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Regular Article

Rapid differentiation of human dental pulp stem cells to neuron-like cells by high K^+ stimulation

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As human-origin cells, human dental pulp stem cells (hDPSCs) are thought to be potentially useful for biological and medical experiments. They are easily obtained from lost primary teeth or extracted wisdom teeth, and they are mesenchymal stem cells that are known to differentiate into osteoblasts, chondrocytes, and adipocytes. Although hDPSCs originate from neural crest cells, it is difficult to induce hDPSCs to differentiate into neuron-like cells. To facilitate their differentiation into neuron-like cells, we evaluated various differentiation conditions. Activation of K^+ channels is thought to regulate the intracellular Ca^{2+} concentration, allowing for manipulation of the cell cycle to induce the differentiation of hDPSCs. Therefore, in addition to a conventional neural cell differentiation protocol, we activated K^+ channels in hDPSCs. Immunocytochemistry and real-time PCR revealed that applying a combination of 3 stimuli (high K^+ solution, epigenetic reprogramming solution, and neural differentiation solution) to hDPSCs increased their expression of neuronal markers, such as β3-tubulin, postsynaptic density protein 95, and nestin within 5 days, which led to their rapid differentiation into neuron-like cells. Our findings indicate that epigenetic reprogramming along with cell cycle regulation by stimulation with high K^+ accelerated the differentiation of hDPSCs into neuron-like cells. Therefore, hDPSCs can be used in various ways as neuron-like cells by manipulating their cell cycle.

Key words: dental pulp stem cell, high K^+ stimulation, neural differentiation, neuron-like cell

Abbreviations: BDNF, brain-derived neurotrophic factor; BSS, balanced salt solution; DAPI, 4',6-diamidino-2-phenylindole; ESC, embryonic stem cell; GFAP, glial fibrillary acidic protein; hDPSC, human dental pulp stem cell; iPSC, induced pluripotent stem cell; PCR, polymerase chain reaction; PI3K, phosphoinositide 3-kinase; PSD, postsynaptic density protein; TRPC, transient receptor potential canonical

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◀ Significance ▶

It is difficult to induce human dental pulp stem cells (hDPSCs) to differentiate neurons. Activation of K^+ channels is thought to regulate the intracellular Ca^{2+} concentration, allowing for manipulation of the cell cycle to induce the differentiation of hDPSCs. Our findings indicate that cell cycle regulation by stimulation with high K^+ accelerates the differentiation of hDPSCs into neurons.

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Introduction

At present, human-origin cells in biological and medical experiments mainly originate from human skin. However, the use of human-origin neurons, not non-human animal neurons, is strongly demanded in various fields. Human dental pulp stem cells (hDPSCs) are a candidate for this purpose. hDPSCs can be easily obtained from primary teeth lost during childhood or wisdom teeth extracted in adulthood [1]. hDPSCs are multipotent [2,3] and their facile collection supports regenerative medicine [4,5]. Because hDPSCs overcome the ethical issues surrounding embryonic stem cells (ESCs) and the tumorigenic fears related to induced pluripotent stem cells (iPSCs), they are anticipated to become a viable alternative to ESCs and iPSCs [6–9].

hDPSCs are mesenchymal stem cells [10,11] that easily differentiate into osteoblasts, chondrocytes, and adipocytes [12]. hDPSCs are thought to originate from neural crest cells (i.e., neural precursors) [10,12] and differentiate into dopaminergic neuron-like cells [13–15] as well as glutamatergic and GABAergic neuron-like cells [16]. However, inducing hDPSCs to differentiate into neuron-like cells is problematic [17, 18], because the same type of stimulation leads hDPSCs to also differentiate into Schwann cells, astrocytes, oligodendrocytes, and retinal ganglion-like cells [19].

Previous studies evaluated the effects of applying epigenetic reprogramming and neural differentiation media to induce the differentiation of hDPSCs into neuron-like cells [20]. Epigenetic reprogramming inhibits DNA methylation, and thus affords broad multipotency to hDPSCs [21]. Application of a neural differentiation solution to hDPSCs activates cAMP-protein kinase C signaling pathways to specifically promote neural differentiation [20]. Differentiation of hDPSCs into neurons by applying a neural differentiation solution, however, requires at least 10 days [20]. We thus hypothesized that the neural differentiation of hDSPCs could be enhanced by manipulating the cell cycle in addition to applying epigenetic reprogramming and neural differentiation media.

The cell cycle may be controlled by the transmembrane potential, which is regulated by the intracellular K^+ concentration [22]. In addition, a high extracellular K^+ concentration can change the cell membrane potential, leading to Ca^{2+} entry via voltage-dependent Ca^{2+} channels and a cell volume change [23]. This cell volume change further increases Ca^{2+} entry via transient receptor potential canonical (TRPC) channels such as TRPC1 and TRPC6 [24]. A higher concentration of intracellular Ca^{2+} is thought to further promote the rate of differentiation from hDPSCs to neurons according to the above-described scenario. Taken together, we hypothesized that activating the big-conductance K^+ channels would accelerate the neural differentiation of hDPSCs. Therefore, if the cell cycle of hDPSCs is well manipulated by high K^+ stimulation, hDPSCs can be used in various ways as neuron-like cells.

Materials and Methods

Isolating and culturing human hDPSCs

The present study was approved by the Ethics Committee of Nippon Dental University School of Life Dentistry at Tokyo (Approval No. NDU-T2013-10) and the Ethics Review Committee on Human Research of Waseda University (2016–198), and conducted in accordance with the amended Declaration of Helsinki. Written informed consent was obtained from each donor after fully explaining the nature of the procedure and the intended use of the tissue obtained. Human third molars were extracted from 6 women (age: 19–31 years) at Nippon Dental University Hospital. hDPSCs were isolated as reported previously [10,25–28]. The cells were cultured in DMEM/F12 containing 10% fetal calf serum, 1% MEM non-essential amino acids, 100 μM GlutaMax (Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B, and incubated at 37°C in 5% CO_2. Confluent cells were passaged by treatment with 0.25% trypsin-0.02% EDTA. The passage number was 2–8.

Calcium imaging

We used high K^+ stimulation (application of a 50 mM KCl solution) to depolarize the cells and open voltage-dependent Ca^{2+} channels, thereby activating large-conductance Ca^{2+}-activated K^+ channels (i.e., big K^+ channels). To confirm calcium influx induced by high K^+ stimulation, calcium imaging was performed as follows. hDPSCs were seeded at 2.0×10^4 cells/well on a ϕ12 mm cover glass in a 24-well plate and incubated for 24 – 48 h. The cover glass was placed in a Ca^{2+} imaging chamber. The cells were incubated with 4 μM Fluo-4-AM in BSS-Ca^{2+} buffer (130 mM NaCl, 5.4 mM KCl, 1.0 mM $MgSO_4$, 2.0 mM $CaCl_2$, 5.5 mM D-glucose, 20 mM HEPES [pH 7.4]) at 37°C in 5% CO_2 for 20 min. After incubation, the cells were washed with BSS-Ca^{2+} buffer, and then 1 mL BSS-Ca^{2+} buffer was added to the chamber. Calcium imaging was performed with a CCD time-lapse microscope (IX81, U-RX-T, K2-UCB, Olympus, Tokyo, Japan). After observation for 1 min, high K^+ BSS-Ca^{2+} buffer (85.4 mM NaCl, 50 mM KCl, 1.0 mM $MgSO_4$, 2.0 mM $CaCl_2$, 5.5 mM D-glucose, and 20 mM Hepes, pH 7.4) was applied to the chamber for 1 min. The cells were then washed with BSS-Ca^{2+} buffer. As a control, the BSS-Ca^{2+} buffer was used as a stimulant instead of high K^+ BSS-Ca^{2+} buffer.

Neural differentiation

For the immunohistochemistry and real-time PCR

experiments, hDPSCs were passaged at 2.0×10^4 cells/well on a ϕ12 mm cover glass in a 24-well plate and at 2.5×10^5 cells in a 6-cm dish, respectively. After incubating for 24 h, we applied the following 3 differentiation solutions to hDPSCs. (1) Epigenetic reprogramming was performed with DMEM/F12 containing 2.5% fetal bovine serum, 10 μM azacytidine, 10 ng/mL basic fibroblast growth factor-2, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO_2 for 48 h [20]. (2) High K^+ BSS-Ca^{2+} buffer was applied to the cells at 37°C in 5% CO_2 for 20 min. (3) Neural differentiation was performed with DMEM/F12 containing 1% insulin-transferrin-selenium (Thermo Fisher Scientific), 250 μM3-isobutyl-1-methylxanthine, 50 μM forskolin, 1 mM dibutyryl cyclic adenosine monophosphate, 200 nM tissue plasminogen activator, 10 ng/mL nerve growth factor, 30 ng/mL neurotrophin-3, 10 ng/mL basic fibroblast growth factor-22, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO_2 for 72 h.

Immunocytochemistry

hDPSCs were fixed with 4% paraformaldehyde at room temperature for 10 min, and incubated with 0.1% Triton-X in phosphate-buffered saline including 0.05% Tween 20 at room temperature for 10 min. Blocking was performed with 5% normal goat serum at room temperature for 30 min. The hDPSCs were then incubated with the primary antibody, such as anti-β3-tubulin (1:500; GTX129913, GeneTex), or anti-postsynaptic density protein (PSD) 95 (1:500; GTX133091, GeneTex), at 4°C overnight. Simultaneously, the cells were co-immunostained with anti-glial fibrillary acidic protein (GFAP) (1:300; 3670, Cell Signaling Technology, Danvers, MA, USA). After applying the primary antibody, the hDPSCs were incubated with the secondary antibody, such as goat polyclonal antibodies to rabbit Alexa 488 (1:200; ab150077, abcam, Cambridge, UK) or goat polyclonal antibodies to mouse Alexa 568 (1:200; ab175473, abcam), at room temperature for 1 h. Further, the cells were co-stained with 100 μg/mL 4',6-diamidino-2-phenylindole (i.e., DAPI) at room temperature for 1 h. Finally, the hDPSCs were mounted with Immu-Mount (Thermo Fisher Scientific). The cells were observed with a confocal laser microscope (IX81, Fluoview FV1000, U-RF-T, Olympus).

Real-time PCR

After neural differentiation, total RNA was extracted from hDPSCs using an RNeasy Mini kit (Qiagen, Venlo, Netherlands). The RNA was reverse-transcribed to cDNA with ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). Real-time PCR was performed with BrightGreen 5×qPCR MasterMix-ROX (Applied Biological Materials, Richmond, BC, Canada) and the following primers: nestin forward primer: 5'-AGCCCTGACCACTCCAGTTTAG-3' [29], nestin reverse primer: 5'-CCCTCTATGGCTGTTTCTTTCTC-3' [29], β3-tubulin forward primer: 5'-GGCCAAGGGTCACT ACACG-3' [30], β3-tubulin reverse primer: 5'-GCAGTC GCAGTTTTCACACTC-3' [30], GFAP forward primer: 5'-GCGAGGAGAACCGGATCAC-3' [31], GFAP reverse primer: 5'-GTGGCCTTCTGACACAGACTTG-3' [31], GAPDH forward primer: 5'-CTCCTCTGACTTCAACAG CGAC-3' [32], and GAPDH reverse primer: 5'-CCTGTT GCTGTAGCCAAATTCG-3' [32]. The reactions were performed with StepOnePlus (Thermo Fisher Scientific).

Results

Depolarization by high K^+ stimulation

First, to examine whether high K^+ stimulation depolarizes the membrane potential of the hDPSCs used in the present study, we observed the intracellular Ca^{2+} changes to confirm whether hDPSCs have voltage-dependent Ca^{2+} channels (Fig. 1). High K^+ stimulation increased the intracellular Ca^{2+} concentration, confirming that high K^+ stimulation depolarizes hDPSCs and activates Cav1.2 Ca^{2+} channels [33]. This phenomenon suggests that the intracellular K^+ concentration is changed by the activation of voltage-dependent K^+ channels and Ca^{2+}-activated K^+ channels. The presence of K^+ currents in hDPSCs was also supported by previous results [20].

Immunocytochemistry for neuronal and glial markers

To examine whether K^+ channel activation accelerates the differentiation of hDPSCs into neuron-like cells by manipulating the intracellular K^+ and Ca^{2+} concentrations, a high K^+ solution was added to the epigenetic reprogramming and neural differentiation media to depolarize the membrane potential of hDPSCs. Immunocytochemical experiments showed that combined application of these 3 solutions for 5 days induced the differentiation of hDSPCs into neuron-like cells (Fig. 2). The neuronal markers used to confirm the differentiation were β3-tubulin and PSD95; GFAP was used as a marker for astrocytes. The expression of β3-tubulin was increased by short-term stimulation with

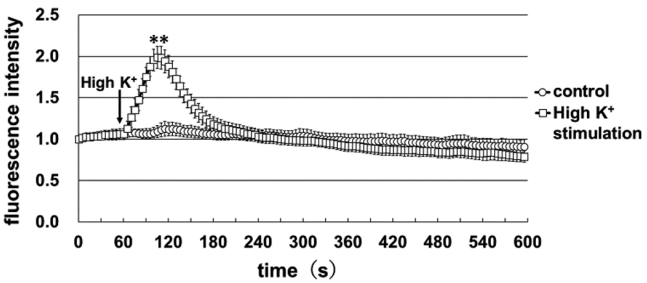

Figure 1 Ca^{2+} responses to high K^+ stimulation in hDPSCs. When 50 mM KCl was applied to hDPSCs, Ca^{2+} influx was observed in the cells. Control indicates the fluorescence in response to the application of normal buffer. n=40 each. Student *t*-test, $^{**}P<0.01$.

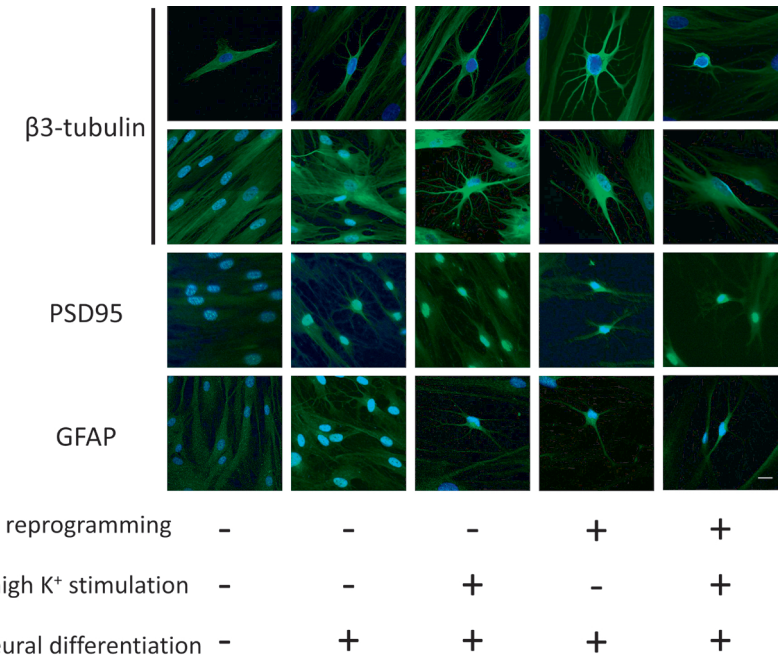

Figure 2 Neuron-like differentiation of hDPSCs by a combination of 3 different conditions. We used epigenetic reprogramming solution, high K^+ solution, and neural differentiation solution as the differentiation solutions, and performed immunohistochemistry. hDPSCs strongly expressed neural markers after neural differentiation. Green indicates β3-tubulin, PSD95, and GFAP. Blue indicates 4',6-diamidino-2-phenylindole (i.e., DAPI). β3-tubulin is shown in 2 lines. Scale bar=20 μm.

a high K^+ solution. β3-Tubulin is an important marker of branched cells, because a critical point for the differentiation of hDPSCs to neuron-like cells is the conversion of the cell morphology from an oval type to a branched type. Interestingly, excitable cells, including neurons and muscle cells, have a branched/slender shape with an interaction between actin and tubulin [34].

mRNA levels of neuronal and glial markers

Expression levels of the neuronal and astrocyte marker genes were examined by real-time PCR. The neuronal markers were nestin and β3-tubulin, whereas the astrocyte marker was GFAP. The mRNA level of β3-tubulin was significantly increased ($P<0.01$), as well as that of nestin seemed increased (not statistically different), by adding high K^+ solution to the epigenetic reprogramming and neural differentiation solutions (Fig. 3). Although GFAP was increased by applying the epigenetic reprogramming solution and high K^+ solution, the increase was small even after cell differentiation ($P<0.05$) (Fig. 3). Therefore, we concluded that high K^+ stimulation promoted the differentiation of hDPSCs to neuron-like cells. Previous experiments demonstrated that neural differentiation of hDPSCs occurs within 10 days without high K^+ stimulation [20]. Therefore, the present findings indicate that the period of differentiation time was cut in half by adding high K^+ stimulation to the differentiation protocol.

Discussion

The cell cycle is associated with the transmembrane potential, which is regulated by a change in the K^+ permeability [22]. Thus, changes in various intracellular K^+ concentrations usually occur with changes in the cell cycle due to cell volume regulation and a membrane potential-dependent transient change in the intracellular Ca^{2+} concentration. The cell volume change is an important parameter for cellular signaling cascades, because changing the cell volume activates TRPC channels to increase the intracellular Ca^{2+} concentration (Fig. 4) [35]. On the other hand, from the viewpoint of the water conditions inside the cells, the cell cycle is thought to depend on a form of intracellular water [36]. Previous studies reported a correlation between the water structure and the chromatin condensation cycle [37]. During the cell division process, intracellular water is in a free form, exhibiting high mobility [38–40]. In this case, it is important to consider that the intracellular Ca^{2+} dynamics change from a wave mode to a diffusion mode [41] according to the water structure. These findings support the notion that K^+ channel activity is important for the cell cycle. The involvement of K^+ channels in cancer therapy through cell cycle regulation is reported for some neurologic and cardiovascular diseases [42], because cancer cells are thought to originate due to a release of cell cycle arrest.

It is important to consider that neurons do not have cell

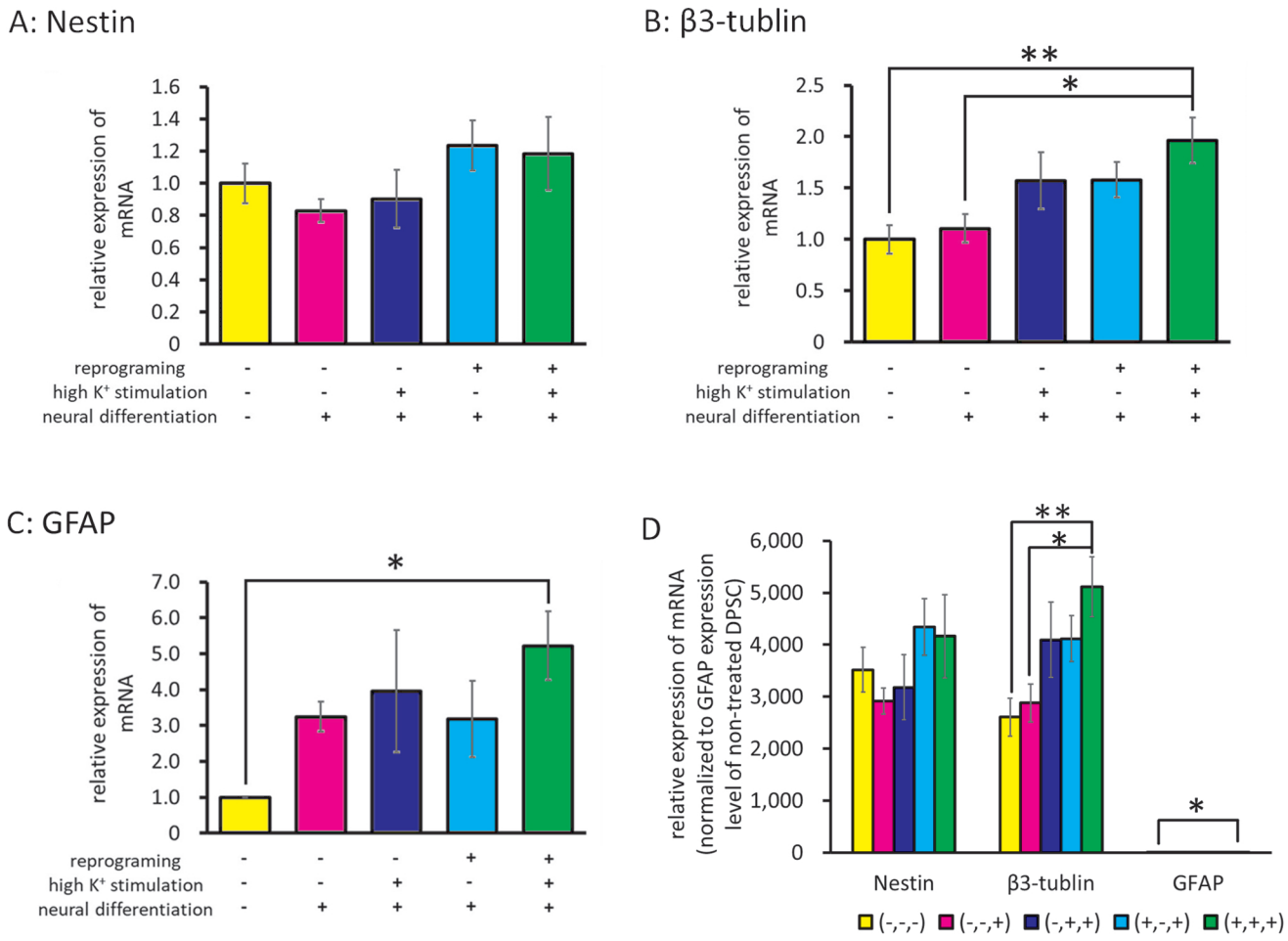

Figure 3 Expression of neuron-specific genes in hDPSCs after combining 3 different conditions (i.e., epigenetic reprogramming solution, high K^+ solution, and neural differentiation solution). Real-time PCR was performed. hDPSCs mostly expressed neuronal markers (i.e., nestin and β3-tubulin) and an astrocyte marker (GFAP). Combined stimulation with epigenetic reprogramming solution and high K^+ solution increased the expression of neuronal markers in hDPSCs. (A) Nestin (n=10). (B) β3-tubulin (n=9). (C) GFAP (n=9 from 3 different subjects). (D) Comparison of the expression among the 4 markers. The *y*-axis shows the relative mRNA amounts normalized by that of GFAP in hDPSCs before treatment, i.e., the GFAP expression level in non-treated hDPSCs was defined as 1. Thus, expression of neuronal markers, such as nestin and β3-tubulin, was 5000-fold higher than that of the astrocyte marker GFAP, although the GFAP expression levels differed significantly between cells not treated with differentiation solutions and cells treated with all 3 differentiation solutions. Bar colors are consistent for each differentiation condition, as shown in (A) to (C). Two-way ANOVA followed by a *post hoc* Holm test, $^{**}P<0.01$ and $^{*}P<0.05$.

cycle, which allows neurons to survive over the life span of the host. Thus, neurons are very suitable models for studies of senescence [43,44]. In addition, many neuroscientists have ascertained that the cytosol of the squid giant axon is a gel, which largely differs from a solution [45–47]. Thus, neurons also seem to be very good models for studying the intracellular water phase. Human neurons are needed for application to medical sciences to clarify why neurons can survive for a long time without a cell cycle and why they contain gel rather than solution. We have therefore attempted to obtain human neurons by inducing hDPSCs to differentiate into neurons. Furthermore, the conversion of hDPSCs into neurons provides another advantage: if we obtain novel methods for producing neurons from hDPSCs, we can learn why the resting membrane potential changes from –25 mV in embryonic cells to –90 mV in adult neurons [48] and begin to understand the roles of K^+ and Ca^{2+} for controlling the cell cycle, which will allow for clarification of the mechanisms of aging, apoptosis, and canceration.

Many attempts have been made to obtain neurons or neuron-like cells isolated from various cell types to study the mechanisms of neurite initiation [49], differences in axons and dendrites [50], axonal growth [51], and intracellular signaling cascades [52]. In our case, however, we need human neurons to study the aging mechanisms of neurons, the relationship between the cell cycle and neurite growth, and the essential mechanisms involved in nerve

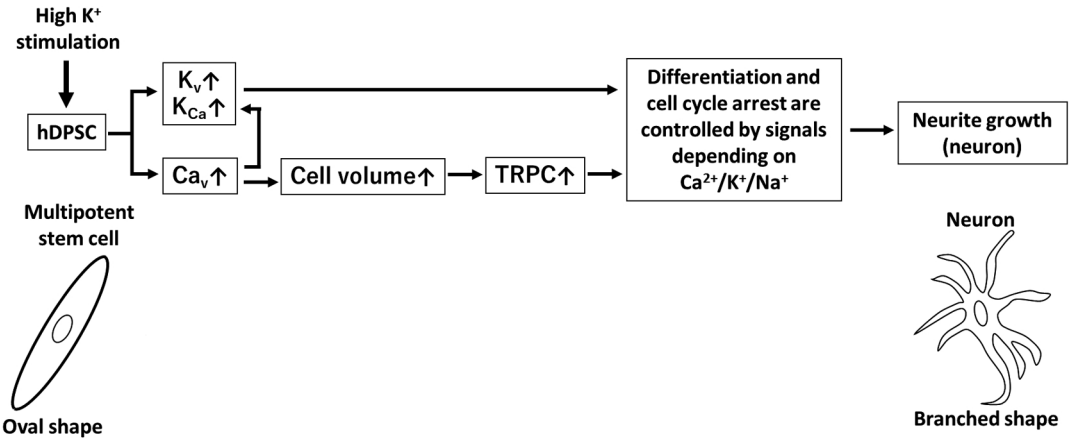

Figure 4 Expected cascades for neural differentiation of hDPSCs by high K^+ stimulation. Kv, KCa, and Cav indicate voltage-dependent K^+ channel, Ca^{2+}-activated K^+ channel and voltage-dependent Ca^{2+} channel, respectively. TRPC is transient receptor potential canonical channel. TRPC passes not only Ca^{2+} but also Na^+. The ratio between Ca^{2+} and Na^+ depends on the subclass of TRPC. Na^+ is also involved in cell signaling.

regeneration and apoptotic neuronal death. Understanding the molecular mechanisms underlying these processes will help to elucidate the basic mechanisms of human aging. Therefore, the present study is the initial attempt in this series of studies.

In conclusion, depolarization of the membrane potential and the associated activation of both K^+ channels and Ca^{2+} channels play important roles in the progression from progenitor cells to differentiated cells. This differentiation process may include the function of brain-derived neurotrophic factor [52], activation of phosphoinositide 3-kinase/Akt signaling [53], interaction between microtubules and actin filaments [54], and a cell volume change [23] with related TRPC channel activation [24]. Thus, the rate of the differentiation of hDPSCs to neuron-like cells is enhanced by the activation of K^+ channels. The present results paved the way for the use of human-origin neuron-like cells in biological and medical experiments.

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Conflicts of Interest

The authors declare no conflicts of interest.

Author Contribution

Y.K.: Investigation, Data analysis, Writing. C.S.: Investigation. Y.T.: Data analysis. M.M.: Resources. T.N.: Resources, Funding acquisition, Writing. K.O.: Interpretation, Writing. T.Y.: Conceptualization, Interpretation, Writing. E.I.: Conceptualization, Interpretation, Writing.

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