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.
Protocol

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

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https://doi.org/10.1016/j.xpro.2022.101386

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; Fujyoshi 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 IgG1 [HI111] (used at 1 μg/mL) | BioLegend | Cat # 301207 |
| R-PE mouse anti-human CD14 IgG1 [63D3] (used at 1 μg/mL) | BioLegend | Cat # 367103 |
| R-PE mouse anti-human CD34 IgG2a [561] (used at 1 μg/mL) | BioLegend | Cat # 343605 |
| R-PE mouse anti-human CD45 IgG1 [2D1] (used at 1 μg/mL) | BioLegend | Cat # 368509 |
| R-PE mouse anti-human CD73 IgG1 [AD2] (used at 1 μg/mL) | BioLegend | Cat # 344003 |
| R-PE mouse anti-human CD90 IgG1 [5E10] (used at 1 μg/mL) | BioLegend | Cat # 328109 |
| R-PE mouse anti-human CD105 IgG1 [P1H12] (used at 1 μg/mL) | BioLegend | Cat # 307605 |
| R-PE mouse IgG kappa [MOPC-21] (used at 1 μg/mL) | BioLegend | Cat # 400113 |
| R-PE mouse IgG2 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 # A5533 |
| Alizarin red S | Nacalai Tesque | Cat # 08456-65 |
| L-Ascorbic Acid 2-Phosphate | FUJIFILM Wako Chemicals | Cat # 323-44822 |
| Dexamethasone | Merck | Cat # A5268 |
| Dulbecco’s Minimum Essential Medium (DMEM), Low Glucose (1.0 g/L) | Nacalai Tesque | Cat # 14249-95 |
| Dulbecco’s Phosphate Buffered Saline (D-PBS) (1X) | Nacalai Tesque | Cat # 11482-15 |
| D-PBS (10X) | Nacalai Tesque | Cat # 386698-25MGCN |
| Ethanol, Absolute | Nacalai Tesque | Cat # 09735-75 |
| Fetal bovine serum (FBS) [170629-0130] | Equitech Bio | Cat # SFBM30-0500 |
| Hydrocortisone | Merck | Cat # 405268-10GMCN |
| Hanks’ balanced salt solution (HBSS) (1X) | Nacalai Tesque | Cat # 410957-1GMCN |
| Indomethacin | Merck | Cat # 29113-95 |
| 3-Isobutyl-1-methylxanthine (IBMX) | Nacalai Tesque | Cat # 0557866001 |
| Isopropanol | Nacalai Tesque | Cat # 39601095 |
| ITS™ Premix | BD Bioscience-Discovery Labware | Cat # 00289-54 |
| Liberase™ MNP-S | Roche | Cat # 20091 |
| Liquid nitrogen | Muto Pure Chemicals | Cat # 05-200-1A |
| Marinol™ 750CPC | Biological Industry | Cat # 05-201-1U |
| MSC NutriStem® XF Basal Medium | Biological Industry | Cat # 05-202-1A |
| 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 (100X) | Nacalai Tesque | Cat # 163-04243 |
| Potassium Dichromate Phosphate | FUJIFILM Wako Chemicals | Cat # 343605 |
| RevertAq® cDNA RT Master Mix | TOYOBO | Cat # 74104 |
| RNasy Mini Kit | QIAGEN | Cat # 02892-54 |

(Continued on next page)
### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE                  | IDENTIFIER      |
|---------------------|-------------------------|-----------------|
| RG1 RNase-free DNase| Promega                | Cat # M6101     |
| STEM CELL BANKER® cryomed| Zenoa               | 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 (TGFβ1) | PeproTech       | Cat # AF-100-21C-10ug |
| Trypan blue solution| Bio-Rad Laboratories | Cat # 1450021   |
| TRYptol® 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

| Biological samples | Human teeth | Oligonucleotides | Other |
|--------------------|-------------|------------------|-------|
|                    | N/A         |                  |       |
|                    |             |                  |       |

#### Oligonucleotides

| Oligonucleotides | SOURCE                           | IDENTIFIER  |
|------------------|----------------------------------|-------------|
| BGLAP            | Thermo Fisher Scientific         | Cat # 4331182|
| COL10A1          | Thermo Fisher Scientific         | Cat # 4331182|
| LPL              | Thermo Fisher Scientific         | Cat # 4331182|
| PPARG            | Thermo Fisher Scientific         | Cat # 4331182|
| rRNA, 18S        | Thermo Fisher Scientific         | Cat # 4331182|
| SOX9             | Thermo Fisher Scientific         | Cat # 4331182|

#### Other

| Other                       | SOURCE                                | IDENTIFIER  |
|-----------------------------|---------------------------------------|-------------|
| 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 |
| CO2 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|

(Continued on next page)
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  |

*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 use.

### 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   |

*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 |

*Note:* Complete growth medium should be prepared just before isolation.

### 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 |

*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 x 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 (1x)                     | 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 | 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 | 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 | 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 | 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 | Final concentration | Amount |
|----------------|---------------------|--------|
| TGFB1          | 1 μg/mL             | 10 μg  |
| D-PBS (1×)     | 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 TGFβ1 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 nM)            | 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  |
| TGFβ1 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.

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**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).
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, mix 10 μL of cell suspension with 90 μL 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.
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 $1–2 \times 10^6$ 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).
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, TrypLETM select without phenol red, into each flask and incubate for 5 min at 37°C with 5% CO₂ in a CO₂ 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 × 10⁵ 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% CO₂ in a CO₂ 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.
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 $2.0 \times 10^6$/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.
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.
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 \( \times \) 10^5 or 3 \( \times \) 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 \( \times \) 10^6 or 1 \( \times \) 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.
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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 × 10⁵ in 200 µL of CGM. Incubate the cells at 37°C with 5% CO₂ in a CO₂ 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 up to 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/lifesience/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.

| Reagent            | Amount   |
|--------------------|----------|
| DNA template       | 2 µL (10 ng) |
| EagleTaq Master Mix| 10 µL    |
| TaqMan probe       | 1 µL     |
| ddH2O              | 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      |
| Denaturation      | 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 $5 \times 10^6$ 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.

ACKNOWLEDGMENTS
We thank all the authors of the original study (Iwanaka et al., 2020) from which this protocol was generated. This work was supported by the grants-in-aid for Early-Career Scientists (JSPS KAKENHI grant number JP19K18945 and JP21K16932 to S.S.) of Japan Society for the Promotion of Science (JSPS).

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.

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