Journal of
Oral Pathology & Medicine

doi: 10.1111/j.1600-0714.2011.01040.x

J Oral Pathol Med (2011) 40: 793–800

wileyonlinelibrary.com/journal/jop

Human dental pulp stem cells derived from different cryopreservation methods of human dental pulp tissues of diseased teeth

Yuk-Kwan Chen[1,2]*, Anderson Hsien-Cheng Huang[1,3]*, Anthony Wing-Sang Chan[4], Tien-Yu Shieh[1,5], Li-Min Lin[1,2]

[1]*Department of Dentistry, College of Dental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan;* [2]*Division of Oral Pathology & Diagnosis, Kaohsiung Medical University Chung-Ho Memorial Hospital, Kaohsiung, Taiwan;* [3]*Grace Dental Clinic, Kaohsiung, Taiwan;* [4]*Yerkes National Primate Research Center, Department of Human Genetics, Emory University School of Medicine, Atlanta, GA, USA;* [5]*Division of Oral & Maxillofacial Surgery, Kaohsiung Medical University Chung-Ho Memorial Hospital, Kaohsiung, Taiwan*

BACKGROUND: Successful isolation of human dental pulp stem cells (hDPSCs) has been documented at least 120 h after tooth extraction. Viable hDPSCs have been isolated chiefly from cryopreserved healthy molar teeth and their undigested dental pulp tissue. Isolation of hDPSCs from diseased but vital teeth after cryopreservation has not been reported. This study aimed to isolate hDPSCs from cryopreserved diseased but vital teeth of various tooth types.

MATERIALS: Fifty tooth samples were divided into group A (*n* = 20) – freshly derived dental pulp tissues, group B (*n* = 20) – liquid nitrogen (liq N_2)-stored dental pulp tissues and group C (*n* = 10) – liq N_2-stored intact teeth.

METHODS AND RESULTS: The success rate for hDPSCs isolation was 100% for groups A and B and only 20% for group C. hDPSCs from all groups demonstrated self-renewal properties and similar multipotent potential characteristics of adipogenic, chondrogenic and osteogenic differentiation. In addition, hDPSCs showed high expression of bone-marrow mesenchymal stem-cell markers (CD29, CD90 and CD105) and very low expression of specific hematopoietic cells markers (CD14, CD34 and CD45).

CONCLUSION: Our results indicate that hDPSCs isolated from diseased but vital teeth of various tooth types can be stored in liq N_2 for future usage.

J Oral Pathol Med (2011) **40**: 793–800

Correspondence: Drs. Yuk-Kwan Chen and Li-Min Lin, Department of Oral Pathology, School of Dentistry, Kaohsiung Medical University, Kaohsiung Medical University Hospital, 100 Shih-Chuan 1st Road, Kaohsiung, Taiwan. Tel: +886 7 31211.1~7007, Fax: 886 7 3210637, E-mail: k0285@ms22.hinet.net

*These authors contributed equally to this paper.

Accepted for publication March 2, 2011

Keywords: cryopreservation; diseased tooth; human dental pulp stem cell

Introduction

Long-term storage of oral tissue specimens in liquid nitrogen (Liq N_2; −196°C) for future usage has been extensively implemented in dental laboratories. Human dental pulp stem cells (hDPSCs) have been reported to sustain their potency after a month of cryopreservation in liq N_2 and a culture is attained up to passage 25 (1). Additionally, Papaccio et al. (2) demonstrated that after storage in liq N_2 for 2 years, hDPSCs were still capable of differentiation into woven bone tissues. Hence, the banking of freshly digested hDPSCs is clinically possible for future usage.

Perry et al. (3) reported that hDPSCs isolation is feasible even 120 h after tooth extraction. Therefore, immediate isolation of hDPSCs for successful banking after tooth extraction may not be absolutely required. However, hDPSCs have been chiefly isolated from cryopreserved healthy molar and premolar teeth, as well as from their undigested dental pulp tissue (3–7); however, to our knowledge, they have not yet been isolated from other tooth types. Thus, the recovery of viable hDPSCs after cryopreservation of intact teeth and their undigested dental pulp tissue suggests that only minimal processing may be needed for the banking of tooth samples with no plans for immediate usage.

As aforementioned, the cryopreservation of tooth types other than permanent premolars and molars for future recovery of hDPSCs has not yet been studied, and additionally, the culturing of hDPSCs from diseased but vital teeth after cryopreservation has not been investigated. The aim of the current study was therefore to

isolate hDPSCs from various tooth types of diseased but vital teeth using two different modalities for the cryopreservation of dental pulp tissues prior to the isolation of stem cells, and then compare these with hDPSCs acquired from freshly derived dental pulp tissues.

Materials and methods

Study population

All samples were obtained with the approval of the Institutional Review Board of our Institution. A cohort of 50 samples (average age, 25.5 years; range, 6–74 years; male to female ratio, 1:18) of diseased but vital teeth were randomly divided into Group A (freshly derived dental pulp tissues, $n = 20$), Group B (liq N_2-stored dental pulp tissues, $n = 20$), and Group C (liq N_2-stored intact teeth, $n = 10$) (Table 1).

The mean age of Group A (20 samples) was 26.5 years, ranging from 6 to 74 years; the mean age of Group B (20 samples) was 23.4 years, ranging from 6 to 49 years; and for Group C (10 samples), the mean age was 27.7 years, ranging from 8 to 52 years. The male to female ratios of Groups A to C were 1:1.5, 1:3, and 1:1, respectively.

Group A: Freshly derived hDPSCs

hDPSCs digestion

Dental pulp tissues were extracted from the samples of group A ($n = 20$) using an endodontic file (Fig. 1A–D). The extracted pulp tissues were digested in collagenase type I (3 mg/ml; Invitrogen, Carlsbad, CA, USA) and dispase (4 mg/ml; Invitrogen) for 1 h at 37°C. The resulting cell suspensions from the tooth samples were filtered through a 40-μm cell strainer (Falcon, BD, Franklin Lakes, NJ, USA) and single-cell suspensions were then cultured in a medium containing α-Minimal Essential Medium (MEM) (Hyclone, Logan, UT, USA), 20% fetal bovine serum (Hyclone), 100 μM L-ascorbic acid-2-phosphate (Sigma, St Louis, MO, USA), 100 units/ml penicillin (Sigma) and 100 μg/ml streptomycin (Invitrogen). hDPSCs were cultured at 37°C with 5% CO_2. Fresh medium was replaced every 2 days and 80% confluence was achieved for optimal cell harvesting (8, 9).

Stem-cell characterization

Colony forming unit (CFU). Third-passage hDPSCs from the extracted pulp tissues for all the successful isolation samples of group A were seeded in a 6-well plate at 40 single cells per well in three replicates. After 2 weeks of culture, the cells were fixed in 10% buffered formalin for 10 min and stained with 3% (v/v) crystal violet (Sigma) for 5 min. The cells were washed twice with distilled water and the number of colonies was counted as previously described (8, 9).

Growth rate analyses. The proliferation rate was determined by plating the isolated hDPSCs at passage 3 at 25 000 cells per well of a six-well plate for all the successful isolation samples of group A. Three replicates of each cell line were made, and cells were passaged and counted at 72 h. A cell count was performed before the

Table 1 The detailed characteristics of the 50 patients included in this study

Sample no.	*Sex*	*Age (years)*	*Tooth type*	*Disease*
Group A (freshly derived dental pulp tissues)				
1	Male	6	81	Root resorption
2	Male	10	73	Root resorption
3	Female	10	53	Root resorption
4	Female	10	54	Root resorption
5	Female	10	84	Root resorption
6	Male	20	19	Supernumerary tooth
7	Female	10	24	Malaligned tooth with inflammation
8	Female	11	14	Malaligned tooth with inflammation
9	Female	11	44	Malaligned tooth with inflammation
10	Male	12	23	Pericoronitis
11	Male	12	13	Pericoronitis
12	Female	13	24	Malaligned tooth with inflammation
13	Female	61	25	Periodontitis
14	Female	30	28	Periodontitis
15	Female	30	38	Pericoronitis
16	Male	37	48	Pericoronitis
17	Male	42	17	Periodontitis
18	Female	60	27	Periodontitis
19	Female	61	27	Periodontitis
20	Male	74	18	Periodontitis
Group B (liquid nitrogen-stored dental pulp tissues)				
21	Female	6	71	Root resorption
22	Male	7	81	Root resorption
23	Female	10	65	Root resorption
24	Male	10	74	Root resorption
25	Female	10	74	Root resorption
26	Male	7	29	Supernumerary tooth
27	Female	10	14	Malaligned tooth with inflammation
28	Female	11	24	Malaligned tooth with inflammation
29	Female	11	34	Malaligned tooth with inflammation
30	Female	13	14	Malaligned tooth with inflammation
31	Female	41	21	Fractured tooth
32	Female	41	22	Fractured tooth
33	Male	49	31	Periodontitis
34	Male	21	38	Impacted tooth
35	Female	28	28	Periodontitis
36	Female	32	48	Pericoronitis
37	Female	33	18	Periodontitis
38	Female	36	48	Periodontitis
39	Female	46	47	Periodontitis
40	Female	46	48	Periodontitis
Group C (liquid nitrogen-stored intact teeth)				
41	Female	8	53	Root resorption
42	Female	8	63	Root resorption
43[a]	Female	13	44	Malaligned tooth with inflammation
44	Female	20	14	Malaligned tooth with inflammation
45	Male	24	18	Malaligned tooth with inflammation
46	Female	33	45	Malaligned tooth with inflammation
47	Male	36	18	Periodontitis
48	Male	41	24	Periodontitis
49[a]	Male	42	48	Periodontitis
50	Male	52	29	Supernumerary tooth

[a]The only two samples in group C from which hDPSCs were successfully isolated.

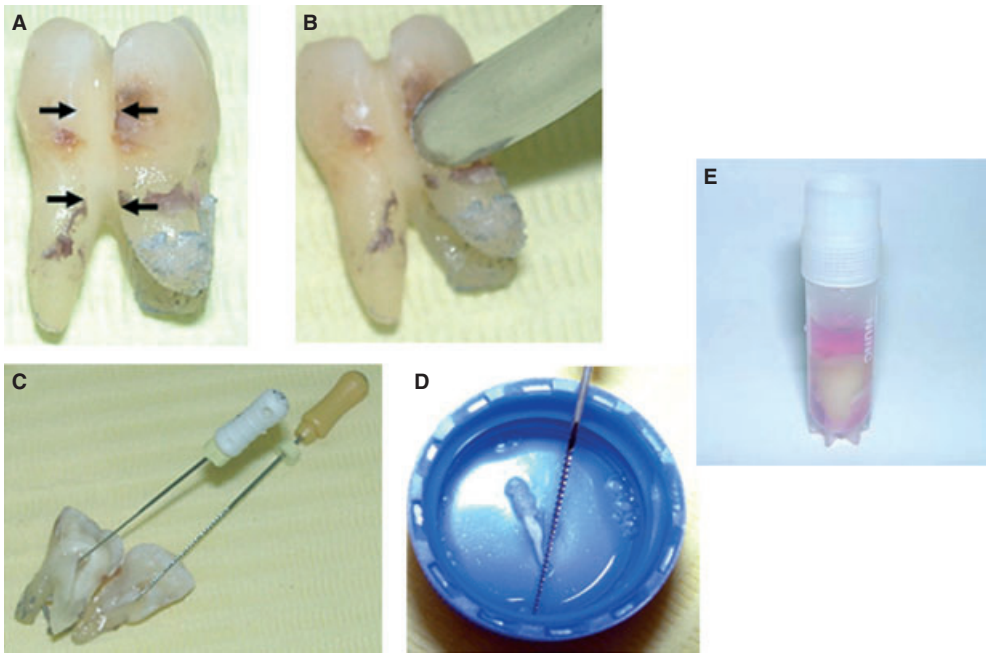

Figure 1 A circumference groove of 0.5–1.0 mm in depth was cut around the entire tooth (arrows) using an aseptic high-speed handpiece bur after cleaning the tooth surface with DPBS (A). The tooth was split using a chisel (B), dental pulp was then exposed (C) and the underlying pulp tissue was extracted using an endodontic file. The tissue was then placed in a container containing Dulbecco's phosphate-buffered saline for subsequent culture (D). Cryopreservation vial containing an intact tooth (E).

next passage and 25 000 cells were plated as described; the process was then repeated for a total of four passages. The proliferation rate was calculated by dividing the total cell number at 72 h of culture by the initial plating number, 25 000 (8, 9).

Adipogenic, osteogenic and chondrogenic differentiation. For adipogenic differentiation, third-passage hDPSCs for all the successful isolation samples of group A were seeded at 400 cells/cm^2 on a 35-mm diameter tissue culture dish for 11 days in hDPSC medium. On day 11, the hDPSC medium supplemented with 5.0 μg/ml insulin, 50 μM indomethacin, 1 μM dexamethasone and 0.5 μM IBMX was replaced, followed by subsequent replacement every 3–4 days for 3 weeks. The culture was then fixed in 4% paraformaldehyde (PFA) followed by Oil-Red-O staining (8, 9). For osteogenic differentiation, the cells were prepared as described above for adipogenic differentiation until day 11. From day 11, the hDPSCs medium supplemented with 1 nM dexamethasone, 20 mM β-glycerolphosphate and 50 ng/ml L-thyroxine sodium pentahydrate was replaced every 3–4 days for 3 weeks. The culture was then fixed in 4% PFA followed by Alizarin Red S staining (8, 9). For chondrogenic differentiation, 2.5×10^5 hDPSCs were centrifuged in a 15-ml conical tube at 1000 *g* for 5 min. The pellet was maintained in the hDPSCs medium supplemented with 10 μg/ml ITS-X (Invitrogen), 5.35 μg/ml linoleic acid, 1.25 μg/ml bovine serum albumin, 1.0 μg/ml dexamethasone and 10 ng/ml TGF-β_3 (Chemicon, Temecula, CA, USA). The medium was replaced with fresh medium every 3–4 days for 4 weeks. The pellets were then fixed in 4% PFA overnight, and paraffin-embedded tissue sections (4 μm) were stained with alcian blue (8, 9).

Flow cytometry. Third-passage hDPSCs for all the successful isolation samples of group A were divided into six fluorescence-activated-cell sorting (FACS) round-bottom tubes (Becton Dickinson Falcon, Sunnyvale, CA, USA) at 2×10^5 cells/tube and stained with IgG-fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated anti-CD14, -CD29, -CD34, -CD45, -CD90 or -CD105 (Beckman Coulter, Marseille, France, 20 μl each). After a 20-min incubation at ambient temperature in the dark, cells were washed twice with 2 ml FACS washing solution of phosphate-buffered saline (PBS) containing 0.1% FBS and 0.1% NaN_3 and centrifuged for 5 min at 230 *g*. The supernatant was then removed and cells were fixed with 1% formaldehyde (in PBS). Respective immunoglobulin G (IgG) isotype-matched controls (Beckman Coulter) were used as negative controls. All data were analyzed as previously described (9, 10).

Reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR for third-passage hDPSCs derived from all the successful isolation samples of group A was carried out using a DNA thermal cycler (TaKaRa MP, Tokyo, Japan). First, total RNA was obtained using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), followed by reverse transcription of the mRNA according to the procedure provided by SuperScript III (Invitrogen). The resulting cDNA was subsequently used for PCR amplification. The PCR reactions were performed using specific oligoprimer sets designed to determine the expression of stem-cell markers (Oct4,

Rex-1 and Nanog) and differentiation markers (Nestin and Osteonectin) (Table 2). The amplification cycle consisted of 3 min at 94°C, 25 s at 94°C, 30 s at 62°C and 50 s at 72°C, repeated 40 times, followed by 3 min at 72°C. Five oligoprimer sets were employed and GAPDH was used as the positive control (9, 10).

Additionally, RT-PCR of PPARγ messenger RNA, indicating the adipogenic lineage of the hDPSCs of the diseased tooth samples, was performed. Briefly, the PCR conditions were 60.5°C annealing with the forward oligoprimer (5'-CAG TGG GGA TGT CTC ATA A-3') and reverse oligoprimer (5'-CTT TTG GCA TAC TCT GTG AT-3') for 35 cycles, producing a PCR product of 391 bp. GAPDH was again used as the positive control (9).

Group B: Liq N_2-stored dental pulp tissues

As shown in Table 1, the dental pulp of 20 tooth samples was exposed and the underlying pulp tissues were extracted using an endodontic file (Fig. 1A–D). The pulp tissues were then directly immersed in 1.0 ml culture medium with 10% dimethyl sulfoxide (DMSO) in 2.0 ml cryovials for 2 h at 4°C to attempt full equilibration of the permeable tissues. These vials were then subjected to a dump-freezing process at −1°C/min for over 8 h in −80°C and stored in liq N_2 for 30 days prior to recovery for isolation procedures. For recovery of cells, vials were thawed in a 37°C water bath, and the thawed pulp tissues were washed with PBS and then transferred to hDPSC culture medium. hDPSCs were then isolated and third-passage hDPSCs for all the successful isolation samples of group B subsequently characterized as stated for the samples of group A.

Table 2 Oligoprimers used for stem-cell and differentiation markers

Oligoprimers	*Sequences*	*PCR products (bp)*
Oct4	Forward: 5'-GAC AAC AAT GAA AAT CTT CAG GAG A-3' Reverse: 5'-TAC AGA ACC ACA CTC GGA CCA-3'	205
Rex-1	Forward: 5'-AGA ATT CGC TTG AGT ATT CTG A-3' Reverse: 5'- GGC TTT CAG GTT ATT TGA CTG A-3'	470
Nanog	Forward: 5'-TCT CTC CTC TTC CTT CCT CCA-3' Reverse: 5'-GGA AGA GTA GAG GCT GGG GT-3'	389
Nestin	Forward: 5'-TGG CAA GGC GAC TGG GCG A-3' Reverse: 5'-CCC TCT ATG GCT GTT TCT TTC TCT-3'	810
Osteonectin	Forward: 5'-ATC TTC TTT CTC CTT TGC CTG G-3' Reverse: 5'-GCA CAC CTC TCA AAC TCG CC-3'	323
GAPDH	Forward: 5'-ATG GGG AAG GTG AAG GTC GG-3' Reverse: 5'-CCA TCA CGC CAC AGT TTC CC-3'	596

Group C: Liq N_2-stored intact teeth

As indicated in Table 1, 10 intact tooth samples were directly immersed into 1.0 ml culture medium with 10% DMSO in 2.0-ml cryovials for 2 h at 4°C to achieve complete equilibration of the permeable tissues (Fig. 1E). These vials were then subjected to a dump-freezing process at −1°C /min for over 8 h at −80°C and stored in liq N_2 for 30 days prior to recovery for the isolation of hDPSCs.

For the recovery of hDPSCs, vials were thawed in a 37°C water bath and the thawed teeth were washed with PBS and then transferred to hDPSCs culture medium. The teeth were subsequently processed as described in group A to extract and digest the pulp tissue to isolate the hDPSCs. The stem-cell characterization analyses described for the samples of group A were then performed for third-passage hDPSCs for all the successful isolation samples of group C.

Statistical analyses

Analyses of the differences between groups in terms of distributions and growth rates were performed by MANOVA using JMP 6.0 (SAS Institute Inc., Cary, NC, USA).

Results

The success rate for the isolation of hDPSCs was 100% for groups A and B, but successful isolation and subsequent tissue culturing was obtained for only two samples of group C (i.e. a success rate of 20%).

After the first or second passage, a homogenous population of hDPSCs was established and maintained for groups A–C. The morphology of the resulting cell cultures of groups A–C was predominantly long and spindle-shaped (Fig. 2A). After 14 days of culture, the CFU efficiency was 80% for all samples of groups A–C (Fig. 2B,C).

The hDPSCs of groups A–C had a high proliferation rate during the early passages, but decreased gradually in culture. For group A, at passage 3, hDPSCs proliferated at 14.8 ± 6.1-fold per 72 h and by passage 6 the rate had decreased to 3.8 ± 1.1-fold per 72 h. For group B, hDPSCs proliferated at 15.5 ± 8.2-fold per 72 h and by passage 6 the rate had decreased to 3.9 ± 1.3-fold per 72 h. For group C, the average proliferation rate of third-passage hDPSCs was 9.2 ± 3.7-fold per 72 h and by passage 6 the rate had decreased to 4.5 ± 0.2-fold per 72 h (Fig. 3). Because of the low success rate for group C (20%, only two samples), this group was excluded from the statistical analyses. There was no statistically significant difference in proliferation rate between group A and group B as analyzed by MANOVA ($P > 0.05$). However, there were no significant differences between the proliferation rates of hDPSCs respectively with age, gender and tooth position for group A (MANOVA, $P > 0.05$, respectively). Similarly, for group B, no significant differences between the proliferation rates of hDPSCs with age, gender and tooth position respectively were noted (MANOVA, $P > 0.05$, respectively). Furthermore, in

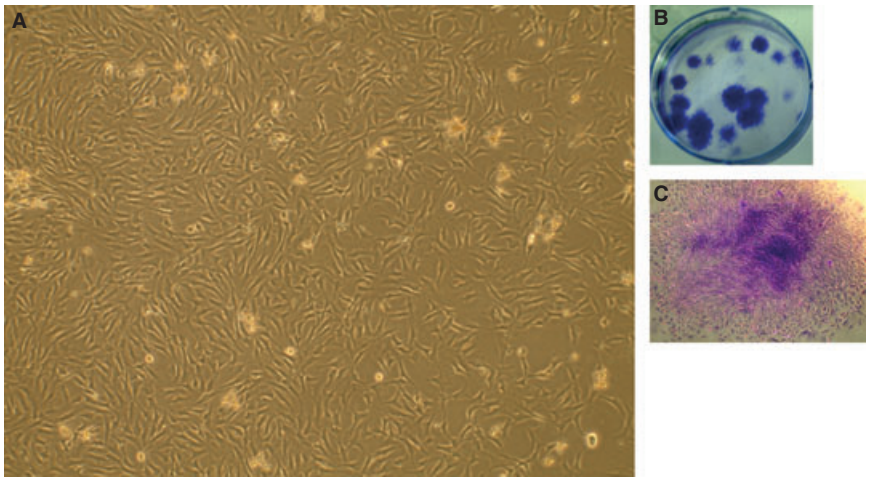

Figure 2 Representative successful tooth sample of group C. A homogenous population of predominantly spindle-shaped human dental pulp stem cells (hDPSCs) was obtained (×100) (A); colony forming units of hDPSCs, naked eye (B), ×40 (C). Similar results were obtained for groups A and B.

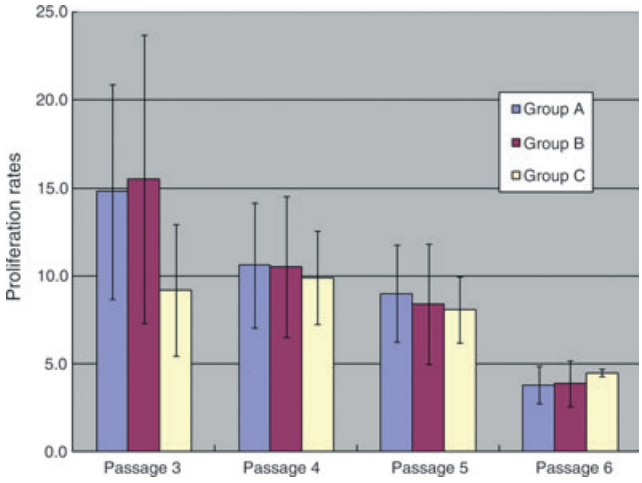

Figure 3 Proliferation rates for the tooth samples of groups A–C.

the MANOVA statistical analyses of the combined groups A and B, neither age, gender, tooth position nor cryopreservation condition were statistically significant with proliferation rate ($P > 0.05$, respectively). Noteworthy, hDPSCs from all the successful isolation samples of groups A–C, up to passage 15 cultures could be maintained in the current study. Hence, there seems to be no passage limit difference for group C respectively with group A and group B.

The isolated hDPSCs for all successful isolation samples (groups A–C) showed positive results in terms of osteogenic differentiation with Alizarin red S staining (Fig. 4A). Adipogenesis was confirmed by the formation of fat droplets upon Oil-Red-O staining (Fig. 4B). Furthermore, chondrogenic differentiation was shown to be positive for the hDPSCs by alcian blue staining (Fig. 4C). Additionally, the expression of stem-cell (Nanog, Rex-1 and Oct-4) and differentiation (osteonectin, nestin and PPARγ) markers was identified for the isolated cell cultures (groups A–C) by RT-PCR analysis using specific primer sets (Fig. 5). Finally, hDPSCs derived from groups A–C revealed a high expression of bone-marrow mesenchymal stem-cell markers (CD29, CD90, CD105) and a very low expression of hematopoietic cells markers (CD14, CD34, CD45) (Fig. 6).

Discussion

hDPSCs have been reported to exist in inflamed human dental pulp (11); however, the degree of severity of pulpitis does not exactly correlate clinically with tooth vitality, especially for multi-root canals in which part or all of the pulp tissue has actually become necrotized. Hence, to avoid controversy regarding the experimental design, pulpitis teeth were excluded in the current study.

Periodontitis, inflamed malaligned teeth, pericoronitis and root resorption are common dental diseases that may result in tooth extraction. Supernumerary teeth, with an incidence varying between 0.45% and 3% (12), may also undergo extraction. Hence, tooth extraction caused by these dental diseases is a daily clinical practice. However, the incidence of complicated crown fractures with dental pulp exposure ranges from 4.6% to 24.4% (13–15). Dental pulp tissue removal during root canal therapy due to pulp exposure for crown fractures is also a routine dental practice. Unfortunately, the extracted teeth are often discarded as contaminated waste and the extirpated dental pulp tissues are also usually thrown away. Hence, attempting to cryopreserve the extracted teeth and the extirpated pulp tissues for hDPSCs isolation would be beneficial.

Reviewing the English-language literature, to our knowledge, previous research into the cryopreservation of hDPSCs has chiefly focused on healthy permanent third molars and premolars (3–7). To our knowledge, there is a lack of detailed data focusing on the exploration of hDPSCs from the other types of tooth,

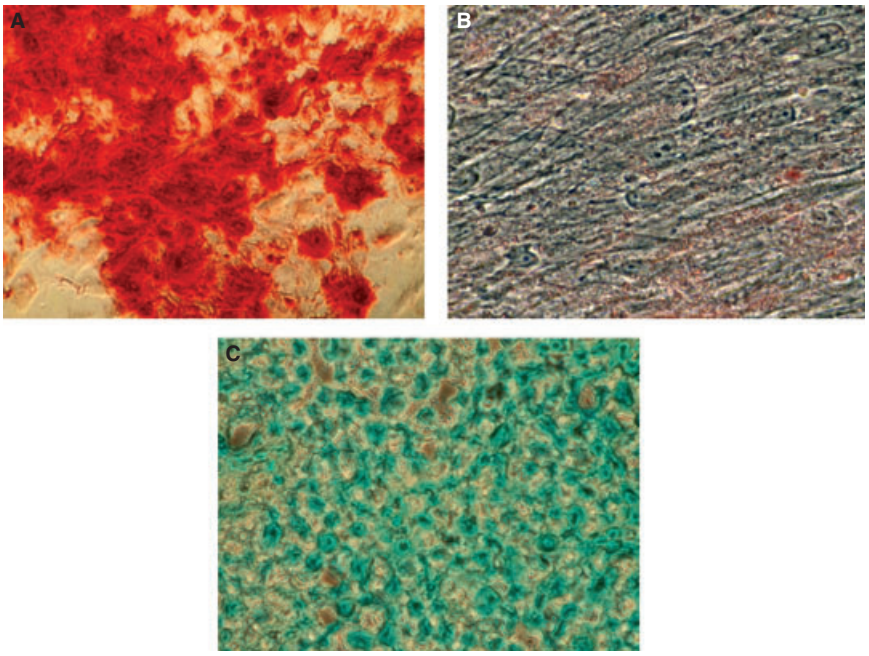

Figure 4 Representative successful tooth sample of group C. hDPSCs expressed osteogenic differentiation with Alizarin Red S staining (×200) (A); adipogenic differentiation with Oil-Red-O staining (×200) (B); and chondrogenic differentiation with alcian blue staining (×100) (C). Similar results were obtained for groups A and B.

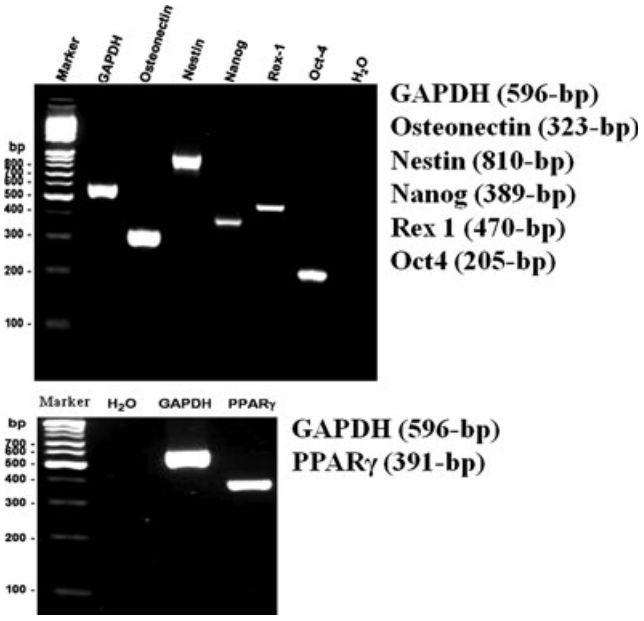

Figure 5 Representative successful tooth sample of group C. Reverse transcription-polymerase chain reaction of isolated hDPSCs revealed stem-cell (Oct-4, Nanog and Rex-1) and differentiation markers (Osteonectin, Nestin and PPARγ). Similar results were obtained for all samples of groups A and B.

particularly for diseased but vital teeth. The isolation of hDPSCs from a cohort of diseased but vital human teeth encompassing periodontitis, supernumerary teeth, inflamed malpositioned teeth, root resorption and pericoronitis, as well as fractured teeth, as successfully demonstrated in the current study, certainly further expands the potential sources of hDPSCs. Additionally, previous study results taken together with our findings indicate that immediate processing for successful banking of hDPSCs after extraction may not be absolutely required, which will aid the development of future protocols for the clinical banking of hDPSCs.

However, we found that the successful recovery rate of hDPSCs after storage of intact teeth in liq N_2 was only 20%. These data are similar to the results of Woods et al. (4), who were able to establish cultures from seven of 10 frozen whole teeth, but only two were readily expandable and functioned similarly to freshly isolated cells. The significant discrepancy in the recovery rate of hDPSCs between cryopreserved dental pulp tissues (group B, 100%) and cryopreserved intact teeth (group C, 20%) is probably caused by the fact that a higher level of cryopreservation, protecting against agent penetration, could have been attained for the isolated dental pulp tissues as compared with intact teeth. It has been shown that water is readily capable of being released and penetrating isolated dental pulp tissues during the development and melting of ice in tissue that has not been entrapped within the inflexible dentin structure of the intact tooth (3, 4). Recently, Zhurova et al. (16) showed that hDPSCs expressed the gap junction-forming protein (Connexin-43) and upon intracellular ice formation retained membrane integrity; however, they lost the ability to proliferate. Nevertheless, for the present study, despite the two different cryopreservation methods having different rates of recovery on primary culture, hDPSCs obtained by these two modalities had similar growth rates (passage 3 sub-culture) and potency

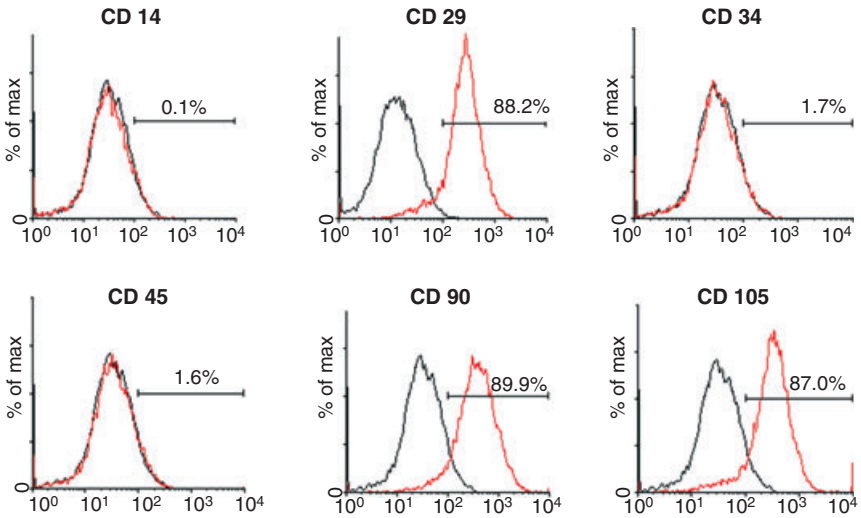

Figure 6 Representative successful tooth sample of group C. Flow cytometry of the isolated human dental pulp stem cells revealed high expression of CD29, CD90 and CD105 and very low expression of CD14, CD34 and CD45. Similar results were obtained for all samples of groups A and B.

properties. However, Lee et al. (7) recently reported the use of a novel programmable freezer coupled with a magnetic field for the cryopreservation of intact premolars, revealing a successful hDPSCs isolation rate of 73%.

Although there have been low rates of successful isolation of hDPSCs from intact teeth after cryopreservation, whole teeth have been successfully cryopreserved and thawed for the purpose of reimplantation (17, 18). Temmerman et al. (5) proved that cryopreservation of human pulp tissue would be possible if the cryoprotective agent could reach the entire pulp. Consequently, if a novel cryopreservation modality could be invented, as demonstrated by Lee et al. (7), improvements in cryopreservation efficiency could be attained. Then, the banking of whole extracted teeth may be feasible, with multipotent hDPSCs being cultured only when needed for clinical use. Moreover, future research in this area, such as the cryopreservation of cell-infiltrated scaffolds, would further shed new light on the application of potential storage techniques and the banking of hDPSCs.

Finally, although it was not the main objective of the present study, our data also indicated that there are no differences between hDPDCs derived from young or old patients regarding growth rate and stem-cell features such as the ability to undergo osteogenic, chondrogenic, and adipogenic differentiation.

In conclusion, we have demonstrated that hDPSC cultures could be established by isolating cryopreserved dental pulp tissues from various tooth types, as well as from diseased but vital teeth, using a digestion and culture protocol after thawing.

References

1. Zhang W, Walboomers XF, Shi S, Fan M, Jansen JA. Multilineage differentiation potential of stem cells derived from human dental pulp after cryopreservation. *Tissue Eng* 2006; **12**: 2813–23.
2. Papaccio G, Graziano A, d'Aquino R, et al. Long-term cryopreservation of dental pulp stem cells (SBP-DPSCs) and their differentiated osteoblasts: a cell source for tissue repair. *J Cell Physiol* 2006; **208**: 319–25.
3. Perry BC, Zhou D, Wu X, et al. Collection, cryopreservation, and characterization of human dental pulp-derived mesenchymal stem cells for banking and clinical use. *Tissue Eng Part C Methods* 2008; **14**: 149–56.
4. Woods EJ, Perry BC, Hockema JJ, Larson L, Zhou D, Goebel WS. Optimized cryopreservation method for human dental pulp-derived stem cells and their tissues of origin for banking and clinical use. *Cryobiology* 2009; **59**: 150–7.
5. Temmerman L, Beele H, Dermaut LR, Van Maele G, De Pauw GA. Influence of cryopreservation on the pulpal tissue of immature third molars in vitro. *Cell Tissue Bank* 2010; **11**: 281–9.
6. Ding G, Wang W, Liu Y, et al. Effect of cryopreservation on biological and immunological properties of stem cells from apical papilla. *J Cell Physiol* 2010; **223**: 415–22.
7. Lee SY, Chiang PC, Tsai YH, et al. Effects of cryopreservation of intact teeth on the isolated dental pulp stem cells. *J Endod* 2010; **36**: 1336–40.
8. Huang AH, Chen YK, Lin LM, Shieh TY, Chan AW. Isolation and characterization of dental pulp stem cells from a supernumerary tooth. *J Oral Pathol Med* 2008; **37**: 571–4.
9. Huang AH, Chen YK, Chan AW, Shieh TY, Lin LM. Isolation and characterization of human dental pulp stem/stromal cells from nonextracted crown-fractured teeth requiring root canal therapy. *J Endod* 2009; **35**: 673–81.
10. Huang AH, Snyder BR, Cheng PH, Chan AW. Putative dental pulp derived stem/stromal cells promote proliferation and differentiation of endogenous neural cells in the hippocampus of mice. *Stem Cells* 2008; **26**: 2654–63.
11. Alongi DJ, Yamaza T, Song YJ, et al. Stem/progenitor cells from inflamed human dental pulp retain tissue regeneration potential. *Regen Med* 2010; **5**: 617–31.
12. Leco BMI, Martin MJF, Martinez GJM. An observational study of the frequency of supernumerary teeth in a population of 2000 patients. *Med Oral Patol Oral Cir Bucal* 2007; **12**: E134–8.

13. Stockwell AJ. Incidence of dental trauma in the Western Australian School Dental Service. *Commun Dent Oral Epidemiol* 1988; **16**: 294–8.
14. Davis GT, Knott SC. Dental trauma in Australia. *Aust Dent J* 1984; **29**: 217–21.
15. Oulis CJ, Berdouses ED. Dental injuries of permanent teeth treated in private practice in Athens. *Endod Dent Traumatol* 1996; **12**: 60–5.
16. Zhurova M, Woods EJ, Acker JP. Intracellular ice formation in confluent monolayers of human dental stem cells and membrane damage. *Cryobiology* 2010; **61**: 133–41.
17. Schwartz O, Rank CP. Autotransplantation of cryopreserved tooth in connection with orthodontic treatment. *Am J Orthod Dentofacial Orthop* 1986; **90**: 67.
18. Paulsen HU, Andreasen JO, Schwartz O. Tooth loss treatment in the anterior region: autotransplantation of premolars and cryopreservation. *World J Orthod* 2006; **7**: 27.