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Differentiation of stem cells from human deciduous and permanent teeth into spiral ganglion neuron-like cells

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ABSTRACT

Objective: Stem cells from pulp tissue are a promising cell-based therapy for neurodegenerative patients based on their origin in the neural crest. The aim of this study was to differentiate and evaluate the ability of human dental pulp stem cells from permanent teeth (DPSC) and stem cells from human exfoliated deciduous teeth (SHED) to differentiate into spiral ganglion neurons.

Design: After isolation and characterization of mesenchymal stem cell properties, DPSC and SHED were treated with the neurotrophins brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and glial cell-derived neurotrophic factor (GDNF). The differentiation was identified by immunostaining and qRT-PCR analysis of neuronal markers and measuring intracellular calcium activity.

Results: After 2 weeks of induction, morphological changes were observed in both DPSC and SHED. The differentiated cells expressed neuron-specific class III beta-tubulin, GATA binding protein 3 (GATA3) and tropomyosin receptor kinase B, protein markers of spiral ganglion neurons. These cells also showed upregulation of the genes encoding these proteins, namely *GATA3* and neurotrophic receptor tyrosine kinase 2. Intracellular calcium dynamics that reflect neurotransmitter release were observed in differentiated DPSC and SHED.

Conclusion: These results demonstrate that dental pulp stem cells from permanent and deciduous teeth can differentiate into spiral ganglion neuron-like cells.

1. Introduction

According to the World Health Organization (2015), approximately 360 million people suffer from hearing loss worldwide. This impairment results from many factors: including aging, heredity (genetic alterations), and environment stimuli (loud noise, ototoxic drugs). Most cases of deafness are caused by permanent sensorineural hearing loss due to inner ear hair cell loss or spiral ganglion (SG) neuron damage. Stem cells are a promising tool for regenerative medicine due to their abilities of self-renewing and differentiation (Doetschman, Eistetter, Katz, Schmidtm, & Kemler, 1985). Embryonic stem cells survived, migrated toward the cochlear nucleus, and differentiated into neurons after co-culturing with auditory brainstem slices (Glavaski-Joksimovic et al., 2009). Adult human nasal mesenchymal-like stem cells directly differentiated into spiral ganglion neurons (SGNs) after an experimentally-induced lesion. Thus, Stem cells appear to be a good candidate for cell-based therapies aimed at restoring hearing abilities.

Human pulp tissue is an enriched source of stem cells that can be divided into two types, including dental pulp stem cells from permanent teeth (DPSC) and stem cells from human exfoliated deciduous teeth (SHED) (Gronthos, Mankani, Brahim, Robey, & Shi, 2000; Miura et al., 2003). Stem cells from human pulp tissue raise fewer ethical concerns as compared to embryonic stem cells. In addition, the isolation procedure of stem cells from pulp tissue is less invasive. Based on the neural crest origin of teeth (Miletich & Sharpe, 2004), DPSC and SHED express the neural crest marker HNKI and the early neuronal marker nestin. Several studies have reported both the *in vitro* and *in vivo* potential of the neuronal differentiation of DPSC and SHED and their applications for neurodegenerative diseases. These cells possessed neuronal plasticity toward dopaminergic-neurons after a midbrain-cue (Majumdar, Kanafi, Bhonde, Gupta, & Datta, 2016) and exhibited therapeutic benefits for Parkinson's disease after transplantation in a rat model (Fujii et al., 2015). However, there are no studies have been conducted to assess the potential of DPSC and SHED for differentiation into SGNs.

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According to embryonic development, tooth and SGNs both share the same origin of neural crest contribution (Coate & Kelley, 2013; Miletich & Sharpe, 2004). Therefore, DPSC and SHED are the good candidates for the restoration of SGNs.

In the present study, we aim to differentiate DPSC and SHED into SGNs using neurotrophins. The ability of differentiation was determined by observing neuronal morphology, examining the expression of SGN-specific genes and proteins and determining neuronal activities.

2. Materials and methods

2.1. Tooth sample collection

This study was approved by the Ethical Committee on Human Rights Related to Human Experimentation of the Faculty of Dentistry at Mahidol University (IRB 2014/041.2110). Seven non-carious deciduous and 7 permanent teeth from healthy subjects were collected at the Faculty of Dentistry, Mahidol University. Teeth with previous restorations, history of trauma, and/or signs of pulpal pathology were excluded from this study.

2.2. Isolation and culturing of dental pulp stem cells

The dental pulp stem cells were obtained using outgrowth methods as described in Jeon et al. (2014). In brief, fragments of dental pulp tissues were extracted and submerged in culture medium comprising Dulbecco's modified Eagle's medium (DMEM) with high glucose (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Biochrom GmbH, Berlin, Germany), and 1% penicillin/streptomycin (Gibco, Rockville, MD, USA) at 37 °C in a humidified atmosphere containing 5% CO_2. The media was changed every 2 days, and the cell cultures were monitored regularly with an inverted phase-contrast microscope. Upon reaching 80% confluence, cells were harvested using 0.05% Trypsin/EDTA (Gibco) and sub-cultured for further experiments. For each assay, the stem cells of three different donors were used.

2.3. Characterization of dental pulp stem cells

2.3.1. Cell surface markers analysis

For the cell surface antigen analysis, DPSCs and SHED (5×10^5) were labeled with monoclonal antibodies specific for human markers associated with mesenchymal and hematopoietic lineages. Namely, mouse anti-human antibodies against the following antigens were used: CD73, CD90, CD105, and CD34 (all purchased from BioLegend, San Diego, CA, USA). To determine the level of nonspecific binding, fluorochrome conjugated isotype control antibodies were used. Flow cytometry was performed using a FACSCanto flow cytometer (BD Biosciences, San Jose, CA, USA).

2.3.2. Colony-forming unit fibroblast

DPSC and SHED were seeded in triplicates into a 6-well plate at 500 cells/well. After 14 days, cultures were stained with 0.1% toluidine blue O (Scharlab S.L., Barcelona, Spain) and colony morphology was inspected under a light microscope. Colonies larger than 2 mm in diameter were counted using ImageProPlus 7.0 software (Media Cybernetics, Rockville, MD, USA).

2.3.3. Differentiation

After 28 days in complete commercial medium (HyClone™ AdvanceSTEM™ Osteogenic Differentiation Kit, Hyclone) for osteogenesis, calcium depositions were detected in the extracellular matrix. Cultures were stained with 40 mM alizarin Red S solution (Sigma). On the other hand, after 28 days in complete medium (HyClone™ AdvanceSTEM™ Adipogenic Differentiation Kit, Hyclone) for adipogenesis, cells were stained with a fresh 0.35% Oil Red O solution. The differentiated cells were observed and imaged using an inverted microscope.

2.4. Neural induction and spiral ganglion neuron-like differentiation

DPSC and SHED at passages 3–6 were induced to form neurospheres by culturing in neural induction medium consisting of DMEM/F12 (Gibco), 20 ng/mL bFGF (Gibco), 20 ng/mL EGF (Gibco), B-27 (Gibco), 100 U/mL Penicillin, and 100 µm/mL Streptomycin (Gibco) in low attachment culture dishes. Half of the medium was changed every other day, and cells were cultured for 5 days. Neurospheres were incubated in Accutase (Gibco) for 10 min to dissociate into single cells and then seeded onto poly-L-ornithine (Sigma) and laminin (Sigma) coated 24-well plates with a density of 2×10^4 cells/cm^2. The cells were cultured for 14–18 days in SGN induction medium consisting of DMEM/F12, 20 ng/mL brain-derived neurotrophic factor (BDNF), 20 ng/mL neurotrophin-3 (NT-3), 20 ng/mL glial cell-derived neurotrophic factor (GDNF) (all from Sigma), supplemented with 2% N-2 (Gibco), 2% B-27 supplement (Gibco), 100 U/mL Penicillin, and 100 µm/mL Streptomycin (Gibco). The medium was changed every other day.

2.5. Immunocytochemistry

All specimens were fixed in 4% paraformaldehyde (Sigma) in PBS for 60 min at RT, followed by 20% ice-cold methanol (Sigma) in PBS for 5 min at RT and washing with PBS. Subsequently, cells were permeabilized with 0.5% Triton X-100 (Sigma) in PBS overnight at 4 °C and then blocked with 15% BSA (Sigma) and 5% normal goat serum (Sigma) for 12 h at 4 °C. Cells were incubated overnight at 4 °C with the following primary antibodies diluted in 5% BSA in PBS with 0.05% Tween-20 (Sigma): β-tubulin III (Tuj1; BioLegend) (1:400), GFAP (Santa Cruz Biotechnology, Inc, Dallas, TX, USA) (1:500), tropomyosin receptor kinase B (TrkB; Santa Cruz Biotechnology, Inc) (1:500), and GATA binding protein 3 (GATA3; BioLegend) (1:1000). Cells were then incubated with secondary antibodies for 4 h at RT: goat anti-mouse Alexa fluor-488 (1:1000; Life Technologies, Waltham, MA, USA) and donkey anti-goat Alexa fluor-594 (1:1000; Life Technologies). Nuclei were counterstained with 20 µg/mL 4′,6-diamidino-2-phenylindole (DAPI; Sigma) for 10 min. The specimens were placed onto glass slides, mounted with mounting medium, covered with a coverslip, and sealed with nail polish.

2.6. Real-time polymerase chain reaction

Total RNA was isolated using a NucleoSpin RNA XS kit (Macherey-Nagel, Bethlehem, PA, USA) and reverse transcribed into cDNA using iScript RT Supermix (Bio-Rad, Hercules, CA, USA). PCR was performed using KAPA SYBR® FAST qPCR Kits (Sigma). Three sets of primer sequences were used: *NTRK2*, GAGCATCATGTACAGGAAAT and CTTGATGTTCTTCCTCATGT; *GATA3*, GTACAGCTCCGGACTCTTCCC and CTGCTCTCCTGGCTGCAGACA; and *GAPDH*, GTCAGTGGTGGACC TGACCT and AGGGGAGATTCAGTGTGGTG.

2.7. Intracellular calcium activity

Calcium influx is necessary for neurotransmitter transmission between two neurons. The calcium indicator dye fluo3 AM was added to the medium at a 3 µM concentration with 0.08% pluronic acid. Cells were incubated at 37 °C for 60 min. After incubation, cells were washed with medium and PBS and maintained in Tyrode's solution prior to observation under the microscope. Intracellular calcium activity was stimulated with 50 mM KCl, and cells were recorded video for 2 min using a confocal laser scanning microscope (Olympus). Snapshots of the video recording were analyzed to determine the calcium dye intensity in cells using cellSens dimension software.

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