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Culturing and Neuronal Differentiation of Human Dental Pulp Stem Cells

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Abstract

A major issue in studying human neurogenetic disorders, especially rare syndromes affecting the nervous system, is the ability to grow neuronal cultures that accurately represent these disorders for analysis. Although there has been some success in generating induced pluripotent stem cells (iPSC) from both skin and blood, there are still limitations to the collection and production of iPSC from these biospecimens. We have had significant success in collecting and growing human dental pulp stem cells (DPSC) from exfoliated teeth sent to our laboratory by the parents of children with a variety of rare neurogenetic syndromes. This protocol outlines our current methods for the growth and expansion of DPSC from exfoliated (baby) teeth. These DPSC can be differentiated into a variety of cell types including osteoblast, chondrocytes and mixed neuron and glial cultures. Here we provide our protocol for the differentiation of early passage DPSC cultures into neurons for molecular studies.

Keywords

Dental Pulp Stem Cells; SHED teeth; Deciduous Teeth; Stem Cells; Neurogenetics; Rare disorders

Introduction

One of the greatest challenges to the study of neurogenetic syndromes affecting the brain is the inability to access live neurons from a sufficient number of individuals with these disorders for various molecular and functional studies. One approach to overcome this challenge has been the collection of skin biopsies from individuals with neurogenetic disorders to create fibroblast cell lines which are then induced into pluripotency, becoming induced pluripotent stem cells (iPSCs), and finally differentiated down neuronal lineages in culture (Marchetto et al.). However, there are still significant problems with this approach: 1) fibroblasts must be obtained through a fairly invasive skin biopsy which causes undue pain and distress in developmentally disabled or autistic children; 2) induction of fibroblasts into stem cells (reprogramming) and then neuronal lineages is a laborious expensive task

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that may not maintain epigenetic marks on the DNA that are essential to proper gene regulation in the native neuronal tissue (Pick et al., 2009); 3) viral vectors used to induce iPSC to pluripotency are immunogenic making downstream therapeutic uses for these neurons improbable; 4) it is difficult to obtain large numbers of these iPSC lines from a broad spectrum of individuals in order to overcome the inherent genetic heterogeneity among samples from a given syndrome; and 5) individuals with rare genetic syndromes may be geographically dispersed and may not be able to travel to adequately equipped laboratories.

In this unit we describe a non-invasive, cost effective approach using dental pulp stem cells (DPSC), which we routinely use in our laboratory to evaluate neurogenetic disorders. Dental pulp stem cells are multipotent neuronal precursors (i.e. cells already destined to become neurons and therefore do not need reprogramming) derived from neural-crest cells that reside deep in the dental pulp and can easily be collected from normally shed deciduous “baby” teeth. In this unit we describe how to collect exfoliated teeth from remote locations for the establishment of primary DPSC lines in the laboratory. The process of generating neurons from DPSC is now well established (Arthur et al., 2008; Kiraly et al., 2011; Kiraly et al., 2009; Nosrat et al., 2004), and we have used it to investigate the cellular and gene expression changes that occur in neurogenetic disease. Here we outline both the initiation and culturing of DPSC from pulp tissue and the differentiation of these DPSC into mixed neuronal and glial cultures that we used for molecular analysis of neurogenetic disease.

BASIC PROTOCOL 1: Collection and Transportation of Exfoliated Teeth

We have developed a successful procedure for remote tooth collection. As long as the tooth arrives within 48-72hrs of the time it fell out it will be viable for growing DPSC. Here we provide the basic protocol for remote collection of exfoliated teeth.

Materials

Transport Media (see reagents and media)

15ml Falcon Tubes

Liquid biohazard shipping bag (SafPak) and box (Cat# STP-210EXMT)

Parafilm™

1. Once a subject is identified for our study, a “tooth kit” is sent to the family, which contains 10ml of the Transport Media in a 15ml Falcon tube sealed at the top with Parafilm™.
- The box also contains return instructions, a biohazard bag with diaper, a pre-filled FedEx form and any paperwork needed (consent forms or surveys, etc.) to be returned with the tooth. The families are instructed to store the tube of media at 4°C (in the refrigerator) but they often do not and yet results are still adequate (Urraca et al., 2015).

2.

Families are instructed that once the tooth falls out it must be placed in the Transport Media within 20 minutes and sent to the lab within 24hrs.

Teeth have been viable for up to 72hrs, but optimal DPSC recovery occurs within the first 48hrs in our hands.

Upon receiving the tooth, if the tooth is not clean, aspirate the transport media from the tooth and add 5.0 ml of Washing Media. Rinse it well in the tube with a sterile Pasteur pipette, discard the wash and repeat two times.

The Extraction Protocol should be performed immediately on new teeth, if possible, but the maximum time for storage in our hands is 72hrs from the time the tooth came out of the mouth.

BASIC PROTOCOL 2: Dental Pulp Extraction Protocol

DPSC reside deep in the pulp of the tooth. Teeth must first be broken or bored to access the pulp tissues before culturing. This protocol describes accessing and digesting the pulp, culturing differentiated pulp cells and production of DPSC cultures for differentiation or storage.

Materials

Sterile forceps

Petrie dishes

Washing Media (see reagents and media)

DPSC Culture Media (see reagents and media)

Dispase II (From Roche Cat# 4942078001)

Collagenase, Type I (10mg/mL) (ThermoFisher Cat# 17100017)

12-well culture plates, Poly-D-lysine coated (Fisher Scientific Cat# 08-774-270)

NOTE: All Solutions and equipment coming in contact with living cells must be sterile and aseptic technique should be used.

1.

Upon receiving a tooth carefully inspect the media for contaminants, remove the media, and under sterile conditions break open the tooth using a dental tool designed to crack open teeth or a small hand held grinding tool like a Dremmel™.

If the media appears very cloudy or fungus can be seen growing in the tube, it may be best to discard this particular tooth and ask for another so as not to contaminate all of the cultures currently in the incubator.

2. Pull out the pulp (red material in the center of the tooth) using a sterile pair of forceps and place the pulp in a sterile petri dish. Cover the pulp completely with 10ml of pre-warmed 37 °C Washing Media.

3. Place the petri dish with the pulp under a 4-6× dissection microscope and mince the pulp into small fragments using a sterile razorblade.

4. Transfer the minced pulp with the Washing Media to a 15 ml centrifuge tube and spin at 2000 RPM for 5 minutes to pellet the pulp. Aspirate the washing media and re-suspend the pelleted pulp in 4 ml of Culturing Media containing 3 mg/ml collagenase and 1 to 4mg/ml of Dispase II. Mix well by pipetting up and down several times. Incubate minced pulp at 37°C for 1 hour.

5. Following enzymatic digestion, centrifuge the tube at 2000 RPM for 5 minutes. Aspirate the supernatant and re-suspend the pellet in 1.0 ml of culturing media. Re-suspend the pellet well and seed the digested pulp in a Poly-D-lysine coated 12-well tissue culture plate. Incubate overnight in a 37°C incubator with 5% CO₂.

6. The next day remove the supernatant from the well and spin down the floating unattached cells. Re-suspend the pellet in 1.0 ml of fresh media. Seed cells in another well of the 12-well plate. Add 1.0 ml of fresh DPSC Culture Media to the original well. Cultures will have visible debris and floating cells (Figure 1a).

Some of the floating unattached cells will attach to the surface when transferred to the fresh well. These “floating cells” also contain dental pulp stem cells (DPSC).

7. Cells derived from the dental pulp are visible usually within a week (Figure 1b). Wash the cells with 1.0 ml of washing media and change the media two times a week until just before they reach confluency (Figure 1c).

8. When the cells reach sub-confluence (Figure 1c) passage both the well seeded with the digested pulp (original well) and the well seeded with the floating cells (second well). These wells can be combined at this stage.

This process can take anywhere from 2 weeks to one month, so the cells should be observed carefully at least every other day to three days until they look like Figure 1c.

9. DPSC cultures are passaged at a ratio of 1:3. One part is sub-cultured for passage, parts 1 and 2 are frozen for future use at this passage.

BASIC PROTOCOL 3: Passage, Freezing and Thawing of DPSC Cultures

The cells obtained from the dental pulp form a monolayer on the small 12-well plate. Here we describe expanding these DPSC onto larger plates, passaging the DPSC and freezing them for long term storage of early passages in a cryobank.

Materials

DPSC Culture Media (see reagents and media)

Washing Media (see reagents and media)

HyClone HyQTase (GE Life Sciences - HyClone Cat# SSV30030.01)

12-well Poly-D-Lysine coated culture plates (Fisher Scientific Cat# 08-774-270)

Freezing Media: DPSC culture media supplemented with 15% DMSO

Nalgene® Cryo 1°C freezing container

NOTE: All solutions and equipment coming into contact with DPSC must be sterile and aseptic technique must be used for best results.

NOTE: All culture incubations are carried out in a humidified 37°C, 5% CO₂ incubator (Thermo Scientific Heracell 150i) in our laboratory.

1. Culture DPSC on poly-D-lysine coated surfaces only. Upon reaching sub-confluency, carefully aspirate the DPSC Culture Media, wash the cells 2× with Washing Media and add HyQtase to cover the surface of the cells. Incubate at 37 °C for 5 minutes.
2. When the cells have detached from the surface of the plate stop the reaction by adding 2 volumes of DPSC Culture Media. Transfer the cells to a centrifuge tube and spin the tubes 1100 RPM for 5 minutes.
3. Remove the supernatant. Add DPSC Culture Media to sub culture the cells at a ratio of 1:3. One part is sub-cultured for passage, parts 1 and 2 are frozen for future use at this passage.

At this step, DPSC should be frozen for use later as a representation of this particular passage. Since these are primary cultures one should consider using these cells for experiments between passage 2 and passage 5 for optimal results.

4. To freeze DPSC for long term storage in a liquid Nitrogen cryobank, detach cells from the surface of the culture plate using HyQtase as in the protocol for passaging the cells. Add 2 volumes of DPSC Culture Media, transfer the cells to a

centrifuge tube and spin the tubes at 1100 rpm to pellet the cells. Aspirate the supernatant and re-suspend the pellet in Freezing Media at a density of 5×10^5 cells per ml.

5. Transfer the re-suspended cells to cryo tubes. Store the tubes in a Nalgene® Cryo 1°C container at -80 °C for 24 hours.

6. The next day, transfer the tubes to a cryopreservation storage unit (-196 °C) for long term storage.

Basic Protocol 4: Differentiation of DPSC into Mixed Neuronal Cultures

We have adapted the three step protocol of Kiraly *et al.* (Kiraly et al., 2009) for the induction of neurons from DPSC. The neurons produced by this protocol are positive for neuronal markers such as TUJ1 (Figure 2b) and NeuN as well as glial markers like GFAP and undergo expression changes indicative of neuronal differentiation including the expression of synapse associated and ion channel transcripts (Urraca et al., 2015). Cells from morphologically homogenous culture of dental pulp stem cells (passage 1-4) are seeded on poly-D-lysine coated 6-well plates for imaging and electrophysiological studies or T-25 flasks for molecular studies.

Materials

DPSC Culture Media (see reagents and media)

6-well Poly-D-Lysine coated culture plates (Fisher Scientific Cat# 08-774-268)

T-25 Poly-D-Lysine coated culture Flasks (Fisher Scientific Cat# 08-774-332)

Epigenetic Reprogramming Media (see reagents and media)

Neural Differentiation Media (see reagents and media)

Neural Maturation Media (see reagents and media)

0.2µm Filter sterilizing units

Step I - Epigenetic Reprogramming—NOTE: All the media for the induction of the DPSC to Neurons has to be prepared fresh before addition to the cells.

1. Aspirate the media from sub-confluent DPSC in plates or flasks. Wash the cells with Washing Media one time.
2. Add freshly prepared Epigenetic Reprogramming Media which has been pre-warmed to 37 °C and incubate the cells in this media for 48 hrs in a 37 °C and 5% CO₂ incubator.

Step II – Neural Differentiation

1. Aspirate the media from the reprogrammed cells. Wash the cells with Washing Media 1×.

2. Add the freshly prepared Neural Differentiation Media and incubate the cells in this media for 3 days in a 37 °C incubator.

The cells will begin to acquire the morphology of neurons on day 2 of incubation in the Neural Differentiation Media (Figure 2a). Some cells will already have elongated projections and a pyramidal appearance, some will look like astrocytes and some will have long complex projections (Figure 2b). We estimate approximately 30% of the cells may be glial-like in this mixed culture.

Step III – Neural Maturation

1. At the end of the 3-day neural induction treatment, wash the cells with Phosphate Buffered Saline (PBS). Change the media to the Maturation Media.
2. After preparing the fresh Maturation Media, filter sterilize the media and incubate the cells in the above media for 3 days.
3. Change half the media every 3 days and replenish with half fresh media. The neurons are maintained in this media for 3 to 6 weeks, until they reach maturity. At this stage the DPSC derived neurons are positive for neuronal marker MAP2 and most of the cultures show only residual levels of glial marker GFAP (Urraca et al., 2015).

Reagents and Solutions

Transport Media

DMEM:F12 (50:50) with HEPES buffer (Biowhittaker Cat# 12-719F)

1% Antibiotic Antimycotic Solution (Pen/Strep/Amphotericin B) (100×) (Sigma Cat# A-5955)

Store at 4 °C

Washing Media

DPBS (No Calcium, no Magnesium (Cellgro Cat# 21-031-CV)

1% Antibiotic Antimycotic Solution (Pen/Strep/Amphotericin B) (100×) (Cat# Sigma A-5955)

Store at 4 °C

DPSC Culture Media

DMEM:F12 (50:50)(Cellgro Cat# 10-090-CV)

20% Fetal Bovine Serum (FBS) (ThermoFisher Cat# 16000044)

10% Newborn Calf Serum (NCS) (GE Life Sciences - HyClone Cat# SH30118.03)

1% Antibiotic Antimycotic Solution (Pen/Strep/Amphotericin B) (100×) (Sigma Cat# A-5955)

Store at 4 °C

Epigenetic Reprogramming Media

DMEM:F12 (50:50)(Cellgro Cat# 10-090-CV)

10uM 5-Azacytidine (Acros Biomedical Cat# 226620010)

10ng/ml bFGF (ThermoFisher Cat# 13256029)

2.5% FBS (ThermoFisher Cat# 16000044)

Prepare Fresh – Do not store.

Neural Differentiation Media

250um IBMX (Acros Cat# 228420010)

50uM Forskolin (Acros Cat# BP2520-10)

200nM TPA (Phorbol 12-myristate 13-acetate) (Sigma Cat# P8139)

1mM dbcAMP (Sigma Cat# D0627)

10ng/ml NGF (ThermoFisher Cat# 13257019)

10ng/ml bFGF (ThermoFisher Cat# 13256029)

30ng/ml NT-3 (PeproTech Cat# 450-03)

1% insulin-transferrin-sodium selenite premix (ITS) (ThermoFisher Cat# 41400045)

Prepare Fresh – Do not store.

Neural Maturation Media

Neurobasal-A Medium (ThermoFisher Cat# 10888022)

1mM dbcAMP (Sigma Cat# D0627)

1% N2 supplement (ThermoFisher Cat# 17502048)

1% B27 (ThermoFisher Cat# 17504044)

30ng/ml NT-3 (PeproTech Cat# 450-03)

1× Glutamax (ThermoFisher Cat# 35050061)

Prepare Fresh – Do not store.

Commentary

Background Information

Dental pulp stem cells have been generated from pulp for some time. The majority of these studies involving DPSC, however, have focused on the differentiation of DPSC into chondrocytes for dental repair, with the eventual goal of re-growing teeth from multipotent DPSC cultures (Gronthos et al., 2011; Gronthos et al., 2002). Our approach to collect DPSC from exfoliated teeth to study neurogenetic disease is rather unique, although other groups have experimented with the development of neurons from DPSC and using primary DPSC for *in vivo* stem cell therapy, even differentiating DPSC *in vivo* in the rat brain stimulated by local lesions (Kiraly et al., 2011). The purpose of refining the Kiraly *et al* differentiation method was to produce a standard protocol for molecular analysis that would allow us to study neuronal gene expression and function from a variety of neurogenetic syndromes and a large number of individuals. The method described here is the approach currently used in the lab on a daily basis for the collection, culture and differentiation of dental pulps to study human neurogenetic disorders.

Critical Parameters

By our estimates, it costs approximately \$500 in reagents and labor to process a single exfoliated tooth and produce a frozen viable DPSC lines from this individual. Although this may not sound expensive, in our experience many families want to contribute to our tooth projects because it gives them an opportunity to directly contribute to research without having to leave their own homes. This can create problems with the subject contributing the biospecimen is not actually the target of recruitment for a particular disorder one is interested in studying. As such, we have developed a strict operating procedure by which potential subjects in our studies MUST produce a genetics report describing the syndrome for which they have been recruited before we ever send a “tooth kit” to the family. In addition, depending on the research study, one may want to stratify subjects into groups – for example with and without autism spectrum disorder in our studies. This can also often be done remotely using questionnaires sent to the parents (in our case the SRS and SCQ for ASD assessments (Granader et al., 2010)). These questionnaires can be included in the “tooth kit” along with the consent form to allow for easy collection of additional clinical data on the biospecimen.

Even though the dental pulp resides deep within the tooth there are channels from the tooth root to the pulp that must be kept moist for optimal results. It must be stressed to parents or family members collecting teeth that the tube of media should be kept in the refrigerator until use and that under no circumstances can we use teeth that have dried out for the production of DPSC. It can also not be over-emphasized that once the tooth is collected we must receive the tube within 48 hrs. We have had teeth come from distant locations that arrived at 72 hrs post exfoliation, but these cases are rare and one must consider the cost benefit in this situation. Since we have no control over when or how many teeth fall out, and because of occasional extraction procedures, we will get multiple teeth from one individual

in a single tube. It is best to process and culture these teeth individually to avoid contamination of all of the cultures by fungal or bacterial contaminants. Finally, it is best to discard teeth when the tube arrives with mold or fungus growing in the culture. All attempts are made to stop this from happening by adding anti-bacterial and anti-fungal reagents to the transportation media, but sometimes these tubes are kept at room temperature by the families for an extended period of time rendering these reagents less effective.

When culturing the DPSC, cells must be grown in Poly-D-Lysine coated plates. If the cells grow past ~85% confluency they will come off the plates, therefore they must be passaged when they are sub-confluent (Figure 1c).

When culturing neurons it is critical to start these cultures in small wells or at high density for the differentiation steps, but care must be taken not to over-crowd the cells as they will likely detach from the plate. In our hands 20,000 cells/cm² is a good working density for differentiation of DPSC into neurons.

Finally, when thawing DPSC from the liquid Nitrogen cryobank or passaging cells, it is important to start DPSC cultures on a 12-well culture plate. If the cells are seeded too sparsely they will not expand since cell-cell interactions at this stage promotes cell propagation.

Troubleshooting

Cloudy or contaminated media surrounding the exfoliated tooth—If the tube of media containing the tooth appears turbid, wash the tooth three times with Washing Media prior to extraction of the pulp. If the DPSC culture is cloudy, add the 1% Pen/Strep/Amphotericin B solution to the culture for one week. Discard this preparation if the culture does not clear up within a week.

Floating particulate matter in new DPSC cultures—Most of the free particulate matter from the tooth enamel can be removed by washing the cultured pulp two times per week with Washing Media, however, it is best to just ignore these bits of enamel as they do not affect outcome. In fact, one should be careful to keep any floating cells early in the process and re-seed them into another well as there may be a large population of DPSC in this supernatant.

Difficulty growing DPSC lines from frozen stocks—When thawing cells from the Liquid Nitrogen cryobank, it is very important to thaw them as fast as possible, therefore have the tubes of culture media pre-warmed to 37 °C and labeled (ready to add the freshly thawed cells). Some cell lines take longer than other cell lines to grow after freezing and may need several media changes to remove the dead cells from the culture. We change the media two times a week to culture freshly thawed DPSC lines.

Neurons dying during maturation steps—Most DPSC lines begin to take on neuronal morphology on day 2 of incubation in the Neural Differentiation Media but sometimes some DPSC lines do not. These lines can be incubated for an additional day in Neural Differentiation Media. When the cultures are in Neural Maturation Media the color of the

media may change (turn yellow) before the end of the typical three-day incubation period. In these cases, the Neural Maturation Media should be changed.

Anticipated Results

Seeding of DPSC cultures from pulp—The process of growing DPSC from dental pulp can take anywhere from 1 to 2 weeks depending on the condition of the tooth and other unknown factors. Each pulp is different and it can take more than two weeks before colonies are visible on the plate. This means it sometimes can take up to 4 weeks for the DPSC to be ready for passage from the time the tooth arrived in the laboratory. Do not give up on cultures less than 4 weeks old unless they appear contaminated with fungus and risk the health of other samples in the incubator.

Once confluent cells from the pulp established and are passaged, it takes 1 week for the cells to reach a sub-confluent stage in a 12-well plate. Most of the cell lines grow well until passage 6, the growth curve slows down from passage 6-12, they stop growing around passage 12 (Figure 1 in (Urraca et al., 2015)).

48 hours after the addition of the Epigenetic Reprogramming Media to the cells, the cells take on an elongated morphology. They start to look different than the DPSC that were seeded. In 24 hours after the addition of the Neural Differentiation media, the cells take on the neuronal morphology. We keep them 3 to 4 weeks in the maturation media. After 4 weeks, many cell lines start come of the plate leading to cell death.

Time Considerations

There are several time sensitive steps in this overall approach to the collection and differentiation of DPSC into neurons. Under ideal conditions, the entire protocol from the arrival of the exfoliated tooth to experiments using mature neurons will take at least 6-8 weeks. However, even before the tooth arrives in the lab it should be emphasized to the families collecting the exfoliated tooth that 1) the tooth must be placed in the transportation media within 20 min of exfoliation and 2) it must be sent the laboratory within the first 48 hrs for best results. If the tooth must be stored prior to shipment it should be refrigerated. It takes approximately 1-2 weeks for DPSC to grow from minced dental pulp. Cultures incubated for 4 weeks without any sign of DPSC growth should be discarded. Finally, the process of generating neuronal cultures from expanded DPSC cultures takes at least at least 4-6 weeks. Half of this time is spent maturing the neurons (a process that takes at least 3 weeks, but optimally 5 weeks). After 5 weeks in maturation media the neurons will begin to detach and some will die. The longest culture time we have tried is 10 weeks.

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Figure 1. Generation of DPSC from dental pulp

A) Typical pulp culture on day one. Note the debris (dark matter). No DPSC are visible at this stage. B) After about one week colonies of DPSC appear. C) DPSC cultures at approximately 80% confluence prior to passage.

Figure 2. Differentiation of DPSC into neuronal cultures

A) low magnification image of neuronal differentiated cultures matured for approximately 3 weeks. Note different morphologies including astrocyte like cells and pyramidal neuron looking cells. B) Higher magnification culture stained with neural tubulin antibody TUJ1.