JOURNAL OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE
J Tissue Eng Regen Med (2014)
Published online in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/term.1945

RESEARCH ARTICLE

Cryopreservation of human dental follicle tissue for use as a resource of autologous mesenchymal stem cells

Bong-Wook Park[1], Si-Jung Jang[2], June-Ho Byun[1], Young-Hoon Kang[1], Mun-Jeong Choi[1], Won-Uk Park[3], Won-Jae Lee[2] and Gyu-Jin Rho[2,4]*

[1]*Department of Oral and Maxillofacial Surgery, School of Medicine and Institute of Health Science, Gyeongsang National University, Jinju, Republic of Korea*
[2]*OBS/Theriogenology and Biotechnology, Gyeongsang National University, Jinju, Republic of Korea*
[3]*Department of Ceramic Engineering, Gyeongsang National University, Jinju, Republic of Korea*
[4]*Research Institute of Life Sciences, Gyeongsang National University, Jinju, Republic of Korea*

Abstract

The main purpose of this study was to develop a cryopreservation method for human dental follicle tissue to maintain autologous stem cells as a resource. A modified cryoprotectant, consisting of 0.05 M glucose, 0.05 M sucrose and 1.5 M ethylene glycol in phosphate-buffered saline (PBS) was employed, with a slow-ramp freezing rate. We observed > 70% of cell survival rate after 3 months of tissue storage. Isolated and cultured human dental stem cells (hDSCs) from cryopreserved dental follicles expressed mesenchymal stem cell markers at a level similar to that of hDSCs from fresh tissue. They also successfully differentiated *in vitro* into the mesenchymal lineage, osteocytes, adipocytes and chondrocytes under specific inductions. Using immunohistochemistry, the early transcription factors OCT4, NANOG and SOX2 were moderately or weakly detected in the nucleus of both fresh and cryopreserved dental follicles. In addition, p63, CCND1, BCL2 and BAX protein expression levels were the same in both fresh and cryopreserved tissues. However, the positive-cell ratio and intensity of p53 protein was higher in cryopreserved tissues than in fresh tissues, indicating direct damage of the freeze–thawing process. Real-time PCR analysis of hDSCs at passage 2 from both fresh and cryopreserved dental follicles showed similar levels of mRNA for apoptosis- and transcription-related genes. Based on these results, a newly developed cryoprotectant, along with a slow ramp rate freezing procedure allows for long-term dental tissue preservation for later use as an autologous stem cell resource in regenerative cell therapy. **Copyright © 2014 John Wiley & Sons, Ltd.**

Received 25 February 2014; Revised 12 May 2014; Accepted 16 June 2014

Keywords **tissue cryopreservation; human dental follicle tissue; autologous stem cell source; mesenchymal stem cells (MSCs); regenerative medicine**

1. Introduction

Tissue engineering techniques using stem cells have been the focus for reconstructing large tissue defects with minimal side-effects. Among many other influencing factors in tissue engineering, the use of autologous cells is highly favoured to prevent an immune reaction and the transmission of disease (Kang *et al*., 2010; Park *et al*., 2012a). However, when patients need stem cells for clinical applications, it is often difficult to obtain quality autologous stem cells, due to old age or disease. Therefore, there is a need for the development of a preservation technique that can safely maintain stem cell characteristics for the long term, allowing autologous stem cells to be harvested when patients are young and healthy.

Various cryopreservation protocols for stem cells have been applied, such as conventional cryopreservation with dimethyl sulphoxide (DMSO), vitrification (rapid freezing) and programmed slow freezing (Li *et al*., 2010). However, long-term cryopreserved stem cells may not be suitable for clinical application, due to cell damage, contamination, genetic variation caused by cryoprotectant toxicity or cell dehydration due

**Correspondence to: Gyu-Jin Rho, OBS/Theriogenology and Biotechnology, College of Veterinary Medicine, Gyeongsang National University, 501 Jinju-daero, Jinju 660-701, Republic of Korea. E-mail: jinrho@gnu.ac.kr*

Copyright © 2014 John Wiley & Sons, Ltd.

tosudden temperature changes (Almansoori *et al.*, 2012; Karlsson and Toner, 1996; Ock and Rho, 2011; Rowley *et al.*, 2003). More importantly, the genetic and epigenetic safety of long-term preserved cells has not been established, and intracellular ice formation during the freeze–thawing process has been reported to be a major cause of cell death (Rowley *et al.*, 2003). Moreover, the isolation and culture of stem cells need much economic effort, due to expensive media and supplements, and the use of fetal bovine serum (FBS) can also affect the internal characteristics of cells (Latinwo *et al.*, 2008). Therefore, in the present study, we focused on the development of a cryopreservation method for untreated and undifferentiated tissue as potential source for stem cells.

Although various tissues have been studied for use as a stem cell resource, dental tissues, especially the dental follicle, pulp and apical papilla of immature wisdom teeth, are one of the best sources of mesenchymal stem cells (MSCs) for tissue engineering and regenerative medicine (Gronthos *et al.*, 2000; Morsczeck *et al.*, 2005; Park *et al.*, 2009, 2012b). Interestingly, various stem cell isolation methods from dental tissues have been introduced, such as enzymatic tissue dissociation, traditional plastic adherence and the magnetic-activated cell sorting technique (Morsczeck *et al.*, 2005; Mrozik *et al.*, 2010; Jiang *et al.*, 2012; Tran *et al.*, 2014). However, any significant differences among these cell isolation methods have not been reported (Mrozik *et al.*, 2010; Tran *et al.*, 2014). Primitive and multipotent dental tissues can be obtained around the age of 20, in contrast to that of other stem cell sources, such as umbilical cord blood or matrix, which requires early collection. If these dental tissues are preserved long-term without losing their multipotency, they could be used as an autologous stem cell source for demanding applications in regenerative medicine. The present study investigated cryopreservation methods for dental follicle tissue to be used as a stem cell source. We also established an optimal cryopreservation method, using programmed slow freezing with a modified cryoprotectant, which was originally developed for the cryopreservation of ovarian tissue (Andersen *et al.*, 2008; Schmidt *et al.*, 2003).

2. Materials and methods

2.1. Chemicals and media

All chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA) and media from Gibco (Invitrogen, Grand Island, NY, USA), unless otherwise specified. For all media, the pH was adjusted to 7.4 and the osmolality to 280 mOsm/kg.

2.2. Cryopreservation of human dental follicles harvested from extracted wisdom teeth

Dental follicles were harvested from 25 patients (average age 19.5 years; 14 females and 11 males) after immature impacted wisdom teeth extraction at the Department of Oral and Maxillofacial Surgery at Gyeongsang National University Hospital, under approved guidelines (GNUH IRB-2012-09-004), with informed consent from patients (Figure 1). Tissues were transferred to the laboratory in Dulbecco's phosphate-buffered saline (D-PBS) on ice within 2 h.

Under aseptic condition, the dental follicles were washed with D-PBS containing 1% penicillin–streptomycin (10 000 IU and 10 000 μg/ml, respectively; Pen–Strep, Gibco), minced into 1–3 mm^2 explants using fine scissors (Solco Biomedical™, Pyeongtaek, Korea) and divided into five experimental groups, each group composed of tissues from five donors: Fresh (control); conventional method with 10% DMSO (Conv-DMSO); cocktail solution consisted of 0.05 M glucose, 0.05 M sucrose and 1.5 M ethylene glycol in PBS (Conv-Cock); and programmed method with 10% DMSO (Prog-DMSO) or cocktail solution (Prog-Cock).

For this study, two different cryoprotectants were used: 10% DMSO solution diluted with advanced Dulbecco's modified Eagle's medium (A-DMEM; 1:1 v/v) supplemented with 10% FBS, and cocktail solution (Cock) consisting of 0.05 M glucose, 0.05 M sucrose and 1.5 M ethylene glycol in PBS.

In the conventional method (Conv), minced dental follicle tissues from single donors in 1.8 ml cryovials (Thermoscientific, Roskilde, Denmark) containing 1 ml cryoprotectant were cooled at approximately –1°C/min from 25°C to –80°C in a freezing container (Nalgene, Rochester, NY, USA), then plunged directly into liquid nitrogen (LN_2). In the programmed method (Prog), tissues in cryovials containing 1 ml cryoprotectant were cooled at a pre-set freezing rate in a programmable controlled-rate freezer (Kryo 360, Planer Ltd, Middlesex, UK). The dental follicle tissues were equilibrated for 30 min at 1°C, then cooled following the programmed protocol in order: –2°C/min to –9.0°C, then –9.0°C to –9.1°C and held for 5 min; then –0.3°C/min to –40°C; then –10°C/min to –140°C. The cryovials were subsequently plunged into LN_2. The detailed combinations of cryopreservation method and cryoprotectants are shown in Table 1.

The cryopreserved dental follicle tissues in each method were stored in liquid nitrogen for at least 3 months. The cryovials were then thawed by immersing them in a circulating water bath at 37°C for 1 min and the tissues were then washed twice by centrifugation with A-DMEM supplemented with 10% FBS and 1% Pen–Strep at 300 × *g* for 5 min, for further analysis.

2.3. Isolation and culture of hDSCs from fresh and cryopreserved dental follicle tissues

Human dental stem cells (hDSCs) were isolated from fresh and cryopreserved dental follicle tissues with a minor revision from previous reports (Jeon *et al.*, 2011). Briefly, the tissues were digested in D-PBS containing 1 mg/ml collagenase type I (Sigma-Aldrich) at 37°C with gentle agitation for 40 min. After washing with D-PBS and centrifugation at 500 × *g* for 5 min, the digested tissues were mechanically dissociated with 1 ml culture medium by repeated gentle pipetting. After adding 9 ml culture medium, the dissociated samples were filtered through a 40 μm cell strainer (BD Falcon, Franklin Lakes, NJ, USA) to harvest single-cell

Copyright © 2014 John Wiley & Sons, Ltd.

DOI: 10.1002/term

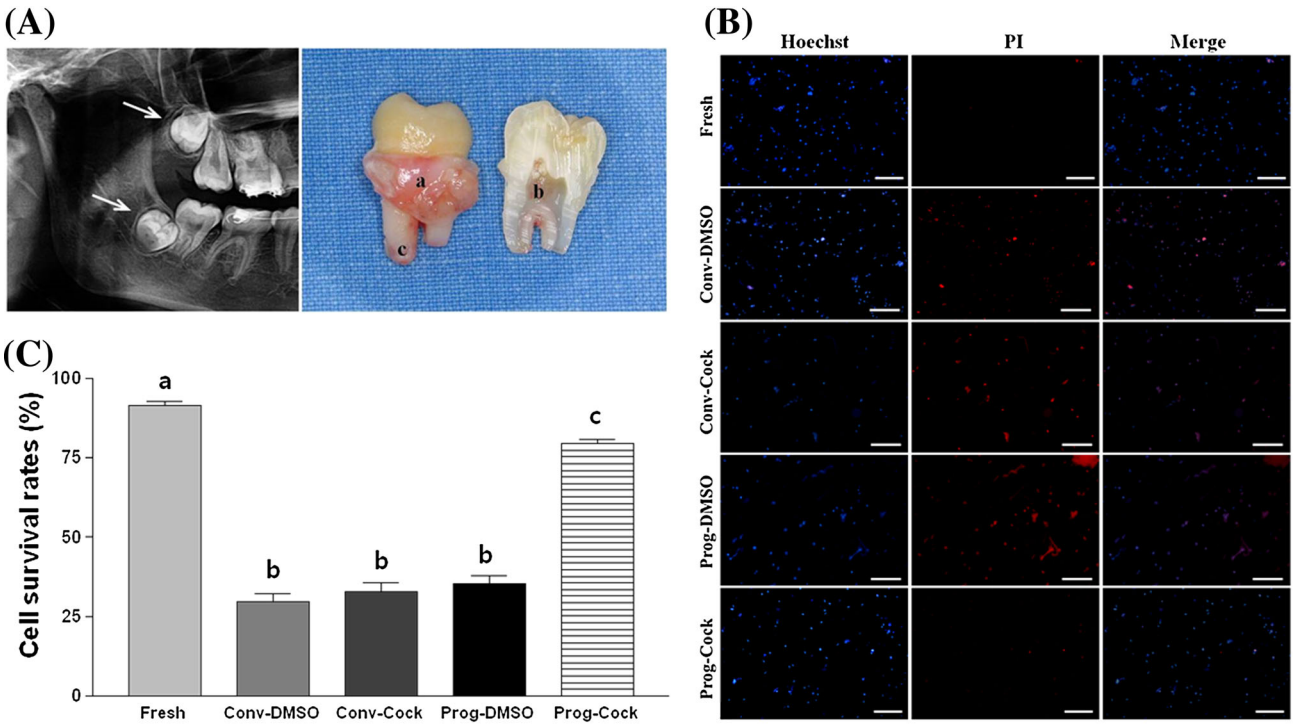

Figure 1. Immature impacted wisdom teeth and cell survival rates of dental tissue cryopreservation. (A) Radiological view of impacted wisdom teeth on maxilla and mandible (arrows) and harvested dental tissues, dental follicle (a), dental pulp (b) and root apical papilla (c) from extracted wisdom teeth. (B) Hoechst 33342 and propidium iodide (PI) staining in isolated single cells from fresh tissue and each cryopreserved dental tissue; scale bar = 100 μm. (C) Calculated cell survival rates by PI:Hoechst ratio in isolated single cells from dental tissues of the Fresh (control), Conv-DMSO, Conv-Cock, Prog-DMSO and Prog-Cock groups. the Fresh group showed a significantly higher cell survival rate; however, survivability in the programmed method with cocktail solution (Prog-Cock) was significantly higher than those in other cryopreservation methods: data represent mean ± SD of three independent experiments; different letters (a–c) denote statistical differences between groups ($p < 0.05$)

Table 1. Summary of cryoprotectants, freezing methods, and cell survival rates in the present study

Experimental group	Cryoprotectant	Freezing method	Cell survival rate (%)
Control	No	No freezing (fresh tissue)	91.3 ± 2.1
Conv-DMSO	10% DMSO and 10% FBS in ADMEM	Conventional: –1°C/min from 25°C to –80°C, then plunge into LN_2	29.7 ± 4.5
Conv-Cock	0.05 M glucose, 0.05 M sucrose and 1.5 M ethylene glycol in PBS	Conventional	32.7 ± 5.0
Prog-DMSO	10% DMSO and 10% FBS in ADMEM	Programmed: hold for 30 min at 1°C, –2°C/min to –9.0°C, hold for 5 min, –0.3°C/min to –40°C, –10°C/min to –140°C, then plunge into LN_2	35.3 ± 4.2
Prog-Cock	0.05 M glucose, 0.05 M sucrose and 1.5 M ethylene glycol in PBS	Programmed	79.3 ± 2.5*

*Prog-Cock group showed significantly higher cell viability than other tissue cryopreservation methods ($p < 0.05$).

populations. A final density of 5×10^5 cells in 4 ml A-DMEM containing 10% FBS and 1% Pen–Strep were cultured in 25T-flasks (Nunc™, Roskilde, Denmark) at 37°C in a humidified atmosphere with 5% CO_2. The medium was changed every 3 days.

2.4. Cell survival and proliferation rates of hDSCs from fresh and cryopreserved dental follicle tissues

Cells isolated from fresh, conventional and programmed cryopreserved dental follicle tissues after incubation with collagenase I were stained with propidium iodide (PI; Sigma-Aldrich) for dead cells and Hoechst 33342 (Sigma-Aldrich) for all cells, as previously reported (Moore *et al.*, 1998). The stained cells were observed under a fluorescent microscope (Nikon Eclipse Ti-U, Nikon Instruments, Tokyo, Japan), and the cell survival rate was calculated in each experimental group.

To investigate the proliferation rates of hDSCs derived from fresh and cryopreserved tissues, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT; Sigma-Aldrich) assay was performed according to the manufacturer's protocol for 12 days. Briefly, 5×10^3 hDSCs at passage 2 were seeded into 12-well plates (Nunc™) in 1 ml

Copyright © 2014 John Wiley & Sons, Ltd.

DOI: 10.1002/term

A-DMEM supplemented with 10% FBS and 1% Pen–Strep. MTT assays were performed by adding 200 μl 12 mM MTT stock solution into each well and incubating at 37°C for 4 h. After removing all media, the insoluble formazan, a product formed when MTT is metabolized by viable cells, was dissolved in 100 μl DMSO and incubated for 10 min. The resulting mixture from each well was transferred into a 96-well plate (Nunc™). Subsequently, the chemiluminescent absorbance in each well was measured at 570 nm, using a Versa Max™ microplate reader (Molecular Devices, Sunnyvale, CA, USA).

From the results of this cell survival assay, only hDSCs derived from fresh and programmed cryopreserved dental follicle tissues with cocktail cryoprotectant (Prog-Cock) groups were used for further analysis.

2.5. Characterization of hDSCs from fresh and cryopreserved dental follicle tissues

hDSCs at passage 2 were analysed for the expression of surface and intracellular markers by flow cytometry (BD FACSalibur; Becton Dickson, Frankin Lakes, NJ, USA) in triplicate, as previously described (Park *et al.*, 2012b). Briefly, after harvesting hDSCs using 0.25% trypsin–EDTA, cells were fixed in 3.7% formaldehyde solution and labelled directly with fluorescein isothiocyanate (FITC)-conjugated antibodies [anti-mouse CD44 (1:100; BD Pharmingen, BD Bioscience, Franklin Lakes, NJ, USA), anti-human CD90 (1:100; BD Pharmingen), anti-human CD34 (1:100; BD Pharmingen), anti-mouse CD105 (1:100; Santa Cruz Biotechnology, Dallas, TX, USA) and anti-human CD45 (1:100; BD Pharmingen)]. To evaluate vimentin expression, fixed cells were incubated in 0.1% Triton X (Sigma-Aldrich) for permeabilization. Then, primary antibody [mouse anti-vimentin (1:100; Sigma-Aldrich)] and FITC-conjugated secondary antibody [goat anti-mouse IgG (1:100; BD Pharmingen™)] were sequentially applied to hDSCs.

hDSCs at passage 2 were induced into osteogenic, adipogenic and chondrogenic lineages by culturing with specific induction media, as previously described (Jeon *et al.*, 2011; Park *et al.*, 2012a). Osteogenic medium consisted of DMEM supplemented with 0.1 μM dexamethasone, 50 μM ascorbate-2-phosphate and 10 mM glycerol-2-phosphate. Adipogenic medium consisted of DMEM supplemented with 1 μM dexamethasone, 10 μM insulin, 100 μM indomethacin and 500 μM isobutylmethylxanthine. Chondrogenic differentiation was induced in chondrogenic medium (HyClone Advance STEM, Thermo Fisher Scientific, Waltham, MA, USA). *In vitro* differentiations were conducted for 21 days and media were changed twice a week. Differentiated cells were stained with von Kossa and alizarin red, oil red O and Alcian blue 8GX to confirm the formation of osteocytes, adipocytes and chondrocytes, respectively, as previously reported (Jeon *et al.*, 2011; Kang *et al.*, 2010). In addition, osteogenic, adipogenic and chondrogenic differentiated cells were further evaluated by real-time PCR for the expression of lineage-specific genes (Table 2).

2.6. Real-time polymerase chain reaction (RT–PCR)

The expression of transcriptional factors, apoptosis-related genes and lineage-specific markers were analysed from cultured hDSCs (passage 2) by RT–PCR. Total RNA was isolated using RNeasy Mini Kit (Qiagen, CA, USA), according to the manufacturer's protocol. cDNA synthesis was performed for 60 min at 37°C with oligo-dT primer, using Omniscript reverse transcription Kit (Qiagen). Real-time PCR was carried out on a Rotor gene Q (Qiagen), using Rotor Gene™ SYBR green PCR kit (Qiagen). A total of 50 ng cDNA was added to 12.5 μl SYBR Green mix, 5.5 μl RNase-free water and 2.5 μl each of forward and reverse primers at 1 pM (final volume 25 μl). The assay was set up for denaturation at 95°C for 10 min, followed by 40 PCR cycles at 95°C for 10 s, 60°C for 6 s and 72°C for 4 s, followed by a melting curve from 60°C to 95°C at 1°C/s, and then cooling at 40°C for 30 s, according to the manufacturer's recommended protocol. Melting curves and cycle threshold values (C_T values)

Table 2. Primers used for evaluating *in vitro* differentiations of hDSCs from fresh and cryopreserved dental follicle tissues by RT–PCR

Gene (marker)	Primer sequences	Product size (bp)	Accession no.
OPN (osteo)	F: TTGCAGCCTTCTCAGCCAA R: GGAGGCAAAAGCAAATCACTG	74	NM_001040058
ON (osteo)	F: GTGCAGAGGAAACCGAAGAG R: AAGTGGCAGGAAGAGTCGAA	202	NM_003118.2
FABP4 (adipo)	F: TGAGATTTCCTTCATACTGGG R: TGGTTGATTTTCCATCCCAT	128	NM_001442
PPARγ2 (adipo)	F: TGCTGTCATTATTCTCAGTGGA R: GAGGACTCAGGGTGGTTCAGC	124	NM_015869
COL2A1 (chondro)	F: ATGGCTGCACGAAACATACC R: TCCACACCGAATTCCTGCTC	133	NM_001844.4
SOX9 (chondro)	F: ATGGAGCAGCGAAATCAACG R: CAAAGTCCAAACAGGCAGAGAG	118	Z46629.1
RPL13a (reference)	F: AAGTACCAGGCAGTGACAG R: CCTGTTTCCGTAGCCTCATG	100	NM_012423

OPN, osteopontin; *ON*, osteonectin; *FABP4*, fatty acid binding protein 4; *PPARγ2*, peroxisome proliferator activated receptor-γ2; *COL2A1*, collagen type II α1; *SOX9*, transcription factor SOX-9; *RPL13a*, ribosomal protein L13a; osteo, osteocyte; adipo, adipocyte; chondro, chondrocyte.

Copyright © 2014 John Wiley & Sons, Ltd.

DOI: 10.1002/term

of each sample were analysed using Rotor-Gene Q Series Software (Qiagen). Product sizes were confirmed on 1.5% agarose gel. The ribosomal protein L13a (*RPL13a*) reference gene was amplified for each sample to verify the presence of cDNA and as an internal control to calculate the relative level of target gene expression, using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). The primers used for the study are shown in Tables 2 and 3.

2.7. Immunohistochemical analysis of fresh and cryopreserved dental follicle tissues

Fresh and freeze–thawed dental tissues were fixed with 10% neutral buffered formalin for 24 h and embedded in a paraffin block, sliced into 4 μm sections and mounted on SuperfrostPlus microscope slides (Fisher Scientific, Rochester, NY, USA). The sections were maintained at room temperature for 12 h. After hydration, immunohistochemical staining for transcription factors, apoptosis- and cell proliferation-related proteins were conducted using an automated immunostainer (BenchMark XT, Ventana Medical System Inc., Tucson, AZ, USA) and detection was done using the Ultraview DAB kit (Ventana Medical System), according to the manufacturer's protocol. Briefly, sections were deparaffinized using EZ Prep solution, then CC1 standard (pH 8.4 buffer contained Tris/borate/EDTA) was treated for antigen retrieval for 60 min at 100°C. The slides were incubated at 37°C for 4 min with DAB inhibitor (3% H_2O_2) to block endogenous peroxidase activity. Then, each primary antibody was allowed to incubate at 37°C for 30 min, followed by secondary antibody consisting of Univeral HRP Multimer, and reacted for 8 min at 37°C. The slides were finally treated with DAB + H_2O_2 substrate for 8 min, followed by haematoxylin II and bluing reagent at 37°C for nucleus counterstaining.

Tissue slices were semi-quantitatively analysed for antibody deposition in cellular components. Using previously reported methods, positive immunostaining intensities were graded as +++, ++, + and – for strong, moderate, weak and negative staining, respectively, and ± was used to represent focal and questionable weak staining (Byun *et al*., 2008). According to the results of the semi-quantitative analysis of staining intensities, the positive staining ratio of p53 protein was calculated in both fresh and cryopreserved dental tissues. At least three different specimens were used to calculate the ratio of positive cells. The primary antibodies and immunohistochemical staining results are summarized in Table 4.

2.8. Statistical analysis

For each set of data, independent experiments were repeated at least three times, with data representing mean ± standard deviation (SD) of all repeats. The statistical differences between experimental groups were determined by one-way analysis of variance (ANOVA), followed by Tukey test for multiple comparisons or unpaired *t*-test for single comparison of experimental data to the control value, using GraphPad Prism analysis software. Results were considered significant at $p < 0.05$, and these differences were denoted by a star or different letters, respectively.

3. Results

3.1. Survival rate of cells isolated from fresh and cryopreserved dental follicle tissues

Survival rates were determined using PI and Hoechst 33342 staining in cells isolated from dental tissues that were either fresh (Fresh control group) or cryopreserved (Conv-DMSO, Conv-Cock, Prog-DMSO and Prog-Cock). The calculated cell survival rates were 91.3 ± 2.1%, 29.7 ± 4.5%, 32.7 ± 5.0%, 35.3 ± 4.2% and 79.3 ± 2.5%

Table 3. List of primers used for evaluation of transcription factors and apoptosis-related genes in cultured hDSCs by RT–PCR

Gene	Primer sequence	Product size (bp)	Accession no.
OCT4	F: AAGCAGCGACTATGCACAAC R: AGTACAGTGCAGTGAAGTGAGG	140	NM_002701.3
SOX2	F: TCCACACTCACGCAAAAACC R:AGTCCCCCAAAAAGAAGTCCAG	77	NM_003106.3
NANOG	F: GCAGATGCAAGAACTCTCCAAC R: CTGCGTCACACCATTGCTATTC	133	NM_024865.2
BAK	F: GGCACCTCAACATTGCATGG R: CAGTCTCTTGCCTCCCCAAG	144	NM_001188.3
BCL2	F: GGCTGGGATGCCTTTGTG R: CAGCCAGGAGAAATCAAACAGA	66	NM_000633.2
p21	F: TGGCAGTAGAGGCTATGGA R: AACAGTCCAGGCCAGTATG	178	NM_000389.4
p53	F: ACCCAGGTCCAGATGAAG R: GCAAGAAGCCCAGACGGAAA	174	NM_000546.5
RPL13a	F: AAGTACCAGGCAGTGACAG R: CCTGTTTCCGTAGCCTCATG	100	NM_012423

OCT4, octamer-binding transcription factor 4; *SOX2*, SRY-box 2; *BAK*, Bcl2-antagonist/killer 1; *BCL2*, B cell lymphoma 2; *p21*, cyclin-dependent kinase inhibitor 1; *p53*, tumour protein 53; *RPL13a*, ribosomal protein L13a.

Copyright © 2014 John Wiley & Sons, Ltd.

DOI: 10.1002/term

Table 4. Antibodies and their dilution rates and result of semi-quantitative analysis for immunostaining intensity in fresh and cryopreserved dental tissues

Name	Type	Company	Dilution	Immunostaining intensity	
				Fresh	Cryo
OCT4	Goat IgG	Santa Cruz Biotechnology	1:100	++	++
NANOG	Goat IgG	Santa Cruz Biotechnology	1:100	++	++
SOX2	Goat IgG	Santa Cruz Biotechnology	1:100	+	+
p53*	Mouse monoclonal	Dako	1:2000	+/–	+++
p63	Mouse monoclonal	Novocastra	1:50	++	++
CCND1	Rabbit monoclonal	Dako	1:400	+/–	+/–
BCL2	Mouse monoclonal	Neomarkers	1:50	+	+
BAX	Rabbit IgG	Santa Cruz Biotechnology	1:100	+/–	+/–

p53, tumour protein 53; p63, tumour protein 63; CCND1, cyclin D1; BCL2, B cell lymphoma 2; BAX, BCL-2-associated X protein.
Company and location: Santa Cruz Biotechnology, Dallas, TX, USA; Dako, Glostrup, Denmark; Novocastra, Lieca Biosystem, Buffalo Grove, IL, USA; Neomarkers, Fremont, CA, USA.
*Only p53 protein showed different expression intensity and positive immunostaining cell ratio between fresh and cryopreserved dental tissues. The calculated positive cell ratio was 4.1 ± 1.3% in fresh tissue but 12.5 ± 2.0% in cryopreserved tissue ($p < 0.05$).

in Fresh, Conv-DMSO, Conv-Cock, Prog-DMSO and Prog-Cock groups, respectively (Figure 1). The Fresh control group had significantly ($p < 0.05$) higher survivability in comparison to the cryopreserved groups; however, cell survivability using the programmed method with cocktail solution (Prog-Cock) was significantly ($p < 0.05$) higher than those in other cryopreservation methods. Among Conv-DMSO, Conv-Cock and Prog-DMSO, the survivability did not differ. Based on these observations, only the programmed cryopreservation with cocktail cryoprotectant method (Prog-Cock) was chosen for subsequent experiments.

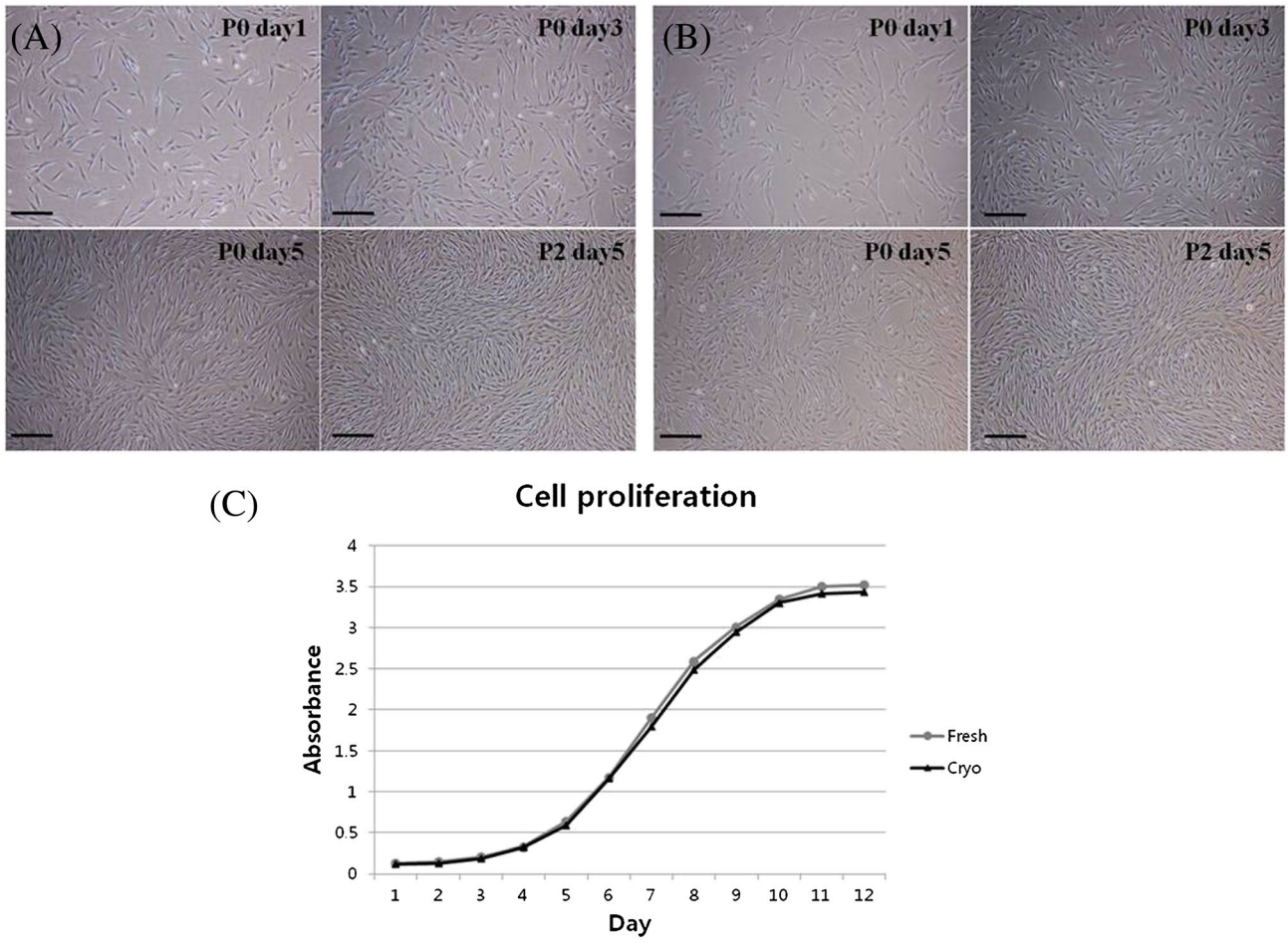

Figure 2. Culture morphology of hDSCs from (A) fresh and (B) cryopreserved dental tissues and (C) cell proliferation assay; scale bar = 100 μm. (A, B) hDSCs from both fresh and cryopreserved dental tissues revealed the same culture characteristics, with plate adherence and fibroblast-like morphology. (C) hDSCs from fresh and cryopreserved tissues showed the same proliferation assays

Copyright © 2014 John Wiley & Sons, Ltd.

DOI: 10.1002/term

3.2. Proliferation and characterization of hDSCs from fresh and cryopreserved dental follicle tissues

hDSCs from fresh and cryopreserved (Prog-Cock) dental tissues showed similar growth patterns, with a plate-adherent homogeneous fibroblast-like appearance. Cell proliferation assessed by MTT assay also showed similar curves in two groups (Figure 2). hDSCs from fresh and cryopreserved tissues revealed the same level of positive expression for MSC-specific markers (CD44, CD90, CD105 and vimentin) but almost no expression for haematopoietic markers (CD34 and CD45) by FACS analysis (Figure 3). In addition, hDSCs from both fresh and cryopreserved dental tissues were successfully differentiated *in vitro* into the mesenchymal lineage, osteocytes, adipocytes and chondrocytes under controlled induction media, which were identified by specific staining (Figure 4A). In addition, the mRNA levels of lineage-specific genes in differentiated cells were shwn to be about two- to five-fold higher than those in pre-differentiated hDSCs, whereas no difference was revealed between the Fresh and cryopreserved tissue groups (Figure 4B). These results demonstrated that the hDSCs from fresh and cryopreserved dental tissues possess the same characteristics as mesenchymal stem cells.

3.3. Immunohistochemical analysis with fresh and cryopreserved dental follicle tissues

Fresh and cryopreserved dental tissues were fixed, embedded in paraffin blocks and immunostained for the detection of early transcription factors, apoptosis-, cell proliferation- and immune-related proteins. In both tissues, nuclear expression of OCT4, NANOG and SOX2 was detected in some cells, even though positive cells were not abundantly detected. Interestingly, although these three proteins are mainly detected in the nuclei of

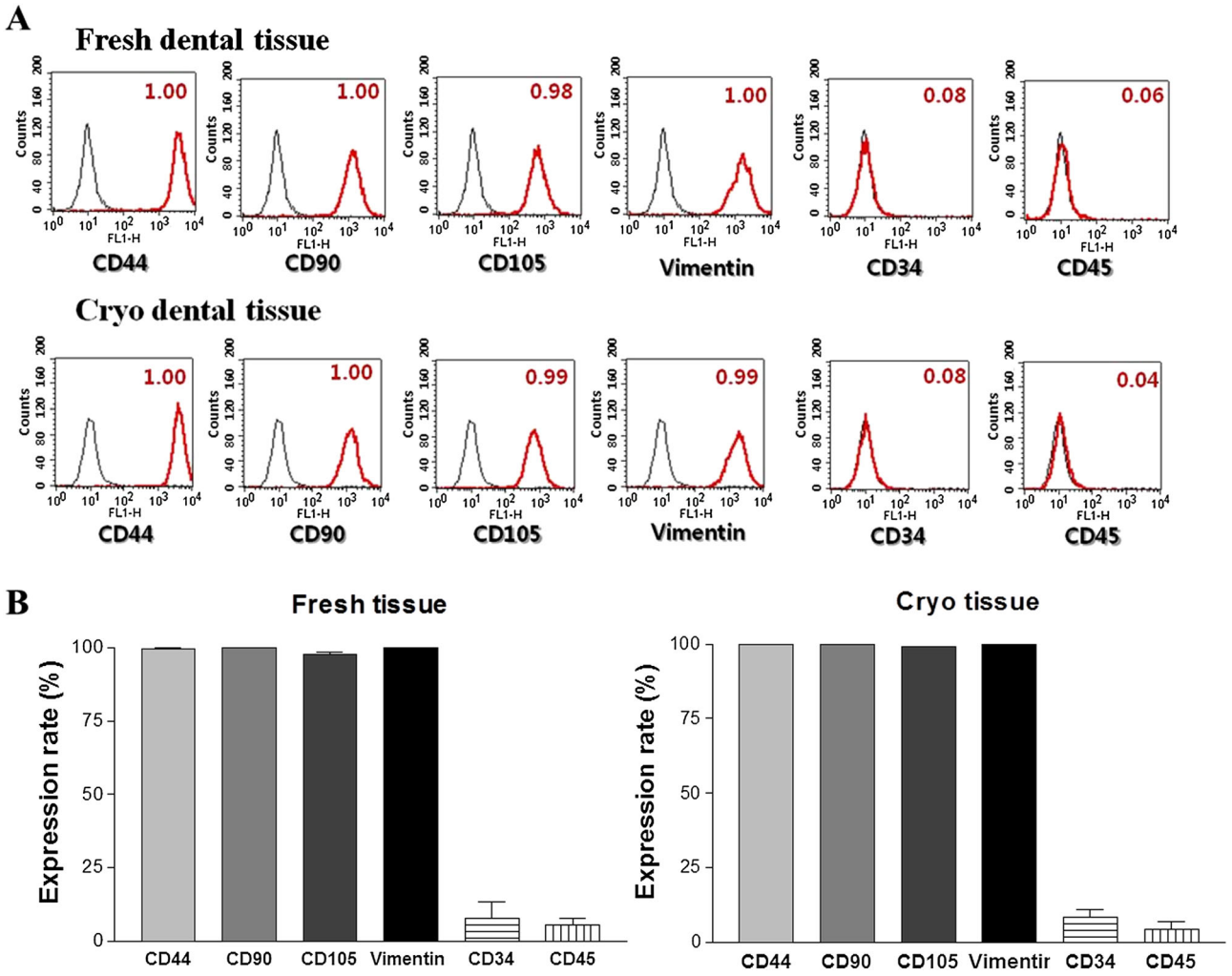

Figure 3. Fluorescence-activated cell sorting (FACS) analysis of hDSCs derived from fresh and cryopreserved dental tissues. (A) Results of FACS analysis for mesenchymal stem cell (MSCs) and haematopoietic cell markers in hDSCs at passage 2. (B) Statistical analysis of (A) showed that hDSCs from both fresh and cryopreserved tissues revealed same positive expression levels for MSC-specific markers (CD44, CD90, CD105 and vimentin), whereas a relatively low level of expression was detected for haematopoietic cell markers (CD34 and CD45). Data represent mean ± SD of three independent experiments

Copyright © 2014 John Wiley & Sons, Ltd.

DOI: 10.1002/term

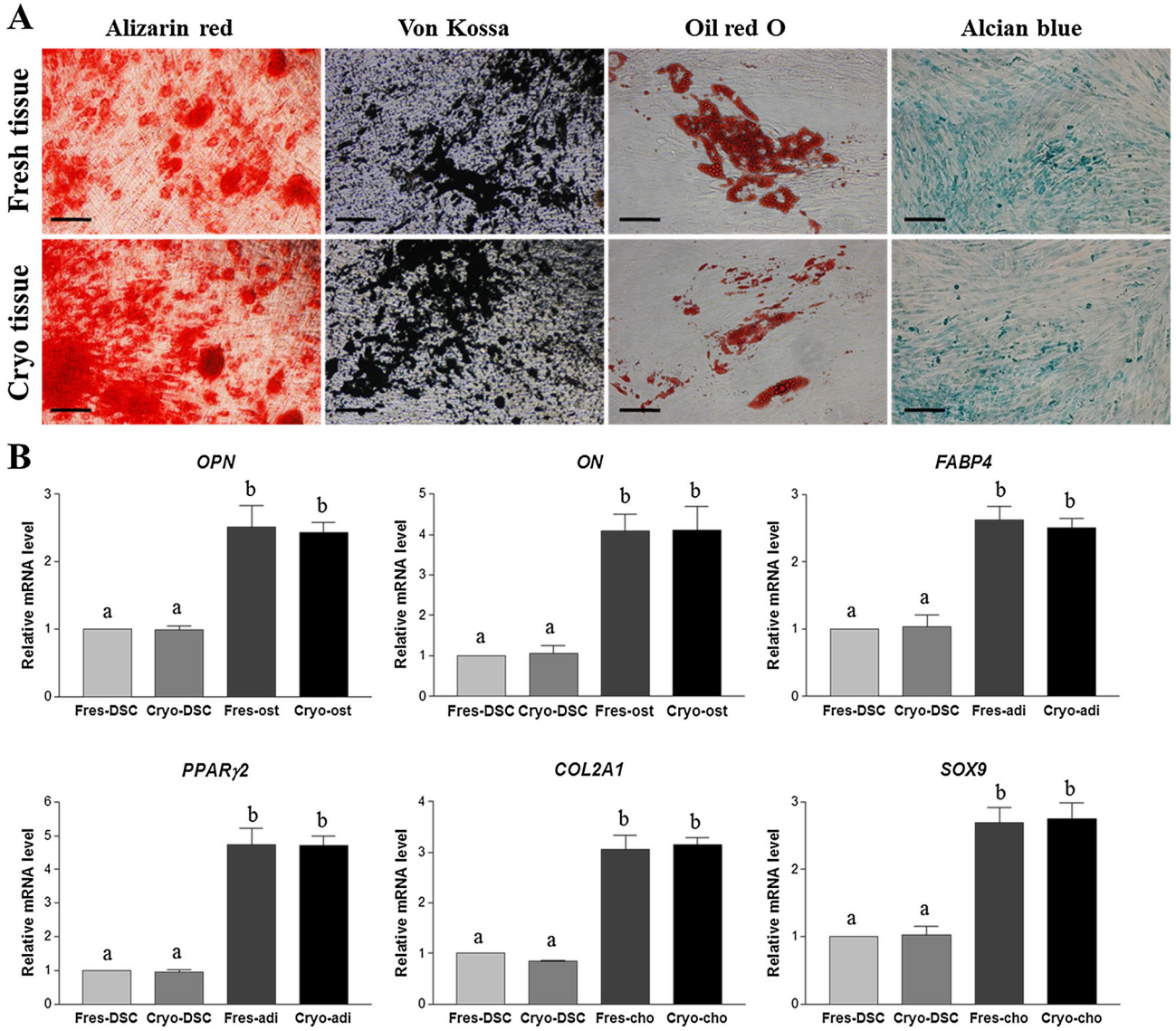

Figure 4. ***In vitro*** **differentiation of hDSCs from fresh and cryopreserved dental tissues. (A) Cultured hDSCs from both tissues were shown to have the same *in vitro* differentiation characteristics into mesenchymal lineages, osteocytes (alizarin red and von Kossa), adipocytes (oil red O) and chondrocytes (Alcian blue) under controlled induction media for 21 days; scale bar = 100 μm. (B) In real time-PCR, the mRNA levels of lineage-specific genes (*OPN* and *ON*, osteocyte-specific; *FABP4* and *PPARγ2*, adipocyte-specific; and *COL2A1* and *SOX9*, chondrocyte-specific markers, respectively) in differentiated cells were shown to be about two- to five-fold higher than those in predifferentiated hDSCs, whereas no difference was revealed between fresh and cryopreserved dental tissue groups; Fres-DSC, hDSCs from fresh dental follicles; Cryo-DSC, hDSCs from cryopreserved dental follicles; Fres-ost, induced osteocytes from Fres-DSC; Cryo-ost, induced osteocytes from Cryo-DSC; Fres-adi, induced adipocytes from Fres-DSC; Cryo-adi, induced adipocytes from Cryo-DSC; Fres-cho, induced chondrocytes from Fres-DSC; Cryo-cho, induced chondrocytes from Cryo-DSC. Data represent mean ± SD of five independent experiments; different letters (a, b) denote statistical differences between groups ($p < 0.05$)**

cells, NANOG was also occasionally observed in the cell cytoplasm of dental tissues (Figure 5).

Both fresh and freeze–thawed dental tissues were analysed by immunohistochemistry for p53, p63, CCND1, BCL2 and BAX protein expression. Only the cell apoptosis and tumour suppressor gene, *p53*, showed significantly ($p < 0.05$) increased expression intensity and higher positive-cell ratio in cryopreserved tissue (12.5 ± 2.0%) compared to fresh tissue (4.1 ± 1.3%). Other apoptosis- and cell proliferation-related proteins showed no difference in their expression levels or patterns in fresh and cryopreserved tissues. The epithelial stem cell marker p63 was positively expressed in both fresh and cryopreserved dental tissues. The anti-apoptosis-related protein BCL2 was weakly expressed, and the pro-apoptotic protein BAX showed questionable weak expression in both dental tissues. In addition, the cell mitosis-related gene *CCND1* had very low to no expression in both tissues (Figure 6).

3.4. RT–PCR for transcription- and apoptosis-related genes in hDSCs

Total RNA was isolated in hDSCs at passage 2 from fresh and cryopreserved dental tissues and real-time PCR was performed to determine the expression level of

Copyright © 2014 John Wiley & Sons, Ltd.

DOI: 10.1002/term

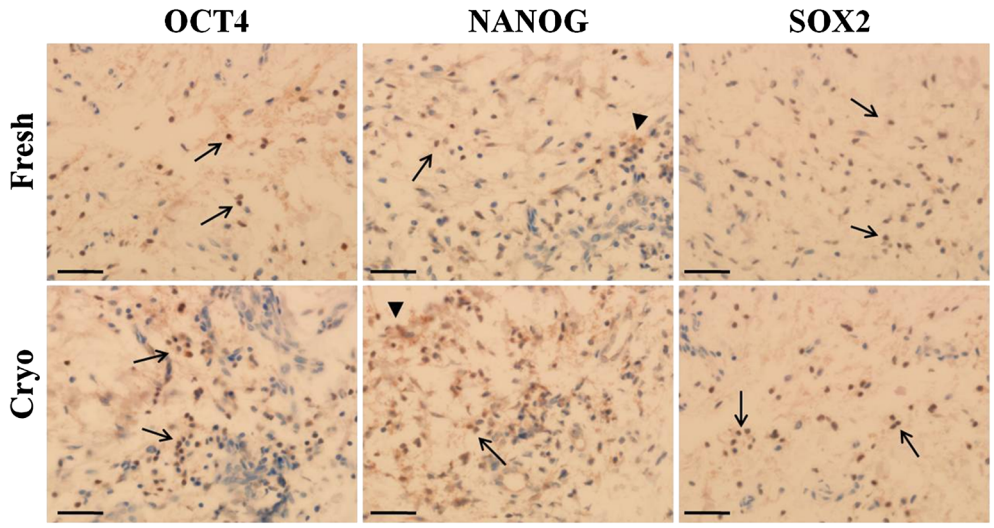

Figure 5. Immunohistochemical expression of the transcription factors OCT4, NANOG and SOX2 in fresh and cryopreserved dental tissues; scale bar = 50 μm. In some cells of both dental tissues, the nuclear expressions of these three proteins were detected with similar patterns (arrows). Interestingly, NANOG was also occasionally observed in the cell cytoplasm in both dental tissues, which may be related to the pluripotency of stem cells (arrowhead)

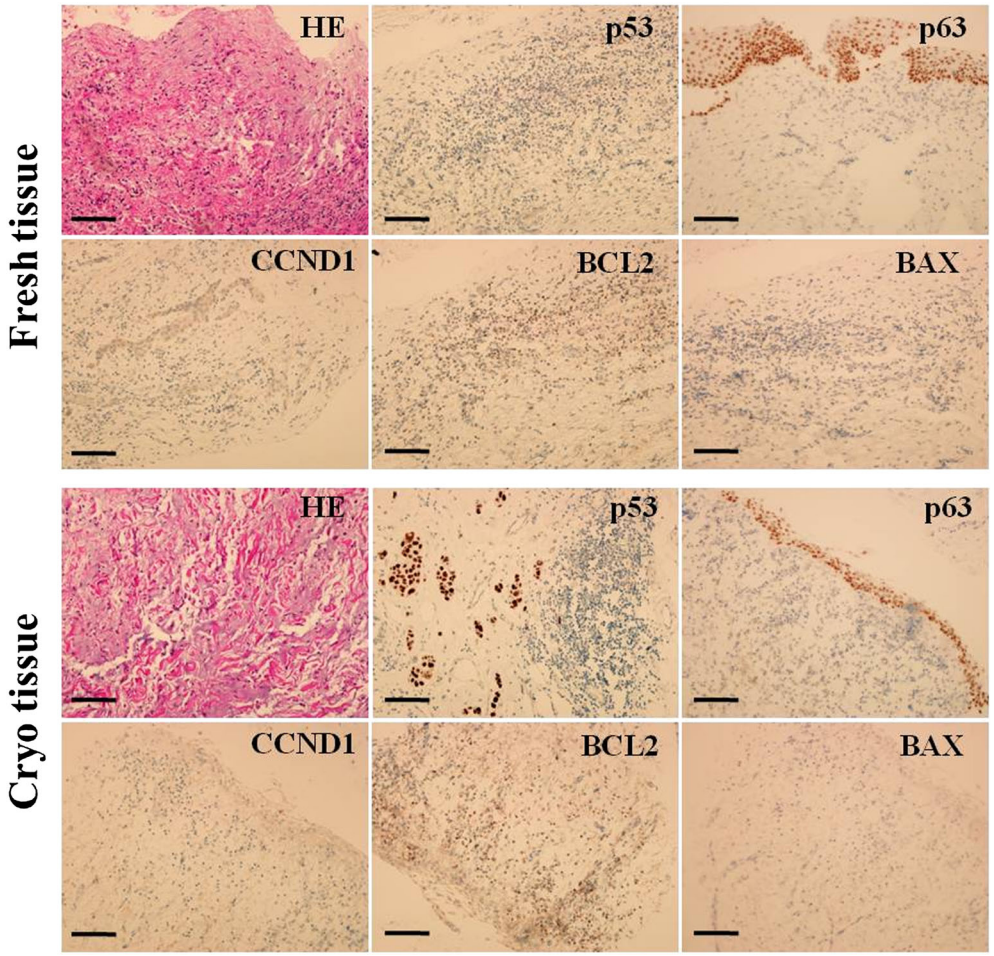

Figure 6. Histological features and immunohistochemical analyses for cell proliferation- and apoptosis-related proteins in fresh and cryopreserved dental tissues; scale bar = 100 μm. Only p53 was differently detected in the two dental tissues; it was highly expressed in cryopreserved tissue. Others, e.g. epithelial stem cell marker (p63), apoptosis-related genes (*BCL2* and *BAX*) and cell mitosis-related gene (*CCND1*) were similarly strongly, moderately or weakly detected in fresh and cryopreserved dental tissues

Copyright © 2014 John Wiley & Sons, Ltd.

DOI: 10.1002/term

transcription- and apoptosis-related genes. hDSCs from both fresh and cryopreserved dental tissues showed the same mRNA levels for the transcription factors *NANOG*, *OCT4* and *SOX2*. Moreover, real-time PCR revealed no statistical significant differences in mRNA levels for the pro-apoptotic genes *BCL2*, *p21* and *p53* and the anti-apoptotic gene *BAK* in hDSCs from both tissues (Figure 7).

4. Discussion

The main purpose of this study was to develop a method for human dental tissue cryopreservation for potential use as an autologous stem cell source for clinical applications. In the literature, several tissue cryopreservation methods for different purposes have been reported. Some researchers have tried to cryopreserve periodontal ligament and dental pulp tissue to serve as a stem cell source (Chen *et al*., 2011; Ma *et al*., 2012; Seo *et al*., 2005). These researchers successfully isolated multipotent stem cells from their long-term preserved dental tissues containing 10% DMSO and FBS (10% or 90%) as their cryoprotectants. Interestingly, dental pulp or ligament tissue was cryopreserved under the magnetic field programmed freezer or after digging micro-channels into the tooth with laser beam (Abedini *et al*., 2011; Lee *et al*., 2010, 2012; Gioventu *et al*., 2012). This magnetic cryopreservation has an advantage, as it can be used in whole-tooth cryopreservation for future tooth auto-transplantations. However, the key cryoprotectants of this magnetic tooth cryopreservation also include 5% or 10% DMSO with FBS or human albumin. Although adding DMSO as a cryoprotectant showed better cell viability than other agents, such as ethylene glycol or propylene glycol (Woods *et al*., 2009; Ding *et al*., 2010), the use of DMSO and a xenogenic agent (e.g. FBS) as a cryoprotectant has severe drawbacks. These could be toxic to cells, which may also influence cellular characteristics and alter genetic and/or epigenetic properties (Karlsson and Toner, 1996; Latinwo *et al*., 2008; Ock and Rho, 2011; Thome *et al*., 1994; Zambelli *et al*., 1998). Due to these drawbacks, there is a need for the development of a safer cryopreservation method for dental tissues that can both minimally affect cell characteristics and enhance cell survival rate. A milestone in tissue cryopreservation was the development of an ovarian tissue cryopreservation method for the purpose of restoring fertility in cancer patients (Andersen *et al*., 2008; Schmidt *et al*., 2003). These studies demonstrated cell safety in long-term preserved ovarian tissues and successful pregnancies following auto-transplantation of freeze–thawed ovarian tissue. In the present study, we modified the ovarian tissue cryopreservation method for dental follicle tissue storage to use as a stem cell source. The conventional cryopreservation of dental tissue with 10% DMSO and 10% FBS had a cell survival rateof about 30%, as shown in the present study, as opposed to the paper published by Woods *et al*. (2009), in which a ca. 87% positive cell growth rate was reported (seven cases from eight donors) after undigested dental pulp cryopreservation for 1 month. These differences may be due to tissue sizes

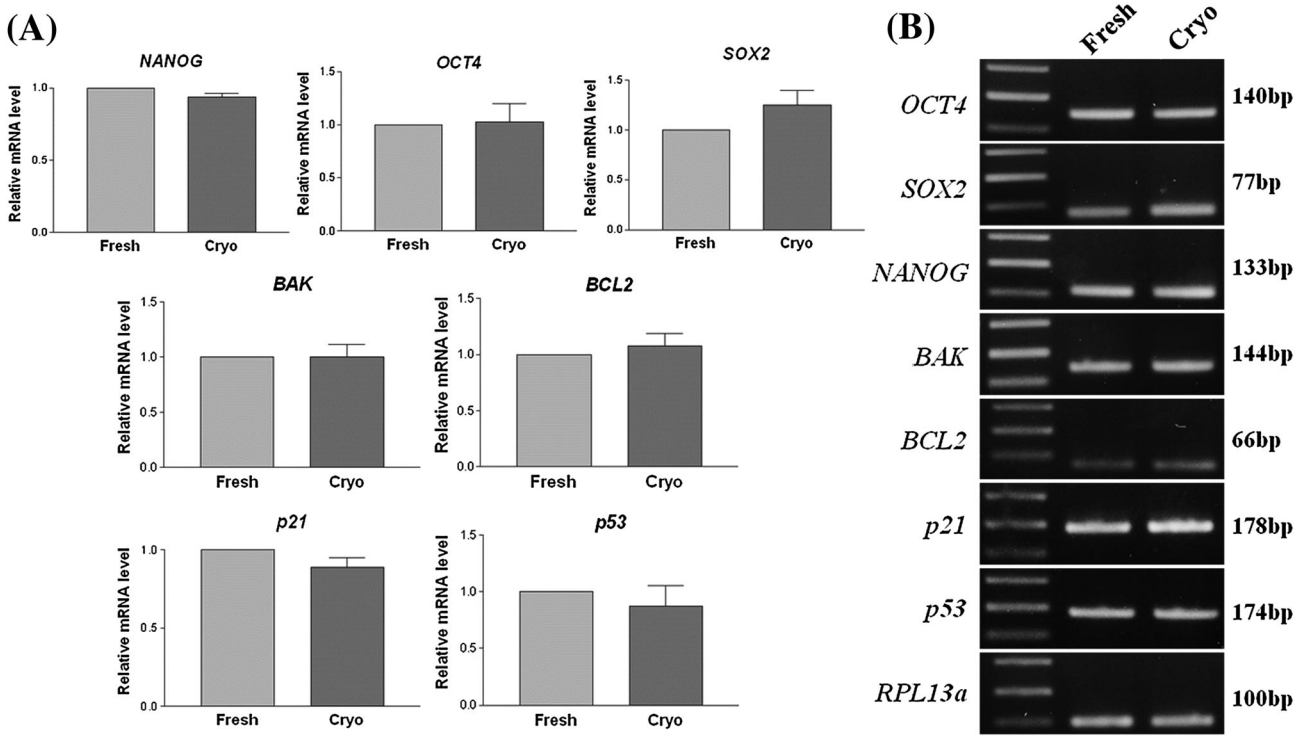

Figure 7. Real-time PCR on cultured hDSCs (passage 2) derived from fresh and cryopreserved dental tissues. (A) Cultured hDSCs from both tissues showed the same mRNA levels for the apoptosis-related genes *BAK*, *BCL2*, *p21* and *p53*, and transcription factors, *NANOG*, *OCT4* and *SOX2*; data represent mean ± SD of four independent experiments. (B) Representative images of RT–PCR products to show product sizes of (A)

Copyright © 2014 John Wiley & Sons, Ltd.

DOI: 10.1002/term

and longevity of storage, or a different calculation method for cell survival rate.

Immunohistochemical analysis of the transcription factors OCT4, NANOG and SOX2 revealed nuclear expression of these proteins in both fresh and cryopreserved dental follicle tissues with a similar pattern, even though their expression was not abundant or strong. Interestingly, these transcription-related proteins were mainly detected in the nucleus, while NANOG was occasionally observed in the cell cytoplasm in both dental tissues. Our finding was similar to previous reports, in which OCT4 and SOX2 showed nuclear staining and NANOG was occasionally observed in the cytoplasm, which might be related to the pluripotency of stem cells and invasive or recurrent cancer cells (Gu *et al*., 2012; Luo *et al*., 2013).

Immunohistochemical analyses for cell proliferation and apoptosis-related proteins indicated no difference in expression patterns in fresh and cryopreserved dental tissues, except for p53 protein. The epithelial stem cell marker p63 was also positively expressed in the epithelial tissues of fresh and cryopreserved dental tissues. The anti-apoptosis related protein BCL2 and the pro-apoptotic protein BAX showed similar expression patterns in both tissues. In addition, the cell mitosis-related gene, *CCND1*, had very low to no expression in both tissues. These findings suggest that the tissue cryopreservation method employed in our present study did not significantly influence the multipotency of dental tissue or the cell mitosis rate or alter the protein expression level of anti-apoptotic or pro-apoptotic factors. Only p53 protein showed a different expression pattern, in which the expression was increased in cryopreserved dental tissue, whereas it was not detected in fresh tissue. Increased expression of p53 in freeze–thawed dental tissue may be due to direct cell damage caused by the freeze–thaw process, instead of an alternative apoptotic pathway. The reason for this speculation is that apoptosis is an active process that requires time for induction and execution, as previously reported in cryopreserved ovarian tissue (Ebrahimi *et al*., 2010; Hussein *et al*., 2006; Mazoochi *et al*., 2009). However, with real-time PCR analysis, cultured hDSCs derived from fresh and cryopreserved dental follicle tissues showed the same levels of mRNA for apoptosis- and transcription-related genes, indicating similar MSC characteristics in both types of hDSCs. Based on the results of our present study, a newly developed method of programmed slow-freezing cryopreservation with cryoprotectant was found suitable for long-term dental follicle tissue preservation, which can potentially be used as an autologous stem cell source for future applications.

Conflict of interest

The authors have declared that there is no conflict of interest.

Acknowledgements

This study was supported by the National Research Foundation of Korea (Grant No. NRF-2012R1A1A4A01009879) and the Ministry of Food and Drug Safety (Grant No. 12182MFDS666), Republic of Korea.

References

Abedini S, Kaku M, Kawata T *et al*. 2011; Effects of cryopreservation with a newly-developed magnetic field programmed freezer on periodontal ligament cells and pulp tissues. *Cryobiology* **62**: 181–187.

Almansoori KA, Prasad V, Forbes JF *et al*. 2012; Cryoprotective agent toxicity interactions in human articular chodrocytes. *Cryobiology* **64**: 185–191.

Andersen CY, Rosendahl M, Byskov AG *et al*. 2008; Two successful pregnancies following autotransplantation of frozen/thawed ovarian tissue. *Hum Reprod* **23**: 2266–2272.

Byun JH, Lee JH, Choi YJ *et al*. 2008; Co-expression of nerve growth factor and p75NGFR in the inferior alveolar nerve after mandibular distraction osteogenesis. *Int J Oral Maxillofac Surg* **37**: 467–472.

Chen YK, Huang AH, Chan AW *et al*. 2011; Human dental pulp stem cells derived from different cryopreservation methods of human dental pulp tissues of diseased teeth. *J Oral Pathol Med* **40**: 793–800.

Ding G, Wang W, Liu Y *et al*. 2010; Effect of cryopreservation on biological and immunological properties of stem cells from apical papilla. *J Cell Physiol* **223**: 415–422.

Ebrahimi B, Valojerdi MR, Eftekhari-Yazdi P *et al*. 2010; *In vitro* maturation, apoptotic gene expression and incidence of numerical chromosomal abnormalities following cryotop vitrification of sheep cumulus–oocyte complexes. *J Assist Reprod Genet* **27**: 239–246.

Gioventu S, Andriolo G, Bonino F *et al*. 2012; A novel method for banking dental pulp stem cells. *Transfus Apher Sci* **47**: 199–206.

Gronthos S, Mankani M, Brahim J *et al*. 2000; Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. *Proc Natl Acad Sci U S A* **97**: 13625–13630.

Gu TT, Liu SY, Zheng PS. 2012; Cytoplasmic NANOG-positive stromal cells promote human cervical cancer progression. *Am J Pathol* **181**: 652–661.

Hussein MR, Bedaiwy MA, Falcone T. 2006; Analysis of apoptotic cell death, Bcl-2, and p53 protein expression in freshly fixed and cryopreserved ovarian tissue after exposure to warm ischemia. *Fertil Steril* **85**: 1082–1092.

Jeon BG, Kang EJ, Kumar BM *et al*. 2011; Comparative analysis of telomere length, telomerase and reverse transcriptase activity in human dental stem cells. *Cell Transplant* **20**: 1693–1705.

Jiang L, Peng WW, Li LF *et al*. 2012; Isolation and identification of CXCR4-positive cells from human dental pulp cells. *J Endod* **38**: 791–795.

Kang EJ, Byun JH, Choi YJ *et al*. 2010; *In vitro* and *in vivo* osteogenesis of porcine skin-derived mesenchymal stem cell-like cells with a demineralized bone and fibrin glue scaffold. *Tissue Eng A* **16**: 815–827.

Karlsson JOM, Toner M. 1996; Long-term storage of tissues by cryopreservation: critical issues. *Biomaterials* **17**: 243–256.

Latinwo LM, Badisa VL, Odewumi CO *et al*. 2008; Comparative evaluation of cytotoxicity of cadmium in rat liver cells cultured in serum-containing medium and commercially available serum-free medium. *Int J Mol Med* **22**: 89–94.

Lee SY, Chiang PC, Tsai YH *et al*. 2010; Effects of cryopreservation of intact teeth on the isolated dental pulp stem cells. *J Endod* **36**: 1336–1340.

Lee SY, Sun CH, Kuo TF *et al*. 2012; Determination of cryoprotectant for magnetic cryopreservation of dental pulp tissue. *Tissue Eng C* **18**: 397–407.

Li Y, Tan J, Li L. 2010; Comparison of three methods for cryopreservation of human embryonic stem cells. *Fertil Steril* **93**: 999–1005.

Copyright © 2014 John Wiley & Sons, Ltd.

Livak KJ, Schmittgen TD. 2001; Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**: 402–408.

Luo W, Li S, Peng B *et al*. 2013; Embryonic stem cells markers SOX2, OCT4 and Nanog expression and their correlations with epithelial–mesenchymal transition in nasopharyngeal carcinoma. *PLoS One* **8**: e56324.

Ma L, Makino Y, Yamaza H *et al*. 2012; Cryopreserved dental pulp tissues of exfoliated deciduous teeth is a feasible stem cell resource for regenerative medicine. *PLoS One* **7**: e51777.

Mazoochi T, Salehnia M, Pourbeiranvand S *et al*. 2009; Analysis of apoptosis and expression of genes related to apoptosis in cultures of follicles derived from vitrified and non-vitrified ovaries. *Mol Hum Reprod* **15**: 155–164.

Moore A, Donahue CJ, Bauer KD *et al*. 1998; Simultaneous measurement of cell cycle and apoptotic cell death. *Methods Cell Biol* **57**: 265–278.

Morsczeck C, Gotz W, Schierholz J *et al*. 2005; Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth. *Matrix Biol* **24**: 155–165.

Mrozik K, Gronthos S, Shi S *et al*. 2010; A method to isolate, purify, and characterize human periodontal ligament stem cells. *Methods Mol Biol* **666**: 269–284.

Ock SA, Rho GJ. 2011; Effect of dimethyl sulfoxide (DMSO) on cryopreservation of porcine mesenchymal stem cells (pMSCs). *Cell Transplant* **20**: 1231–1239.

Park BW, Hah YS, Choi MJ *et al*. 2009; In vitro osteogenic differentiation of cultured human dental papilla-derived cells. *J Oral Maxillofac Surg* **67**: 507–514.

Park BW, Kang DH, Kang EJ *et al*. 2012a; Peripheral nerve regeneration using autologous porcine skin-derived mesenchymal stem cells. *J Tissue Eng Regen Med* **6**: 113–124.

Park BW, Kang EJ, Byun JH *et al*. 2012b; *In vitro* and *in vivo* osteogenesis of human mesenchymal stem cells derived from skin, bone marrow and dental follicle tissues. *Differentiation* **83**: 249–259.

Rowley SD, Feng Z, Chen L *et al*. 2003; A randomized phase III clinical trial of autologous blood stem cell transplantation comparing cryopreservation using dimethylsulfoxide vs dimethylsulfoxide with hydroxyethyl starch. *Bone Marrow Transplant* **31**: 1043–1051.

Schmidt KLT, Ernst E, Andersen AN *et al*. 2003; Survival of primordial follicles following prolonged transportation of ovarian tissue prior to cryopreservation. *Hum Reprod* **12**: 2654–2659.

Seo BM, Miura M, Sonoyama W *et al*. 2005; Recovery of stem cells from cryopreserved periodontal ligament. *J Dent Res* **84**: 907–912.

Thome S, Craze J, Mitchell C. 1994; Dimethylsulfoxide-induced serum hyperosmolality after cryopreserved stem-cell graft. *Lancet* **344**: 1431–1432.

Tran HL, Doan VN, Le HT *et al*. 2014; Various methods for isolation of multipotent human periodontal ligament cells for regenerative medicine. *In Vitro Cell Dev Biol Anim*. DOI: 10.1007/s11626–014–9748-z.

Woods EJ, Perry BC, Hockema JJ *et al*. 2009; Optimized cryopreservation method for human dental pulp-derived stem cells and their tissues of origin for banking and clinical use. *Cryobiology* **59**: 150–157.

Zambelli A, Poggi G, Da Prada G *et al*. 1998; Clinical toxicity of cryopreserved circulating progenitor cells infusion. *Anticancer Res* **18**: 4705–4708.

Copyright © 2014 John Wiley & Sons, Ltd.