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# Characterization of neurons from immortalized dental pulp stem cells for the study of neurogenetic disorders

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ABSTRACT

A major challenge to the study and treatment of neurogenetic syndromes is accessing live neurons for study from affected individuals. Although several sources of stem cells are currently available, acquiring these involve invasive procedures, may be difficult or expensive to generate and are limited in number. Dental pulp stem cells (DPSCs) are multipotent stem cells that reside deep the pulp of shed teeth. To investigate the characteristics of DPSCs that make them a valuable resource for translational research, we performed a set of viability, senescence, immortalization and gene expression studies on control DPSC and derived neurons. We investigated the basic transport conditions and maximum passage number for primary DPSCs. We immortalized control DPSCs using *human telomerase reverse transcriptase* (*hTERT*) and evaluated neuronal differentiation potential and global gene expression changes by RNA-seq. We show that neurons from immortalized DPSCs share morphological and electrophysiological properties with non-immortalized DPSCs. We also show that differentiation of DPSCs into neurons significantly alters gene expression for 1305 transcripts. Here we show that these changes in gene expression are concurrent with changes in protein levels of the transcriptional repressor REST/NRSF, which is known to be involved in neuronal differentiation. Immortalization significantly altered the expression of 183 genes after neuronal differentiation, 94 of which also changed during differentiation. Our studies indicate that viable DPSCs can be obtained from teeth stored for ≥72 h, these can then be immortalized and still produce functional neurons for in vitro studies, but that constitutive hTERT immortalization is not be the best approach for long term use of patient derived DPSCs for the study of disease.



## 1. Introduction

In order to understand the molecular and physiological changes in neurons of the brains of individuals with disorders ranging from intellectual disability to autism, it is essential to study live neurons that accurately represent the conditions in the brain in the disease state. A significant challenge to the study of both rare and common neurogenetic syndromes is the inability to access live neurons for study in the laboratory setting. One recent approach to overcome this problem has been the collection of skin biopsies from individuals with neurogenetic disease in order to create fibroblast cell lines, which are then induced into pluripotency using viral constructs to become pluripotent stem cells (iPSCs), before finally being differentiated down neuronal lineages in culture (Marchetto et al., 2010). This approach is limited by the fact that iPSCs can be difficult to generate, are often restricted in their downstream differentiation potential and can take a long time to differentiate into representative neurons (Arbab et al., 2014). One solution to these problems is to obtain biospecimens of multipotent neuronal precursor cells, which are already destined to become neurons and therefore do not need reprogramming. Mammalian dental pulp is a neural crest-derived tissue and has been shown to contain a potent population of stem cells with neurogenic potential both in vitro and in vivo (Abraham and Verfaillie, 2012; Achilleos and Trainor, 2012). These stem cells can respond to local microenvironment cues in the mammalian brain to become a variety of central nervous system cell types (Kiraly et al., 2011) and have been shown to respond to neuronal

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differentiation signals in culture (Mezey, 2003). Normally exfoliated or extracted primary teeth (baby teeth) are a good source for dental pulp stem cells (DPSCs), and are easy to collect even from remote locations. This is an important practical consideration for the study of rare disorders, where the logistics required to create a single collection of cell lines from a variety of remote locations can be prohibitive, and more so when generating enough samples to overcome the normal genetic heterogeneity and gene expression variation found in human populations.

The process of generating neurons from tooth pulp neural precursors is now well tested in control samples (Kiraly et al., 2011; Arthur et al., 2008; Kiraly et al., 2009; Nosrat et al., 2004), but it has never been used before to establish a large repository of samples for the study of neurogenetic syndromes. Currently, there are no standardized protocols for long-term storage and transportation of extracted teeth for the production of DPSCs. The evaluation of factors such as storage conditions, transport time and cellular senescence of primary DPSCs is crucial to the development of DPSCs as a resource for the broader study of neurogenetic disease. In this study, DPSCs have been grown under various conditions and immortalized with a *human telomerase reverse transcriptase* (*hTERT*) retrovirus soon after processing. Here, we assess how storage conditions, processing time, and the timing of immortalization affect the success of the immortalization process. In addition, immortalized DPSCs were evaluated for their ability to efficiently form DPSC neurons that electrophysiologically and morphologically resemble non-immortalized DPSC neurons. Finally, using whole genome RNA-seq analysis, we established that the immortalized DPSCs and DPSC neurons are molecularly similar to non-immortalized primary DPSCs at the gene expression level. These studies indicate that teeth can be obtained from distant locations as much as 72 h away and still produce viable DPSCs which, when immortalized using *hTERT*, are similar in many ways to non-immortalized DPSCs. These are the first steps in the development of a protocol to successfully obtain DPSCs from any individual with any neurogenetic syndrome anywhere in the world with the eventual goal of creating a repository of immortalized DPSC lines that can be used to study nervous system disorders at the molecular and physiologic level.

## 2. Materials and methods

### 2.1. Generation of DPSC lines

Teeth were obtained through the Department of Pediatric Dentistry at the University of Tennessee Health Science Center (UTHSC). The UTHSC Institutional Review Board approved this study and informed consent was obtained from the parent or legal guardian of all participants. Participants did not receive a dental cleaning prior to tooth donation in the clinic. Immediately following extraction of a loose tooth in the clinic, the tooth was placed in transportation media (DMEM/F12 50/50 mix with HEPES (Fisher Scientific) and 100 U/mL penicillin, 100 μg/mL streptomycin). DPSCs were isolated and culture as previously described with slight modifications (Kiraly et al., 2009). Briefly, the pulp was minced and digested in a solution of 3 mg/mL Collagenase type I and 4 mg/mL Dispase II for 1 h at 37 °C. Cells were seeded in poly-D-lysine 12-well dishes and maintained under standard conditions (37 °C, 5% $CO_2$) in DMEM/F12 1:1, 10% fetal bovine serum (Fisher Scientific), 10% newborn calf serum (NCS) (Fisher Scientific) and 100 U/mL penicillin, 100 μg/mL streptomycin (Pen/Strep). Sub-confluent cultures were passaged regularly with 0.1 μM HyQTase (HyClone).

### 2.2. Evaluation of cellular senescence in DPSCs

Senescence testing was performed following the Senescence Cell Histochemical Staining Kit protocol (Sigma-Aldrich). The assay is based on a histochemical stain for senescence-associated β-galactosidase (β-gal) activity at pH 6. Each DPSC line was washed twice in 1× PBS, 400-μL-fixation buffer was added to each DPSC line and incubated for 7 min at room temperature. The staining solution had 250 μL of stock X-gal solution, and 400 μL of staining solution was added to each sample according to the manufacturer's protocol. The samples were incubated at 37 °C without $CO_2$ until the cells appeared blue (approximately 18 h). After the incubation the cells were rinsed twice in 1× PBS and counterstained with Eosin (Sigma-Aldrich) for 8 min. Cells were viewed under a light microscope and cells stained with blue were counted as senescent. For each time point, at least 500 cells were counted to determine the percentage of senescent cells.

### 2.3. Immortalization of primary DPSCs

Immortalization was performed as described previously (Egbuniwe et al., 2011). $1 \times 10^4$ cells were plated in a poly-D-lysine coated 12-well dish and infected with pBABE-*hTERT-Puro*® retrovirus at a multiplicity of infection of 20 plus 6 μg/mL Polybrene for 24 h. The virus was removed and replaced with growth media for a 72–90 hour period. After this period, the infected DPSCs were selected by adding 1 μg/mL puromycin for 7–10 days. The DPSCs that survived this process were immortalized DPSCs and were ready for differentiation into neurons.

### 2.4. Neural differentiation

DPSCs were converted to neurons according to a previously published protocol (Kiraly et al., 2009). 20,000 cells/$cm^2$ were seeded in poly-D-lysine coated 12 well plates or T-25 flasks in DMEM/F12 (1:1), 2.5% fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin, and cultured for 24 h. Epigenetic reprogramming was performed by exposing the DPSCs to 10 μM 5-azacytidine (Acros Scientific) in DMEM/F12 containing 2.5% fetal calf serum (Fisher Scientific) and 10 ng/mL bFGF for 48 h. Neural differentiation was induced by exposing the cells to 250 μM IBMX, 50 μM forskolin, 200 nM TPA (Sigma-Aldrich), 1 mM db-cAMP, 10 ng/mL bFGF, 10 ng/mL NGF (Invitrogen), 30 ng/mL NT-3 (Peprotech), and 1% insulin–transferrin–sodium selenite premix (ITS) in DMEM/F12 for 3 days. At the end of the neural induction treatment, the cells were washed with 1× PBS. Neuronal maturation was performed by maintaining the cells in Neurobasal A media (Invitrogen) supplemented with 1 mM dbcAMP, 1% N2, 1% B27, and 30 ng/mL NT-3 and 1× Glutamax for 3 weeks.

### 2.5. Western blot analysis

Protein was extracted from DPSCs and neurons using radioimmunoprecipitation assay (RIPA) lysis buffer for DPSCs or neuronal protein extraction reagent for neurons (Thermo Fisher Scientific). Samples were resolved on a NuPage 1.5 mm 4–12% Bis–Tris or a 3–8% Tris–Acetate gel according to manufacturers instructions (Invitrogen) and transferred to Immobilon-FL PVDF membrane (Millipore). The membrane was blocked with 5% BSA, and 0.2% Tween-20 in Phosphate Buffered Saline (PBS). Primary antibodies used include α-GAPDH (dilution 1:5000 from Novus Biologicals catalog#: IMG-3073), α-REST (dilution 1:2500 from Bethyl Laboratories Inc. catalog#: A300-540A), α-MAP2 (dilution 1:10,000 from Abcam catalog#: ab5392) and α-GFAP (dilution 1:1500 from Abcam catalog#: ab7260). Infrared (IR) labeled secondary antibodies purchased from Li-Cor (Lincoln, NE) and used in a 1:5000 dilution (α-rabbit 680, α-goat 800 and α-chicken 680). The blot was imaged and analyzed using the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE). After adjustment for background fluorescence, lanes were normalized using the signal from the α-GAPDH loading control as the reference channel and signal intensity was then adjusted based on the calculated normalization factor assigned to each lane in the channel.