Isolation and Characterization of Human Dental Pulp Stem/Stromal Cells From Nonextracted Crown-fractured Teeth Requiring Root Canal Therapy

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Abstract

Introduction: Human dental pulp stem/stromal cells (hDPSCs) in adults are primarily derived from the pulp tissues of permanent third molar teeth in existing literatures, whereas no reports exist, to our knowledge, on deriving hDPSCs from a tooth without the need for surgical procedure. The aim of this study was to raise a novel idea to source hDPSCs from complicated crown-fractured teeth requiring root canal therapy. Methods: hDPSCs were harvested from the pulp tissues for two complicated crown-fractured teeth requiring root canal therapy, retaining the teeth for subsequent prosthodontic rehabilitation, in a 41-year-old woman who had suffered a motorcycle accident. Pulp tissue from the left lower deciduous canine of a healthy 10-year-old boy (the positive control) was also removed because of high mobility and used as a positive control; removal was performed

together using an endodontic file, with the informed consent of the patient (Fig. 1A). The dental pulp tissues of both complicated crown-fractured teeth were extracted 4 days later using an endodontic file, with the informed consent of the patient (Fig. 1C) and institutional review board approval from our institution (KMUH-IRB-980016). After endodontic treatment (Fig. 1D), porcelain crowns for each of the two crown-fractured teeth were fabricated (Fig. 1E).

A deciduous tooth (left lower canine) was also removed from a healthy 10-year-old boy because of high mobility and used as a positive control; removal was performed

Materials and Methods

Isolation and Culture of hDPSCs

A 41-year-old woman suffered complicated crown fractures with pulp exposure of the left upper central and lateral incisors because of a motorcycle injury (Fig. 1A and B). The dental pulp tissues of both complicated crown-fractured teeth were extracted 4 days later using an endodontic file, with the informed consent of the patient (Fig. 1C) and institutional review board approval from our institution (KMUH-IRB-980016). After endodontic treatment (Fig. 1D), porcelain crowns for each of the two crown-fractured teeth were fabricated (Fig. 1E).

Key Words

Crown fracture, dental pulp, dental pulp stem cell
under local anesthesia after the consent of the patient’s parents with institutional review board approval from our institution (KMUH-IRB-980016). The surface of the deciduous tooth was first cleaned with methanol (70% alcohol). The deciduous tooth was then kept on ice in Dulbecco’s phosphate-buffered saline (DPBS; Invitrogen, Carlsbad, CA) and delivered to the laboratory for the isolation of hDPSCs. A groove of 0.5 to 1.0 mm in depth was cut around the circumference of the tooth using a sterile handheld high-speed drill. The dental pulp was then exposed by splitting the tooth with a chisel along the groove, and the pulp tissue was extracted using an endodontic file.

The extracted pulp tissues of the two complicated crown-fractured teeth and the deciduous tooth were then digested in collagenase type I (3 mg/mL, Invitrogen) and dispase (4 mg/mL, Invitrogen) for 1 hour at 37°C (11). The resulting cell suspensions were filtered through a 40-μm cell strainer (Falcon, BD, Franklin Lakes, NJ), and the single cell suspensions were then cultured in a DPSC medium containing α-MEM (Hyclone, Logan, UT), 20% fetal bovine serum (Hyclone), 100 μmol/L L-ascorbic acid-2-phosphate (Sigma, St Louis, MO), 100 U/mL penicillin (Invitrogen), and 100 μg/mL streptomycin (Invitrogen). The hDPSCs derived from the two complicated crown-fractured teeth and the deciduous tooth were cultured at 37°C with 5% CO₂; the medium was refreshed every 2 days, and 80% confluence was obtained for optimal cell harvesting.

**Proliferation Rate of hDPSCs**

The proliferation rates of the hDPSCs obtained from the complicated crown-fractured teeth and the deciduous tooth were determined by plating 25,000 hDPSCs (passage 3) per well on a six-well plate; each had three replicas and was passaged and counted at every 72 hours. Cells were counted before the next passage, and 25,000 cells were replated for a total of four passages. The proliferation rate was then calculated by dividing the total cell number after every 72 hours of culture by the initial plating number (25,000).

Data analyses for the differences in different tooth groups and different proliferation rates on passages were performed by a one-way analysis of variance in Statistical Analysis Systems (version 14; SPSS, Chicago, IL).

**Colony-forming Unit Assay**

The 3-passage hDPSCs from both the complicated crown-fractured teeth and the deciduous tooth were seeded in a six-well plate at
40 single cells per well with three replicas. After 2 weeks in culture, the
cells were fixed in 10% buffered formalin for 10 minutes and stained
with 3% (v/v) crystal violet (Sigma) for 5 minutes; they were then
washed twice with distilled water and the number of colonies enumer-
ated. Only colonies larger than 2 mm in diameter were counted: those
any smaller could be fall-off cells or satellite cells that were growing
instead of the cell originally seeded. The percentage colony-forming
efficiency was expressed as the total number of colonies divided by
the initial number of cells that were seeded and multiplying by 100 (11).

Reverse–transcriptase Polymerase Chain Reaction

To determine whether the hDPSCs of the complicated crown-fract-
tured teeth and the deciduous tooth shared similar expression patterns
of stem cells and differentiation markers, as has been previously
described for hDPSCs (1) and BMSCs (2), reverse-transcriptase poly-
merase chain reaction (RT-PCR) was performed using a DNA thermal
cycler (TaKaRa MP, Tokyo, Japan). First, total RNA was obtained using
an RNeasy Plant Mini Kit (QIAGEN, Valencia, CA) followed by reverse
transcription of messenger RNA according to the procedure provided
by SuperScript III (Invitrogen). The resulting complementary DNA
was subsequently used for PCR amplification. The PCR reactions
were performed using specific oligoprimer sets (11), which were de-
signated with reference to the published complementary DNA sequences
in GenBank (Bethesda, MD). The amplification cycle consisted of 3
minutes at 94°C, 25 seconds at 94°C, 30 seconds at 62°C, and 50
seconds at 72°C repeated 40 times, followed by 3 minutes at 72°C.
Five oligoprimer sets were designed in order to determine the expres-
sion of stem cell markers (Oct4, Rex-1, and Nanog; Table 1) and differ-
entiation markers (Nestin and Osteonectin, Table 1). All oligoprimer
sets were designed to cross the junction between two exons with the forward
primer located at the 3' region of the leading exon, whereas the reverse
primer was located at the 5' region of the following exon in order to
minimize the possibility of a false-positive or contamination of genomic
DNA. In addition, glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
was used as the positive control (Table 1) and H2O as the negative
control.

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<th>Oligoprimer</th>
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| Oct4        | Forward: 5'-GAC AAC AAT GAA AAT CTT CAG GAG A-3'  
Reverse: 5'-TAC AGA ACC ACA CTC GGA CCA-3' | 205bp |
| Rex-1       | Forward: 5'-AGA ATT CCG TTG AGT ATT CTG A-3'   
Reverse: 5'-GGC TTT CAG GTT ATT TGA CTG A-3' | 470bp |
| Nanog       | Forward: 5'-TCT CTC TCT TTT CTT CCT CCA-3'  
Reverse: 5'-GGA AGA GTA GAG GCT GGG GT-3' | 389bp |
| Nestin      | Forward: 5'-TGG CAA GGC GAC TGG GGC A-3'  
Reverse: 5'-GCC AAG GTA GAG GCT GGG GT-3' | 810bp |
| Osteonectin | Forward: 5'-ACT TTC TCT TCT TGG CCA-3'  
Reverse: 5'-GCC CAC TCT CTC AAC TCG CC-3' | 323bp |
| GAPDH       | Forward: 5'-ATG GGG AAG GTG AAG GTC GG-3'  
Reverse: 5'-CCA TCA CGC CAC AGT TTC CC-3' | 596bp |

Figure 2. The human DPSCs obtained from both the complicated crown-fractured teeth (A, central incisor; B, lateral incisor) and (C) the deciduous tooth were spindle shaped in morphology (×100).
Differentiation of hDPSCs

Adipogenic differentiation. To differentiate toward adipogenic lineage, the three-passage hDPSCs of both the complicated crown-fractured teeth and the deciduous tooth were seeded at 400 cells/cm² in a 35-mm tissue culture dish and cultured for 11 days in DPSC medium until confluence was achieved. The DPSC medium was subsequently supplemented with 5 μg/mL insulin (Sigma), 50 μmol/L indomethacin (Sigma), 1 μmol/L dexamethasone (Sigma), and 0.5 μmol/L isobutyl-1-methyl xanthine (Sigma) and the cultures placed in an incubator at 37°C and 5% CO₂ for another 21 days, with medium changes three times per week. As a control, DPSC medium only was used. At the end of the cultivation period, the cells were then fixed in 10% buffered formalin for 10 minutes and stained with Oil-Red-O (Sigma) to stain the lipid vacuoles (11). Furthermore, RT-PCR of PPARγ messenger RNA indicating adipogenic lineage for the hDPSCs (3-passage) of both the complicated crown-fractured teeth and the deciduous tooth were performed. Briefly, PCR conditions were 60.5°C annealing with forward oligoprimer (5’-CAG TGG GGA TGT CTC ATA A-3’) and reverse oligo-primer (5’-CTT TTG GCA TAC TCT GTG AT-3’) for 35 cycles producing a PCR product of 391 bp. These primers can detect both PPARγ-1 and -2 (14). GAPDH was used as the positive control and H₂O as the negative control (Table 1).

Chondrogenic differentiation. A total of 2.5 x 10⁵ hDPSCs from both the complicated crown-fractured teeth and the deciduous tooth were centrifuged in a 15-mL conical tube at 1,000 rpm for 5 minutes. The pellet was maintained in DPSC medium supplemented with 10 μg/mL ITS-X (Invitrogen), 5.35 μg/mL linoleic acid, 1.25 μg/mL bovine serum albumin (BSA), 1.0 μg/mL dexamethasone, and 10 ng/mL TGF-β3 (Chemicon, Billerica, MA). The medium was replaced every three to four days for four weeks. As a control, DPSC medium only was used. The pellets were then fixed in 4% paraformaldehyde (PFA) PFA overnight, and paraffin-embedded sections (4-5μm) were stained with Alcian blue (15).

Figure 3. The human DPSCs obtained from both the complicated crown-fractured teeth (A and D [× 40]; central incisor; B and E [× 100]: lateral incisor) and the deciduous tooth (C and F [×100]) were able to form colonies; they were stained with crystal violet.

Figure 4. The proliferation rates for the complicated crown-fractured teeth (A: central incisor and B: lateral incisor) and (C) the deciduous tooth.
Osteogenic differentiation. The hDPSCs from the two complicated crown-fractured teeth and the deciduous tooth were prepared as described previously, with the exception that 1 nmol/L dexamethasone (Sigma), 20 mmol/L β-glycerolphosphate (Sigma), and 50 ng/ml L-thyroxine sodium pentahydrate (Sigma) were supplemented in the DPSC medium on day 11. The culture was incubated at 37°C and 5% CO₂ for another 21 days, with medium changes three times per week. As a control, DPSC medium only was used. At the end of the cultivation period, the DPSCs of both the crown-fractured teeth and the deciduous tooth were fixed in 10% buffered formalin for 10 minutes and stained with alizarin red S (Wako, Osaka, Japan) for 15 minutes at room temperature so that the mineral matrix of the bone could be observed (11).

Flow Cytometry

DPSCs of both the complicated crown-fractured teeth and the deciduous tooth were divided into 6 fluorescence-activated cell sorting round-bottom tubes (Becton Dickinson Falcon, Sunnyvale, CA) at 2 × 10⁵ cells/tube and stained with immunoglobulin G–fluorescein isothiocyanate or phycoerythrin-conjugated anti-CD14, -CD29, -CD34, -CD45, -CD90, -CD105 (Beckman Coulter, Villepinte, France, 20 μL each). After a 20-minute incubation at ambient temperature in the dark, cells were washed twice with 2 mL fluorescence-activated cell sorting wash solution of phosphate-buffered saline containing 0.1% phosphate buffered solution (PBS) FBS and 0.1% Na₂SO₄ and centrifuged 5 minutes at 230g. Supernatant was removed, and cells were fixed with 1% formaldehyde (in phosphate-buffered saline). Respective immunoglobulin G isotype-matched controls (Beckman Coulter) were used as negative controls (16). All data were acquired using a Coulter Epics XL (Beckman Coulter) and analyzed using EXPO32 ADC software (Beckman Coulter) and WinMDI version 2.8 (Windows Multiple Document Interface for Flow Cytometry; freeware developed by Joe Trotter, downloadable at methods.info/software/flow/winmdi.html).

Results

The hDPSCs from both the complicated crown-fractured teeth and the deciduous tooth proliferated rapidly, with a spindle-shaped morphology (Fig. 2A-C). After the third passage, homogeneous populations of hDPSCs of both the crown-fractured teeth and the deciduous tooth were obtained and maintained in culture based on the cell morphology. We found that on average 80% of the hDPSCs derived from the two complicated crown-fractured teeth and 83% of the hDPSCs from the deciduous tooth were capable of forming colonies (Fig. 3A–C).

In addition, the hDPSCs from both the complicated crown-fractured teeth and the deciduous tooth had high proliferation rates during the early passages, but the rate decreased gradually in culture. At passage 3, hDPSCs of both the complicated crown-fractured teeth proliferated at 11.70 ± 0.26 folds and 12.37 ± 0.71 folds, respectively, and by passage 7 the rate was decreased to 5.67 ± 0.71 folds and 2.70 ± 0.17 folds, respectively (Fig. 4A and B). Moreover, at passage 3, hDPSCs of the deciduous tooth proliferated at 9.33 ± 0.76 folds, and by passage 7 the rate was declined to 5.07 ± 0.15 folds (Fig. 4C). In different tooth groups, the analysis of variance result is nonsignificant (p = 0.442); however, the differences in proliferation rates on different passages, the test of homogeneity of variances, was significant (p = 0.016). In addition, using the Welch analysis of variance to analyze the proliferation rates on different passages indicated a significant difference (p = 0.018). Then, the post hoc comparison (Tamhane
test) indicated significant differences among passage 3 to 4 versus passage 5 to 6 and passage 3 to 4 vs passage 6 to 7.

Furthermore, the hDPSCs of both the complicated crown-fractured teeth and the deciduous tooth were able to differentiate into adipogenic (Fig. 5A–D), chondrogenic (Fig. 6A–C), and osteogenic (Fig. 7A–C) lineages. We also determined that the hDPSCs of the two complicated crown-fractured teeth and the deciduous tooth expressed stem cells and differentiation markers (Fig. 8A–C). In addition, hDPSCs...
from both the complicated crown-fractured teeth and the deciduous tooth showed high expression for common BMSC markers including CD29, CD90, and CD105 (Fig. 9A–C). However, markers specific for hematopoietic cells such as CD14, CD34, and CD45 revealed very low expression (Fig. 9A–C).

**Discussion**

To date, three kinds of human mesenchymal stem cells have been derived from teeth: DPSCs, mostly from primary incisors and permanent third molar teeth (1); stem cells from exfoliated deciduous teeth (SHED) (17); and stem cells from immature apical papilla (18). Obtaining stem cells from immature apical papilla, although feasible, is a rather uncommon dental practice, whereas SHED has the main drawback of being a limited source because, despite the occasional retained deciduous tooth, nearly all primary teeth are exfoliated during adulthood. hDPSCs are relatively convenient stem cell resources resulting from pulp exposure and endodontic treatment. Not only can they be obtained from primary incisors and permanent third molar teeth in existing literatures, but also, as aforementioned, we extended the source of hDPSCs in our previous study by successfully isolating and characterizing hDPSCs from a supernumerary tooth (11). The incidence of supernumerary teeth varies between 0.45% and 3% (19, 20). In the current report, we have further shown the feasibility of deriving hDPSCs from a complicated crown-fractured tooth with exposed pulp. In addition, we have shown that these hDPSCs possess the adipogenic, chondrogenic, and osteogenic differentiation capacity as well as show the stem cell markers. Thus, their expression profiles and differentiation capabilities are similar to those of BMSCs (1, 2). These findings may enhance the development of a novel biologically based new generation of clinical materials for regenerative dental and medical treatments (3, 6). Significantly, the ability to isolate hDPSCs from a pulp-exposed crown-fractured tooth without requiring extraction, as shown in the
Figure 9. The human DPSCs from both the complicated crown-fractured teeth (A: central incisor and B: lateral incisor) and (C) the deciduous tooth showed high expression for surface markers CD29, CD90, and CD105 but very low expression for CD14, CD34, and CD45 (black line, isotype control; red line, marker of interest; Max, maximum).
present study, further extends the potential source and possibilities for hDPSCs. The incidence of complicated crown fractures with pulp exposure ranges from 4.6% to 24.4% (20–22); moreover, hDPSC acquisition from this source has the additional advantage that hDPSCs can be obtained without invasive surgical procedures. It is, of course, unethical to perform an experiment to obtain hDPSCs from a nondiseased vital tooth; however, in line with and further expanding on the findings presented in this report, it is at least theoretically indicated that, in the future, with the progress of regenerative dental and medical treatments, a person could opt to have his/her own hDPSCs removed from his/her own tooth whenever necessary for regenerative dental and medical treatments without the necessity of tooth extraction, which indicates an immense resource of personal hDPSCs.

In conclusion, this report describes the successful isolation and characterization of hDPSCs from the pulp tissue of complicated crown-fractured teeth without the need for tooth extraction. Therefore, pulp exposed in complicated crown-fractured teeth might represent a valuable source of hDPSCs.

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References