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Invited Review

INVITED REVIEW

# Dental pulp stem cells for the study of neurogenetic disorders

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## Abstract

Dental pulp stem cells (DPSC) are a relatively new alternative stem cell source for the study of neurogenetic disorders. DPSC can be obtained non-invasively and collected from long-distances remaining viable during transportation. These highly proliferative cells express stem cell markers and retain the ability to differentiate down multiple cell lineages including chondrocytes, adipocytes, osteoblasts, and multiple neuronal cell types. The neural crest origin of DPSC makes them a useful source of primary cells for modeling neurological disorders at the molecular level. In this brief review, we will discuss recent developments in DPSC research that highlight the molecular etiology of DPSC derived neurons and how they may contribute to our understanding of neurogenetic disorders.

## Introduction

To understand the complex molecular and physiological pathways underlying neurogenetic syndromes, it is often useful to have an *in vitro* model that accurately recapitulates the live neurons in the disease state. The use of induced pluripotent stem cells (iPSC) to model the function of cortical neurons from patient derived fibroblasts or blood cells is now well-documented (1–4). However, there are notable drawbacks to using these de-differentiated, reprogrammed cells as *in vitro* models for molecular studies. Recent studies indicate that iPSC may not accurately represent changes associated with neurological pathogenesis since residual epigenetic marks associated with their original cell type which can lead to inappropriate gene expression in the newly derived iPSC neurons (5). This residual epigenetic signature, along with genomic instability (6), tumorigenic potential (7), and a high mutational load (8) raises concerns for the use of iPSC to model neurogenic disorders that often have complicated genetic and epigenetic etiologies which can alter the molecular changes indicative of the particular syndrome.

Dental pulp stem cells (DPSC) (9,10) provide an alternative to iPSC and overcome some of the problems associated with epigenetic changes and re-programming (11,12). Another significant advantage to using DPSC is that they can be easily obtained from exfoliated or extracted teeth and then transported within 48 h to the laboratory (13). The easy collection and transport means that families of children with various neurogenetic syndromes are able to directly contribute to the investigation of these disorders without ever traveling to the laboratory. It also means that many more teeth representing many more individuals with these syndromes can be collected than for iPSC, which can be difficult to reprogram and often yield clones of a single individual for molecular studies (14–16).

DPSC have been shown to differentiate into functionally active neurons in the presence of varying neurotrophic factors (17–19). DPSC have been shown to differentiate into various neural cell types including dopaminergic cells (20–22), glutamatergic and GABAergic cells (23), as well as glial and Schwann cells (24,25). Additionally, the ability of pre-differentiated DPSC to integrate into the brains of normal rats and a rat model of traumatic cortical lesion which speaks to their therapeutic

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potential (26). Kiraly *et al.* showed that these engrafted DPSC retained their neuronal immunohistochemical and electrophysiological characteristics weeks after transplantation. In the traumatic cortical lesion rat model, the pre-differentiated DPSC even migrated to the cortex in response to injury. Pre-differentiated DPSC have also been shown to improve neurological function in a Parkinsonian rat model. These dopaminergic-like neurons increased brain dopamine levels and the neuroprotection of endogenous dopaminergic neurons (27). These studies suggest great promise for using DPSC in translational research.

## Stem Cells of the Dental Pulp

Stem cells are classified into three distinct categories: embryonic stem cells (ESC), adult (post-natal) stem cells (ASC), and induced pluripotent stem cells (iPSC). ASC encompass a broad range of different stem cells including neural (NSC) and mesenchymal stem cells (MSC) (28). MSC have been shown to differentiate into several terminal cell types such as osteoblasts, adipocytes, and chondrogenic cells (29), making them prime candidates for cell based therapies. MSC have been isolated from a variety of tissues including bone marrow (BMSC) and dental pulp (DPSC).

DPSC reside deep in the dental pulp at the center of the tooth in the pulp cavity and is a part of the dentin-pulp complex (Fig. 1). The regenerative quality of this complex relies on activity within the dental pulp. Gronthos *et al.* discovered a unique stem cell population derived from this pulp tissue that resembled BMSC (9). They found that these cells had self-renewal capabilities and were able to differentiate into adipocytes and neural-like cells (10). Following the initial discovery of DPSC, a separate population of stem cells arising from the pulp of deciduous ("baby") teeth was characterized. Miura *et al.* isolated stem cells from human exfoliated deciduous teeth (SHED) (30) that shared similarities with MSC found in umbilical cord blood. They also discovered that compared to DPSC, SHED had higher proliferation rates, increased cell population doublings, and a distinct morphology. This difference is not shocking given the different developmental origins between deciduous and adult teeth. Deciduous teeth develop much earlier in the prenatal period than adult teeth. This difference likely effects the stem cell niches in which these cells reside. These microenvironments are controlled by genetic, epigenetic, and environmental forces that regulate the activities of the stem cells (31). Where a particular stem cell resides and the cell type from which it is derived can contribute to the variability in gene expression observed among stem cells isolated from different tissues.

DPSC have now been compared to other stem cell types used for neurogenetic investigations including ESC, iPSC, and other MSC. Karoaz *et al.* were among the first to compare DPSC to BMSC (32). They found that DPSC had similar differentiation capabilities as BMSC and expressed both neural and glial specific markers. They also found that DPSC are more developed and metabolically active cells than BMSC, leading to the conclusion that DPSC demonstrate better neural and epithelial stem cell properties. More recently, more robust comparisons utilizing several types of stem cells have been performed. Umbilical cord, dental pulp, and menstrual blood are all relatively accessible sources of MSC. Ren *et al.* compared these three sources on the basis of morphology, proliferation, and differentiation capabilities (33). Their results show that while stem cells isolated from the umbilical tissue (UC) had higher proliferative capacities,

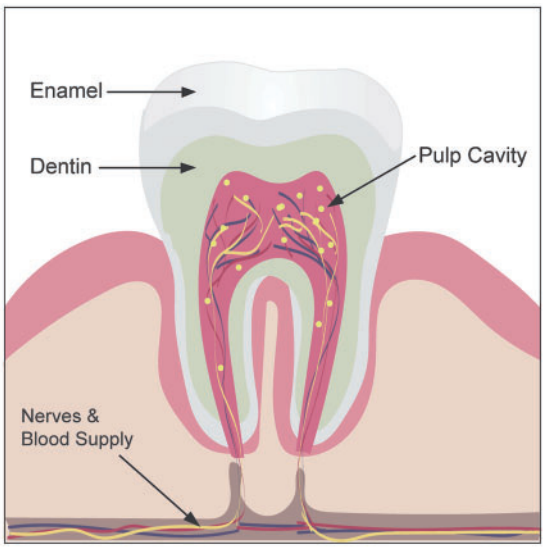


**Figure 1.** Longitudinal section of a tooth depicting the pulp cavity where the dental pulp resides. DPSC are found within the dental pulp and are represented here as yellow dots.

DPSC demonstrated less cellular senescence and higher osteogenic capabilities than either the UC or menstrual blood cells.

Similarly, Kang *et al.* compared gene expression between DPSC and UC (34). They found that genes related to growth factor activity, receptor activity, and signal transduction were significantly upregulated in DPSC compared to UC. In contrast, genes related to cell proliferation, angiogenesis, and immune responses were expressed in higher levels in the UC. These studies complement each other since the higher proliferation rates of UC correlates with the higher expression of genes related to cell proliferation and the lower senescence of the DPSC likely correlates to the increased expression of genes related to growth factor activity.

Isobe *et al.* compared the differentiation capacity among DPSC, SHED, BMSC, and synovial fluid cells (SFC), all considered MSC (35). Multipotent capacity was significantly different among these stem cell sources. BMSC and SFC had higher osteogenic and chondrogenic capacity, while DPSC and SHED demonstrated increased neurogenesis. They also observed that SHED showed higher proliferation rates compared to both DPSC and BMSC. Another study comparing DPSC, SHED, and BMSC found that DPSC and SHED express higher levels of embryonic stem cell markers, Oct-4 and STRO-1, than BMSC. As in other studies, BMSC showed higher osteogenic capabilities than SHED and DPSC. They also present notable differences between DPSC and SHED. SHED had higher osteogenic capabilities and expression of embryonic stem cell markers than DPSC. Between DPSC and SHED, SHED demonstrated higher expression of these markers (36). Both studies concluded that SHED likely represent a more primitive cell type than DPSC. These comparison studies both found that the levels of MSC-maker expression between the cell types is similar, but the differentiation potential is significantly different, even between SHED and DPSC.

Comparative studies between DPSC and iPSC, the main stem cell culture used to study neurogenetic syndromes, are still limited. However, there have been multiple studies focused on the efficiency and reliability of re-programming DPSC into iPSC (37–40). DPSC express many ESC markers even prior to reprogramming, thus it has been proposed that these cell types could be a more efficient source of creating iPSC than cells traditionally

used for iPSC generation (39). A transcriptomic analysis of iPSC-derived neurons from both SHED and fibroblasts revealed lower expression of genes involved in hindbrain development (*HOX* and *IRX* families) and higher expression of genes involved in forebrain development (*FOXP2, OTX1, and LHX2)* in the SHED derived iPSC neurons (41). FOXP2 is involved in language and communication networks and has been implicated in ASD pathology, leading to the conclusion that DPSC derived iPSC may be appropriate for the study of neurodevelopmental disorders (41).

Analyzing the genetic and functional differences between iPSC and DPSC is crucial for determining the best approach to modeling both molecular and neurophysiological changes that occur in neurons in the disease state (38). Since reprogrammed cells often retain epigenetic marks from their original cell type, understanding the consequences of reprogramming or cellular origin on epigenetic marks is essential while developing reliable disease models. Dunaway *et al*. analyzed how accurately SHED modeled early life and whole genome DNA methylation patterns compared to iPSC (12). They found that the primitive origin of SHED allows these cells to more accurately model the embryonic inner cellular mass and the placenta. They concluded that compared to iPSC, DPSC are a more reliable model for the study of genome architecture and gene expression changes that occur in neurodevelopmental disease (12).

There is great potential for the use of stem cells to directly treat human disease by returning healthy stem cells with neurogenetic potential to the brain (42,43). In order for stem cells cultured *ex vivo* to be considered for therapeutic use, however, they must meet certain criteria including assessment of *in vivo* tumorigenic potential. While the tumorigenic potential of iPSC is well established (44,45), our lab recently showed that immortalized DPSC do not form tumors *in vivo* (11). The tumorigenic potential of both spontaneous and induced immortalized DPSC was assessed. Karyotype analysis revealed that while the spontaneously immortalized DPSC showed no genomic instability. The genomic instability of spontaneously immortalized stem cells from other tissue sources has been shown in multiple studies (6,46). The absence of this genomic instability and tumorigenic potential adds to the growing evidence that DPSC may represent a better stem cell source for both disease modeling and cell therapies.

## Multipotent Differentiation Provides Cellular Specificity for a Variety of Syndromes

As neurodevelopmental disorders often involve multiple neuronal and non-neuronal cell types that can contribute to the phenotype, developing target treatments without access to the specific neuronal cell types can limit the effectiveness of these studies. Neural stem cells (NSC) differentiate into neurons and glia *in vivo* and *in vitro*, thus they represent a mixed population of cells that may represent the varied cell types found in the brain. DPSC, which arise for the neural crest, are an excellent proxy for NSC to study in the lab setting. When given the appropriate environmental cues, DPSC differentiate into functional neurons showing neuronal morphology and responsive sodium and potassium channels (47). Arthur *et al*. first differentiated DPSC into functional neurons using a protocol typically used to differentiate ESC (18). Using fibroblast growth factor or epidermal growth factor signaling, they were able to differentiate DPSC into neurons that exhibited functional sodium channels. Kiraly *et al*. also developed a protocol for differentiating DPSC into a neural population (19). In their protocol, the simultaneous activation of protein kinase C and cyclic AMP pathways induced neural differentiation. The DPSC-derived neurons had morphological characteristics similar to neurons derived from other sources (Fig. 2). They were able to show genetic markers associated with neural differentiation and functional sodium and potassium channels, demonstrating strong evidence that the DPSC had differentiated into functional neurons. Using the protocol established by Kiraly *et al*, we assessed the global gene expression changes resulting from DPSC into mixed neuronal cultures (19). We found that the differentiation process significantly changes gene expression and that these expression changes occur through the transcription factor RE1-Silencing Transcription (REST). The differentiated DPSC also positive for the neuronal marker MAP2 and expression for the glial marker GFAP was decreased in the mixed neuron/glia cultures versus undifferentiated DPSC but still present (48).

Differentiating DPSC into specific neural subtypes is crucial for modeling neurogenetic disorders as different syndromes affect different neural sub-types in a distinct manner. Several protocols have been published for differentiating DPSC and SHED to dopaminergic cells, or DA neurons. Wang *et al*. were the first to present a protocol for DA differentiation of SHED (20). This protocol results in about 10% of the cells expressing tyrosine hydroxylase (TH), the enzyme that converts tyrosine to L-DOPA, an essential dopamine precursor. Although dopaminergic induction resulted in a relatively small proportion of TH+ neurons, they also showed that when these DA-like cells were introduced into a Parkinson disease mouse model some Parkinson-like behavioral abnormalities were corrected (20). Kanafi *et al*. published a two-step protocol using the embryonic midbrain cues, SHH and FGF8 (22). They found that over 70% of cells were TH+ after induction and maturation. In a related study, they found that while this protocol induced the DPSC into functional DA-like cells, it was not efficient in differentiating the SHED to a terminal DA fate (49). Fujii *et al*. differentiated SHED into DA-like neurons using a BDNF stimulating protocol (27). Compared to other dopaminergic protocols (SHH and FGF8), they discovered that a BDNF protocol resulted in the most TH+ cells (~75%). Because BDNF specifically is involved in the differentiation of new neurons and synapses, they concluded that a protocol with early neurogenesis signals and later maturation of DA neurons was required for efficient dopaminergic differentiation from SHED. This could provide a potential solution for the differentiation of SHED to DA-like neurons.

Although there is little research published showing differentiation of DPSC along neuronal lineages other than DA, one group has been able to differentiate DPSC into a neuronal population that shows increased glutamatergic and GABAergic-like cells (23). Depletion of PIN1 results in suppression of neurogenesis, while overexpression results in enhanced neurogenesis. PIN1 functions in regulating key signaling molecules involved in cell growth and differentiation. Cho *et al*. observed that upon differentiation, these neuronal populations displayed increased glutamatergic and GABA-ergic markers, while dopaminergic and glial marker expression was decreased resulting in a more glutamatergic and GABA-ergic like neuronal population (23). These results imply that we may soon develop protocols for a variety of neuronal cell types (dopaminergic, GABAergic, hypothalamic, cortical, etc.), which can be generated from patient-derived DPSC.

## Practical Use of Primary DPSC Cultures

All primary cultures, even stem cells, eventually undergo cellular senescence. Long term culturing of primary cells typically

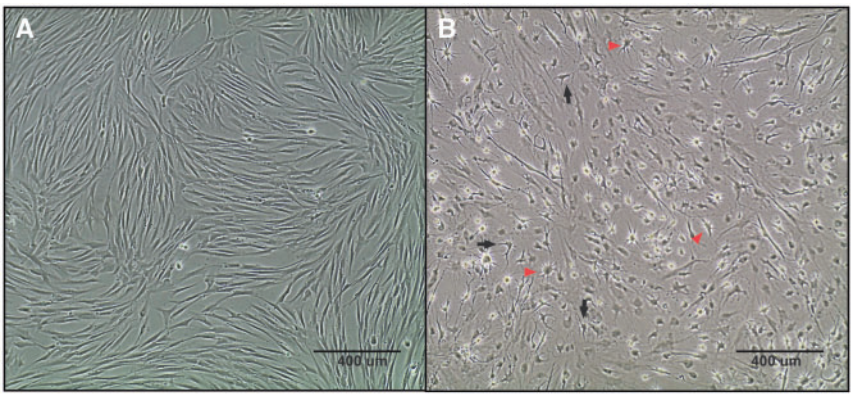


**Figure 2.** Morphological changes during neuronal differentiation of DPSC. (**A**) Undifferentiated DPSC have a spindle-shape similar to fibroblasts. (**B**) DPSC were differentiated and allowed to mature for 3 weeks, resulting in a mixed culture containing both neuron-like and glial-like cells. The neurons (black arrows) show a pyramidal-like, neuronal morphology with shorter projections similar to dendrites and a longer axonal projection on the opposing side. The glial cells (red arrows) show a typical star-shaped morphology with a rounded cell body and small projections extending from the perimeter. Both images were taken at a 10x magnification using phase-contrast microscopy.

causes senescence through telomere shortening or replicative senescence (50), which results in a DNA damage response. Senescent cells remain metabolically active, but undergo irreversible growth arrest that is accompanied by characteristic changes in gene expression. Even in stem cells, cellular senescence occurs during prolonged culture periods and results in the loss of differentiation potential and gene expression changes (51). DPSC also undergoes age-dependent molecular and genetic changes that result in reduced proliferative capacity and differentiation potential (52). Donor age has also been shown to positively correlate with cellular senescence and impaired regenerative abilities. In order for DPSC to be useful for long-term studies and clinical applications, it will be necessary to overcome cellular senescence in primary DPSC cultures.

The introduction of human telomerase reverse transcriptase (*hTERT*) into cells is one way to immortalize DPSC. In order for *hTERT* immortalization to be considered a practical solution cellular senescence in DPSC an investigation into how permanent *hTERT* expression effects gene expression and differentiation potential of DPSC (53). Recently, our lab analyzed the effects of *hTERT* immortalization on the gene expression of SHED derived neurons (48). Molecular analysis revealed that some of the transcripts that are upregulated during differentiation into neurons were suppressed by constitutive immortalization with *hTERT*. Thus, constitutive *hTERT* expression is therefore not ideal for neurogenetic studies. Although the *hTERT* immortalized SHED demonstrated characteristics of functional neurons, they do not accurately represent the expression patterns of donor neurons. An inducible immortalization system driving *hTERT* expression in the DPSC stage, but turning it off during neuronal differentiation could be a solution. Identifying a protocol that allows for the immortalization of DPSC without affecting the molecular and genetic characteristics of the neurons derived from these cells is critical for the utility of the DPSC model of disease.

## Conclusions

There are several reasons to consider DPSC for neurogenetic studies versus stem cells from other tissue sources. DPSC can be obtained non-invasively and even shipped across long distances, remaining viable in culture media for as long as 72 h (48). Especially for rare genetic syndromes, the ability to collect samples from distant subjects, even from other countries, is invaluable since it is often difficult to obtain large sample sizes for molecular studies of these disorders. More comprehensive comparison studies of various stem cell types, including DPSC, iPSC, ESC, and other MSC, derived from a single donor may help to elucidate the functional and genomic differences between stem cell types. The differentiation potential of DPSC into a variety of neuronal cell types is still largely unexplored. Future studies will require an understanding of the developmental origins and molecular characteristics of DPSC in order to identify the signaling mechanisms needed to differentiate DPSC down different neuronal lineages. Finally, the immortalization of donor DPSC for long-term propagation *in vitro* is required for scaling up the use of DPSC in the study of various disorders of the nervous system. Although the use of DPSC for the study of neurogenetic disorders is relatively new, there is great potential for this stem cell resource both in the study of disease and potentially as a source for therapeutic stem cell treatments targeted to the nervous system.

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*Conflict of Interest statement*. None declared.

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