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-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 of hDPSCs, suggesting that enhanced PIN1 expression induced glutamatergic and GABAergic differentiation but suppressed 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 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. 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.

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. Differentiation of SHED into dopaminergic neuron-like cells in vitro has been reported. Neurogenic-differentiated murine DPSCs expressed markers for cholinergic, GABAergic, and glutamatergic neurons, indicating a mixture of CNS and peripheral nervous system cell types. Although hDPSCs...
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-1+ cell population was sorted from the primary hDPCs by a magnetic activating cell sorting method and was isolated and cultured according to the manufacturer’s instructions.

**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 CO₂ 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.
Transcript levels of nestin increased early, with highest expression 1 day after NM treatment, after which levels decreased. Expression of βIII-tubulin mRNA increased until day 7, with a significant reduction on day 14. Expression of mRNA levels of NeuN increased in a time-dependent manner after exposure to NM until 14 days, showing a similar expression pattern to glial cell marker GFAP and PIN1 mRNA (Fig. 1B). Confocal microscopic images revealed that nestin protein was present in undifferentiated hDPSCs for 1 day, whereas PIN1, βIII-tubulin, NeuN, and GFAP proteins were expressed in differentiated cells for 7 or 14 days (Fig. 1C). PIN1 and NeuN were co-expressed in the nucleus of differentiated hDPCs (Fig. 1C).

Effects of PIN1 Inhibition and Overexpression on Neural Differentiation of hDPSCs

To investigate the role of PIN1 in neurogenic differentiation of hDPSCs, PIN1 expression was inhibited by the PIN1 inhibitor juglone and overexpressed by Ad-PIN1 in NM-stimulated hDPSCs. Juglone concentration-dependently decreased the number of Nissl bodies, a mature neuron marker (Fig. 2A), and downregulated mRNA expression of nestin, βIII-tubulin, and NeuN (Fig. 2C). To examine whether inhibition of PIN1 transcripts by juglone is nonspecific cytotoxic effects in higher doses, cell viability was analyzed by MTT assay. Juglone did not significantly affect NM-induced cell viability (Fig. 2D). Treatment of hDPSCs with Ad-PIN1 enhanced NM-induced upregulation of nestin, βIII-tubulin, and NeuN but blocked GFAP expression (Fig. 2G). Although the number of Nissl body–stained cells did not change significantly (Fig. 2F), flow cytometry analysis revealed that the number of NeuN-positive cells was significantly higher in the Ad-PIN1–treated cells than in negative control and cells transfected with control adenovirus expressing β-galactosidase (Ad-LacZ) (Fig. 2H). In contrast, GFAP expression was enhanced by juglone in a concentration-dependent manner (Fig. 2C) but attenuated by Ad-PIN1 (Fig. 2G).

Because the NM-induced increase in GFAP mRNA level was inhibited by Ad-PIN1, immunofluorescence staining and flow cytometry for GFAP were performed to confirm the expression of this glial marker protein. GFAP-positive cells were rarely detected in either the control or Ad-PIN1 groups (Fig. 3A). The number of GFAP-positive cells decreased significantly in response to treatment with Ad-PIN1 on the basis of flow cytometric analysis (Fig. 3B).

Effects of PIN1 Overexpression on the Ability of hDPSCs to Differentiate into Neurons with Different Neurochemical Phenotypes

To determine the neuronal population types obtained, the expression of the glutamatergic marker VGluT1, GABAergic marker GABA, and dopaminergic marker TH were assessed by confocal microscopy (Fig. 4A). Immunoreactivity for VGluT1, GABA, and TH was observed in both NM-treated control cells and Ad-PIN1–infected cells (Fig. 4A). The percentage of double positive cells (VGluT1+ NeuN+, GABA+ NeuN+, or TH+ NeuN+) was evaluated by flow cytometry. As shown in Figure 4B, PIN1 overexpression significantly increased the
percentages of glutamatergic and GABAergic cells but decreased the proportion of dopaminergic cells.

**Discussion**

hDPSCs can undergo differentiation to neural progenitors such as glial or neuronal cells in vitro (4, 6). Because of the clinical advantages of hDPSCs as an alternative source of stem cells, we investigated whether glial or specific neural phenotypes can be derived from CD34+/c-kit+/STRO-1+ hDPSCs by modulation of PIN1 expression.

hDPSCs constitutively express the neural precursor marker nestin, which is an intermediate filament that is expressed in neural stem or NPCs (4, 6). βIII tubulin is the only phosphorylated tubulin and is considered to be an early/intermediate neuronal-specific marker expressed during neuronal differentiation of hDPSCs (7). NeuN is a mature neuronal marker and has been observed in more than half of neuronally committed hDPSCs (8). Furthermore, GFAP has been shown to be a marker of glial cells among hDPSCs (4). To neurally differentiate hDPSCs, we exposed hDPSCs to NM, which has previously been used to induce DPSCs (25) and MSCs (26) to become neurogenic cells. Culture in NM resulted in morphologic changes of the majority of the cells toward a neuron-like morphology, and co-expression of neurogenic or glial cell markers was found in hDPSCs, consistent with previous studies (25, 27).

Our findings confirm that hDPSCs have the potential to differentiate into neuronal and glial cell types. Moreover, we demonstrated peak upregulation of nestin at 1 day (proliferation stage), βIII-tubulin at 7 days (early differentiation stage), and NeuN and GFAP at 14 days (late differentiation stage) in NM-induced differentiated hDPSCs. The sequential expression of these markers according to neural differentiation stage is similar to that reported in a previous study of the differentiation of human adult skin-derived neuronal precursors into mature neurons (28).

PIN1 level was found to be strongly increased during neuronal differentiation of NPCs (23). However, the expression pattern of PIN1 in differentiating cells may vary depending on the types of stem cells and the lineages of cellular differentiation (17). The present study showed that PIN1 mRNA level was time-dependently elevated throughout the process of neurogenic differentiation of hDPSCs, suggesting the functional involvement of PIN1 in both early and late developmental stages of neurogenesis from hDPSCs. Consistent with these results, time-dependent PIN1 upregulation was observed during adipogenic differentiation of hDPSCs (17) and NPC differentiation (25).

Stem cells can differentiate first into neuron-glia progenitors and later into glial cells and functional neurons (29). However, the signaling mechanisms by which neuronal or glial cell fates are decided in hDPSCs are not well-understood. We found that differentiation of hDPSCs into neurons was inhibited by the PIN1 inhibitor juglone but promoted by...
PIN1 overexpression, as evidenced by the formation of Nissl bodies and upregulation of neural marker genes such as nestin, βIII-tubulin, and NeuN. These results are consistent with previous data that the number of βIII-tubulin–positive neurons increased in response to PIN1 overexpression but decreased in PIN1 knockout NPCs (23).

Concurrently, NM-induced glial differentiation was promoted by a PIN1 inhibitor but inhibited by PIN1 overexpression, which was confirmed by GFAP expression as determined by RT-PCR, immunochemistry, and flow cytometric analysis. Thus, our results indicate that PIN1 overexpression promotes neurogenic differentiation and inhibits glial differentiation,

**Figure 2.** (Continued). Cells were pretreated with Ad-PIN1 for 5 hours and post-treated with NM for 7 days (E–H). Adenoviral vector containing LACZ (Ad-LACZ) served as a control. Cell viability was evaluated by MTT assay (D). Differentiation was assessed by (A and B) Nissl staining, (C and G) RT-PCR, and (H) flow cytometric analysis. Quantification of % of Nissl-positive cells in each group (B and F). (H) Histogram indicates percentage of NeuN-positive cells among all hDPSCs. Data were obtained from 3 independent experiments. #P < .05 versus NM alone control.

**Figure 3.** Effects of PIN1 overexpression on glial marker GFAP expression in hDPSCs. Expression was assessed by double immunofluorescence (A) and flow cytometric analysis (B). Cells were pretreated with Ad-PIN1 for 5 hours and post-treated with NM for 7 days. Representative images revealed GFAP-positive cells with relatively plump cytoplasm and multiple dendrites (scale bar = 25 μm). Histogram indicates percentage of GFAP-positive cells among total hDPSCs. Data were obtained from 3 independent experiments. #P < .05 versus control. FITC, fluorescein isothiocyanate.
whereas PIN1 inhibition has the opposite effects in hDPSCs. However, the number of GFAP+ cells did not change in PIN1 knockout NPCs, which suggests that PIN1 knockout might specifically impair neuronal but not glial differentiation (23). Further analysis of the mechanism of regulation of neural and glial cell fates in hDPSCs is necessary.

NPCs can generate heterogeneous progeny; clones of mature neurons can synthesize multiple neurotransmitters such as GABA, glutamate, dopamine, or some combination (30). The differentiation of SHED into dopaminergic neuron-like cells in vitro has been reported (11). Glutamatergic and GABAergic axons are abundant in the dental pulp and appear to play a role in mediating pulpal pain during inflammation and injury (31, 32). In addition, murine DPSC-derived neural cells expressed markers for GABAergic and glutamatergic neuron but did not express dopaminergic neuron marker (12). To the best of our knowledge, this study presents the first evidence of glutamatergic, GABAergic, and dopaminergic cells as percentage of NeuN-positive hDPSCs. Data were obtained from 3 independent experiments. Scale bar = 50 μm. *P < .05 versus control. FITC, fluorescein isothiocyanate.

Figure 4. Effects of PIN1 overexpression on neuronal phenotype of differentiating hDPSCs. Cells were pretreated with Ad-PIN1 for 5 hours and post-treated with NM for 7 days. Neuronal phenotype was examined by double immunofluorescence (A) and flow cytometric analysis (B). Histogram indicates percentage of glutamatergic, GABAergic, and dopaminergic cells as percentage of NeuN-positive hDPSCs. Data were obtained from 3 independent experiments. Scale bar = 50 μm.
Regenerative Endodontics

Trigeminal nucleus caudalis (Vc), relaying nociceptive somatosensory information derived from the face, mouth, and dental pulp, contains glutamatergic and GABAergic projection neurons and interneurons that exert crucial modulatory effects on primary sensoryafferent inputs from this region (34). Long-term consequences of peripheral injury and inflammation lead to neuropathic changes (central sensitization) in trigeminal nucleus that play a central role in acute and chronic pain. Therefore, therapeutic targets for pain management and development of therapeutic procedures should be focused on manipulating peripheral inputs and central processes within this region (Vc). Neuropathic pain pathogenesis and maintenance involve interactions among neurons, inflammatory immune cells, glial cells, and a wide cascade of proinflammatory and anti-inflammatory cytokines (35, 36).

Recently, many researchers have tried to relieve neuropathic pain by using stem cells with various origins, not only treating pain but also repairing the damaged nervous system by replacing injured or lost neural cells in experimental animal model (37). Intravenous neural stem cell delivery in sciatic nerve injury model was reported to induce a significant reduction of neuropathic symptoms, proinflammatory cytokines, and spinal cord fos expression in addition to increase of activated anti-inflammatory cytokines, improvement of nerve morphology, and altering microenvironment within the spinal cord (38). MSCs are also being studied as a therapeutic agent in the treatment of neuropathic orofacial pain or atypical odontalgia besides CNS disorders (39). Neuropathic pain results from inflammatory nerve damage and is maintained by glial activation in the spinal cord after peripheral nerve injury (40). Because PIN1 upregulation can promote neurogenesis but inhibit glial cell differentiation of hDPSCs, the transplantation of PIN1-modulated hDPSCs might be an alternative treatment option in neurogenic orofacial pain by regenerating injured peripheral nerves without excessive glial proliferation.

In conclusion, the present study is the first to elucidate the dual functions of PIN1: activation of glutamatergic and GABAergic neuronal differentiation and suppression of glial differentiation of hDPSCs. Our findings suggest that PIN1-modulated hDPSCs may serve as useful sources of neurogenesis and gliogenesis in degenerative disorders of the CNS.

Acknowledgments

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