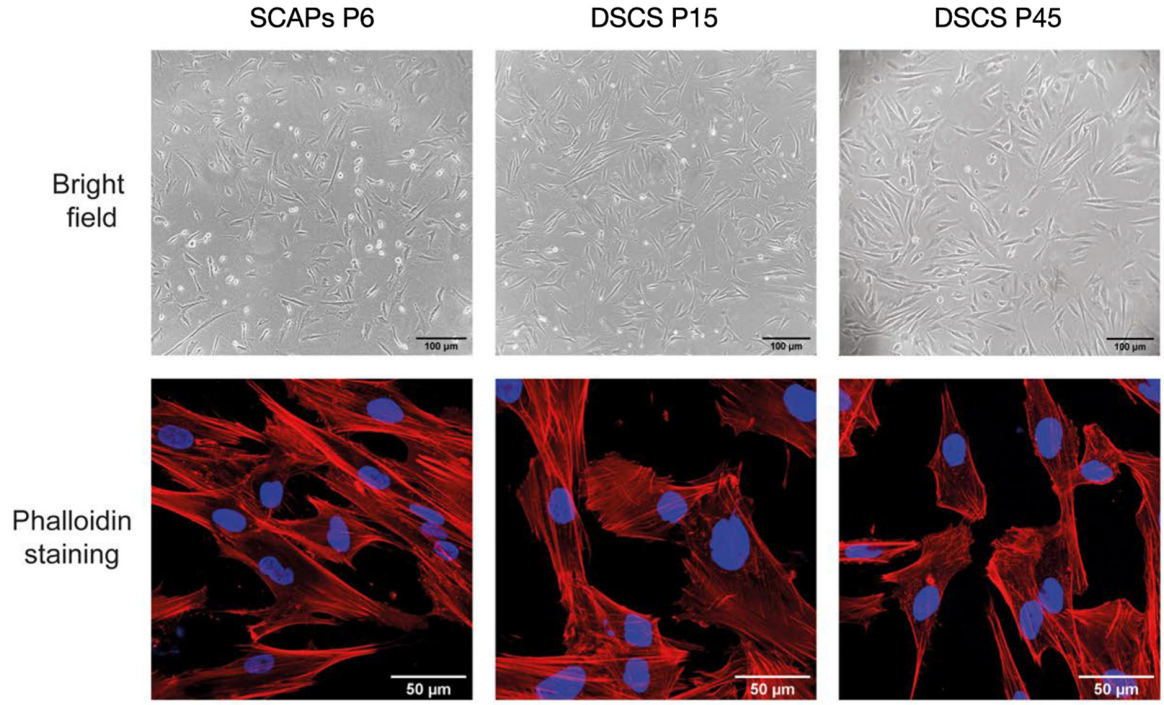

FIGURE 4 Phase contrast and confocal laser scanning microscopy images of the primary cells at passage 6 (P6) and DSCS cells at early [passage 15 (P15)] and late [passage 45 (P45)] passage.

DISCUSSION

Caries and dental trauma are high-incidence dental conditions that, when untreated, lead to severe complications including infection and arrest of root development. Many adult stem cells have been studied for tissue regeneration, and among them, SCAPs are of interest because they can induce root development even after apical periodontitis has occurred (Chrepa et al., 2017; Lin et al., 2018). Given their anatomical location, SCAPs are believed to be the principal source of stem cells during regenerative endodontic therapy (Cui et al., 2021; Lovelace et al., 2011). In the disease and during regenerative endodontic therapy, SCAPs face conditions of necrosis, infection and periapical inflammation, are exposed to different substances (irrigants and intracanal medications) that can affect their viability, and must be tested *in vitro* before clinical use (Althumairy et al., 2014; Ruparel et al., 2012; Trevino et al., 2011). SCAPs have shown potential for promoting repair after spinal cord injury in rats, which is an area in need of further study (De Berdt et al., 2015; Yang et al., 2017). Unfortunately, SCAPs isolated from the apical papilla are a limited source, have a low yield and become senescent after multiple passages, expressing changes in morphology (increased size and a flattened morphology) and multilineage differentiation potential.

In this study, a primary culture of human apical papilla stem cells (DSCS cells) was immortalized through lentiviral infection with SV40LT. SV40LT has shown efficient immortalization of various cell types, including some dental stem cells such as DPSC cells (Li et al., 2021). Cell immortalization through pBABE-puro SV40 LT infection occurs through the interaction of LT antigen with p53 and the Rb family of tumour suppressor proteins (Ahuja et al., 2005), which are responsible for the initial *in vitro* growth arrest (Piñeiro-Ramil et al., 2019). It has also been reported that SV40LT inhibits apoptosis through the activation of PI3K/Akt signalling. Therefore, cells expressing LT antigen can be propagated for an extended period in culture but do eventually enter replicative senescence, and reconstitution of telomerase activity in transformed cells is needed to achieve stable immortalization (Darimont et al., 2002). Nevertheless, in the present study, the cell line retained its proliferative capacity and typical MSC characteristics even at late passages. We observed that SV40 immortalized MSCs retain their proliferation and differentiation potential even without the incorporation of hTERT gene. MSCs from the apical papilla have higher telomere length and telomerase activity than other MSCs, such as the ones from the dental pulp and follicle (Jeon et al., 2011). Additionally, Ruparel reported a primary cell culture of human apical papilla that did not show signs of senescence even at late passages. In this study, the cells of the apical papilla presented high levels of the mesenchymal stem cell markers CD73, CD90 and CD105 and low levels of the cell differentiation markers, maintaining

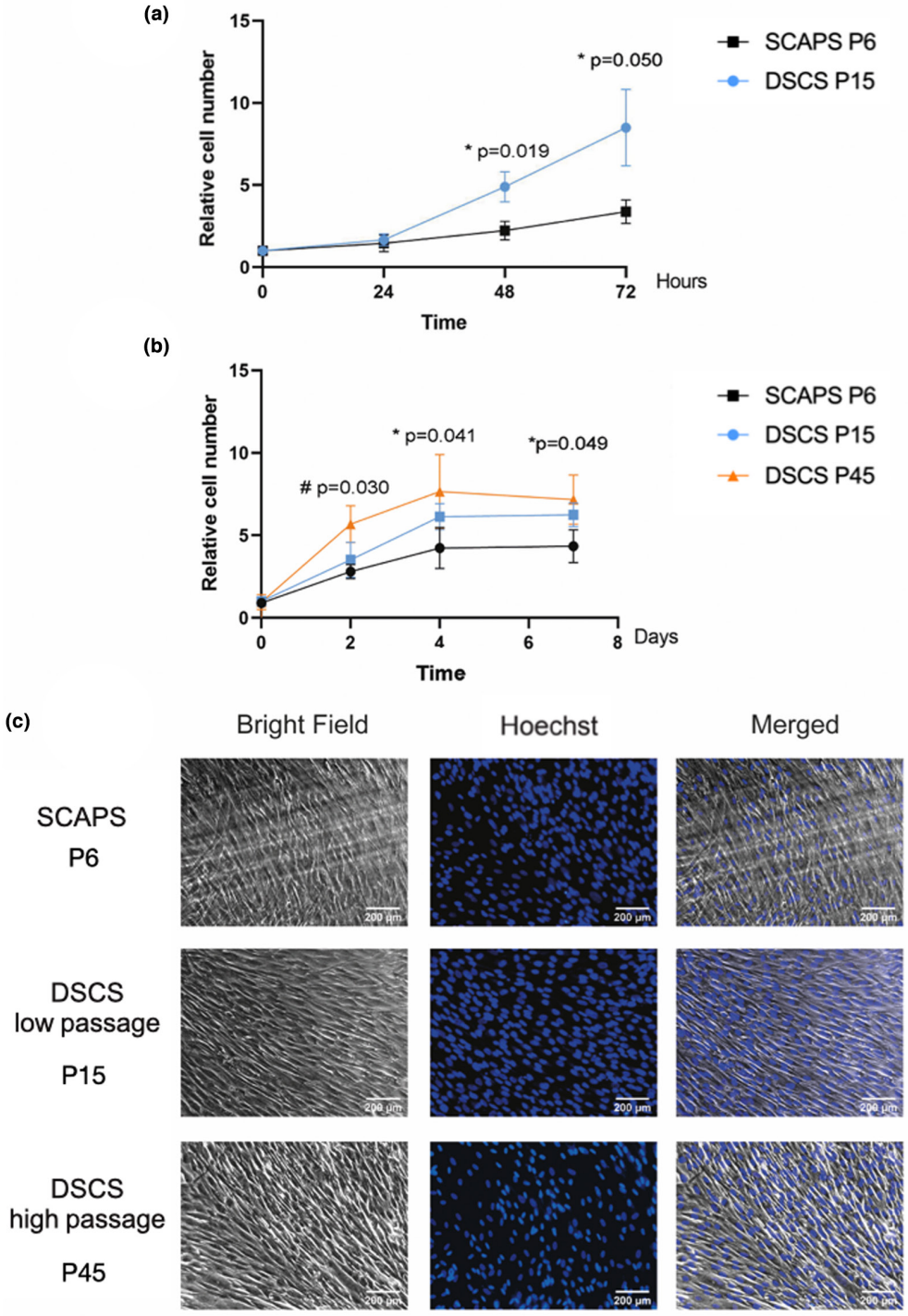

FIGURE 5 SV40 induces increased cell proliferation. (a) Growth curve of proliferation of primary cells [passage 6 (P6)] and DSCS cells [passage 15 (P15)] expressed as a ratio of the number of cells normalized by the initial cell number per each condition to control at point 0 (4 h after seeding). Doubling time was 23.46 h for DSCS cells and 40.31 h for SCAPs. *$p \leq .05$. (b) Growth curve of number of the SCAPs, and DSCS at low [passage 15 (P15)] and high [passage 45 (P45)] passage. At day 4, proliferation decreased in all groups. (c) Images of confluence of P6 SCAPs, P15 DSCS cells and P45 DSCS cells showing monolayer growth and absence of multilayer formation.

proliferation after 20 passages (Ruparel et al., 2013). We also have to consider that SV40LT can secondarily alter telomerase and cause maintenance of telomere length (Li et al., 2021; Toouli et al., 2002).

In common with other studies (Artigas et al., 2017), it was shown in this study that the SV40 immortalized cells retained the ability to grow reaching confluence over >45 passages [150 population doubling (PD)], while the

primary culture became senescent after 15 passages (45 PD). Therefore, immortalization through SV40 infection provides a useful method for obtaining a large number of cells that retain most of the characteristics of primary cultures for biological studies (Gong et al., 2011; Huang et al., 2015; Lee et al., 2015; Piñeiro-Ramil et al., 2020). Consistent with previous reports (Gong et al., 2011; Piñeiro-Ramil et al., 2020), we found that DSCS cells retained their morphology (spindle shape) and the expression of surface markers even at late passages.

There is extensive evidence in the literature that cell proliferation and differentiation are negatively correlated. However, DSCS cells retained their multilineage differentiation capacity despite their proliferative capacity. DSCS cells had the higher basal expression of chondrogenic and osteogenic gene markers compared with undifferentiated SCAPs. p53 modulates osteoblastic gene expression; therefore, inhibition of p53 by SV40LT results in the upregulation of genes implicated in bone development (Artigas et al., 2017). SV40LT is known to activate PI3K, a signalling pathway that governs bone formation (Gámez et al., 2016). The Rb family, which is also suppressed by SV40LT, has a principal role in regulating cell cycle progression and differentiation of neurons, muscle, adipose tissue and retina (Khidr & Chen, 2006). In MSCs, Rb plays a critical role in cell fate decisions. On the one hand, it binds to Runt-related transcription factor 2 (RUNX2) to promote osteoblast differentiation, while on the other hand, it inhibits peroxisome proliferator-activated receptor γ subunit; the master activator of adipogenesis (Calo et al., 2010). Therefore, inactivation of the Rb family may be related to alterations in adipocyte and osteoblast differentiation, explaining our results when comparing adipogenesis in primary and immortalized cultures. Other clonal effects can also contribute to the differences in terms of bone and chondrocyte differentiation. Further studies are needed to confirm the exact nature of the relationship involved.

In summary, this study established an immortalized human multipotent stromal cell line from SCAPs (DSCS cells) with higher proliferative capacity and retained the expression of mesenchymal surface markers and multipotency. This new cell line could be a valuable tool for investigating the role and mechanisms of SCAPs in different regeneration fields.

AUTHOR CONTRIBUTIONS

Diana Sanz-Serrano involved in data curation, investigation, formal analysis, methodology and writing—original draft preparation. Cristina Sánchez-de-Diego involved in data curation, investigation, formal analysis, methodology and writing—original draft preparation. Montse Mercade involved in conceptualization, supervision, writing—review and editing and funding acquisition. Francesc Ventura involved in conceptualization, supervision, methodology, writing—review and editing and funding acquisition.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

ETHICAL APPROVAL

All the procedures received the approval of the Ethical Committee of the Hospital Odontologic of the Universitat de Barcelona (Ceim HOUB protocol number 2018-021-1).

DATA AVAILABILITY STATEMENT

Data available on request due to privacy/ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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