

Original article

Human dental pulp stem cells as a potential feeder layer for human embryonic stem cell culture

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Background: Human embryonic stem (hES) cells are pluripotent, and can differentiate into three germ layers. Traditionally, cultures of hES cells are maintained in a system containing mouse embryonic fibroblasts as a feeder layer for support of undifferentiated growth. However, contamination by animal cells limits the use of hES cells.

Objective: We evaluated the use of human dental pulp stem cells (hDPSCs) as a feeder layer for hES cell culture. It should be possible to obtain a new source of human mesenchymal stem cells for feeder cells to maintain undifferentiated growth of hES cells.

Methods: hDPSCs from removed impacted wisdom teeth (third molars) were extracted, cultured, and characterized for mesenchymal stem cell properties. Furthermore, hDPSCs were used as a feeder layer for culturing Chula2 and Chula5 hES cell lines. Finally, hES cell lines grown on hDPSCs feeders were examined embryonic stem cell properties.

Results: We found that hDPSCs, which have mesenchymal properties, can support undifferentiated growth of hES cell lines. After prolonged culture (passage 17), these hES cell lines still maintain ES cell properties including typical morphology seen in hES cells, the expression of pluripotency markers (Oct4, Sox2, Nanog, Rex1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81), embryoid body formation and retention of a normal karyotype.

Conclusion: hDPSCs, derived from the pulp tissue of impacted third molars, are a potential source of human feeder cells for the culture of undifferentiated hES cells.

Keywords: Dental Pulp Stem Cell, human embryonic stem cell, human feeder layer, pluripotent

Embryonic Stem (ES) cells are pluripotent and can maintain undifferentiated proliferation and potentially develop into all three embryonic germ layers after prolonged culture [1-4]. Human ES (hES) cells are initially derived from the culture of inner cell masses of in vitro fertilized embryos in the blastocyst stage [1]. Recently, derived hES cell lines have been used for studies [5-7]. As distinct from hES cells, mouse ES cells can be maintained in prolonged growth in an undifferentiated state cultured on gelatin-coated plates together with addition of exogenous leukemia inhibitory factor (LIF) [8-10]. Prolonged culture of hES cells needs to maintain undifferentiated growth

based on mouse embryonic fibroblasts for feeder layer cells [1]. In turn, the use of animal feeder layers is associated with contamination of hES cells with animal cells, which limits the use of hES cells in clinical studies. At present, many studies have reported conditions for culture of hES cells based on human feeder layers including human fetal muscle cells and fetal skin cells [11], human foreskin fibroblasts [12, 13], human adult marrow cells [14], human placenta derived-cells [15], human amniotic mesenchymal cells [16], and human ES cell derived-mesenchymal stem cells [17]. Moreover, feeder free conditions such as conditioned medium [18, 19] and defined culture medium are reported to maintain undifferentiated growth [20-24].

Bone marrow-derived mesenchymal stem cells (hMSCs) can be used as a source of human feeder

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layer cells [14]. It has been shown that these cells produce many essential cytokines required for long-term cultures of ES cells including TGF- β and IGF2 [25]. Therefore, it should be possible to obtain a new source of hMSCs for feeder cells to maintain undifferentiated growth of hES cells. Human dental pulp stem cells (hDPSCs) were isolated from pulp tissues of various types of teeth [26-29]. The studies also revealed that dental pulp derived-stem cells have similar attributes to mesenchymal stem cells (MSCs) including the expression of protein markers and the ability to differentiate into various types of cells such as odontocytes, osteocytes, adipocytes, and neurons [30-34]. Among various types of teeth used for human dental pulp cell isolation, impacted third molars, which are considered biological waste, are a relatively easily acquired source of mesenchymal stem cells [35, 36].

We isolated and characterized hDPSCs from removed impacted wisdom teeth (third molars). Then, we evaluated the undifferentiated growth of two hES cell lines, Chula2 and Chula5 [37] on hDPSCs feeder layers. The results showed that hDPSCs can support the undifferentiated growth of hES cells in prolonged culture. We found that hDPSCs are a novel potential source of human feeder cells for culture of undifferentiated hES cells.

Materials and methods

Collection of hDPSCs

Impacted wisdom teeth (third molars) were removed and collected from patients at the dental department of Lerdsin General Hospital under the approval of Lerdsin General Hospital's ethics committees. All participants who donated impacted third molars for this study provided their signed written informed consent. The procedure of stem cell isolation was modified from that previously described [26]. Briefly, pulp tissue was removed from the pulp chamber using sterile technique and enzymatically digested with a solution of 3 mg/mL collagenase type I (Gibco, Grand Island, NY, USA) for 1 h at 37°C. The digestion was stopped by adding 5 mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin G-streptomycin and centrifuged at 1200 rpm for 10 min at 4°C. The cell pellet was resuspended in the same culture medium and then plated into a 10 cm petri dish. The cell culture was incubated in an atmosphere of air with 5% CO₂ at 37°C. The culture medium was changed every three days until the cells became

confluent.

Flow cytometry analysis of hDPSCs

For phenotypic characterization of hDPSCs, cells were harvested and washed in PBS buffer. Then, 10⁶ cells were resuspended in 100 μ L PBS and incubated with FITC-, PE-, or PE-Cy 7 conjugated monoclonal antibodies against CD 34, CD 45, HLA-DR, HLA-ABC, CD79a, CD 29, CD 33, CD 44, CD 73, CD 90, and CD 11b for 30 min at room temperature.

All antibodies were obtained from BD Biosciences, San Diego, USA. After incubation, excess antibodies were washed off by adding phosphate-buffered saline (PBS) and centrifugation at 2000 rpm for 5 min. Antibody incubated-cells were resuspended in 200 μ L fluorescence-activated cell sorting (FACS) solution and flow cytometry was performed by using a FACS Calibur (BD Biosciences, San Diego, USA). The data were analyzed with Cell Quest Pro software (BD Biosciences).

Differentiation of hDPSCs

We studied hDPSCs for their potential to differentiate to mesenchymal stem cells by using the human MSC functional identification kit (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, 3.7 \times 10⁵ cells were cultured in α minimal essential medium (α MEM; Gibco) supplemented with 10% FBS, 100 units/mL penicillin G-streptomycin until reaching 100% confluency. Adipogenic or osteogenic differentiation medium was used to replace spent α MEM in each plate to induce adipogenesis or osteogenesis twice a week for 3 weeks. For chondrogenic differentiation, 2.5 \times 10⁵ cells were transferred to 15 mL conical tubes and centrifuged at 200 \times g for 5 min at room temperature. After discarding the supernatant, cells were resuspended in chondrogenic differentiation medium and centrifuged at 200 \times g for 5 min at room temperature. The cap of the tube was loosened to allow gas escape and chondrogenic differentiation medium was replaced twice weekly for 3 weeks. Immunohistochemistry of adipocytes, osteocytes, and chondrocytes was determined with anti-FABP-4, anti-human osteocalcin, and anti-human aggrecan respectively to confirm the differentiation. Lipid vacuoles and mineral matrix were also stained with Oil Red O and alizarin red (Sigma Chemical Co., St. Louis, MO, USA) to distinguish adipocytes and osteocytes respectively.

Feeder cell preparation

Commercial human foreskin-derived fibroblasts (hFFs; CRL-2429, American Type Culture Collection, Manassas, VA, USA) were cultured and maintained according to standard protocols as previously described [37]. To use hFFs and hDPSCs as the feeder layer, confluent hFFs and hDPSCs were inactivated with 10 μ g/mL mitomycin C (Sigma-Aldrich, St. Louis, MO, USA) for 3 hours, dissociated with 0.05% trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) and plated on a 0.1% gelatin coated-dish (Sigma-Aldrich) at a density of 5×10^4 cells/cm².

Culture of hES cells

Chula2 and Chula5 hES cell lines [37] were cultured on either HFFs or hDPSC feeders in the hES cell culture medium consisting of knockout DMEM supplemented with 20% knockout serum replacement (KSR), 1% Glutamax, 1% nonessential amino acids, 1% penicillin–streptomycin, 0.1 mM 2-mercaptoethanol (all from Invitrogen), and 8 ng/mL basic fibroblast growth factor (bFGF; R&D Systems).

For propagation of hES cells, colonies of hES cells were mechanically cut into small pieces every 5–7 days using a 23G needle, detached from the culture dish and plated onto a new feeder layer. The culture medium was changed daily.

Immunohistochemical analysis of hES cells

Immunohistochemistry for pluripotency marker expression of hES cells was determined. Cells were rinsed briefly in PBS and fixed with 4% formaldehyde for 15 min at room temperature. After washing three times in PBS for 5 min each, nonspecific-binding sites on cells were blocked with 5% normal goat serum in PBS, 0.1% Tween 20 for 30 min and incubated with 1:200 anti-human Oct-4, SSEA-3, SSEA4, TRA-1-60, TRA-1-81, α -fetoprotein (AFP), Brachyury (Bry), and nestin in 1% bovine serum albumin (BSA) in PBS, 0.1% Tween 20 overnight at 4°C. All primary antibodies were from Cell Signaling (Danvers, MA, USA). Subsequently, they were incubated with biotinylated secondary antibodies diluted with Alexa Flour 488-conjugated avidin (Invitrogen) for 30 min. Images were obtained using a fluorescence microscope (Olympus DP 71, Tokyo, Japan).

Reverse transcription–polymerase chain reaction

hES cells were separated from feeder cells by mechanically cutting colonies with 23G needle. Total RNA was isolated from hES cells with PureZol

(Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol as previously described [38]. cDNA synthesis was conducted with 1 μ g of isolated RNA using a Protoscript first strand cDNA synthesis kit (New England Biolabs, Ipswich, MA, USA). One microgram of cDNA was used for the polymerase chain reaction (PCR) with the corresponding primers Oct4: 5'-GGAAGGTATTTCAGCCAAACG-3', 5'-CCCTGAGAAAGGAGACCCA-3', Sox2: 5'-CCAGCGCATGGACAGTTAC-3', 5'-TGGAGTGGGAGGAA GAGGTA-3', Nanog: 5'-ACCAGTCCCAAAGGCA AAC-3', 5'-GAGTAAAGGCTGGGGTAGGTAG-3', Rex1: 5'-AACGGGCAAAGACAAGACAC-3', 5-TCCAAAGAACATTCAAGGGAG-3', and β -actin: 5'-CTTTCTACAATGAGCTGCGTG-3', 5'-TCATGAGGTAGTCTGTCAGG-3' with a Stratagene Mx3000P PCR system (Stratagene, La Jolla, CA, USA) and *EXTaq* DNA polymerase (Takara, Shiga, Japan). The PCR products were electrophoresed and visualized under UV light.

Embryoid body (EB) formation and spontaneous differentiation

To test the differentiation potential of hES cells, undifferentiated colonies were manually cut into small pieces, picked up and cultured in ultra-low adhesion culture dishes (Corning) as previously described [39]. Differentiation medium comprised of knockout DMEM supplemented with 20% KSR, 2mM L-glutamine, MEM nonessential amino acids, 100 units/mL penicillin G–streptomycin (all media components from Gibco, Grand Island, NY, USA), and 0.1 mM 2-mercaptoethanol (Sigma-Aldrich) without bFGF. After 7–14 days, some EBs were transferred to gelatin-coated dishes and cultured further for spontaneous differentiation.

Karyotype analysis of hES cells

G-banding technique was used to determine the karyotype of hES cells. Briefly, undifferentiated hES cells were incubated with 10 ng/mL of karyoMAX colcemid (Invitrogen) for 3 hours at 37°C, 5% CO₂. The cells were then trypsinized, treated with 0.075% of KCl solution (Biological Industries, Kibbutz Beit-Haemek, Israel) and fixed (3:1 of methanol: acetic acid). Metaphases were spread on microscope slides and stained by using a standard G banding technique. Chromosomes were classified according to the International System for Human Cytogenetic Nomenclature (ISCN). Approximately 15–20 metaphases were analyzed.

Results

hDPSCs expressed fibroblast-like morphology and protein markers of mesenchymal stem cells and differentiated into adipocytes, osteocytes and chondrocytes

After plating the cell suspension of the digested human dental pulp to the culture, the primary cells grew as clonogenic at day 7 (**Figure 1a**) and reached confluence around day 14 with a fibroblast-like

morphology (**Figure 1b**). To determine the protein marker expression of hDPSCs, flow cytometric analysis was performed. As previously reported, hDPSCs expressed mesenchymal protein markers including CD29, CD44, CD73, CD90, and HLA-Dr, but other protein markers such as CD11b, CD34, D45, CD79a, and HLA-ABC were not expressed in these cells (**Figure 1c**).

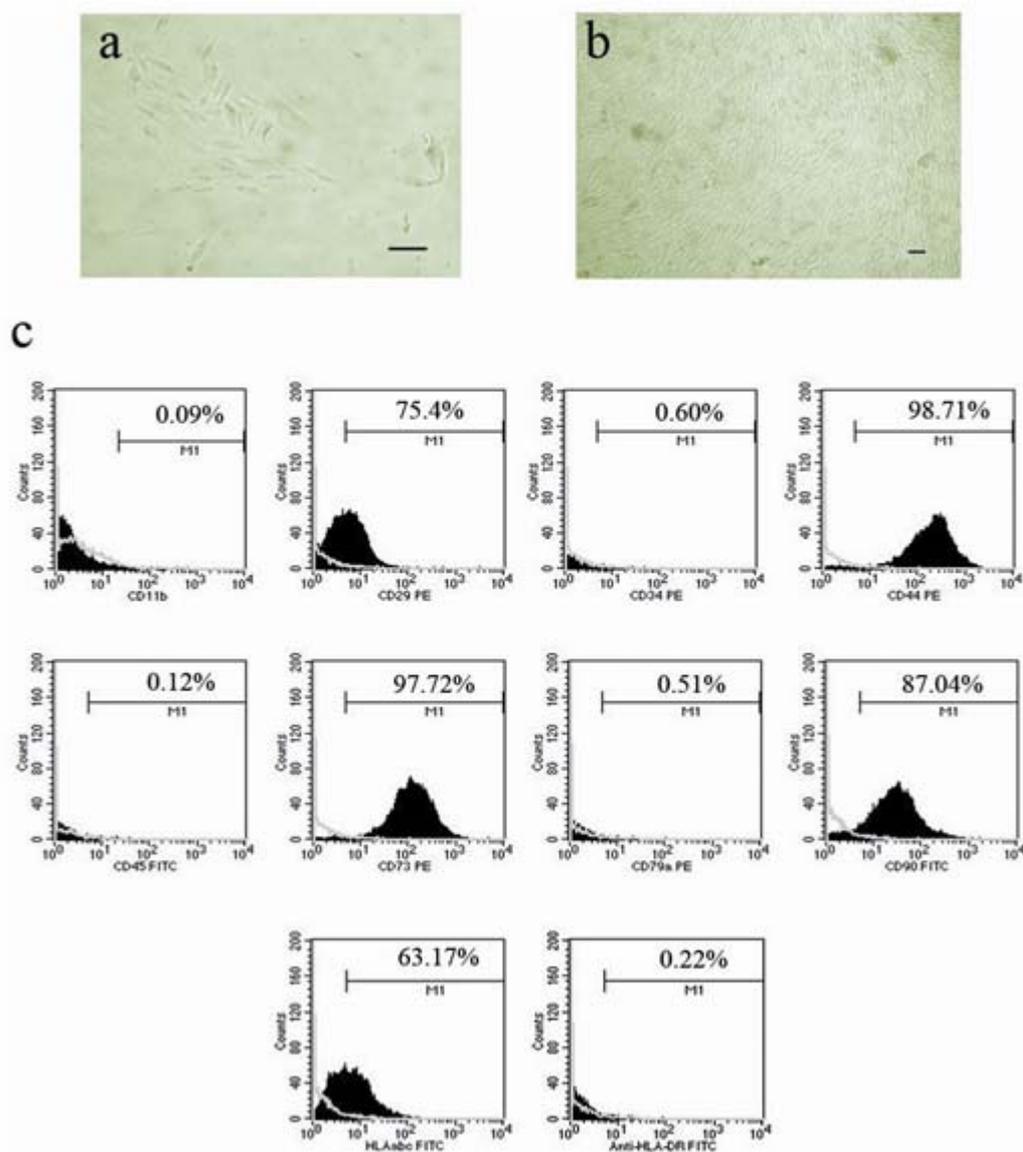


Figure 1. Morphology and surface marker expression of human dental pulp stem cells (hDPSCs). After plating enzymatically digested dental pulp on 10 cm petri dishes, hDPSCs grew as clonogenic formations at day 7 (a). They reached confluence in a monolayer with fibroblast-like morphology at day 14 (b). Flow cytometric analysis showed that hDPSCs expressed mesenchymal protein markers, CD29, CD44, CD73, CD90 and HLA-Dr, but not CD11b, CD34, CD45, CD79a, or HLA-ABC (c). Scale bars: (a) 50 μ m and (b) 100 μ m.

hDPSCs induce differentiation into adipocytes, osteocytes and chondrocytes

The differentiation potency of hDPSCs was also tested to determine the ability of hDPSCs to differentiate into multilineage cell types. After three weeks in each induction medium, the differentiated cells were confirmed by staining with specific antibodies and dyes. We could detect the expression of FABP-4 (**Figure 2a**), osteocalcin (**Figure 2c**), and aggrecan (**Figure 2e**) from adipocytic, osteocytic, and chondrocytic differentiated hDPSCs respectively. Oil Red O and alizarin red staining also showed that lipid droplets (**Figure 2b**) and mineral matrix (**Figure 2d**) were detected in adipocytic and osteocytic differentiated cells. These results confirmed that these cells have the ability to differentiate into adipocytes, osteocytes and chondrocytes.

Culturing hES cells on hDPSCs feeders maintained undifferentiated growth including the

typical morphology and expression of pluripotent markers of hES cells after long-term expansion

Chula2 and Chula5 hES cell lines, which were cultured on hDPSCs, showed typical morphology during cell growth and were studied for expression of pluripotent surface markers (SSEA-3, SSEA-4, TRA-1-60, TRA-1-81) and intracellular marker (Oct4). The hDPSCs had the ability to support the undifferentiated growth of hES cells as the colonies of these cells had a tightly distinct border and monolayer growth. Within a colony that contained tightly packed individual cells, which had large nuclei and dominant nucleoli (**Figure 3a**). The expression of pluripotent cell-specific markers was determined by immunofluorescence staining. The results showed that both hES cells cultured on hDPSCs feeders expressed pluripotent cell-specific markers, Oct-4A, SSEA-3, SSEA4, TRA-1-60, and TRA-1-81 (**Figure 3b**). This condition was maintained during the undifferentiated growth of hES cells for at least 17 passages.

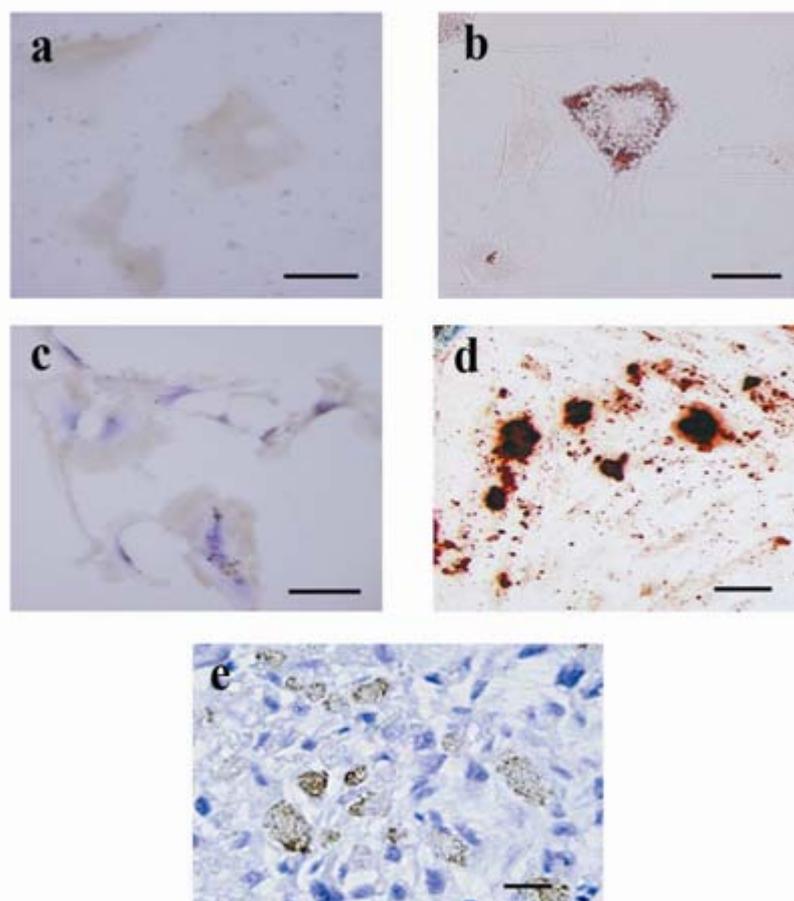


Figure 2. Human dental pulp stem cells (hDPSCs) are able to differentiate into adipocytes, osteocytes, and chondrocytes. Adipocyte differentiated hDPSCs expressed FABP-4 (**a**) and lipid droplets (**b**). Immunohistochemistry and alizarin red staining. This showed that osteocyte-differentiated hDPSCs expressed osteocalcin (**c**) and mineral matrix (**d**). Immunohistochemistry in chondrocytic-differentiated hDPSCs showed expression of aggrecan (**e**). Scale bars: 50 μ m.

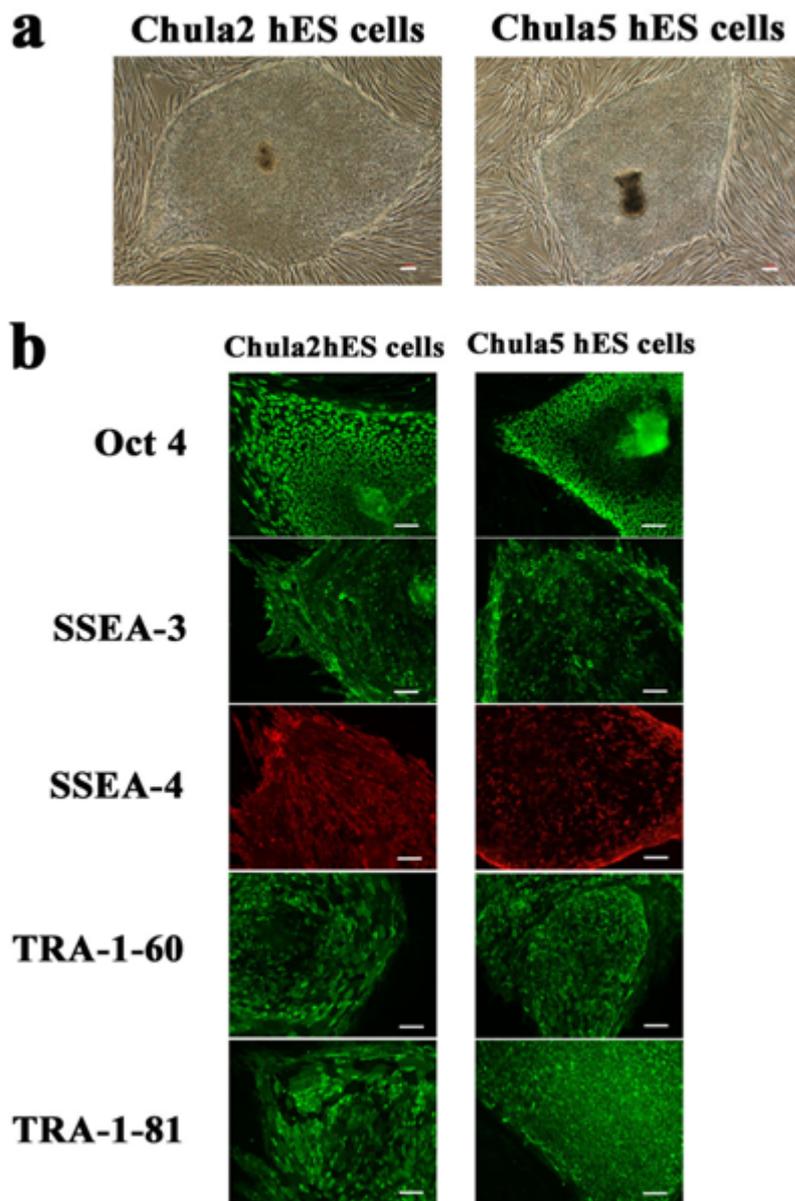


Figure 3. Morphology and pluripotent markers expression of human embryonic stem (hES) cells grown on human dental pulp stem cell (hDPSC) feeders. hES cell lines cultured on hDPSCs maintained the undifferentiated morphology of hES cells as a tightly distinct border and a monolayer growth (a). Immunohistochemical staining also showed that hES cells cultured on hDPSCs expressed pluripotent markers, Oct4, SSEA-3 SSEA-4, TRA-1-60, and TRA-1-81 (b). Scale bars: 100 μ m.

Reverse transcription–PCR analysis also showed that hES cells grown on hDPSCs feeder expressed pluripotent markers

Reverse transcription–PCR analysis was performed to confirm the expression of pluripotent cell-specific marker genes in Chula2 and Chula5 hES cells. Reverse transcription–PCR analysis showed that both hES cell lines cultured on hDPSCs feeders expressed pluripotent cell-specific marker genes

including Oct 4, Sox 2, Nanog, and Rex 1 as compared to hES cell lines cultured on hFFs feeders. However, there were differences in intensity of mRNA expression of both hES cell lines cultured on different feeders. Expression of Oct 4 and Rex 1 mRNA in both hES cell lines was slightly increased when cultured on hDPSC feeders. By contrast, transcription of Sox 2 and Nanog genes was slightly increased when cultured on hFFs feeders (**Figure 4**).

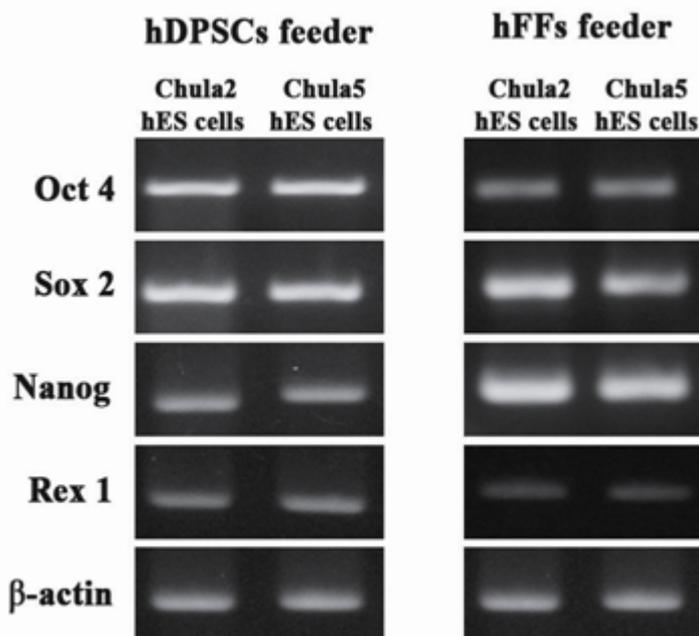


Figure 4. Reverse transcription–PCR analysis showed the expression of pluripotent cell-specific genes in Chula2 and Chula5 human embryonic stem (hES) cells grown on human dental pulp stem cell (hDPSC) feeders. Representative reverse transcription–PCR analysis of pluripotent cell-specific marker genes in Chula2 and Chula5 hES cells cultured on hDPSC feeders, indicating that both hES cell lines cultured on hDPSCs feeder expressed pluripotent cell-specific marker genes including Oct 4, Sox 2, Nanog, and Rex 1. Expression of Oct 4 and Rex 1 mRNA in both hES cell lines was slightly increased when cultured on hDPSC feeder. By contrast, mRNA level of Sox 2 and Nanog was slightly lower than hES cell lines cultured on hFF feeders.

hES cells cultured on hDPSCs feeder had ability to form embryoid body and could spontaneously differentiate

Chula2 and Chula5 hES cell lines cultured on hDPSCs feeder were examined in vitro for differentiation potency. After 7 days in suspension culture without bFGF, the isolated hES cells from the culture on hDPSC feeders at passage 17 had embryoid bodies (EBs) formation (**Figures 5a and f**). Furthermore, after culturing for 2 weeks, EBs could adhere to the plate and become spontaneously differentiated when observed under a microscope (**Figures 5b and g**). Immunocytochemical analysis showed that these cells differentiated into all three germ lines including endoderm (AFP) (**Figure 5c and h**), mesoderm (Bry) (**Figure 5d and i**) and ectoderm (Nestin) (**Figures e and j**).

hES cells cultured on hDPSC feeders maintained normal karyotypes of hES cells cultured on hDPSC feeders

The chromosomal stability of hES cells grown on hDPSCs was seen through conventional karyotyping.

The result showed that both hES cell lines cultured on hDPSCs feeder maintained normal chromosome contents of 46 XY and 46 XX at passage 17 (**Figure 6a and b**).

Discussion

Our work reports a novel adult human feeder source that supports the undifferentiated long-term growth of hES cells. We isolated and characterized hDPSCs derived from the dental pulp of impacted third molars. Previous reports have shown successful isolation of stem cells from dental pulp of various types of teeth such as wisdom teeth, exfoliated deciduous teeth, supernumerary teeth, and neonatal teeth [26-29]. These studies indicated that dental pulp derived stem cells have similar attributes to MSCs including a fibroblast-like morphology, the expression of MSC specific protein markers such as STRO-1, CD29, CD44, D105, and CD146, but not hematopoietic specific protein markers (CD34 and CD45). Moreover, these cells have the ability into differentiate to multiple lineages (odontogenic, chondrogenic, adipogenic and neurogenic) [26-31]. Our

results revealed that isolated hDPSCs in this study also had the properties of MSCs as well as expressing CD29, CD44, CD73, CD90 and HLA-Dr, but not CD11b, CD34, CD45, CD79a or HLA-ABC, and could differentiate to adipocytes, osteocytes and chondrocytes.

We used these hDPSCs as feeder cells for supporting the undifferentiated expansion of hES cells for long-term culture. To avoid contamination by feeder cells, we harvested colonies of hES cells by manual cutting [38]. Characterization of hES cells grown on hDPSCs feeders revealed that hES cells maintain embryonic stem cell properties including the typical morphology of hES cells and expression of pluripotency markers (Oct4, Nanog, Sox2, Rex1 SSEA-3 SSEA-4, and TRA1-60), embryoid body formation, and can spontaneously differentiate and retain normal karyotype during passage 1–17.

Studies by Cheng et al. suggest that using human MSC from bone marrow as a feeder layer could

support 13 passages of undifferentiated growth of hES cells [14]. MSC differentiated from hES cells could be used as an autologous feeder layer for supporting undifferentiated growth of autologous hES cells without addition of bFGF-2 for more than 30 passages [17]. The undifferentiated growth of hES cells was regulated by paracrine signaling. The roles of feeder cells to maintain the undifferentiated growth of hES cells are to produce adhesion molecules and extracellular matrix for supporting attachment of cells and secrete growth factors for cell growth and survival [21]. Recent studies suggest that TGF- β signaling molecules as TGF- β , activin and nodal, and insulin-like growth factor II (IGF-II) have a vital role for maintaining pluripotency in response to bFGF level [21, 25, 39–41]. Montes and colleagues reported that bone marrow derived MSCs could express bFGF receptors and secrete TGF- β and culture of hES cells with MSC conditioned medium and could maintain the undifferentiated growth [25].

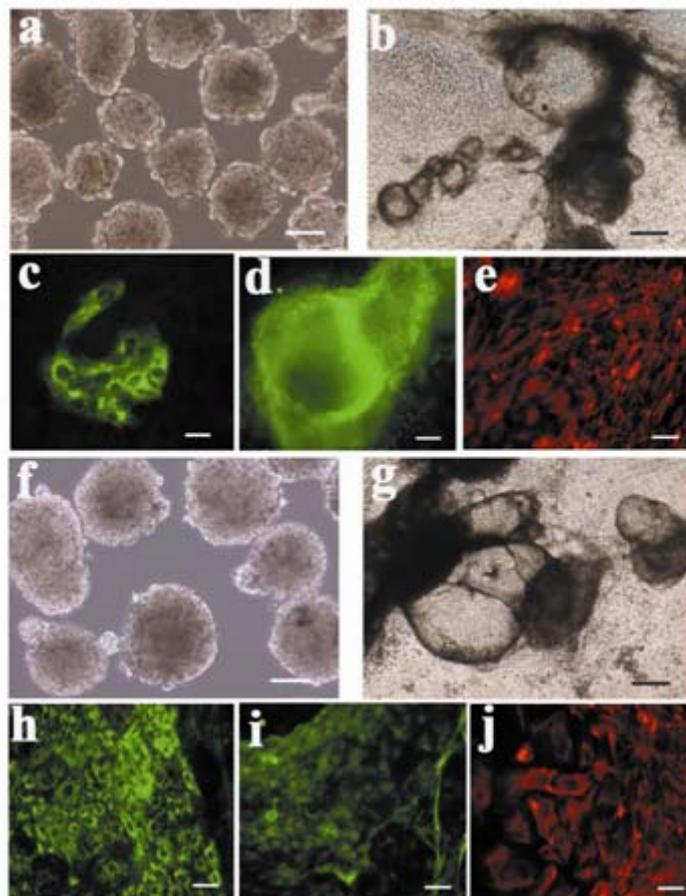


Figure 5. Embryoid body formation and spontaneous differentiation of human embryonic stem (hES) cells grown on human dental pulp stem cell (hDPSC) feeders. Both Chula2 hES and Chula5 hES cell lines cultured on hDPSC feeder had the ability to form embryoid bodies (EB) after 7 days in hanging drop suspension culture (**a and f**). After culturing these EBs on gelatin-coated plate for 2 weeks, they spontaneously differentiated (**b and g**). Immunocytochemical staining showed that these differentiated cells expressed endoderm (α -fetoprotein) (**c and h**), mesoderm (Brachyury) (**Fig 5d and i**) and ectoderm (nestin) (**e and j**) lineages. Scale bars: (**a–b and d–j**) 50 μ m, (**c**) 20 μ m.

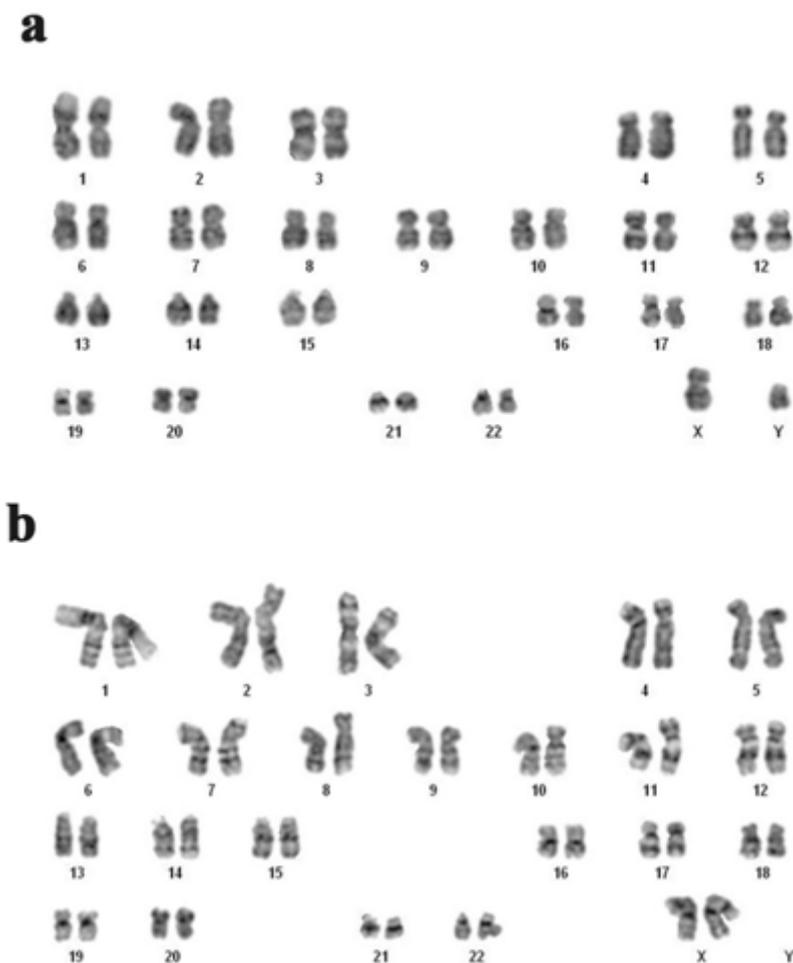


Figure 6. Normal karyotypes of human embryonic stem (hES) cell lines cultured on human dental pulp stem cell (hDPSC) feeders. Chula2 and Chula5 hES cell lines cultured on hDPSCs feeder maintained normal chromosome contents of 46 XY (a) and 46 XX (b) at passage 17.

hDPSCs were first isolated from pulp tissue of third molars by Gronthos and colleagues [26]. Recently, many investigators have isolated DPSCs from the pulp tissue of various types of teeth [27-29]. These results presented here revealed that dental pulp derived-stem cells have similar attributes to MSCs including the expression of protein markers and the ability to differentiate into multilineages [30-34]. Moreover, another study suggests that hDPSCs express TBF- β family members such as activin, TBF- β 1, and TBF- β 2 [42]. Although, bone marrow derived MSCs have the ability to maintain undifferentiated growth of hES cells, a slow proliferation after multiple passaging, the pain of bone marrow aspiration, and ethical concerns are disadvantages of using these cells [14, 43]. Compared with bone marrow-derived MSC, this current work clearly demonstrates that hDPSCs had a high proliferation rate. These cells are a newly-

discovered potential source of human feeder cells to support the undifferentiated growth of hES cells.

In conclusion, hDPSCs, newly discovered human feeder cells described here, were obtained by removing impacted third molars. This human feeder cell source is from an easily accessible source of biological waste without huge ethical concerns. The results from this experiment showed that the use of these cells for feeders takes advantage of an alternative feeder source for hES cell culture with xeno-free conditions.

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