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RESEARCH ARTICLE

Human dental pulp stem cells derived from cryopreserved dental pulp tissues of vital extracted teeth with disease demonstrate hepatic-like differentiation

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Abstract

Reviewing the literature, hepatic differentiation of human dental pulp stem cells (hDPSCs) from cryopreserved dental pulp tissues of vital extracted teeth with disease has not been studied. This study is aimed to evaluate the hypothesis that hDPSCs from cryopreserved dental pulp tissues of vital extracted teeth with disease could possess potential hepatic differentiation. Forty vital extracted teeth with disease recruited for hDPSCs isolation, stem cell characterization and hepatic differentiation were randomly and equally divided into group A (liquid nitrogen-stored dental pulp tissues) and group B (freshly derived dental pulp tissues). Samples of hDPSCs isolated from groups A and B but without hepatic growth factors formed negative controls. A well-differentiated hepatocellular carcinoma cell line was employed as a positive control. All the isolated hDPSCs from groups A and B showed hepatic-like differentiation with morphological change from a spindle-shaped to a polygonal shape and normal karyotype. Differentiated hDPSCs and the positive control expressed hepatic metabolic function genes and liver-specific genes. Glycogen storage of differentiated hDPSCs was noted from day 7 of differentiation-medium culture. Positive immunofluorescence staining of low-density lipoprotein and albumin was observed from day 14 of differentiation-medium culture; urea production in the medium was noted from week 6. No hepatic differentiation was observed for any of the samples of the negative controls. We not only demonstrated the feasibility of hepatic-like differentiation of hDPSCs from cryopreserved dental pulp tissues of vital extracted teeth with disease but also indicated that the differentiated cells possessed normal karyotype and were functionally close to normal hepatic-like cells. Copyright © 2013 John Wiley & Sons, Ltd.

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1. Introduction

Adult stem cells can be obtained from various tissues (Lee *et al*., 2000; Toma *et al*., 2001; Zuk *et al*., 2002; Kögler *et al*., 2004), including dental tissues (Seo *et al*., 2004; Gronthos *et al*., 2000), which can easily be obtained from the daily clinical practice of tooth extraction. Human dental pulp stem cells (hDPSCs) have been demonstrated to possess osteogenic, adipogenic and chondrogenic differentiation (Gronthos *et al*., 2000; Miura *et al*., 2003), suggesting a potential for multi-differentiation of dental mesenchymal cells.

The incidence of hepatoma related to hepatitis B and C continues to increase in developed countries (El-Serag *et al*., 2003). Chronic liver injury occurs following viral infection, caused by persistent inflammation and fibrosis and subsequent development into liver cirrhosis and

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hepatoma. Stem cell-based therapy is of potential use in these liver diseases. The investigation of alternative sources for hepatocytes is certainly an important area of research. Considering the potential alternative sources of hepatic-like cells for stem cell-based therapy, human adipose tissue-derived stromal cells transplanted into carbon tetrachloride (CCl_4)-injured severe combined immunodeficiency (SCID) mice have been found to be able to differentiate into hepatocytes *in vivo* (Moriya *et al*., 2008), and umbilical cord blood stem cells have also been demonstrated to be capable of differentiating into hepatocytes after transplantation into CCl_4-injured rats (Tang *et al*., 2006). On the other hand, hDPSCs are potentially superior to other types of adult stem cell, because teeth are easy to access and vital teeth with disease have the possibility of being extracted throughout life. Hence, further understanding of the potential hepatic differentiation of hDPSCs is an issue of potential interest today.

Cryopreservation provides a potentially valuable aspect of applying tissues for future recovery of stem cells; the isolated cells will then be available for future clinical usage. Reviewing the English-language literature, to our knowledge, the potential hepatic differentiation of hDPSCs from cryopreserved human dental pulp tissues of vital extracted teeth with disease (which were usually discarded after extraction) has not yet been studied. Consequently, the current study aimed to investigate this.

2. Materials and methods

2.1. Study population

A cohort of 40 samples of vital human teeth with disease (residual root, supernumerary tooth, periodontitis, pericoronitis and tooth fracture) was extracted with the approval of the Institution Review Board of our Institution (KMUH-IRB-980016), and informed consent was obtained from all subjects who participated in the study. The tooth samples were randomly and equally divided into groups A (liquid nitrogen-stored dental pulp tissues; average age 26.5 years) and B (freshly-derived dental pulp tissues; average age 23.4 years).

2.2. hDPSCs digestion

Dental pulp tissues were extracted from samples of groups A and B using aseptic endodontic files, as previously described (Huang *et al*., 2008). The whole pulp tissues extracted from all of the samples of group A were directly immersed in 1 ml culture medium with 10% dimethyl sulphoxide (DMSO) in 2 ml cryovials for 2 h at 4°C in order to obtain full equilibration of the permeable tissues. These vials were subjected to a dump-freezing process at −1°C/min for over 8 h to −80°C and then stored in liquid nitrogen for 30 days prior to the recovery of hDPSCs.

After cryopreservation for 30 days, the extracted dental pulp tissues were digested as previously described, with modifications (Miura *et al*., 2003; Huang *et al*., 2008), with collagenase type I (3 mg/ml; Invitrogen, Carlsbad, CA, USA) and dispase (4 mg/ml, Invitrogen) for 1 h at 37°C. The cell suspensions obtained from the tooth samples were filtered through a 40 μm cell strainer (Falcon BD, Franklin Lakes, NJ, USA) and the single-cell suspensions were cultured in medium containing α-minimal essential medium (MEM; Hyclone, Logan, UT, USA), 20% fetal bovine serum (FBS; Hyclone), 100 μM L-ascorbic acid-2-phosphate (Sigma, St. Louis, MO, USA), 100 U/ml penicillin (Sigma) and 100 μg/ml streptomycin (Invitrogen). The hDPSCs were then cultured at 37°C with 5% CO_2. On the other hand, all the extracted dental pulp tissues of group B were freshly digested, following the procedures described for group A without the cryopreservation step.

2.3. Stem cell characterizations

The passage 3 hDPSCs obtained from the extracted pulp tissues of groups A and B were used for stem cell characterization experiments, including colony forming unit (CFU), growth rate analyses, adipogenic, osteogenic and chondrogenic differentiation, flow cytometry (bone marrow mesenchymal stem cell markers CD29, CD90 and CD105; and haematopoietic cell markers CD14, CD34 and CD45), reverse transcription–polymerase chain reaction (RT–PCR; Table 1) as well as western blot analyses (Nestin, Oct-4 and Rex-1) with procedures and conditions as previously described (Huang *et al*., 2009; Snyder *et al*., 2011). Then the relative expression level of Nestin, Oct-4 and Rex-1 upon western blot analyses was measured and normalized to the expression level of the positive control.

2.4. Hepatic differentiation

2.4.1. Negative controls for hepatic differentiation

Each of the samples of the passage 3 hDPSCs derived from groups A and B, respectively, was treated with basal medium with penicillin/streptomycin (Invitrogen) and glutamine (Invitrogen) but without any growth factor or supplements, and these treated samples were used as hepatic differentiation negative controls.

2.4.2. Positive control for hepatic differentiation

A well-differentiated hepatocellular carcinoma (HCC) cell line (BCRC 60169, Food Industry Research and Development Institute, Taiwan) was commercially available and employed as the positive control for hepatic differentiation.

2.4.3. Identification of morphological change

For hepatic differentiation, the passage 3 hDPSCs of the samples of groups A and B at $1.0\times10^4/cm^2$ were serum-deprived

Table 1. Various oligoprimers used for the present experiment

Oligoprimers	Sequences	PCR products (bp)
Targeting for stem-cell and differentiation markers		
Oct4	Forward: 5′-GACAACAATGAAAATCTTCAGGAGA-3′ Reverse: 5′-TACAGAACCACACTCGGACCA-3′	205
Rex-1	Forward: 5′-AGAATTCGCTTGAGTATTCTGA-3′ Reverse: 5′-GGCTTTCAGGTTATTTGACTGA-3′	470
Nanog	Forward: 5′-TCTCTCCTCTTCCTTCCTCCA-3′ Reverse: 5′-GGAAGAGTAGAGGCTGGGGT-3′	389
Nestin	Forward: 5′-TGGCAAGGCGACTGGGCGA-3′ Reverse: 5′-CCCTCTATGGCTGTTTCTTTCTCT-3′	810
Osteonectin	Forward: 5′-ATCTTCTTTCTCCTTTGCCTGG-3′ Reverse: 5′-GCACACCTCTCAAACTCGCC-3′	323
GAPDH	Forward: 5′-ATGGGGAAGGTGAAGGTCGG-3′ Reverse: 5′-CCATCACGCCACAGTTTCCC-3′	596
Targeting for PPARγ mRNA indicating adipogenic differentiation		
PPARγ	Forward: 5′-CAGTGGGGATGTCTCATAA-3′ Reverse: 5′-CTTTTGGCATACTCTGTGAT-3′	391
Targeting for hepatic differentiation markers		
αFP	Forward: 5′-TGCAGCCAAAGTGAAGAGGGAAGA-3′ Reverse: 5′-CATAGCGAGCAGCCCAAAGAAGAA-3′	216
Alb	Forward: 5′-TGCTTGAATGTGCTGATGACAGGG-3′ Reverse: 5′-AAGGCAAGTCAGCAGGCATCTCATC-3′	161
CK-18	Forward: 5′-TGGTACTCTCCTCAATCTGCTG-3′ Reverse: 5′-CTCTGGATTGACTGTGGAAGT-3′	148
TO	Forward: 5′-ATACAGAGACTTCAGGGAGC-3′ Reverse: 5′-TGGTTGGGTTCATCTTCGGTATC-3′	299
G-6P	Forward: 5′-GCTGGAGTCCTGTCAGGCATTGC-3′ Reverse: 5′-TAGAGCTGAGGCGGAATGGGAG-3′	350
C/EBPα	Forward: 5′-CCTTCAACGACGAGTTCCTG-3′ Reverse: 5′-GCTTGGCTTCATCCTCCTC-3′	300
HNF1α	Forward: 5′-GTGGCGAAGATGGTCAAGTC-3′ Reverse: 5′-ACCTGTGGGCTCTTCAATCA-3′	233
CYP1A1	Forward: 5′-CTACCTACCCAACCCTTCCC-3′ Reverse: 5′-CAGGCTGTCTGTGATGTCCC-3′	132

αFP, α-fetoprotein; Alb, albumin; CK-18, cytokeratin 18; TO, tryptophan 2,3-dioxygenase; G-6P, glucose 6-phosphatase; C/EBPα, CCAAT/enhancer-binding proteins α; HNF1α, hepatocyte nuclear factor-1α; CYP1A1, cytochrome P450 family-1, subfamily-A, polypeptide 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

for 2 days in Iscove's modified Dulbecco's medium (IMDM; Invitrogen) supplemented with 20 ng/ml epidermal growth factor (Sigma) and 10 ng/ml β-fibroblast growth factor (FGF; Sigma), prior to induction by a two-step protocol (Lee *et al*., 2004). Differentiation was induced by treating hDPSCs with step 1 differentiation medium, consisting of

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IMDM supplemented with 20 ng/ml HGF (Sigma), 10 ng/ml β-FGF and nicotinamide 0.61 g/l for 7 days, and then the step 1 differentiation medium was stopped. The experiment was subsequently followed by treatment with step 2 maturation medium, consisting of IMDM supplemented with 20 ng/ml oncostatin M (Sigma), 1 μM dexamethasone and 50 mg/ml ITS^+ premix (Falcon) for days 8–42.

2.4.4. Hepatic RT–PCR

In order to evaluate the expression of hepatic differentiated passage 3 hDPSCs of the samples of groups A and B, as well as hepatic negative and positive controls, RT–PCR was performed for hepatic metabolic function genes (α-fetoprotein, *αFP*; albumin, *Alb*; cytokeratin-18, *CK-18*; tryptophan 2,3-dioxygenase, *TO*; glucose 6-phosphatase, *G-6P*) and liver-specific genes (CCAAT/enhancer-binding proteins α, *C/EBPα*; hepatocyte nuclear factor-1α, *HNF1α*; cytochrome P450 family-1, subfamily-A, polypeptide-1, *CYP1A1*), using the specific oligoprimer sets shown in Table 1. *GAPDH* was used as the positive control. The amplification cycle consisted of 3 min at 94°C for 40 s, 56°C for 50 s and 72°C for 60 s for 35 cycles, after initial denaturation at 94°C for 5 min. Then, the relative expression level of various hepatic markers was measured and normalized to *GAPDH* mRNA level.

2.4.5. Periodic acid–Schiff (PAS) staining for glycogen

Culture dishes containing cells from samples of the passage 3 hepatic differentiated hDPSCs of groups A and B, as well as hepatic negative and positive controls, were fixed in 4% formaldehyde, permeabilized with 0.1% Triton X-100 for 10 min, and either incubated or not with diastase for 1 h at 37°C. The samples were then oxidized in 1% periodic acid for 5 min, rinsed three times in deionized distilled water (dH_2O), treated with Schiff's reagent for 15 min and rinsed in dH_2O for 5 min. The samples were subsequently counterstained with Mayer's haematoxylin for 1 min, rinsed in dH_2O and assessed under a light microscope.

2.4.6. Immunofluorescence for the uptake of low-density lipoprotein (LDL)

A Dil-Ac-LDL staining kit was purchased from Biomedical Technologies (Stoughton, MA, USA) and assays for the samples of the passage 3 hepatic differentiated hDPSCs of groups A and B, as well as hepatic negative and positive controls, was performed according to the manufacturer's instructions by placing into culture medium, 1:200 by volume. LDL uptake was observed under fluorescence microscope.

2.4.7. Immunofluorescence for Alb

For the staining of Alb, the passage 3 hepatic differentiated hDPSCs from the samples of groups A and B, as well as hepatic negative and positive controls, were fixed overnight in 4% formaldehyde at 4°C and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 10 min. The samples were then incubated with anti-BSA rabbit IgG fraction (A-11133, Invitrogen) primary antibodies (1:200) for 1 h, followed by secondary antibodies AlexaFluor 488 (1:200) anti-rabbit IgG for 1 h. Between incubations, the samples were washed with PBS.

2.4.8. Urea assay

Urea concentrations in the passage 3 hepatic differentiated hDPSCs from the samples of groups A and B and hepatic positive and negative controls were measured by colorimetric assay (urea assay kit, BioChain, Hayward, CA, USA) according to the manufacturer's recommendations and analysed using a BioPhotometer ELISA Detector (Dynex MRX v 1.31) at a reading optical density of 490 nm. The same number of cells (1.0×10^4 cells/cm^2) were used in each assay. The step-2 hepatocyte maturation medium described above was used as a negative control.

Statistical comparisons were performed using JMP 9.0 software (SAS, Cary, NC, USA). The means of the urea concentrations in the different weeks of differentiation were analysed using ANOVA and Tukey HSD tests. The data were regarded as significant when $p<0.0001$.

2.4.9. Western blot

Total proteins were extracted and concentrated for analysis of the passage 3 hepatic differentiated hDPSCs of groups A and B using the Bradford assay (Bio-Rad, Hercules, CA, USA), as well as from the HCC cell line (used as the control). Equal amounts of the protein were boiled prior to polyacrylamide gel electrophoresis; the proteins were transferred onto a polyvinylidene difluoride membrane (cat. no. IPVH 00010, Millipore Immobion P, Millipore, Billerica, MA, USA), using Bio-Rad's Transblot. The membrane was then blocked, treated with primary antibodies (HNF4α 1:2000; cat. no. GTX100311, GeneTex, Irvine, CA, USA) and γ-tubulin (1:10 000; cat. no. T6557, Sigma-Aldrich) secondary antibody, and detected using Amersham's ECL kit (Amersham, Pittsburgh, PA, USA).

2.4.10. Karyotyping

Karyotypings of the passage 3 hepatic differentiated hDPSCs of groups A and B were done in accordance with the protocol available online (http://www.cellmigration.org/resource/komouse/protocols/karyotyping_feb06.pdf), with some modifications.

3. Results

3.1. Stem cell characterizations

A 100% successful isolation rate of hDPSCs was obtained for the samples of groups A and B, and for both groups

the resulting hDPSCs had a high initial proliferation rate and were of a predominantly long and spindle-shaped morphology, similar to human bone marrow stem cells (hBMSCs) (Figure 1A). A homogeneous population of hDPSCs was established and maintained after passages 1 or 2. After 2 weeks of culture, the CFU efficiency was 80% (Figure 1B). All isolated hDPSCs were capable of differentiating into osteogenic (Figure 1C), chondrogenic (Figure 1D) and adipogenic (Figure 1E) lineages under specific experimental conditions and also expressed stem cells markers (*Nanog*, *Rex-1* and *Oct-4*) and differentiation markers (*Osteonectin*, *Nestin* and *PPARγ*) upon RT–PCR (Figure 1F).

All DPSCs obtained from groups A and B revealed high expression of CD29, CD90 and CD105, all of which are common hBMSC markers. However, markers specific for haematopoietic cells, including CD14, CD34 and CD45, were found to be of very low expression by flow cytometry (Figure 1G). In addition, all DPSCs obtained from groups A and B expressed stem cell markers (Rex-1 and Oct-4) and the differentiation marker Nestin upon western blot analyses (Figure 1H1, H2). Finally, the hDPSCs had a high proliferation rate during the early passages, but this rate reduced steadily in culture. At passage 3, hDPSCs of group A proliferated at 14.8±6.1-fold/72 h, and by passage 6 the rate had decreased to 3.8±1.1-fold/72 h, whilst hDPSCs of group B proliferated at 15.5±8.2-fold/72 h, decreasing to 3.9±1.3-fold/72 h by passage 6. Comparison of the proliferation rates of groups A and B revealed no statistically significant difference (MANOVA, $p>0.05$).

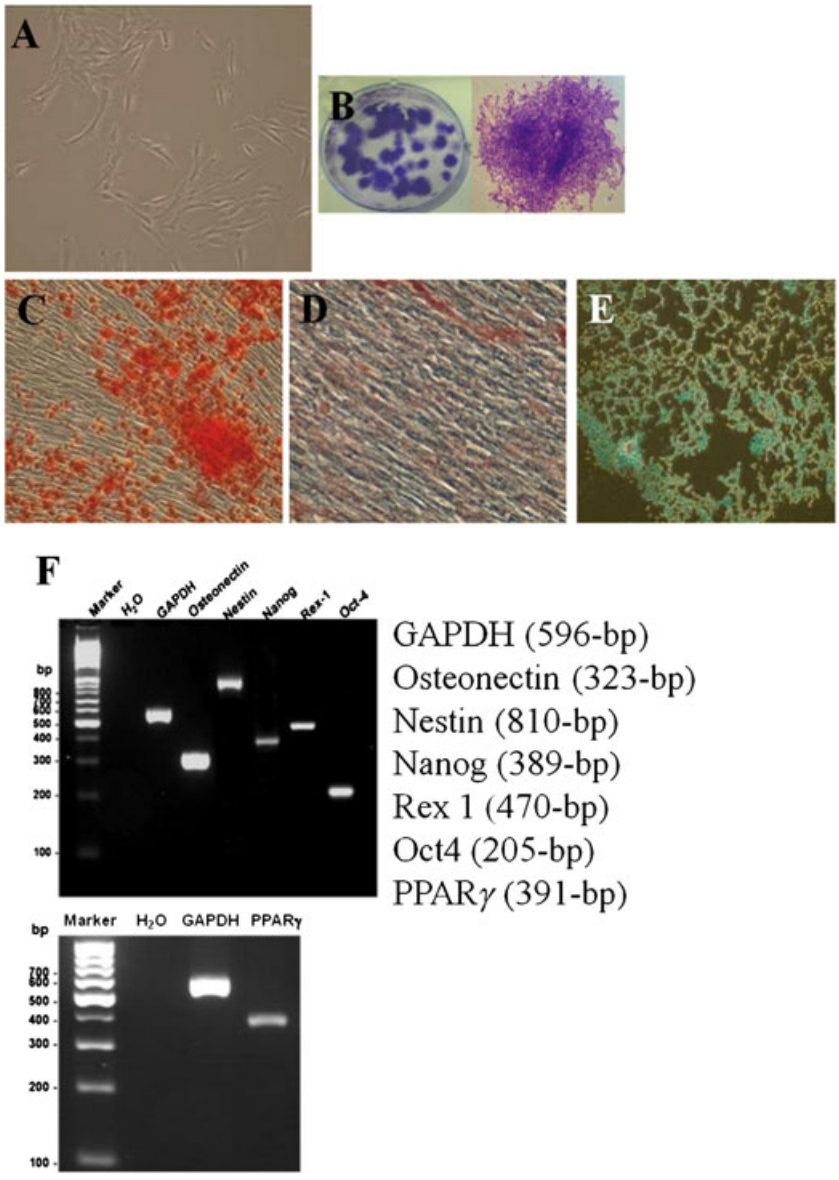

Figure 1. Stem cell characterizations: representative sample(s) of group A (liquid nitrogen-stored dental pulp tissues). (A) A homogeneous population of spindle-shaped cells (7 day culture) was obtained (×100). (B) Colony-forming units of hDPSCs; (left) naked eye, (right) ×40. (C) hDPSCs expressed osteogenic differentiation with alizarin red S staining (×100). (D) Adipogenic differentiation with oil red O staining (×100). (E) Chondrogenic differentiation with Alcian blue staining (×40). (F) RT–PCR of isolated hDPSCs revealed stem-cell (*Oct-4*, *Nanog* and *Rex-1*) and differentiation markers (*Osteonectin*, *Nestin* and *PPARγ*). (G) Flow cytometry of the isolated hDPSCs revealed high expression of CD29, CD90 and CD105 and low expression of CD14, CD34 and CD45. (H1) Western blot analyses revealed stem-cell markers (Rex-1 and Oct-4) and differentiation marker (Nestin); similar results were obtained for group B (freshly-derived dental pulp tissues) (H2)

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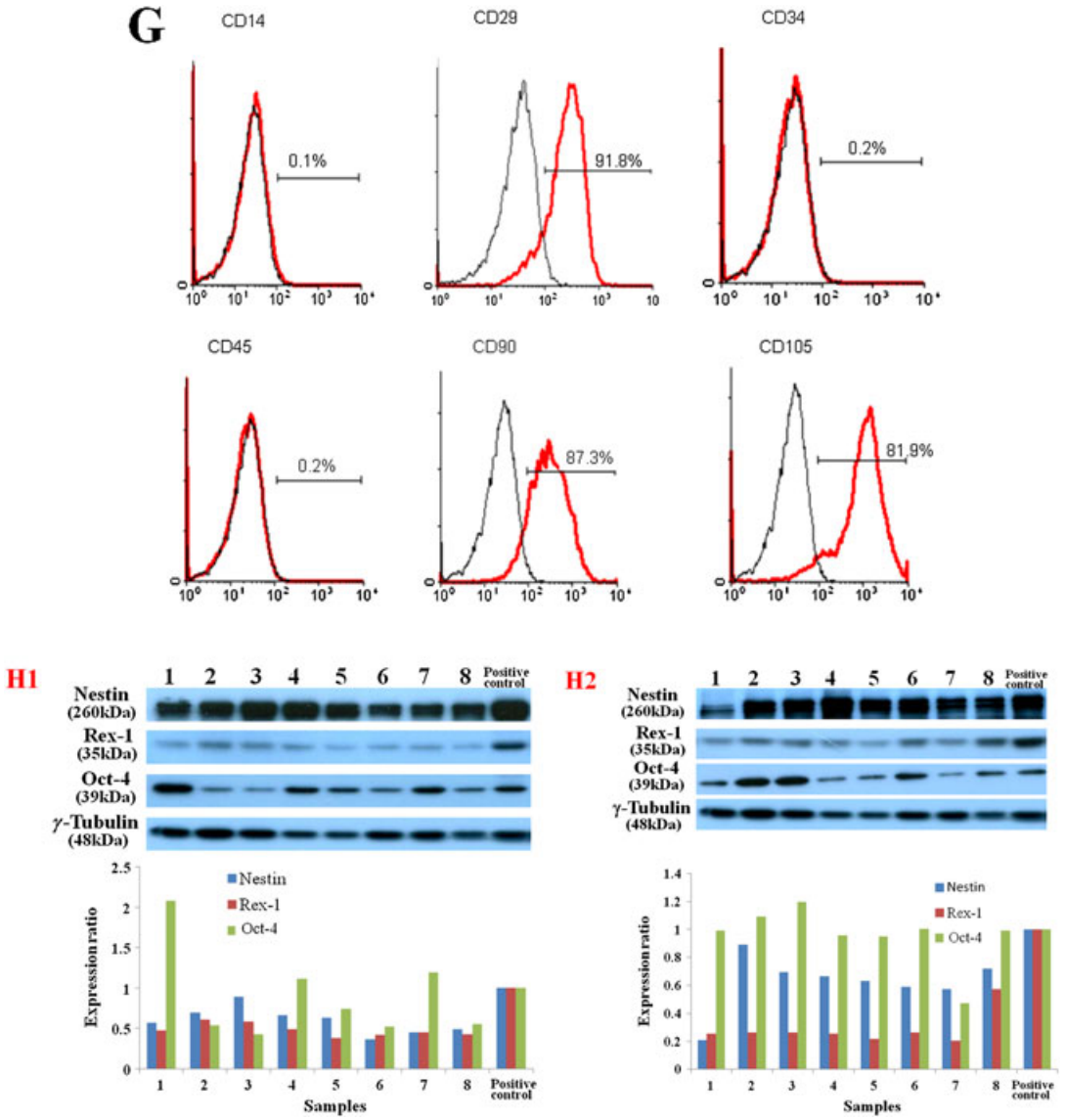

Figure 1. (Continued)

3.2. Hepatic differentiation

After the cell cultures had been exposed to differentiation media for 14 days, polygonal-shaped cells were observed to have clustered together in each of the samples of groups A and B, respectively. The morphology of the cells in the clusters of isolated hDPSC cultures changed from spindle-shaped to polygonal, starting from 14 days and still observed up to 42 days (Figure 2A); in contrast, the morphology of the hepatic negative controls remained unchanged when observed up to 42 days (Figure 3A1).

For all samples of groups A and B at different durations of differentiation, the expression of hepatic metabolic function genes and liver-specific genes by differentiated hDPSCs was positive (Figure 2B1, B2), as was the case for the HCC cell line serving as the positive control for hepatic differentiation (Figure 3B2). On the other hand, the hepatic negative controls demonstrated negative findings when observed up to 42 days (Figure 3B1).

Moreover, for all samples of groups A and B, glycogen storage in the hepatic differentiated hDPSCs cells was assessed by PAS reaction, and positive staining was noted from day 7 of differentiation-medium culture and could still be observed up to day 42 (Figure 2C). Positive immunofluorescence staining of LDL (Figure 2D) and Alb (Figure 2E) was observed from day 14 of differentiation-medium culture and could still be observed up to day 42, while the undifferentiated hDPSCs of hepatic negative controls were negative for PAS staining (Figure 3C1) and immunofluorescence staining for LDL (Figure 3D1) and Alb when observed up to day 42 (Figure 3E1). Significantly, the positive results for PAS, LDL and Alb staining of the hepatic differentiated hDPSCs of groups A and B were all consistent with the data of the HCC cell line (Figure 3C2–E2) that served as the positive control for hepatic differentiation.

On the other hand, we found that urea production in the medium in the hepatic differentiated hDPSCs of groups A and B was noted from week 6 compared with

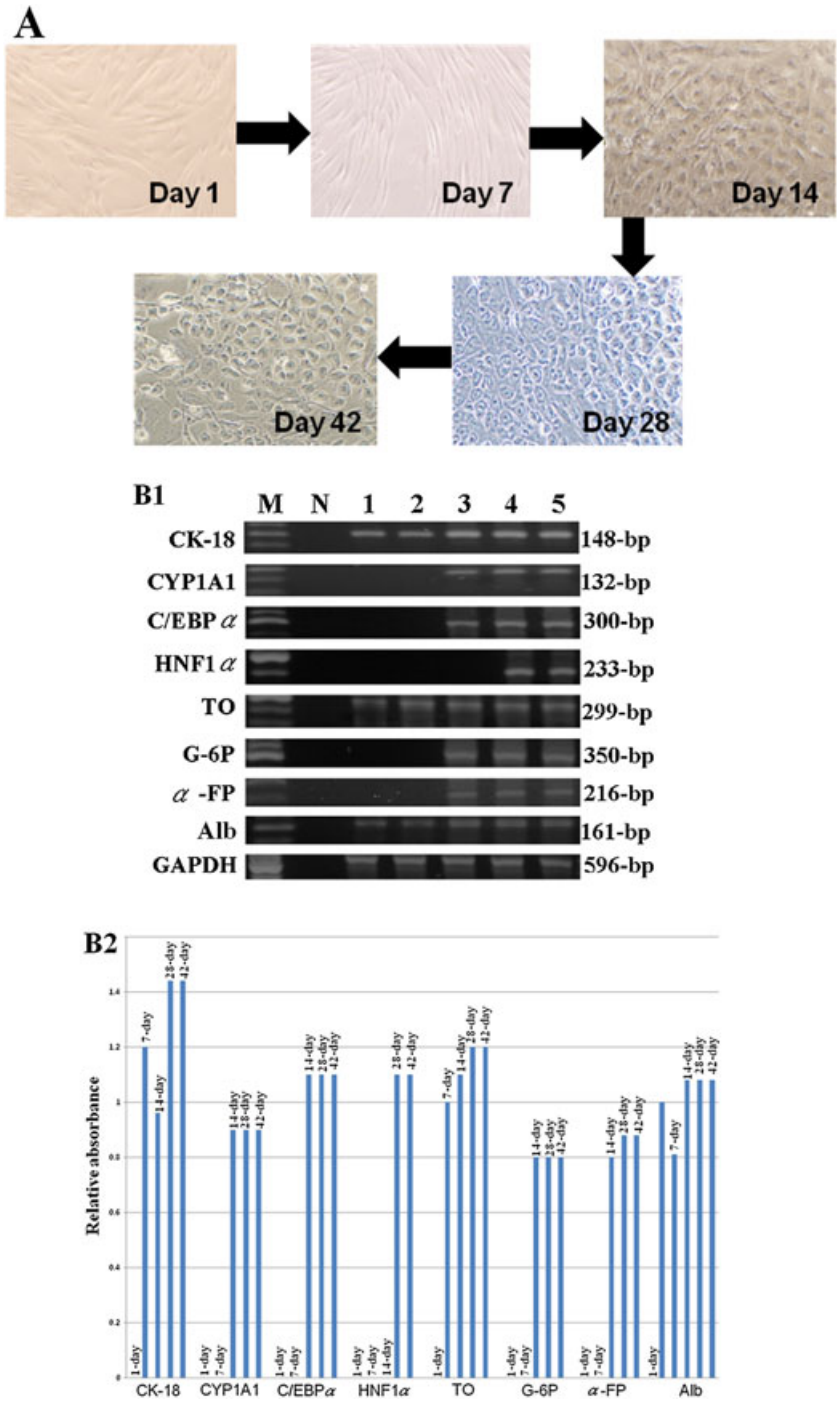

Figure 2. Hepatic characterizations: representative sample(s) of group A (liquid nitrogen-stored dental pulp tissues). (A) Morphological change of human dental pulp stem cells (hDPSCs) upon hepatic differentiation was noted 14 days after the cell cultures were exposed to differentiation media and still be observed up to 42 days (×200). RT–RCR (B1) and relative resorbance (B2): M, molecular weight marker; N, H_2O; lane 1, 1 day; lane 2, 7 days; lane 3, 14 days; lane 4, 28 days; lane 5, 42 days. (C) Periodic acid–Schiff staining for glycogen; hepatic differentiated hDPSCs revealed positive staining starting from day 7 of differentiation medium culture and these could still be observed up to 42 days (×100). (D) Immunofluorescence staining for low-density lipoprotein; hepatic differentiated hDPSCs revealed positive staining, starting from day 14 of differentiation-medium culture and these could still be observed up to 42 days (×200). (E) Immunofluorescence staining for albumin; hepatic differentiated hDPSCs revealed positive staining, starting from day 14 of differentiation-medium culture, and these could still be observed up to 42 days (×200). (F) Mean urea concentrations of all the cryopreserved samples in the different weeks of differentiation (blue line): urea production by hepatic differentiated hDPSCs was noted, starting from week 6 of differentiation-medium culture, and increased as observed up to week 8; the HCC cell line also demonstrated positive findings for urea assay (red line). (G) Western blot revealed hepatocyte nuclear factor (HNF) 4α expression in both sample and control [a well-differentiated hepatocellular carcinoma (HCC) cell line (BCRC 60169, Food Industry Research and Development Institute)]. (H) Genetic analysis revealed 100% normal karyotype. Similar results were obtained for group B (freshly-derived dental pulp tissues)

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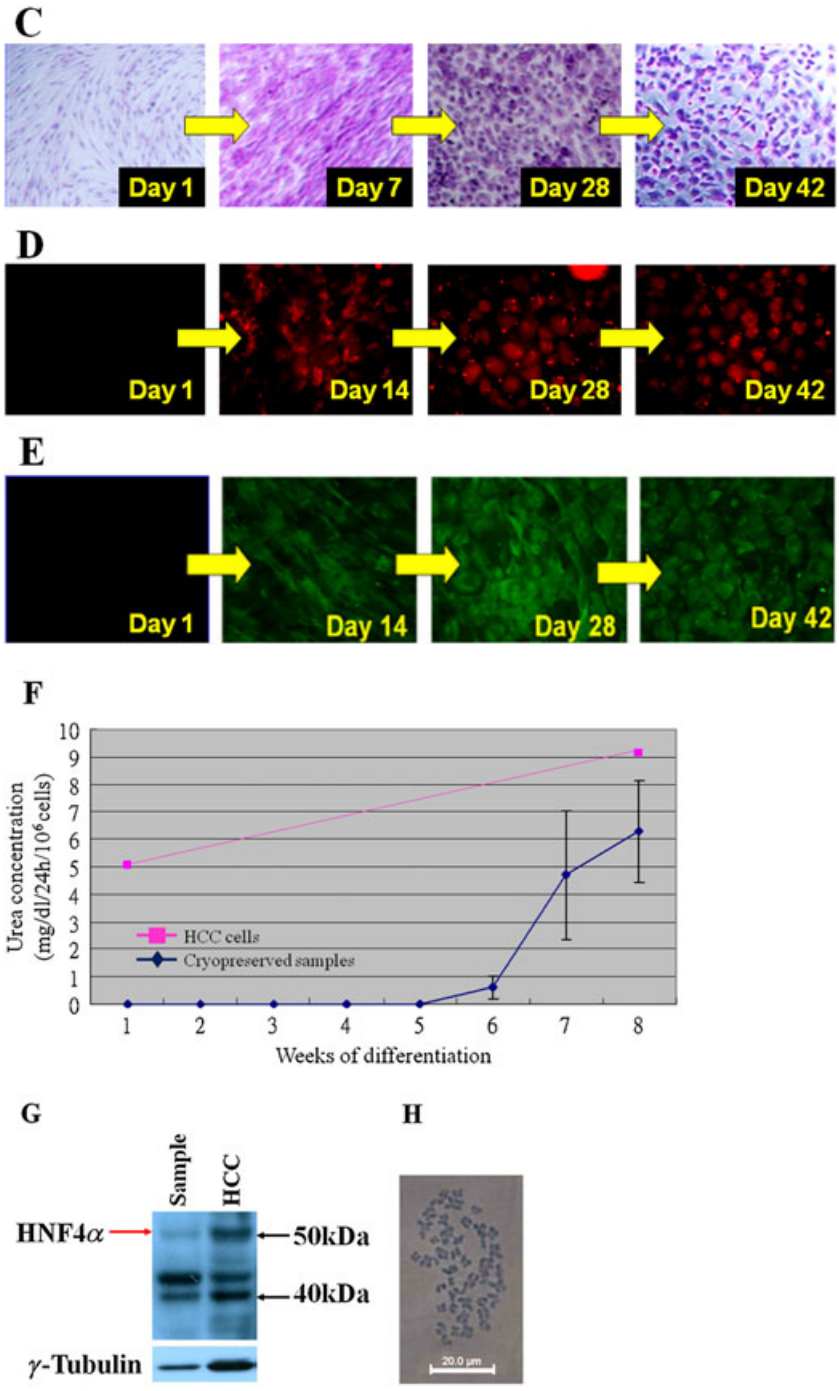

Figure 2. (Continued)

fresh medium supplemented with the same additives, and increased as observed up to week 8 (Figure 2F); statistical significances were noted for comparison of the following pairs: week 8 vs weeks 0–5; week 8 vs week 6; week 7 vs weeks 0–5; week 7 vs week 6; but no statistical significances were observed for comparison of the following pairs: week 8 vs week 7; weeks 0–5 vs week 6. Negative findings for the urea assay were noted for the samples of hepatic negative controls when observed up to week 8 (data not shown), whereas, for the positive control, urea concentrations of 5.04 mg/dl/24 h/10^6 cells and 9.12 mg/dl/24 h/10^6 cells were observed at weeks 1 and 8, respectively (Figure 2F). Finally, HNF4α (Figure 2G) and normal karyotype (Figure 2H) were demonstrated for the hepatic differentiated hDPSCs of groups A and B, respectively.

4. Discussion

Although hDPSCs derived from healthy third molar tooth germs (Ikeda *et al.*, 2008) and deciduous teeth have been observed to be differentiated into hepatic-like cells (Ishkitiev *et al.*, 2010), the first significance of the current

study is that we further extend these previous findings (only obtained in freshly extracted healthy tooth/tooth germ) (Ikeda *et al*., 2008; Ishkitiev *et al*., 2010) to successfully demonstrate the hepatic differentiation of hDPSCs from liquid nitrogen-cryopreserved dental pulp tissues obtained from vital extracted teeth even with disease. Indeed, we had limited experience of obtaining positive outcomes for six samples that had been cryopreserved for>1 year (unpublished data). Since the possibility of extracting vital teeth with disease has always been encountered in daily clinical dental practice, the positive outcome of the current study could provide a novel avenue to increase the potential source of hepatic-like cells in future.

Additionally, the findings of Ikeda *et al*. (2008) and Ishkitiev *et al*. (2010) were more fully confirmed, using a number of additional hepatic cell markers in the present study. After hepatic differentiation, markers αFP, Alb, C/EBPα, HNF1α and especially *CYP1A1* (a key enzyme of the xenobiotic effect of hepatic cells), as well as HNF4α (a key hepatic developmental associated transcription factor for MSC-to-hepatic-like cell conversion), were found to be expressed in cell cultures derived from the present study cohort, indicating that, as reported for MSCs isolated from human bone marrow and umbilical cord blood (Tang *et al*., 2006), the dental pulp tissues of vital extracted teeth with disease have the potential to differentiate into hepatic-like cells, even after cryopreservation. Furthermore, positive urea production and positive glycogen storage in hDPSC-derived hepatic-like cells obtained from cryopreserved dental pulp tissues of vital extracted teeth with disease are

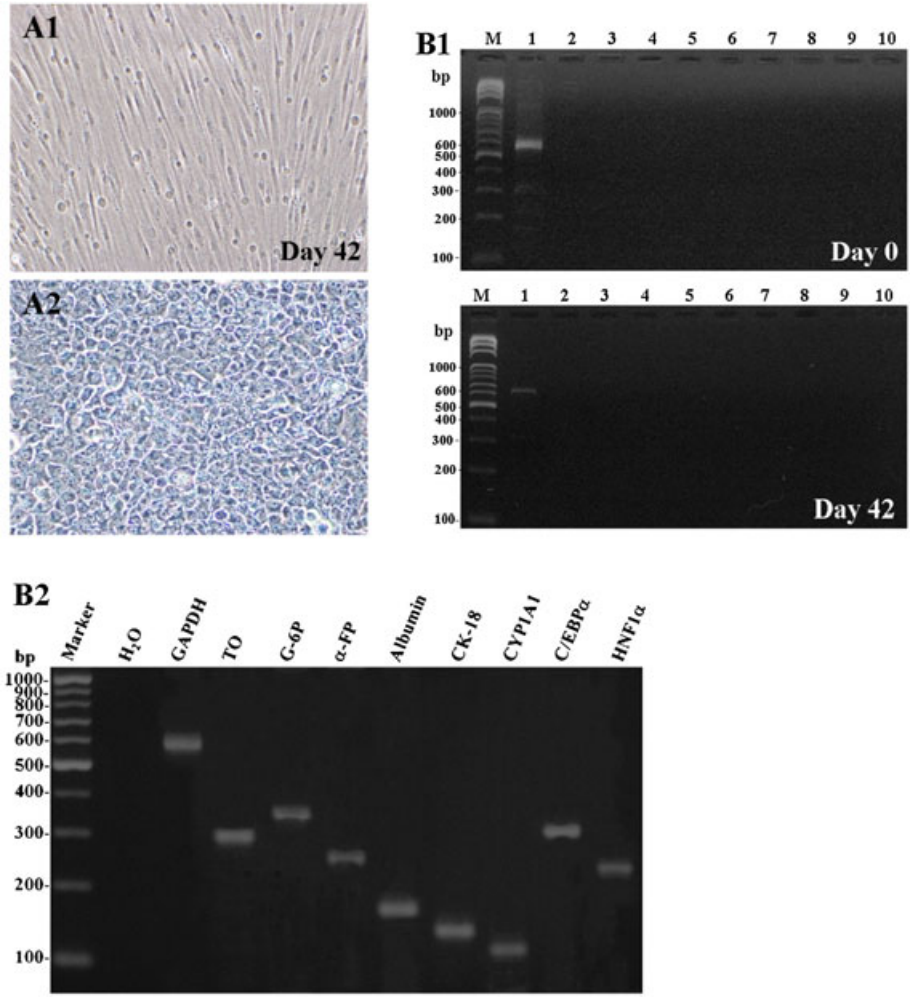

Figure 3. Hepatic differentiations of negative controls and positive control. (A1) Morphology of undifferentiated hDPSCs (negative control) remained unchanged, as observed up to 42 days (×100). (A2) A well-differentiated hepatocellular carcinoma (HCC) cell line (BCRC: 60169, Food Industry Research and Development Institute) used as the positive control for hepatic differentiation (×200). (B1) Undifferentiated hDPSCs demonstrated negative findings upon RT–PCR of various hepatic markers, as observed up to 42 days: M, molecular weight marker; lane 1, GAPDH (596 bp); lane 2, H_2O; lane 3, α-fetoprotein; lane 4, albumin; lane 5, cytokeratin-18; lane 6, tryptophan 2,3-dioxygenase; lane 7, glucose 6-phosphatase; lane 8, CCAAT/enhancer-binding proteins α; lane 9, hepatocyte nuclear factor-1α; lane 10, cytochrome P450 family 1, subfamily A, polypeptide 1]. (B2) RT–PCR for various hepatic markers were positive for the HCC cell line. (C1) Periodic acid–Schiff staining for glycogen; undifferentiated hDPSCs demonstrated negative findings when observed up to 42 days (×100). (C2) Periodic acid–Schiff staining for glycogen was positive for the HCC cell line (×100). (D1) Immunofluorescence staining for low-density lipoprotein; undifferentiated hDPSCs demonstrated negative findings when observed up to 42 days (×200). (D2) Immunofluorescence staining of low-density lipoprotein uptake was positive for the HCC cell line (×200). (E1) Immunofluorescence staining for albumin; undifferentiated hDPSCs demonstrated negative findings observed up to 42 days (×200); (E2) Immunofluorescence staining of albumin was positive for the HCC line (×200)

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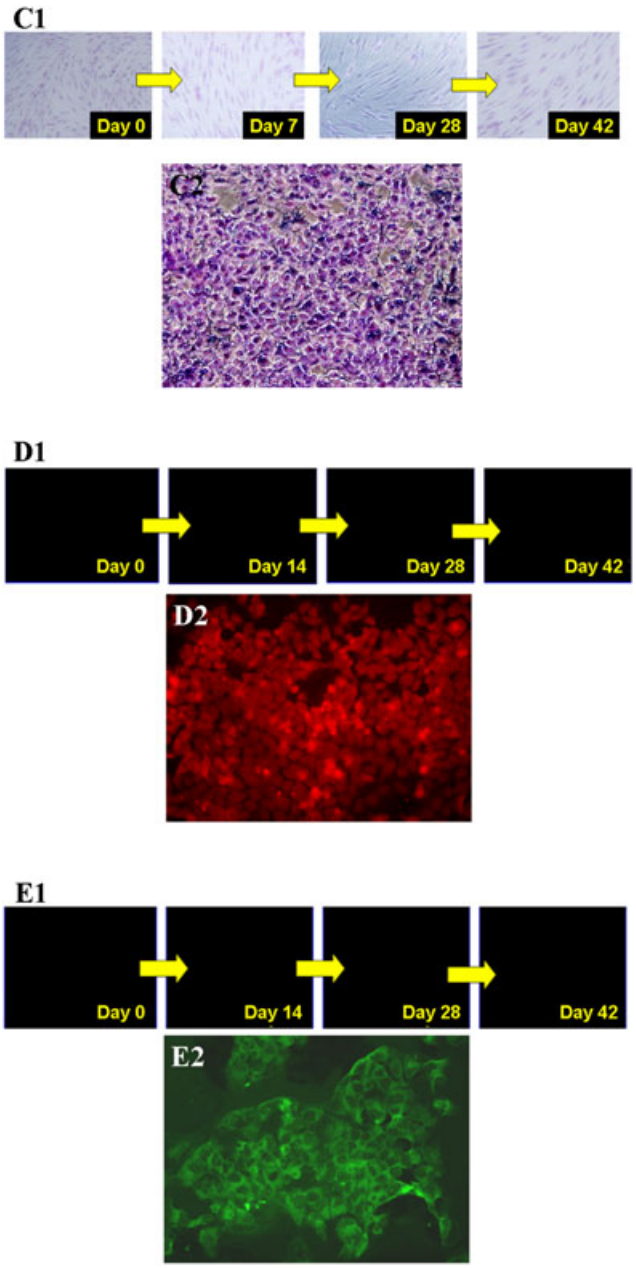

Figure 3. (Continued)

demonstrated in the current study. Additionally, we observed a significant increase in urea production as compared between 6 and 7 weeks of differentiation, but a non-significant increase between 7 and 8 weeks of differentiation, perhaps indicating that the hepatic differentiation of the cryopreserved DPSCs have nearly approached a peak value at 8 weeks of differentiation. On the other hand, the urea concentrations of the current study were observed to be compatible with the values expressed by induced pluripotent stem cells (iPS)-derived hepatic cells in the recent study of Ghodsizadeh *et al.* (2010). Therefore, in summary, the second significance of our study is that the differentiated cells of the present cohort appear to be functionally close to normal hepatic-like cells.

Hepatocyte transplantation is one of the therapeutic options for the treatment of patients with hepatic disorders, including cirrhosis and HCC. Nonetheless, the source of hepatocytes is restricted by the scarcity of appropriate liver donors and, moreover, hepatic transplantation could induce injury to both donor and recipient. Thus, the development of substitute resources of hepatocytes, such as stem cells, is an important area of research. With normal karyotyping of the differentiated cells of the present cohort as well as using appropriate hepatic differentiation positive and negative controls, the third significance of the current study is that we demonstrate that normal karyotype, hepatic-like cells are derived from cryopreserved dental pulp tissues of extracted vital teeth with disease. Hence, taken altogether, this study provides evidence that hDPSCs from cryopreserved dental pulp tissues of vital extracted teeth with disease could be one of the potential candidates of hepatic-like cells.

Finally, to conclusively demonstrate the potential application of hDPSC-derived hepatic-like cells, the drawback that the cell proliferation has been declined with passage should be dealt with. In addition, hDPSCs constitute a minor population of total dental pulp cells and, depending on the quantity of dental pulp tissues collected, a range of 6–20 cells were able to be isolated from one dental pulp tissue in the current study. Hence, cell quantity would be a key issue in spite of excellent proliferation capacity. Nonetheless, our experiments show a new avenue for further investigations, not only to understand the mechanisms involved but also to select precisely the subpopulations of hepatic-like cells which are then expanded *in vitro* to obtain a sufficient quantity of differentiated cells for future potential clinical applications.

Overall, the results obtained from this study allow us to conclude that hDPSCs can not only give rise to hepatic-like cells from the isolated dental pulp tissues of vital extracted teeth with disease (even after cryopreservation of the dental pulp tissues, followed by digestion and culture post-thawing) but also that the differentiated cells possess normal karyotype and are functionally close to normal hepatic-like cells. We believe that if, in future, the issue of hDPSCs being a minor population of total dental pulp cells can be manipulated and a sufficient number of functional hepatic-like cells could be obtained from the dental pulp tissues, then the use of hDPSCs derived from cryopreserved dental pulp tissues of vital extracted teeth with disease as a possible source for autogenic transplantation for tissue engineering-based approaches for the treatment of hepatic conditions would not be impossible.

Conflict of interest

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

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