Protein Interacting with Never in Mitosis A-1 Induces Glutamatergic and GABAergic Neuronal Differentiation in Human Dental Pulp Stem Cells

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Abstract

Introduction: The purpose of this study was to investigate the role of protein interacting with never in mitosis A-1 (PIN1) in the neuronal or glial differentiation of human dental pulp stem cells (hDPSCs) and whether PIN1 can regulate determination of neuronal sub-phenotype. **Methods:** After magnetic-activated cell sorting to separate $CD34^+$/c-kit^+/$STRO\text{-}1^+$ hDPSCs, cells were cultured in neurogenic medium. Differentiation was measured as Nissl staining and marker protein or mRNA expression by reverse transcriptase polymerase chain reaction, immunofluorescence, and flow cytometric analysis. **Results:** PIN1 mRNA levels were upregulated in a time-dependent fashion during neurogenic differentiation. The PIN1 inhibitor juglone suppressed neuronal differentiation but promoted glial differentiation as assessed by the number of Nissl-positive cells and mRNA expression of neuronal markers (nestin, βIII-tubulin, and NeuN) and a glial marker (glial fibrillary acidic protein). Conversely, overexpression of PIN1 by infection with adenovirus-PIN1 increased neuronal differentiation but decreased glial differentiation. Moreover, PIN1 overexpression increased the percentage of glutamatergic and GABAergic cells but decreased that of dopaminergic cells among total NeuN-positive hDPSCs. **Conclusions:** This is the first study to demonstrate that PIN1 overexpression induced glutamatergic and GABAergic neuronal differentiation but suppressed glial differentiation of hDPSCs, suggesting that enhancing PIN expression is important to obtain human glutamatergic and GABAergic neurons from hDPSCs. *(J Endod 2016;42:1055–1061)*

Key Words

Glial cells, human dental pulp stem cells, neural differentiation, neuron, PIN1

Significance

PIN1 overexpression induced glutamatergic and GABAergic neuronal differentiation but suppressed glial differentiation of human dental pulp stem cells, which may serve as useful sources of neuro- and gliogenesis in degenerative disorders of the CNS.

Human dental pulp stem cells (hDPSCs) that originate from adult tooth pulp tissue are neural crest–derived adult stem cells capable of differentiating into a variety of cell lineages such as odontoblasts, osteoblasts, adipocytes, and hepatocytes (1–3). In addition, hDPSCs were able to differentiate into glial or neuronal cells on the basis of cellular morphology, expression of the neural progenitor marker nestin, and expression of the glial marker glial fibrillary acidic protein (GFAP) (4). Furthermore, *in vivo* studies demonstrated that grafted dental pulp cells (DPCs) and stem cells from human exfoliated deciduous teeth (SHED) into the central nervous system (CNS) survived for several months and expressed neuronal markers (5, 6). Culture-expanded hDPSCs exposed to neurogenic medium (NM) differentiated into functionally active mature neurons both *in vitro* and *in vivo* (7). In addition, engrafted hDPSCs integrated into the rat brain showed neuronal properties not only by expressing neuron-specific markers but also by exhibiting voltage-dependent sodium and potassium channels (8). The neural progenitor marker nestin is more highly expressed in undifferentiated hDPSCs than in human adipose stem cells or human skin-derived mesenchymal stem cells (MSCs) (9).

Human neurodegenerative diseases are caused by chronic and progressive loss of specific types of neurons: cerebral cortex glutamatergic and basal forebrain cholinergic neurons in Alzheimer's disease (AD), midbrain dopaminergic neurons in Parkinson's disease (PD), and striatal GABAergic neurons in Huntington's disease. An alternative treatment approach in neurodegenerative disease is transplantation of easily expandable cells that have the capacity to generate those specific neuron types that are lost in the different disorders (10). However, fetal or embryonic cell transplantation has significant ethical, technical, and practical limitations. Recently, hDPSCs have been proposed as promising stem cells for nerve regeneration because of their close embryonic origin and ease of harvest (1, 4).

DPCs from both rats and humans have the phenotypic characteristics of embryonic dopaminergic neurons and protect dopaminergic neurons against the neurotoxin 6-hydroxy-dopamine *in vitro* (6). Differentiation of SHED into dopaminergic neuron-like cells *in vitro* has been reported (11). Neurogenic-differentiated murine DPSCs expressed markers for cholinergic, GABAergic, and glutamatergic neurons, indicating a mixture of CNS and peripheral nervous system cell types (12). Although hDPSCs

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exposed to either dopaminergic or motor NM undergo neuronal differentiation (13), regulatory controls for neuronal differentiation toward specific neurons or glial cells remain to be elucidated in hDPSCs.

Protein phosphorylation of certain serine or threonine residues is a central signaling mechanism in diverse cellular processes (14). Protein interacting with never in mitosis A-1 (PIN1) is involved in cis-trans isomerization of phosphorylated serine/threonine-proline bonds in phosphoproteins, which regulates numerous key signaling molecules involved in cell growth and differentiation (15, 16). We recently demonstrated that PIN1 inhibition can promote odontogenic differentiation of hDPSCs but inhibits adipogenesis (17).

PIN1 has been shown to be involved in neurodegenerative disorders such as AD, PD, and amyotrophic lateral sclerosis (18, 19). PIN1-deficient mice display both tau-related and Abeta-related pathologies and neurodegeneration in an age-dependent manner, resembling AD (20, 21). In contrast, PIN1 overexpression in postnatal neurons effectively suppresses tau-related pathology and neurodegeneration in a mouse model of AD (22). Furthermore, PIN1 depletion suppressed neuronal differentiation, whereas PIN1 overexpression enhanced it without any effects on gliogenesis in neural progenitor cells (NPCs) (23).

Although defective neuronal differentiation in PIN1 knockout NPCs was rescued *in vitro* by overexpression of β-catenin (23), little is known about the role of PIN1 in the neuronal differentiation of hDPSCs. In addition, PIN1 is expressed in most neurons in the brain but is present at an especially low level in those neurons most vulnerable to neurodegeneration in AD (20). Moreover, the major challenge for the development of neuronal replacement therapies for brain diseases is the identification of reliable sources of easily expandable cells with the capacity to generate those specific neuron types that are lost in the different neurodegenerative disorders (10, 20). Therefore, the aim of the present study was to investigate the role of PIN1 on neuronal differentiation toward specific neurons or glial cells of hDPSCs.

Materials and Methods

Cell Culture of hDPSCs

Human dental pulp tissue was obtained from the healthy premolars of young adults undergoing routine extractions at the dental hospital of Kyung Hee University (Seoul, Korea) who provided informed consent. Human dental pulp tissue was isolated, and human dental pulp cells (hDPCs) were separated enzymatically as described previously (1). Primary hDPCs were grown in α-MEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. The hDPSC $CD34^+$/c-kit^+/$STRO\text{-}1^+$ cell population was sorted from the primary hDPCs by a magnetic activating cell sorting method and was isolated and cultured as described previously (3, 24).

Neurogenic Induction of hDPSCs

The hDPSCs were cultured in NM (Sigma-Aldrich, St Louis, MO) supplemented with 1.55 mg/mL glucose, 0.073 mg/mL L-glutamine, 1.69 mg/mL sodium bisulfite, N-2 supplement (R&D Systems Inc, Minneapolis, MN), 20 ng/mL EGF (R&D Systems), and 20 ng/mL FGF (R&D Systems) at 37°C in a 5% humidified CO_2 atmosphere for 2 weeks, as described previously for DPSCs (25).

Preparation of Recombinant PIN1 Adenovirus

An adenovirus encoding PIN1 (Ad-PIN1) (provided by Professor Byung-Hyun Park, Jeonbuk National University, Korea) was created by using the ViraPower Adenovirus Expression System (Invitrogen) according to the manufacturer's instructions.

Nissl Staining

Formalin-fixed cells were washed with phosphate-buffered saline and stained in 0.1% Nissl staining solution (0.12 g cresyl violet acetate with 120 mL distilled water and 0.2 mL glacial acetic acid) at room temperature for 30 minutes. After staining, the specimens were carefully washed in distilled water, dehydrated in alcohols, and mounted in balsam at different time points.

RNA Isolation and Reverse Transcriptase Polymerase Chain Reaction

Total RNA of cells was extracted by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and then reverse-transcribed by using AccuPower RT pre-mix (Bioneer, Daejeon, Korea). cDNA was amplified by using AccuPower PCR PreMix (Bioneer) in a DNA thermal cycler. Primer sequences for genes were reported previously (24). Polymerase chain reaction (PCR) products were subjected to electrophoresis on 1.2% agarose gels stained with ethidium bromide.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde and incubated with Anti-NeuN (Merck Millipore, Darmstadt, Germany), GFAP (Merck Millipore), or nestin (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies overnight at 4°C. Cells were then washed in phosphate-buffered saline and incubated with anti-mouse Alexa Fluor 488 or anti-rabbit Alexa Fluor 594 conjugated secondary antibodies (Molecular Probes Inc, Eugene, OR) for 1 hour at 4°C. Slides were imaged on a confocal microscope (Yokogawa Electric Corporation, Tokyo, Japan). Cells stained with a secondary antibody without the primary antibody served as negative control.

Flow Cytometric Analysis

Cells were fixed in ice-cold 50% methanol and stained by using anti-vesicular glutamate transporter-1 (VGluT1) (Abcam, Cambridge, UK), gamma-aminobutyric acid (GABA) (Abcam), and tyrosine hydroxylase (TH) (Abcam) primary antibodies. Secondary antibodies used were Alexa Fluor 488. Cells stained only with a single secondary antibody were used as negative controls. Ten thousand events were acquired by using a BD FACSVerse flow cytometer (BD Biosciences, San Jose, CA), and data were analyzed by using FACSuite software (BD Biosciences).

Statistical Analysis

The Student t test was used to detect significant differences between groups after determining that the data were normally distributed and exhibited equal variances. Values in all figures are presented as the means $\pm$ standard deviations. P value $< .05$ was considered statistically significant.

Results

Characterization of Neural Differentiation of hDPSCs

To characterize neurogenic differentiation of hDPSCS, cells were treated with NM up to 14 days, and then their morphology was analyzed. The shape of hDPSCs cultured in NM changed from an elongated spindle-shape to rounded or formed nest cells at 7 days, but diverse cellular morphologies including elongated bipolar cells, star-shaped cells forming a network, and round cells with numerous processes at 14 days, consistent with neural cell phenotypes, were observed (Fig. 1*A*).

To further evaluate the neuronal phenotype of hDPSCs, the expression of mRNA and protein levels of the glial cell-specific marker GFAP and neural progenitor marker nestin, early neuronal marker βIII-tubulin, and late neuronal marker NeuN were assessed by reverse transcriptase (RT)-PCR (Fig. 1*B*) and confocal microscopy (Fig. 1*C*).