Published in final edited form as:

Methods Mol Biol. 2019 ; 1922: 21–27. doi:10.1007/978-1-4939-9012-2_3.

Establishment of Stable Cell Lines from Primary Human Dental Pulp Stem Cells

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Summary/Abstract

i.

This protocol is for the isolation of primary human dental pulp stem cells (DPSCs) from adult extracted molars, and for the generation of high titer lentivirus for *in vitro* infection of the DPSCs. Stable cell lines of dental pulp stem cells are generated, maintained in culture, and used for subsequent experiments.

Keywords

ii. Lentivirus; Gene Transfer Techniques; Genetic Transduction; Genetic Recombination; Somatic Stem Cells

1. Introduction

Lentiviral expression vector systems have been used extensively to overexpress proteins in mammalian cells. This method of gene transfer is used for otherwise difficult to transfect target cells, and has allowed the study of protein function in the same (1,2). Briefly, a gene of interest is cloned into a commercially available, replication incompetent lentiviral vector. The remaining genomic material necessary for virion assembly is co-transfected into packaging cell lines, commonly human embryonic kidney cells 293 (HEK 293 cells), which will produce infectious transgenic lentiviruses in culture. This transgenic virus is then used to infect and deliver the gene of interest into a target cell type (1,2,3,4). Because lentiviruses naturally integrate into the host genome, the gene of interest is now stably passed down to daughter cells.

Many different lentiviral transfer plasmids exist into which one can clone their sequence of interest. In this protocol, a lentiviral transfer plasmid is used that contains a CMV promoter-driven gene of interest, an SV40 promoter-driven GFP reporter, and a puromycin resistance selection marker for mammalian cells. Dental pulp stem cells (DPSCs) are used in these studies for their ability to differentiate into dentin-forming odontoblasts. Understanding the molecular pathways by which dental pulp stem cells differentiate into dentin secreting odontoblasts promises an opportunity to use these stem cells for regenerative therapies of injured dentin (5,6,7).

2. Materials

3.1 Isolation of primary human DPSCs

- Adult molars
- Povidone-iodine solution
- Sterilized dental fissure burs
- Type I collagenase (3 mg/ml)
- Dispase (4 mg/ml)
- 70-µm strainer
- Dulbecco's Modified Eagle Medium: 4 g/L D-Glucose, 4mM L-Glutamine, 1mM Sodium Pyruvate, Phenol Red
- Antibiotic-Antimycotic 100x: 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Gibco Amphotericin B
- 0.05% Trypsin-EDTA, Phenol Red
- Dimethyl sulfoxide (DMSO)
- Defined Fetal Bovine Serum (HyClone)
- Mr. Frosty™ Freezing Container (Thermo Scientific™)

Cell Maintenance - DMEM Media (per 500 mL)

- 500 mL Dulbecco's Modified Eagle Medium: 4 g/L D-Glucose, 4mM L-Glutamine, 1mM Sodium Pyruvate, Phenol Red
- 50 mL Defined Fetal Bovine Serum (HyClone)
- 5 mL antibiotic-antimycotic 100x (1% w/v)

3.2 Establishment of Stable Cell Lines from Primary Human DPSCs

- 293FT Cells
- 100mm, 150mm tissue culture dishes
- 6-well plate
- Ultracentrifuge tubes, 38.5 mL, 25 × 89 mm
- Dulbecco's Modified Eagle Medium: 4 g/L D-Glucose, 4mM L-Glutamine, 1mM Sodium Pyruvate, Phenol Red
- Antibiotic-Antimycotic 100x: 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Gibco Amphotericin B
- Phosphate Buffered Saline without Ca²⁺ and Mg²⁺
- Defined Fetal Bovine Serum (HyClone)
- Sodium Phosphate, Dibasic

- Sodium Chloride
- Sodium Hydroxide
- Tris-Cl
- Potassium Chloride
- Glycerol
- Cell Culture Grade Water, Deionized, Ultra Pure, Endotoxin Free, Sterile
- 0.45 μ m Low-protein binding filter flask (Millipore)
- Puromycin Dihydrochloride (10mg/mL in H₂O)

Transfection reagents

- 1mM Tris-Cl, pH 7.05
- Calcium Chloride 2 M solution
- 15% Glycerol PBS Solution (per 50 mL)
- 2X HBS Buffer (per 500 mL): 280 mM NaCl, 10 mM KCl, 1.5 mM Na₂HPO₄2H₂O, 12 mM dextrose, 50 mM HEPES (see Note 1)

3. Methods

3.1 Isolation of Primary Human DPSCs

1. Third molars were collected from adult patients, decontaminated with povidone-iodine solution. Teeth were sectioned longitudinally using sterilized dental burs to reveal the pulp chamber. Exposed pulp tissues were gently separated from the crown and root, collected, and enzymatically digested with type I collagenase (3 mg/ml) and dispase (4 mg/ml) for 1 hour at 37°C. Single-cell suspensions were obtained by passing the cells through a 70- μ m strainer (8).
2. Cells were counted and seeded at a density of 1.8×10^4 /cm². Cell cultures were maintained with Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 1% antibiotic-antimycotic 100x, at 37°C with 5% CO₂. The medium was refreshed the next day after initial cell attachment and thereafter at three times per week. Cells exhibit a fibroblast-like morphology when observed under the microscope.
3. Cells were detached by trypsinization whenever 80–90% confluent using 0.05% Trypsin-EDTA, Phenol Red solution and were re-plated at the same density. Colony-forming units (aggregates of 50 cells) derived from dental pulp tissue averaged 22–70 colonies/10⁴ cells plated, as previously published (8).
4. For storage, cells were trypsinized, centrifuged at 218 x g for 5 minutes, and resuspended in 90% FBS-10% Dimethyl sulfoxide (DMSO). Resuspension was frozen at –80°C (see Note 2). Cells were transferred to liquid nitrogen within a week.

3.2 Establishment of Stable Cell Lines from Primary Human DPSCs

Day 0:

1. Split four 100mm dishes of 95% confluent 293FT cells into five to eight 150mm dishes. For each dish, use 25 mL of DMEM media (see Note 3).
2. Rock the plate gently to evenly distribute the cells.
3. Incubate the dishes at 37°C overnight. The cells should reach 90% confluence in 24 hours.

Day 1:

1. Warm 330 mL of DMEM media to room temperature, and replace cell's old media.
2. Wait at least 4–6 hours to start the transfection.
3. In a 50 mL conical tube, prepare the following mixture:
 - a. 840 $\times$ 5–8 μ l of 1mM Tris-Cl
 - b. 11.1 $\times$ 5–8 μ g of lentivirus plasmid (e.g. pLenti-hDMP1-GFP-2A-Puro or pLenti-GFP-2A-Puro)
 - c. 10.7 $\times$ 5–8 μ g of psPAX2 (Addgene)
 - d. 5.8 $\times$ 5–8 μ g of pMD2.G (Addgene)
 - e. 5.1 $\times$ 5–8 μ g of pHPV17
4. At this point mix thoroughly, using bubbling to mix. Never vortex the mixture (see Note 4).
 - a. Add 120 $\times$ 5–8 μ l of 2 M CaCl₂ solution drop by drop to DNA solution while bubbling the mixture.
 - b. Mix thoroughly by bubbling.
5. Add the above mixed DNA-CaCl₂ solution to 960 $\times$ 5–8 μ l of 2X HBS drop-wise, slowly, while bubbling.
 - a. Wait about 15–20 minutes. Then mix again and pour directly into the 150mm dish of cells, 2 mL per dish of cells. (see Note 5).
6. Put the tissue culture dishes back into the incubator.

Day 2:

1. Warm DMEM media to room temperature.
2. Check the cells using a fluorescent microscope; 20–30% of the cells should be GFP positive.
3. Fifteen to sixteen hours after initial transfection, remove the transfection media from the dishes, leaving only a little bit of media behind. Add 1–2 mL of 15% glycerol PBS solution to dishes and wait for 1 minute. Then wash each dish

twice with 25 mL of 1x PBS. Then add 25 mL of fresh DMEM media to each dish.

4. Put the tissue culture dishes back into incubator for 24 hours.

Day 3:

1. Twenty-four hours post transfection, collect the media (lentivirus medium) and check cells. More than 50% cells should be GFP positive by now.
2. Add 25 mL of DMEM media.
3. Put cells back into the incubator. Cells detach very easily at this point, so be very gentle.
4. Split the target cells (DPSCs) to be infected into a 6-well plate, be sure to have enough cells per well to reach 60% confluence the next day. For stem cells, we use the lowest passage cells to infect virus and establish cell lines.

Day 4:

1. Another 24 hours later, collect the virus containing supernatant into four 50 mL conical tubes and centrifuge all medium for 10 minutes at $872 \times g$.
2. To concentrate lentivirus by ultracentrifugation, divide the filtered virus-containing supernatant among six ultracentrifuge tubes.
3. Centrifuge in a Beckman SW-28 rotor for at least 2 hours at $68,383 \times g$, 4°C .
4. Gently carry the centrifuge tubes back to the tissue culture hood and pour out the supernatant. There should be a tiny semi-transparent pellet at the bottom of each centrifuge tube.
5. Dry the side of each tube with Kimwipe.
6. Add 500 μL of cold serum-free DMEM media to every tube and re-suspend the pellet by swirling and gentle pipetting. Do NOT pipet too much because it will degrade the virus.
7. After re-suspending all virus pellets, pipet the virus solution into an Eppendorf tube and add polybrene, to reach 5–10 $\mu\text{g}/\text{mL}$ final concentration. Maintain at room temperature for 15–20 minutes.
8. Wash the cells to be infected twice with 1xPBS, and add the virus/polybrene/serum-free DMEM medium to the 6-well plate of cells, 1 mL/well. Incubate the cells in the biosafety cabinet for 4 hours. Then add 3 mL of the full nutrient medium to each well.

Day 5:

1. Wash out the virus-containing medium with 1xPBS, twice, and add fresh DMEM media to target cells in 6-well plate.
2. Twenty-four to forty-eight hours later, check the target cells, and finally add puromycin 1–10 $\mu\text{g}/\text{mL}$ to begin the selection. It is recommended to use 1 $\mu\text{g}/\text{mL}$

of puromycin to select PDL, HMSC and DPSC cells and 5–10 µg/ml to select tumor cell lines.

4. Notes

1. To make 2x HEPES-Buffered Saline (HBS) solution: Dissolve 1.6 g of NaCl, 0.074 g of KCl, 0.027 g of Na₂HPO₄·2H₂O, 0.2 g of Dextrose, and 1 g of HEPES in a total volume of 90 mL of distilled H₂O. Adjust the pH to 7.05 with 0.5 N NaOH, and then adjust the volume to 100 ml with distilled H₂O. Filter with 0.22 µm filter. This solution is stable at room temperature for 6 months
2. A freezing container is used to freeze cells at a rate of –1°C/minute in the –80°C freezer. After 24 hours, cells can be transferred to a liquid nitrogen tank. To prevent thawing during transport, dry ice may be placed inside an insulated container and cryovials containing cells may be placed inside. To thaw cells, it is best to thaw quickly. Once again, cryovials can be placed inside an insulated container containing dry ice. Once in the cell culture area, hold the cryovial inside a 37°C water bath, making sure the water line is well below the cap in order to prevent infiltration. Remove cryovial just before all the ice melts and proceed to resuspend in media. Add cryovial contents to 50 ml centrifuge tube and add culture media drop-wise for about 5 ml to allow cells to adjust to the new osmolality. Add remaining media and plate into tissue culture dish.
3. It is important to use low passage 293FT cells for the production of viruses. To make sure the cells are always in the fastest growth phase, never let the cells grow to 100% confluence. Prepare and freeze stocks and use a new vial whenever making more virus.
4. The transfection reagents should never be vortexed. “Bubbling” refers to the use of two pipettes, simultaneously, to mix the transfection reagents. Using one hand, a pipette will be used to introduce air (“bubbles”) to the bottom of the tube. Using the other hand, another pipette will be used to introduce the DNA mixture, drop-wise and very slowly, into the tube.
5. Be careful not to tilt the plates too much, as cells may detach easily.

References

1. Miller AD Retrovirus Packaging Cells. Human Gene Therapy 1, 5–14 (1990). [PubMed: 2081186]
2. Marino MP, Luce MJ, Reiser J (2003) Small- to Large-Scale Production of Lentivirus Vectors In: Federico M (eds) Lentivirus Gene Engineering Protocols. Methods in Molecular Biology, vol 229 Humana Press.
3. Mosimann C & Zon LI Chapter 10 - Advanced Zebrafish Transgenesis with Tol2 and Application for Cre/lox Recombination Experiments in Methods in Cell Biology (eds. Detrich HW, Westerfield M & Zon LI) 104, 173–194 (Academic Press, 2011). [PubMed: 21924163]
4. Austin J Transgenes. in Encyclopedia of Genetics (eds. Brenner S & Miller JH) 1989–1990 (Academic Press, 2001).
5. Couble ML et al. Odontoblast differentiation of human dental pulp cells in explant cultures. Calcif. Tissue Int 66, 129–138 (2000). [PubMed: 10652961]

6. Ishizeki K, Nawa T & Sugawara M Calcification capacity of dental papilla mesenchymal cells transplanted in the isogenic mouse spleen. *Anat. Rec* 226, 279–287 (1990). [PubMed: 2327600]
7. Kuo M, Lan W, Lin S, Tsai K & Hahn L Collagen Gene-Expression in Human Dental-Pulp Cell-Cultures. *Arch. Oral Biol* 37, 945–952 (1992). [PubMed: 1281629]
8. Gronthos S et al. “Postnatal Human Dental Pulp Stem Cells (DPSCs) in Vitro and in vivo.” *Proceedings of the National Academy of Sciences of the United States of America* 9725 (2000): 13625–13630. Print. [PubMed: 11087820]