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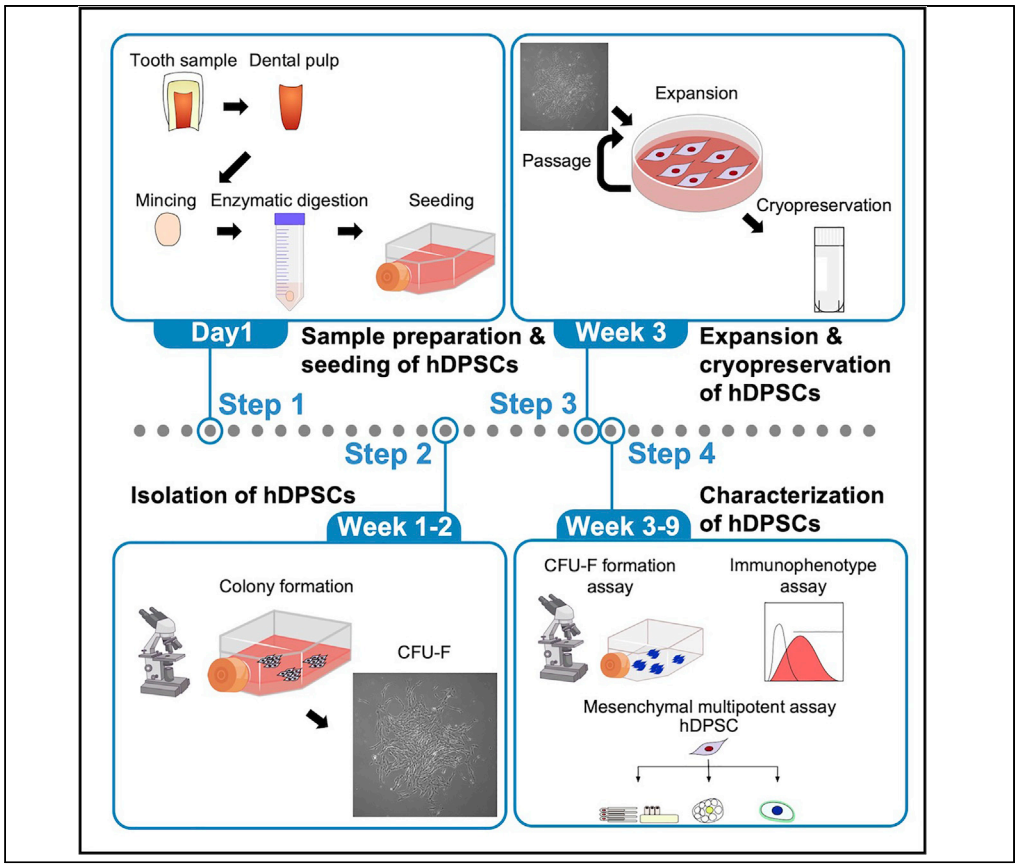
Protocol to generate xenogeneic-free/serum-free human dental pulp stem cells



Human dental pulp stem cell (hDPSCs)-based therapy is a feasible option for regenerative medicine, such as dental pulp regeneration. Here, we show the steps needed to colony-forming unit-fibroblasts (CFU-F)-based isolation, expansion, and cryopreservation of hDPSCs for manufacturing clinical-grade products under a xenogeneic-free/serum-free condition. We also demonstrate the characterization of hDPSCs by CFU-F, flow cytometric, and *in vitro* multipotent assays.

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Highlights

Protocol to generate xenogeneic-free/serum-free hDPSCs

Expansion, cryopreservation, and recovery for biobanking of hDPSCs

CFU-F, flow cytometric, and *in vitro* multipotent assays for characterization of hDPSCs

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Protocol to generate xenogeneic-free/serum-free human dental pulp stem cells

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SUMMARY

Human dental pulp stem cell (hDPSCs)-based therapy is a feasible option for regenerative medicine, such as dental pulp regeneration. Here, we show the steps needed to colony-forming unit-fibroblasts (CFU-F)-based isolation, expansion, and cryopreservation of hDPSCs for manufacturing clinical-grade products under a xenogeneic-free/serum-free condition. We also demonstrate the characterization of hDPSCs by CFU-F, flow cytometric, and *in vitro* multipotent assays. For complete details on the use and execution of this protocol, please refer to Iwanaka et al. (2020).

BEFORE YOU BEGIN

Human dental pulp stem cells (hDPSCs) were identified in dental pulp tissue of deciduous and permanent teeth (Gronthos et al., 2000; Miura et al., 2003) and apical papillae of developing teeth (Sonoyama et al., 2006). hDPSCs exhibit remarkable mesenchymal stem cell (MSC) characteristics, including self-renewal, immunophenotype, and multipotency into osteoblasts, adipocytes, and chondrocytes (Yamaza et al., 2010). We have focused on the stem cell potency of hDPSCs for regenerative medicine; Immunosuppressive function of hDPSCs can be an option for treating systemic lupus erythematosus (SLE) (Ma et al., 2012, 2015; Makino et al., 2013). Transdifferentiation capacities into hepatocytes and cholangiocytes and cell aggregation ability of hDPSCs is applicable in liver and bone regeneration (Yamaza et al., 2015; Tanaka et al., 2018, 2019; Fujiyoshi et al., 2019; Takahashi et al., 2019; Yuniartha et al., 2021). Extracellular vesicles of hDPSCs improve bone density and immune tolerance in osteoporosis and SLE, respectively (Sonoda et al., 2020, 2021a). Pulpitis- and biliary atresia-specific hDPSCs are suggested as a potent autologous option for dental pulp and liver regeneration (Sonoda et al., 2016, 2018; 2021b). Bilirubin-free and pamidronate treatment could rejuvenate deficient hDPSCs under an experimental biliary atresia condition (Yamaza et al., 2018a; 2018b). Thus, hDPSCs-based therapy is considered to be a novel option for regenerative medicine (Sonoda et al., 2015; Taguchi et al., 2019). The employment of fetal bovine serum (FBS) in cell manufacturing faces severe immunological concerns in clinical application (Karnieli et al., 2017). Recently, we have established a protocol for producing clinical graded hDPSCs under a xenogeneic-free/serum-free condition by colony-forming unit-fibroblasts (CFU-F) method (Friedenstein et al., 1974) and provided feasible hDPSC products to treat chronic liver fibrosis in an animal model. (Iwanaka et al., 2020).

Institutional permissions

Ethical approvals and institutional permissions are required for the use of human samples. Procedures for handling dental pulp tissues from human deciduous teeth referred in this protocol were approved by the Kyushu University Institutional Review Board for Human Genome/Gene Research (protocol numbers: 738-01, 02, 03, and 04). All experiments conform to the relevant regulatory standards.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
R-PE mouse anti-human CD11b IgG_1 [HI111] (used at 1 μg/mL)	BioLegend	Cat # 301207
R-PE mouse anti-human CD14 IgG_1 [63D3] (used at 1 μg/mL)	BioLegend	Cat # 367103
R-PE mouse anti-human CD34 IgG_{2a} [561] (used at 1 μg/mL)	BioLegend	Cat # 343605
R-PE mouse anti-human CD45 IgG_1 [2D1] (used at 1 μg/mL)	BioLegend	Cat # 368509
R-PE mouse anti-human CD73 IgG_1 [AD2] (used at 1 μg/mL)	BioLegend	Cat # 344003
R-PE mouse anti-human CD90 IgG_1 [5E10] (used at 1 μg/mL)	BioLegend	Cat # 328109
R-PE mouse anti-human CD105 IgG_1 [43A3] (used at 1 μg/mL)	BioLegend	Cat # 323205
R-PE mouse anti-human CD146 IgG_1 [P1H12] (used at 1 μg/mL)	BioLegend	Cat # 361006
R-PE mouse anti-human HLA-DR IgG_{2a} [L243] (used at 1 μg/mL)	BioLegend	Cat # 307605
R-PE mouse IgG_1 kappa [MOPC-21] (used at 1 μg/mL)	BioLegend	Cat # 400113
R-PE mouse IgG_{2a} kappa [MOPC-173] (used at 1 μg/mL)	BioLegend	Cat # 400213
Chemicals, peptides, and recombinant proteins		
7-AAD viability staining solution	BioLegend	Cat # 42043
Acetic Acid	Nacalai Tesque	Cat # 00212-85
Alcian blue 8GX	Merck	Cat # A5268
Alizarin red S	Merck	Cat # A5533
L-Ascorbic Acid 2-Phosphate	FUJIFILM Wako Chemicals	Cat # 323-44822
Dexamethasone	Merck	Cat # D4902-1G
Dulbecco's Minimum Essential Medium (DMEM), Low Glucose (1.0 g/L)	Nacalai Tesque	Cat # 08456-65
Dulbecco's Phosphate Buffered Saline (D-PBS) (1×)	Nacalai Tesque	Cat # 14249-95
D-PBS (10×)	Nacalai Tesque	Cat # 11482-15
EagleTaq Master Mix	Roche	Cat # 5876486001
Ethanol, Absolute	Nacalai Tesque	Cat# 08948-45
Fetal bovine serum (FBS) [170629-0130]	Equitech Bio	Cat # SFBM30-0500
Hydrocortisone	Merck	Cat # 386698-25MGCN
Hanks' balanced salt solution (HBSS) (1×)	Nacalai Tesque	Cat# 09735-75
Indomethacin	Merck	Cat # 405268-10GMCN
3-Isobutyl-1-methylxanthine (IBMX)	Merck	Cat # 410957-1GMCN
Isopropanol	Nacalai Tesque	Cat # 29113-95
ITS™ Premix	BD Bioscience-Discovery Labware	Cat # 354351
Liberase™ MNP-S	Roche Custom Biotech	Cat # 05578566001
Liquid nitrogen	N/A	N/A
Marinol750cpc	Muto Pure Chemicals	Cat # 20091
MSC NutriStem® XF Basal Medium	Biological Industry	Cat # 05-200-1A
MSC NutriStem® XF Supplement Mix	Biological Industry	Cat # 05-201-1U
Oil red O	Merck	Cat # O0625
Paraffin, Paraplast plus® for tissue embedding	Leica Biosystems	Cat # 39601095
Paraformaldehyde (PFA)	Merck	Cat # 818715
Penicillin-Streptomycin-Amphotericin B Mixed Solution (100×)	Nacalai Tesque	Cat # 02892-54
Potassium Dihydrogen Phosphate	FUJIFILM Wako Chemicals	Cat # 163-04243
ReverTraAce® qPCR RT Master Mix	TOYOBO	Cat # FSQ-201
RNeasy Mini Kit	QIAGEN	Cat # 74104

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
RQ1 RNase-free DNase	Promega	Cat # M6101
STEM CELL BANKER® cryomedium	Zenoak	Cat # CB061
Sodium Hydroxide solution (1 N)	Nacalai Tesque	Cat # 37421-05
Sodium Pyruvate solution (5 mM)	Nacalai Tesque	Cat # 06977-34
Toluidine blue	Merck	Cat # T3260
Transforming Growth Factor beta 1 (TGFB1)	PeproTech	Cat # AF-100-21C-10ug
Trypan blue solution	Bio-Rad Laboratories	Cat # 1450021
TRIzol® reagent	Thermo Fisher Scientific	Cat # 15596026
TrypLE™ select without phenol red	Thermo Fisher Scientific	Cat # 12563011
Turk's solution	Merck	Cat # 1.09277
Xylene	Nacalai Tesque	Cat # 36612-35
Biological samples		
Human teeth	N/A	N/A
Oligonucleotides		
BGLAP [Hs01587814_g1]	Thermo Fisher Scientific	Cat # 4331182
COL10A1 [S00166657_m1]	Thermo Fisher Scientific	Cat # 4331182
LPL [Hs00173425_m1]	Thermo Fisher Scientific	Cat # 4331182
PPARG [Hs0115513_m1]	Thermo Fisher Scientific	Cat # 4331182
rRNA, 18S [Hs99999901_s1]	Thermo Fisher Scientific	Cat # 4331182
RUNX2 [Hs00231692_m1]	Thermo Fisher Scientific	Cat # 4331182
SOX9 [Hs01001343_g1]	Thermo Fisher Scientific	Cat # 4331182
Other		
Automated cell counter, TC20™	Bio-Rad Laboratories	Cat # 1450101J1
Cell counting slide for TC10 ™ /TC20™	Bio-Rad Laboratories	Cat # 1450011
Cell culture dish, 35 mm	Corning, Falcon®	Cat # 354467
Cell culture dish, 60 mm	Corning, Falcon®	Cat # 353002
Cell culture dish, 100 mm	Corning, Falcon®	Cat # 353003
Cell culture e flask, T-75	Corning, Falcon®	Cat # 353135
Cell culture plate, 6-well	Corning, Falcon®	Cat # 353046
Cell culture plate, 96-well, round bottom, PrimeSurface®	Sumitomo Bakelite	Cat # MS-9096U
Cell scraper	Corning, Falcon®	Cat # 353089
Cell strainer, 70 µm	Corning, Falcon®	Cat # 352350
Cell thawing system, ThawSTAR® CFT2	Biolife Solutions	Cat # AST-601
Centrifuge machine	N/A	N/A
Centrifuge Tube, conical, polypropylene, 1.5 mL	Nichiryo	Cat # 00-ETS-CT-15
Centrifuge Tube, conical, polypropylene, 15 mL	Corning, Falcon®	Cat # 352096
Centrifuge Tube, conical, polypropylene, 50 mL	Corning, Falcon®	Cat # 352070
Centrifuge Tube, round bottom, polypropylene, 5 mL	Corning, Falcon®	Cat # 352008
CO_2 incubator	N/A	N/A
Cryogenic vial	Sumitomo Bakelite	Cat # MS-4501G
Dissecting instruments (dental disk, dental probe, endodontic file, forceps, scalpel, surgical blade #23)	N/A	N/A
Flow cytometric (FCM) analyzer	N/A	N/A
Filter, disc, 0.2 µm	Advantech	Cat # 25CS020AS
Filter, membrane, 0.2 µm	Advantech	Cat # C020A047A
Filter, paper, No. 3, 150 mm	Advantech	Cat # 01301150
Filter, paper, No. 5B, 150 mm	Advantech	Cat # 01521150
Flask, T-75	Corning, Falcon®	Cat # 353136
Freezing container	N/A	N/A
Light microscopy, inverted	N/A	N/A
Light microscopy, upright	N/A	N/A
Liquid nitrogen tank	N/A	N/A
PCR plate, 96-well	N/A	N/A
Petri dish, 100 mm	Corning, Falcon®	Cat # 351029

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Realtime-PCR machine	N/A	N/A
Stereoscopic microscopy	N/A	N/A
Vortex mixer	N/A	N/A
Water bath	N/A	N/A

MATERIALS AND EQUIPMENT

Sample transfer medium		
Reagent	Final concentration	Amount
DMEM, Low Glucose (1.0 g/L)	n/a	49.5 mL
Penicillin-Streptomycin-Amphotericin B Mixed Solution (100×)	100 units/mL / 100 μg/mL / 0.25 μg/mL	0.5 mL
Total	**n/a**	**50 mL**
Store at 4°C until for up to 1 month.		

Note: Sample transfer medium is used for maintaining tissue/cell activity during sample transfer. Store each 10 mL of sample transfer medium at 4°C in a 50 mL conical polypropylene centrifuge tube before used.

Tissue digestion medium		
Reagent	Final concentration	Amount
Liberase™ MNP-S	0.45 units/mL	up to product lot
D-PBS (1×)	n/a	6 mL
Total	**n/a**	**6 mL**
Prepare fresh and keep at 4°C until used and prewarm at 37°C before used.		

Note: Tissue digestion medium should be prepared just before isolation.

Complete growth medium (CGM)		
Reagent	Final concentration	Amount
MSC NutriStem® XF Basal Medium	n/a	500 mL
MSC NutriStem® XF Supplement Mix	n/a	3 mL
Total	**n/a**	**503 mL**
Store at 4°C until for up to 1 month and prewarm at 37°C before used.		

PFA solution		
Reagent	Final concentration	Amount
PFA	4%	20.0 g
D-PBS (10×)	n/a	50 mL
Milli-Q water	n/a	480 mL
Total	**4%**	**500 mL**
Store at 4°C until for up to 1 month.		

Note: The following protocol may be helpful for preparing 4% PFA solution.

- Warm 400 mL of Milli-Q water at 60°C.

- Add 20 g of PFA powder and mix well.
- Add 1 mL of 1 N NaOH and mix well.
- Add 50 mL of 10 × D-PBS, mix well, and iced for 30 min.
- Filter using a No. 3 paper filter.
- Adjust to pH 7.2–7.4 and fill up to 50 mL.

Toluidine blue solution		
Reagent	Final concentration	Amount
Toluidine blue	0.4%	2.0 g
Milli-Q water	n/a	500 mL
Total	**0.4%**	**500 mL**
Store at 20°C until for up to 1 month.		

Toluidine blue staining solution		
Reagent	Final concentration	Amount
PFA solution (4%)	1%	50 mL
Toluidine blue solution (0.4%)	0.2%	100 mL
Milli-Q water	n/a	50 mL
Total	**0.4%**	**200 mL**
Store at 20°C until for up to 1 month.		

Note: Toluidine blue staining solution should be mixed and filtered using a No. 5B paper filter just before used.

FCM buffer		
Reagent	Final concentration	Amount
HBSS (1×)	n/a	49 mL
FBS, heat inactivated	2%	1 mL
Total	**n/a**	**50 mL**
Store at 4°C until for up to 1 week.		

Note: FCM buffer should be filtered using a 0.45 μm paper filter after mixed.

L-Ascorbic Acid 2-Phosphate solution		
Reagent	Final concentration	Amount
L-Ascorbic Acid 2-Phosphate	100 mM	322 mg
MSC NutriStem® XF Basal Medium	n/a	100 mL
Total	**n/a**	**100 mL**
Store at −20°C until for up to 1 month.		

Note: Store each 5 mL of L-Ascorbic Acid 2-Phosphate solution at −20°C in a 15 mL conical polypropylene centrifuge tube before used.

Potassium Dihydrogen Phosphate solution		
Reagent	Final concentration	Amount
Potassium Dihydrogen Phosphate	180 mM	984 mg
MSC NutriStem® XF Basal Medium	n/a	40 mL
Total	**n/a**	**40 mL**
Store at −20°C until for up to 1 month.		

Note: Store each 5 mL of Potassium Dihydrogen Phosphate solution at −20°C in a 15 mL conical polypropylene centrifuge tube before used.

Dexamethasone solution		
Reagent	Final concentration	Amount
Dexamethasone	180 mM	1 vial
Ethanol, Absolute	n/a	1 mL
MSC NutriStem® XF Basal Medium	n/a	9 mL
Total	**n/a**	**10 mL**
Store at −20°C until for up to 1 month.		

Note: Dilute Dexamethasone with 1 mL of absolute ethanol well and add 9 mL of MSC NutriStem® XF Basal Medium. Store each 50 µL of Dexamethasone solution at −20°C in a 1.5 mL conical polypropylene centrifuge tube before used.

IBMX solution		
Reagent	Final concentration	Amount
IBMX (50 mM)	50 mM	111 mg
Ethanol, Absolute	n/a	10 mL
Total	**n/a**	**10 mL**
Store at 20°C until for up to 1 month.		

Note: Keep to protect from the light to avoid loss of activity.

Indomethacin solution		
Reagent	Final concentration	Amount
Indomethacin	6 mM	215 mg
Ethanol, Absolute	n/a	100 mL
Total	**n/a**	**100 mL**
Store at 20°C until for up to 1 month.		

Note: Keep to protect from the light to avoid loss of activity.

Hydrocortisone Phosphate solution		
Reagent	Final concentration	Amount
Hydrocortisone (500 mM)	0.5 mM	18 mg
Ethanol, Absolute	n/a	100 mL
Total	**n/a**	**100 mL**
Store at 20°C until for up to 1 month.		

Note: Keep to protect from the light to avoid loss of activity.

TGFB1 solution		
Reagent	Final concentration	Amount
TGFB1	1 µg/mL	10 µg
D-PBS (1_x)	n/a	10 mL
Total	**n/a**	**10 mL**
Store at −20°C until for up to 1 month.		

Note: Store each 100 μL of TGFB1 solution at −20°C in a 1.5 mL conical polypropylene centrifuge tube before used.

Osteogenic induction solution		
Reagent	Final concentration	Amount
MSC NutriStem® XF Basal Medium	n/a	490 mL
MSC NutriStem® XF Supplement Mix	n/a	3 mL
L-Ascorbic Acid 2-Phosphate solution (10 mM)	100 μM	5 mL
Potassium Dihydrogen Phosphate solution (180 mM)	2 mM	5 mL
Dexamethasone solution (100 mM)	10 nM	50 μL
Total	**n/a**	**500 mL**
Store at 4°C until for up to 1 month.		

Adipogenic induction solution		
Reagent	Final concentration	Amount
MSC NutriStem® XF Basal Medium	n/a	481.5 mL
MSC NutriStem® XF Supplement Mix	n/a	3 mL
L-Ascorbic Acid 2-Phosphate solution (10 mM)	100 μM	5 mL
IBMX solution (180 mM)	500 μM	5 mL
Indomethacin solution (6 mM)	60 μM	5 mL
Hydrocortisone solution (500 mM)	500 nM	500 μL
Total	**n/a**	**500 mL**
Store at 4°C until for up to 1 month.		

Chondrogenic induction solution		
Reagent	Final concentration	Amount
MSC NutriStem® XF Basal Medium	n/a	9.44 mL
MSC NutriStem® XF Supplement Mix	n/a	60 μL
L-Ascorbic Acid 2-Phosphate solution (10 mM)	100 μM	100 μL
ITS™ Premix	n/a	100 μL
Dexamethasone solution (100 mM)	100 nM	0.05 μL
Sodium Pyruvate solution (5 mM)	100 μM	200 μL
TGFB1 solution (1 μg/mL)	10 ng/mL	100 μL
Total	**n/a**	**10 mL**
Store at 4°C until for up to 1 month.		

Alizarin red S staining solution		
Reagent	Final concentration	Amount
Alizarin red S	1%	2.5 g
Milli-Q water	n/a	250 mL
Total	**n/a**	**250 mL**
Store at 20°C until for up to 1 month.		

Note: Filter using a No. 3 paper filter before used.

Oil red O staining stock solution		
Reagent	Final concentration	Amount
Oil red O	0.5%	0.5 g
Isopropanol	n/a	100 mL
Total	**n/a**	**100 mL**
Store at −20°C until for up to 1 month.		

Note: Filter using a No. 3 paper filter before used.

Oil red O staining working solution		
Reagent	Final concentration	Amount
Oil red O staining stock solution	0.3%	15 mL
Milli-Q water	n/a	10 mL
Total	**n/a**	**25 mL**
Store at −20°C until for up to 1 month.		

Note: Filter using a No. 3 paper filter before used.

Alcian blue staining solution		
Reagent	Final concentration	Amount
Alcian blue 8GX	1%	1 g
Acetic Acid (3%)	n/a	100 mL
Total	**n/a**	**25 mL**
Store at −20°C until for up to 1 month.		

Note: Adjust pH to 2.5 using acetic acid. Filter using a No. 3 paper filter before used.

STEP-BY-STEP METHOD DETAILS

Sample collection and transfer

Timing: depending on the process in your institution and number of tooth samples

This section describes the transfer condition of collected human samples.

1. Sample transfer.
   a. Extract human tooth samples under a general surgical condition and put them into sample transfer medium in a 5 mL conical polypropylene centrifuge tube (Figure 1).

   Note: Human tooth samples derived from any donors with age and gender are available for generating hDPSCs.

   b. Transport the samples at 4°C within 24 h and maintain the samples at 4°C before used.

Cell preparation and seeding

Timing: 1–2 h, depending on the number of tooth samples

This section describes the procedure to isolate all nucleated cells (ANCs) from human dental pulp tissue.

2. Tissue preparation and digestion.
   a. Tissue preparation.
      i. Discard the transfer medium and wash the samples with 10 mL of D-PBS 3 times (Figures 2A and 2B).

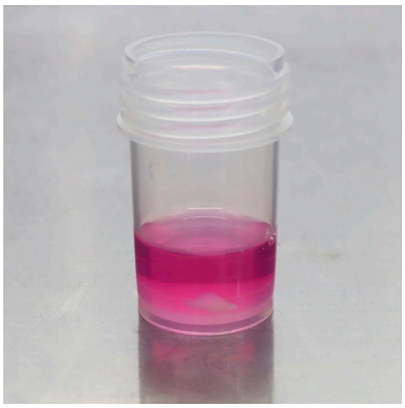

Figure 1. Storing of a tooth sample
An imaged of a deciduous tooth sample stored in a polypropylene vial containing sample transfer medium at 4°C.

ii. Hold the sample using a forceps. Pull-out dental pulp tissue using an endodontic file or dental probe under a stereoscopic microscopy on a 100 mm petri dish (Figure 2C).

Optional: If it is not easy to pull out dental pulp tissue directly, cut the tooth along cementoenamel junction using a dental disk and separate into the crown and root parts (see troubleshooting, problem 1).

iii. Drop 100–200 μL of D-PBS on the sample (Figure 2D).
iv. Mince the tissue sample in a pulp by using a scalpel with surgical blade #23.

⚠ CRITICAL: This step is critical to obtain larger number of dental pulp cells. The tissue should be minced as minute as possible to be in a pulp (Figure 2E).

b. Tissue digestion.
   i. Immerse the pulp tissue sample into 6 mL of tissue digestion medium in a 50 mL conical polypropylene centrifuge tube (Figure 3A).
   ii. Incubate the medium for 30 min at 37°C in a water bath. Mix every 10 min with a Vortex mixer to help break up tissue (Figure 3B).
   iii. Centrifuge at 300 × *g* at 4°C for 6 min. Aspirate the supernatant carefully. Resuspend the cell pellet gently with 1 mL of CGM and add 4 mL of CGM. Repeat this step again.
   iv. Pass the cell suspension through a 70 μm cell strainer.
   v. Centrifuge at 300 × *g* at 4°C for 6 min and aspirate the supernatant carefully. Resuspend the cell pellet gently with 1 mL of CGM and store at 4°C.

3. Cell counting.
   a. To stain ANCs, mix10 μ L of cell suspension with 90 μ L of Turk's solution in a 1.5 mL polystyrene conical centrifuge tube.

Optional: Living cells may be visualized by 0.4% trypan blue cell staining solution.

   b. Count number of ANCs in triplicate with a TC20™ automated cell counter using a cell counting slide for TC10 ™ /TC20™. Determine cell number from the mean of the three measurements.

Note: ANCs are usually obtained 1–5 × 10^6 cells of ANCs can be obtained from one tooth.

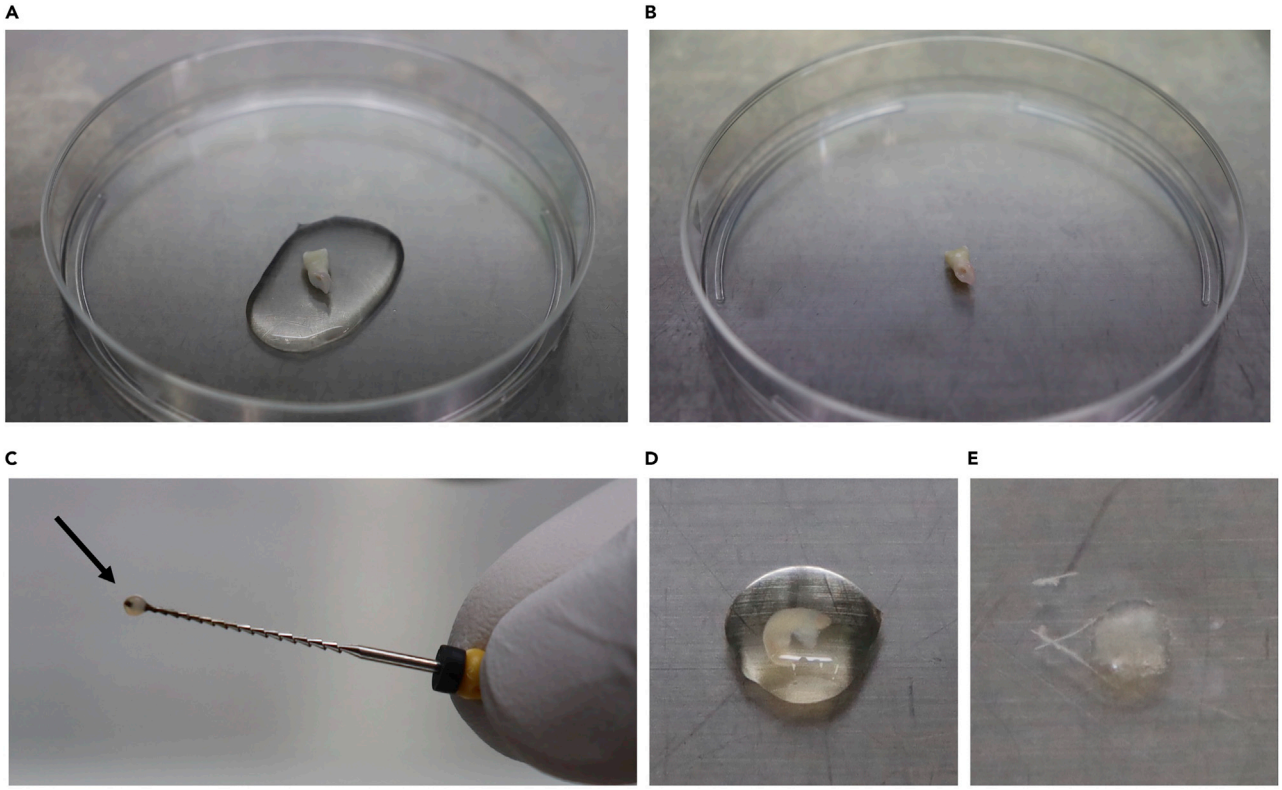

Figure 2. Preparation of tooth sample

(A) An image of a tooth sample washed in 1 mL of Dulbecco's phosphate buffered saline (D-PBS) on a petri dish.
(B) An image of the cleaned tooth sample after washing on the petri dish.
(C) An image of a dental pulp tissue (arrow) pulled from the tooth sample by using an endodontic file.
(D) An image of the pulled dental pulp tissue kept in a droplet of 100 μL of D-PBS on the petri dish.
(E) An image of the dental pulp tissue after minced in the droplet of D-PBS on the petri dish.

CFU-F forming isolation

Timing: 10–14 days, depending on colony forming condition

This section describes the procedure for forming CFU-F from ANCs. This part is most critical section throughout this procedure.

4. Colony forming culture (Figure 4).
   a. Seed ANCs at 1–2 × 10^6 cells in 10 mL of CGM onto T-75 flask. Shake the flasks gently on 8-loop.
   b. Incubate the flasks for 18 h at 37°C with 5% CO_2 in a CO_2 incubator.
   c. Wash the flasks gently with 3 mL of D-PBS 3 times to eliminate unattached cells. Add 10 mL of CGM and maintain the flasks at 37°C with 5% CO_2 in a CO_2 incubator.

   Note: Plastic adherence is one of important characteristics of MSCs.

   Note: The flasks should be left without any handling during initial 3 days to avoid the mechanical detachment of weak-attached cells.

   d. Replace with 10 mL of CGM 7 days after seeding and maintain further 7 days.

   Note: Adherent cell colony formation should be recognized under an inverted light microscopy approximately 10–14 days after seeding (see troubleshooting, problem 2).

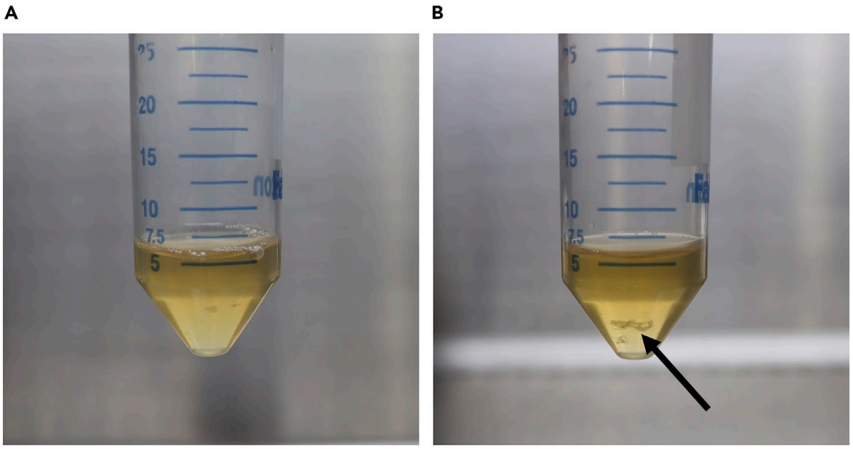

Figure 3. Digesting of dental pulp tissue

(A) An image of the minced dental pulp tissue in 6 mL of tissue digestion medium before digestion.

(B) An image of the dental pulp tissue after digestion for 30 min at 37°C. Arow: digested tissue debris.

Note: Culture period may be different to each sample. Observe the density of the formed adherent colonies carefully under microscopy. When some colonies reach high density, cell dissociation is available for cell expansion.

Cell passage and expansion

Timing: 7–10 days, depending on colony forming condition

This section describes the procedure for expanding CFU-F-forming cells to obtain large amount of hDPSCs.

5. Cell dissociation.
   a. Gently wash the flask twice with 3 mL of D-PBS.
   b. Put 1 mL of cell dissociation reagent, TrypLE™ select without phenol red, into each flask and incubate for 5 min at 37°C with 5% CO_2 in a CO_2 incubator.
   c. Add 2 mL of CGM per flask and suspend gently well to remove colony-forming cells from flask. Transfer the cell suspension into a 50 mL conical polypropylene centrifuge tube through a 70-μm cell strainer and centrifuge at 300 × *g* at 4°C for 6 min on a centrifuge machine.
   d. Discard the supernatant and resuspend the cell pellet with 1 mL of CGM gently. Count the number of ANCs as described in step 3.
6. Cell expansion.
   a. Seed 1.0–2.5 × 10^5 cells per flask into 10 mL of CGM onto T 75 flask and maintain hDPSCs in 10 mL of CGM at 37°C with 5% CO_2 in a CO_2 incubator.
   b. Change CGM twice a week.
   c. Passage or cryopreserve hDPSCs when reached at 70% confluent condition.

Cell cryopreservation

Timing: 6 h, depending on colony forming condition

This section describes the procedure for cryopreservation of expanded hDPSCs.

7. Cell cryopreservation.

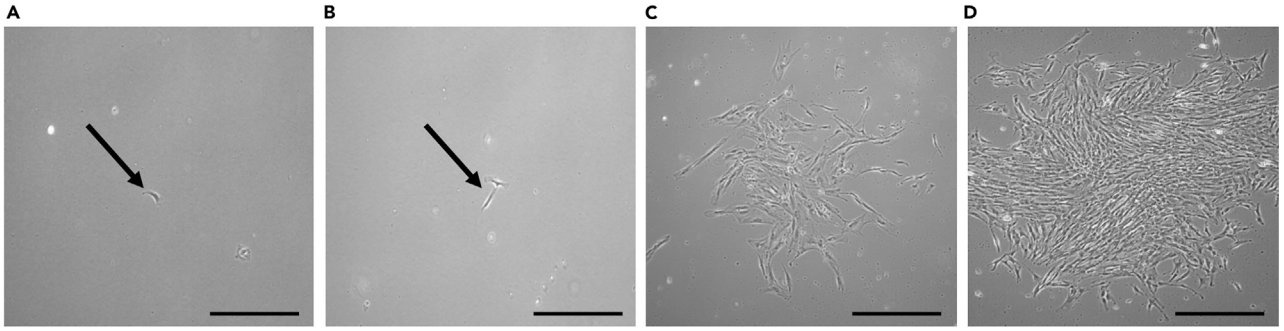

Figure 4. Forming of single attached cell-derived adherent colony

(A) A microscopic image of a single attached cell (arrow) on a culture flask.

(B) A microscopic image of the cell division of single attached cells on the culture flask.

(C) A microscopic image of the immature adherent cell cluster on the culture flask 8 days after cell seeding.

(D) A microscopic image of the mature adherent cell cluster on the culture flask 14 days after cell seeding. Scale bars, 100 μm.

a. Dissociate cultured cells as described in step 5. Resuspend cell pellet 1 mL of STEM CELL BANKER® cryomedium instead of CGM and store at 4°C. Count the number of ANCs as described in step 3.
b. Adjust the concentration to 2.0×10^6/mL with additional cryomedium and divide 1 mL of the cell suspension per cryogenic vial.
c. Keep the vials for 4 h at −80°C in a cell freezing container, then store in liquid nitrogen.

Cell thawing

Timing: ~30 min, depending on colony forming condition

This section describes the procedure for thawing of cryopreserved hDPSCs for further expansion.

8. Cell thawing.
   a. Put a cryogenic vial in a cell thawing system, ThawSTAR® CFT2, and transfer thawed cells into a 15 mL polystyrene conical centrifuge tube with 4 mL of CGM.

   Optional: Cell thawing is done using a water bath at 37°C.

   b. Centrifuge at 300 × *g* at 4°C for 6 min and wash the thawed cells with 5 mL of CGM.
   c. Aspirate the supernatant completely and suspend the cell pellet with 1 mL of CGM gently. Count the number of cells as described in step 3.
   d. Seed the cells as described in step 6.

Colony forming efficiency

Timing: 10–14 days, depending on colony forming condition

This section describes the colony forming ability of hDPSCs by CFU-F method (Figure 5).

9. Colony forming culture.
   a. Cultured ANCs on 100 mm culture dishes as described in step 4.

   Note: Adherent cell colony formation should be recognized under an inverted light microscopy approximately 10–14 days after seeding.

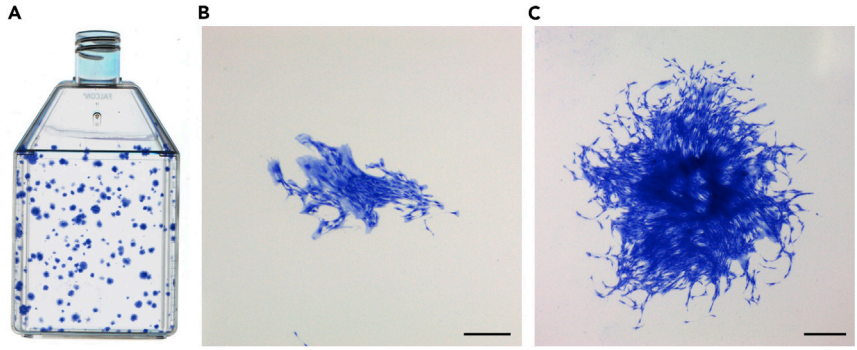

Figure 5. Formation of colony-forming unit-fibroblasts (CFU-F)

(A) A scanning image of CFU-F formation on a culture flask 14 days after cell seeding by Toluidine blue staining.

(B) A microscopic image of one of small-sized CFU-Fs on the culture flask.

(C) A microscopic image of one of large-sized CFU-Fs on the culture flask. B, C: Scale bars, 100 μm.

Note: Culture period may be different to each sample. Observe the density of the formed adherent colonies carefully under microscopy. When some colonies reach high density, cell staining is available. It may be better to check the colony density under an inverted light microscopy every day after day 10.

b. Wash twice with 1 mL of D-PBS and treat with toluidine blue staining solution for 1 day at 20°C.
c. Wash the dishes several times with Milli-Q water gently and dry at 20°C.
d. Capture the colonies using a light microscope and/or a scanner. Number of cell clusters, which contain larger than 50 cells, are scored under an inverted light microscope and calculate the colony-forming efficacy.

Immunophenotype assay by flow cytometric (FCM) analysis

Timing: 2–3 h, depending on culture dishes

This section describes the immunophenotypical characterization of hDPSCs by FCM method (Figure 6).

10. Immunostaining for FCM analysis.
    a. Dissociate cultured hDPSCs as described in step 5, wash with HBSS, and resuspend cell pellets in 1 mL of FCM buffer.
    b. Adjust the concentration of cell suspension to 1.0×10^6 ANCs/mL with additional FCM buffer, resuspend gently well, and iced, at least for 15 min.
    c. Divide the cell suspension into 100 μL into a 5 mL round bottom polypropylene centrifuge tubes and keep on ice.
    d. Incubate with appropriate primary antibody to CD146, CD105, CD90, CD73, CD45, CD35, CD14, CD11b, and human leukocyte antigen DR (HLA-DR) or isotype-matched controls antibody at a concentration of 1.0 μg/mL for 45 min on ice. Tap or shake the centrifuge tubes gently every 10 min.
    e. Centrifuge at 300 × *g* at 4°C for 6 min. Wash the cells twice with 2 mL of FCM buffer as described above.
    f. Put 500 μL of FCM buffer into the centrifuge tube, tap gently, and keep on ice.
    g. Add 5 μL of 7-AAD viability staining solution, tap gently, and incubate for 5 min on ice in the dark.
    h. Analyze the cells on a FCM analyzer. The percentage of positive living cells was determined compared to control living cells stained with corresponding isotype-matched antibodies in which a false-positive rate of less than 1% was accepted.

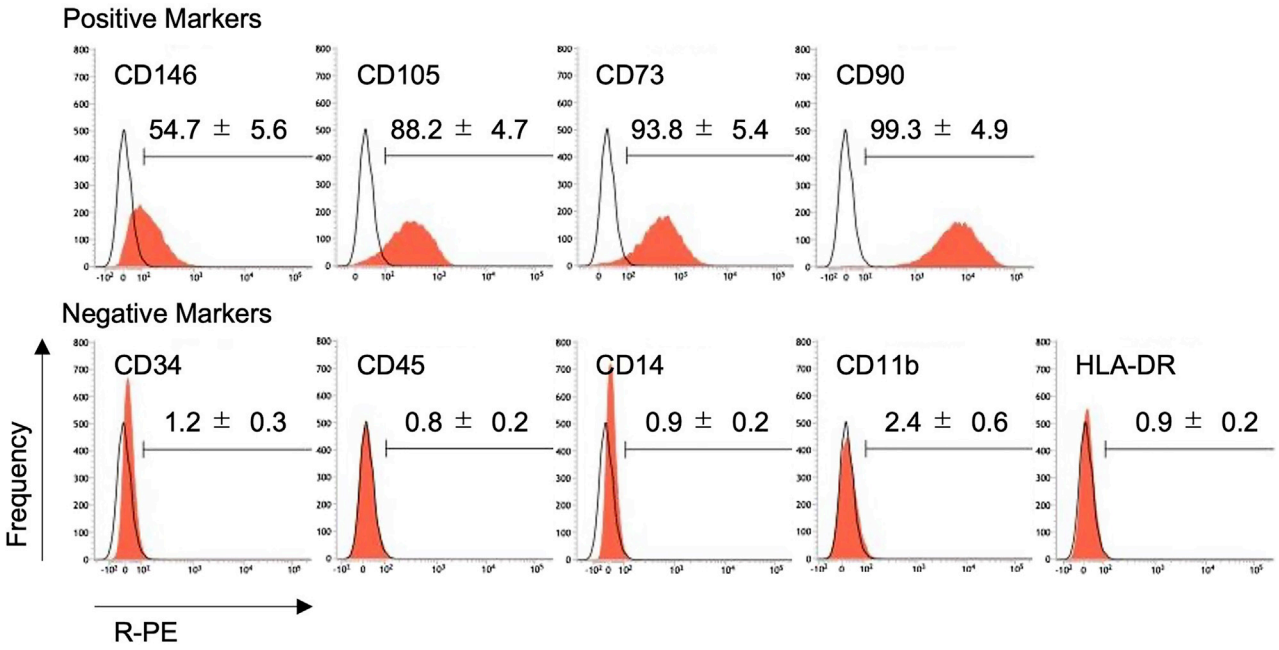

Figure 6. Immunophenotype of human dental pulp stem cells (hDPSCs)

Representative histograms of cell surface antigens were analyzed by flow cytometric analysis. The numbers indicate the mean ± standard error of mean of a positive rate of target markers. Areas filled with red; target antibody-stained histograms; solid lines; isotype-matched control-stained histograms. HLA-DR, human leukocyte antigen DR; R-PE, R-phycoerythrin.

Note: Keep the staining tubes under shield during the staining and storing to protect from the light to avoid loss of fluorescence.

Mesenchymal multipotent assay

Timing: 4–6 weeks

This section describes the *in vitro* multipotent capabilities of hDPSCs into osteoblasts, adipocytes, and chondrocytes.

11. Culture and staining for assessment of odontogenic/osteogenic and adipogenic potential.
    a. Seed expanded hDPSCs into a 60 mm or 35 mm culture dish at 1.0×10^5 or 3×10^4 in 5 or 5 mL of CGM, respectively. Incubate the cells at 37°C with 5% CO_2 in a CO_2 incubator and change the medium twice a week until the cells reach to 100% confluent condition.

    Optional: Seed expanded hDPSCs into a 100 mm culture dish or a well of 6 well plate at 1.0×10^6 or 1×10^4 in 10 or 1 mL of CGM, respectively.

    b. Replace the medium to an equivalent volume of osteogenic or adipogenic inductive medium. Change odontogenic/osteogenic or adipogenic inductive medium twice a week.
    c. Calcium deposition is analyzed by Alizarin red S staining 4 weeks after osteogenic induction.
        i. Aspirate the medium and rinse gently the cultures twice with D-PBS.
        ii. Dehydrate with 1 mL of 60% isopropanol for 1 min and rehydrate in Milli-Q water for 2–3 min.
        iii. Stain the cultures with 1% w/v Alizarin red S staining solution for 3–5 min at 20°C.
        iv. Rinse with Milli-Q water and remove excess Alizarin red S stain dye. Air dry well.
        v. Observe the cultures under an inverted light microscopy (see troubleshooting, problem 3).
    d. Lipid accumulation is analyzed by Oil red O staining 4 weeks after adipogenic induction.

i. Aspirate the medium and rinse gently the cultures twice with D-PBS and fix with 4% PFA for 20 min at 20°C. Rinse with D-PBS.
ii. Hydrate with 1 mL of 60% isopropanol for 1 min.
iii. Aspirate isopropanol solution and stain the cultures with Oil Red O staining solution for 10–20 min at 20°C.
iv. Rinse the cultures with 1 mL of 60% isopropanol and remove excess Oil Red O stain dye. Store the cultures with Milli-Q water at 4°C.
v. Observe the cultures under an inverted light microscopy (see troubleshooting, problem 4).

Note: Do not allow to air dry after Oil red O staining.

12. Culture and staining for assessment of chondrogenic potential.
    a. Seed expanded hDPSCs into a well of PrimeSurface® 96-well plate at 1.0×10^5 in 200 μL of CGM. Incubate the cells at 37°C with 5% CO_2 in a CO_2 incubator and change the medium gently twice a week for 1 week.

Note: Cell spheres should be recognized under a light microscopy 1, 2, 3 days after seeding.

Note: Medium should be changed very carefully after seeding (see troubleshooting, problem 5).

    b. Replace the medium to an equivalent volume of chondrogenic inductive medium. Change the chondrogenic inductive medium gently twice a week.
    c. Cartilage matrix formation is analyzed by Alcian blue staining 4 weeks after chondrogenic induction.
        i. Aspirate the medium and rinse gently the cultures twice with D-PBS and fix with 4% PFA for 60 min at 20°C. Rinse with D-PBS.

Pause point: The PFA-fixed samples can be stored at 4°C upto 1 month before paraffin embedding.

        ii. Dehydrate the samples through an ascending alcohol gradient in 70% (3 min) 80% (3 min), 90% (3 min), and 100% (3 min at 3 times) alcohol and clean with xylene (3 min at 3 times). Immerse them for 8 h in melted paraffin. Embed the samples in paraffin. Cut the embedded materials into 4-μm sections.

Pause point: The paraffin-embedded samples can be stored at 4°C or −20°C before sectioning.

        iii. Dewax the paraffin sections with xylene (3 min at 3 times), rehydrate the samples through a descending alcohol gradient in 100% (3 min at 3 times), 90% (3 min), 80% (3 min), and 70% (3 min) alcohol, and immerse in Milli-Q water. Treated with 3% acetic acid solution for 2 min, and stained with Alcian blue staining solution for 30 min at 20°C.
        iv. Wash in Milli-Q water, dehydrate through an ascending alcohol gradient in 70%, (3 min) 80% (3 min), 90% (3 min), and 100% (3 min, twice) ethanol, clean with xylene (3 min, twice), and treat with a mounting medium. Observe the sections under an upright light microscopy (see troubleshooting, problem 6).

13. Specific gene expression analysis for odontogenic/osteogenic, adipogenic, and chondrogenic potential.
    a. Odontoblasts/osteoblast-specific gene expression are analyzed 1 week after odontogenic/osteogenic induction. Adipocyte- and chondrocyte-specific gene expression are analyzed 4 weeks after adipogenic and chondrogenic induction.
    b. Wash the cultured cells with 1 mL of D-PBS twice and add solution. Collect the cultured cells with TRIzol® reagent using a cell scraper and extract total RNA according to the

manufacturer's instruction (see https://tools.thermofisher.com/content/sfs/manuals/trizol_reagent.pdf).

Pause point: The TRIzol-collected samples can be stored at −80°C before total RNA extraction.

Pause point: The extracted total RNA can be stored at −80°C before total RNA purification.

c. Digest the DNA with RQ1 RNase-free DNase according to the manufacturer's instruction (see https://www.promega.com/-/media/files/resources/protocols/product-information-sheets/g/rq1-rnase-free-dnase-protocol.pdf?rev=e52d482142fb48a68771a17c7dc458f4&sc_lang=en) and purify the digest sample with a RNeasy Mini Kit according to the manufacturer's instruction (see https://www.qiagen.com/us/resources/resourcedetail?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24&lang=en).

Pause point: The purified total RNA can be stored at −80°C before RT-PCR reaction.

d. Synthesize cDNA by reverse transcription of total RNA using a ReverTraAce® qPCR RT Master Mix according to the manufacturer's instruction (see https://www.toyobo-global.com/seihin/xr/lifescience/support/manual/QPK-101.pdf).
e. Amplify the cDNA with EagleTaq Master Mix and TaqMan probes for odontoblast/osteoblast-specific genes (*runt related transcription factor 2* [*RUNX2*] and *bone gamma-carboxyglutamate protein* [*BGLAP*]), adipocyte-specific genes (*peroxisome proliferator-activated receptor gamma* [*PPARG*] and *lipoprotein lipase* [*LPL*]), chondrocyte-specific genes (*SRY-box9* [*SOX9*] and *collagen, type X, alpha 1* [*COL10A1*]), and 18S rRNA on 96-well PCR plates with a real-time PCR machine.

PCR reaction master mix

Reagent	Amount
DNA template	2 μL (10 ng)
EagleTaq Master Mix	10 μL
TaqMan probe	1 μL
ddH_2O	7 μL

Present PCR reactions with the following format (if relevant):

PCR cycling conditions

Steps	Temperature	Time	Cycles
Pre-incubation 1	50°C	120 s	1
Pre-incubation 2	95°C	600 s	1
Denature	95°C	15 s	45
Annealing/Extension	60°C	60 s	
Hold	4°C	forever	

EXPECTED OUTCOMES

Upon completion of this protocol, we generate xenogeneic-free/serum-free hDPSCs by CFU-F method. The generated hDPSCs are available to biobanking for expansion, cryopreservation, and recovery. The generated hDPSCs exhibit MSC characteristics including CFU-F formation, immunophenotype positive to CD146, CD105, CD90, and CD73 and negative to CD45, CD35, CD14, CD11b, and HLA-DR, and multipotency into osteoblasts, adipocytes, and chondrocytes.

LIMITATIONS

hDPSCs can be generated from human dental pulp tissues with this protocol. The number of CFU-F colonies and CFU-F-forming cells (passage 0 cells) obtained from one tooth sample ranges between 10 and 100 colonies and between 1 and 5 × 10^6 cells. Generating hDPSCs might not be a problem but this protocol contains a limitation when performed with human samples. Furthermore, there is a great variability to obtaining cell number of hDPSCs upon sampled tooth conditions (ex. dental caries, pulpitis, caries-treated, aged). When the dental pulp tissues are not minced well, this protocol might not work to obtain enough number of colonies/colony-forming cells.

TROUBLESHOOTING

Problem 1

At step 2, occasionally, we cannot visually find dental pulp in a tooth sample.

Potential solution

Check the sample condition. Deciduous teeth are sometimes treated due to the dental caries. Use another sample if you notice a caries treatment in the sample.

Problem 2

At step 4, we cannot often notice a single attached colony on a culture flask under microscopy a few days or more after cell seeding.

Potential solution

Keep the culture and maintain the above-mentioned conditions because very small numbers of single attached cells are often hard to find. Using a fibronectin-coated flask/dish for cell seeding is an alternative to enhance cell attachment.

Problem 3

At step 11, osteogenic matrix is shrunken or removed under osteogenic condition.

Potential solution

Change to bigger sized culture dish or well and osteogenic medium is changed very carefully.

Problem 4

At step 11, lipid accumulation cannot be found in cultured cells under microscopy after adipogenic induction according to the protocol.

Potential solution

Generally, hDPSCs exhibit a low adipogenic capacity. One- or two-week extension of the culture period may be effective.

Problem 5

At step 12, cell spheres cannot be formed after seeding or formed cell spheres are broken before and after chondrogenic induction.

Potential solution

The cultures should be wasted.

Problem 6

At step 12, cartilage matrix deposition cannot be observed in cultures under microscopy after chondrogenic induction according to the protocol.

Potential solution

Generally, hDPSCs exhibit a low chondrogenic capacity. One- or two-week extension of the culture period may be effective.

RESOURCE AVAILABILITY

Lead contact

For further information or to request reagents, please direct requests to Dr. Takayoshi Yamaza, yamazata@dent.kyushu-u.ac.jp.

Materials availability

The materials used for this study can be created from commercially available materials, as is indicated in key resources table.

Data and code availability

The data sets supporting this protocol, and used in Figures, have not been deposited in a public repository but are available from the corresponding author upon request.

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AUTHOR CONTRIBUTIONS

S.S. and T.Y. conceived this project and prepared the manuscript. S.S. and H.Y. conducted experiments. K.Y. and T.T. participated in method development.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Friedenstein, A.J., Deriglasova, U.F., Kulagina, N.N., Panasuk, A.F., Rudakowa, S.F., Luriá, E.A., and Ruadkow, I.A. (1974). Precursors for fibroblasts in different populations of hematopoietic cells as detected by the *in vitro* colony assay method. Exp. Hematol. *2*, 83–92.

Fujiyoshi, J.K., Yamaza, H., Sonoda, S., Yuniartha, R., Ihara, K., Nonaka, K., Taguchi, T., Ohga, S., and Yamaza, T. (2019). Therapeutic potential of hepatocyte-like-cells converted from stem cells from human exfoliated deciduous teeth in fulminant Wilson's disease. Sci. Rep. *9*, 1535. https://doi.org/10.1038/s41598-018-38275-y.

Gronthos, S., Mankani, M., Brahim, J., Robey, P.G., and Shi, S. (2000). Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc. Natl. Acad. Sci. U.S.A. *97*, 13625–13630. https://doi.org/10.1073/pnas.240309797.

Iwanaka, T., Yamaza, T., Sonoda, S., Yoshimaru, K., Matsuura, T., Yamaza, H., Ohga, S., Oda, Y., and Taguchi, T. (2020). A model study for the manufacture and validation of clinical-grade deciduous dental pulp stem cells for chronic liver fibrosis treatment. Stem Cell Res. Ther. *11*, 134. https://doi.org/10.1186/s13287-020-01630-w.

Karnieli, O., Friedner, O.M., Allickson, J.G., Zhang, N., Jung, S., Fiorentini, D., Abraham, E., Eaker, S.S., Yong, T.K., Chan, A., et al. (2017). A consensus introduction to serum replacements and serum-free media for cellular therapies. Cytotherapy *19*, 155–169. https://doi.org/10.1016/j.jcyt.2016.11.011.

Ma, L., Makino, Y., Yamaza, H., Akiyama, K., Hoshino, Y., Song, G., Kukita, T., Nonaka, K., Shi, S., and Yamaza, T. (2012). Cryopreserved dental pulp tissues of exfoliated deciduous teeth is a feasible stem cell resource for regenerative medicine. PLoS One *7*, e51777. https://doi.org/10.1371/journal.pone.0051777.

Ma, L., Aijima, R., Hoshino, Y., Yamaza, H., Tomoda, E., Tanaka, Y., Sonoda, S., Song, G., Zhao, W., Nonaka, K., et al. (2015). Transplantation of mesenchymal stem cells ameliorates secondary osteoporosis through interleukin-17-impaired functions of recipient bone marrow mesenchymal stem cells in MRL/lpr mice. Stem Cell Res. Ther. *6*, 104. https://doi.org/10.1186/s13287-015-0091-4.

Makino, Y., Yamaza, H., Akiyama, K., Ma, L., Hoshino, Y., Nonaka, K., Terada, Y., Kukita, T., Shi, S., and Yamaza, T. (2013). Immune therapeutic potential of stem cells from human supernumerary teeth. J. Dent. Res. *92*, 609–615. https://doi.org/10.1177/0022034513490732.

Miura, M., Gronthos, S., Zhao, M., Lu, B., Fisher, L.W., Robey, P.G., and Shi, S. (2003). SHED: stem cells from human exfoliated deciduous teeth. Proc. Natl. Acad. Sci. U.S.A. *100*, 5807–5812. https://doi.org/10.1073/pnas.0937635100.

Sonoda, S., Tomoda, E., Tanaka, Y., and Yamaza, T. (2015). Properties and possibilities of human dental pulp-derived stem cells. Arch. Stem Cell Res. *2*, 1012. https://doi.org/10.13140/RG.2.1.2213.1684.

Sonoda, S., Yamaza, H., Ma, L., Tanaka, Y., Tomoda, E., Aijima, R., Nonaka, K., Kukita, T., Shi, S., Nishimura, F., and Yamaza, T. (2016). Interferon-gamma improves impaired dentinogenic and immunosuppressive functions of irreversible pulpitis-derived human dental pulp stem cells. Sci. Rep. *6*, 19286. https://doi.org/10.1038/srep19286.

Sonoda, S., Mei, Y.F., Atsuta, I., Danjo, A., Yamaza, H., Hama, S., Nishida, K., Tang, R., Kyumoto-Nakamura, Y., Uehara, N., et al. (2018). Exogenous nitric oxide stimulates the odontogenic differentiation of rat dental pulp stem cells. Sci. Rep. *8*, 3419. https://doi.org/10.1038/s41598-018-21183-6.

Sonoda, S., Murata, S., Nishida, K., Kato, H., Uehara, N., Kyumoto, Y.N., Yamaza, H., Takahashi, I., Kukita, T., and Yamaza, T. (2020). Extracellular vesicles from deciduous pulp stem cells recover

bone loss by regulating telomerase activity in an osteoporosis mouse model. Stem Cell Res. Ther. *11*, 296. https://doi.org/10.1186/s13287-020-01818-0.

Sonoda, S., Murata, S., Kato, H., Zakaria, F., Kyumoto-Nakamura, Y., Uehara, N., Yamaza, H., Kukita, T., and Yamaza, T. (2021a). Targeting of deciduous tooth pulp stem cell-derived extracellular vesicles on telomerase-mediated stem cell niche and immune regulation in systemic lupus erythematosus. J. Immunol. *206*, 3053–3063. https://doi.org/10.4049/jimmunol.2001312.

Sonoda, S., Yoshimaru, K., Yamaza, H., Yuniartha, R., Matsuura, T., Yamauchi-Tomoda, E., Murata, S., Nishida, K., Oda, Y., Ohga, S., et al. (2021b). Biliary atresia-specific deciduous pulp stem cells feature biliary deficiency. Stem Cell Res. Ther. *12*, 582. https://doi.org/10.1186/s13287-021-02652-8.

Sonoyama, W., Liu, Y., Fang, D., Yamaza, T., Seo, B.M., Zhang, C., Liu, H., Gronthos, S., Wang, C.Y., Wang, S., and Shi, S. (2006). Mesenchymal stem cell-mediated functional tooth regeneration in swine. PLoS One *1*, e79. https://doi.org/10.1371/journal.pone.0000079.

Taguchi, T., Yanagi, Y., Yoshimaru, K., Zhang, X.Y., Matsuura, T., Nakayama, K., Kobayashi, E., Yamaza, H., Nonaka, K., Ohga, S., and Yamaza, T. (2019). Regenerative medicine using stem cells from human exfoliated deciduous teeth (SHED): a promising new treatment in pediatric surgery. Surg. Today *49*, 316–322. https://doi.org/10.1007/s00595-019-01783-z.

Takahashi, Y., Yuniartha, R., Yamaza, T., Sonoda, S., Yamaza, H., Kirino, K., Yoshimaru, K., Matsuura, T., and Taguchi, T. (2019). Therapeutic potential of spheroids of stem cells from human exfoliated deciduous teeth for chronic liver fibrosis and hemophilia A. Pediatr. Surg. Int. *35*, 1379–1388. https://doi.org/10.1007/s00383-019-04564-4.

Tanaka, Y., Sonoda, S., Yamaza, H., Murata, S., Nishida, K., Hama, S., Kyumoto-Nakamura, Y., Uehara, N., Nonaka, K., Kukita, T., and Yamaza, T. (2018). Suppression of AKT-mTOR signal pathway enhances osteogenic/dentinogenic capacity of stem cells from apical papilla. Stem Cell Res. Ther. *9*, 334. https://doi.org/10.1186/s13287-018-1077-9.

Tanaka, Y., Sonoda, S., Yamaza, H., Murata, S., Nishida, K., Kyumoto-Nakamura, Y., Uehara, N., Nonaka, K., Kukita, T., and Yamaza, T. (2019). Acetylsalicylic acid treatment and suppressive regulation of AKT accelerate odontogenic differentiation of stem cells from the apical papilla. J. Endod. *45*, 591–598.e6. https://doi.org/10.1016/j.joen.2019.01.016.

Yamaza, T., Kentaro, A., Chen, C., Liu, Y., Shi, Y., Gronthos, S., Wang, S., and Shi, S. (2010). Immunomodulatory properties of stem cells from human exfoliated deciduous teeth. Stem Cell Res. Ther. *1*, 5. https://doi.org/10.1186/scrt5.

Yamaza, T., Alatas, F.S., Yuniartha, R., Yamaza, H., Fujiyoshi, J.K., Yanagi, Y., Yoshimaru, K., Hayashida, M., Matsuura, T., Aijima, R., et al. (2015). In vivo hepatogenic capacity and therapeutic potential of stem cells from human exfoliated deciduous teeth in liver fibrosis in mice. Stem Cell Res. Ther. *6*, 171. https://doi.org/10.1186/s13287-015-0154-6.

Yamaza, H., Tomoda, E., Sonoda, S., Nonaka, K., Kukita, T., and Yamaza, T. (2018a). Bilirubin reversibly affects cell death and odontogenic capacity in stem cells from human exfoliated deciduous teeth. Oral Dis. *24*, 809–819. https://doi.org/10.1111/odi.12827.

Yamaza, H., Sonoda, S., Nonaka, K., Kukita, T., and Yamaza, T. (2018b). Pamidronate decreases bilirubin-impaired cell death and improves dentinogenic dysfunction of stem cells from human deciduous teeth. Stem Cell Res. Ther. *9*, 303. https://doi.org/10.1186/s13287-018-1042-7.

Yuniartha, R., Yamaza, T., Sonoda, S., Yoshimaru, K., Matsuura, T., Yamaza, H., Oda, Y., Ohga, S., and Taguchi, T. (2021). Cholangiogenic potential of human deciduous pulp stem cell-converted hepatocyte-like cells. Stem Cell Res. Ther. *12*, 57. https://doi.org/10.1186/s13287-020-02113-8.