The Activation of Autophagy Induced by Neural Differentiation of Human Dental Pulp Stem Cells

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Abstract

Human dental pulp stem cells (hDPSCs) are multipotent, and contain neurogenicity. Numerous studies showed that Notch inhibition and DNA demethylation promoted neural differentiation. Here, we investigated the modulation of autophagy during neural differentiation of hDPSCs, induced by DAPT and 5-Azacytidine. hDPSCs were extracted, characterized, and differentiated. The hDPSCs presented typical mesenchymal stem cell phenotypes, in which the majority of the cells expressed CD73, CD90 and CD105. hDPSCs were multipotent, capable of differentiating into mesodermal cells. After treating with DAPT, hDPSCs upregulated the expression of neuronal genes, SOX2, Nestin, and βIII-tubulin, and autophagy genes, LC3I/II and Beclin. These genes were further increased when 5-Azacytidine was co-supplemented into the culture. The inhibition of autophagy by chloroquine impeded neural differentiation of hDPSCs, marked by the downregulation of βIII-tubulin, while the activation of autophagy by VPA instigated the emergence of βIII-tubulin-positive cells. Notch inhibitor and DNA demethylating agent efficiently induced neural differentiation of hDPSCs. During the differentiation process, autophagy was modulated, implying that autophagy should play a significant role during the differentiation of hDPSCs. The blockage and stimulation of autophagy could either hinder or induce the formation of neural-like cells, respectively. Therefore, the refinement of autophagy activity at an appropriate level might improve the efficiency of stem cell differentiation, and shed light for stem cell therapy.

Keywords**:** *human dental pulp stem cells, neural differentiation, Notch signaling, autophagy*

1. Introduction

Stem cells are hoping to be a novel therapeutic materials for restoring injured diseases and tissues to normal (Vishnubhatla, Corteling, Stevanato, Hicks, & Sinden, 2014). One major issue in embryonic stem cell (ESC) research is the current discussion in safety and ethical issues surrounding ESC research, as many groups have made an attempt to identify and characterize adult stem cells (ASCs) for future therapies (Watt & Driskell, 2010). The best characterized ASC populations reside in the bone marrow (BM). BM-derived mesenchymal stem cells (MSCs) are considered as a potential cell source for stem cell therapies due to their plasticity and potent immunosuppressive capabilities (Nombela-Arrieta, Ritz, & Silberstein, 2011). Despite its capacity and potency, difficulty in obtaining BM aspirates from patients becomes a problem; hence, alternative sources of therapeutic MSCs have been sought. Among many types of stem cells, human dental tissue has gained increasing attention as the attractive source for adult stem cells since they are non-invasive and ease of isolation (Karaöz et al., 2011; Lizier et al., 2012; Ranganathan & Lakshminarayanan, 2012; Zhang, Walboomers, Shi, Fan, & Jansen, 2006). Human dental pulp stem cells (hDPSCs) are mesenchymal stem cells and contain a great potential to differentiate into various cells types, including neuronal cells. Numerous studies have showed an increased interest in the success of differentiating hDPSCs into neural lineage under specific conditions (Chang, Chang, Tsai, Chang, & Lin, 2014; Gronthos, Mankani, Brahim, Robey, & Shi, 2000; Hei et al., 2016; Zainal Ariffin et al., 2013). Therefore, neural differentiation from hDPSCs provides a promising cell-based therapy for neurodegenerative diseases, such as Alzheimer's, Parkinson's and Huntington's diseases.

In this study, we focus on the Notch signaling; which has a critical role for directing differentiation of stem cells into several cell types (Boni et al., 2008; Jönsson, Xiang, Pettersson, Lardelli, & Nilsson, 2001; Ramasamy & Lenka, 2010). Notch signaling is activated when Notch receptor binds to its ligand, and results in the cleavage of the Notch intracellular domain (NICD) of the Notch receptor. NICD can then translocate into the nucleus and initiate the transcription of Notch target genes (Engin & Lee, 2010;

Løvschall, Tummers, Thesleff, Füchtbauer, & Poulsen, 2005). One of the most significant approaches to direct cell reprograming is applying the small molecules targeting at different levels of molecular central dogma regulation. Recent developments in neural differentiation have heightened the need for the inhibition of Notch signaling pathway by γ-secretase inhibitor; DAPT was shown to associate with stem cells and neural differentiation (Kageyama, Ohtsuka, Shimojo, & Imayoshi, 2008; Kanungo, Zheng, Amin, Pant, 2008; Wang et al., 2016). In this study, we set out to differentiate harvested hDPSCs toward the neural lineage using a combination of small molecules of differentiation protocols originally designed for hDPSCs. There is a solid connection between epigenetic modifier and the changes of genes expression profile during stem cell differentiation (Sheaffer et al., 2014). DNA demethylating agent can inhibit methylation at gene promotion and is possibly a prerequisite for transcription activation. 5-Azacytidine is a commonly used DNA demethylation compound, which can integrate into DNA and inhibit DNA methylation (Holliday, 1990; Schneider-Stock et al., 2005). The combination of DAPT and 5-Azacytidine might thus particularly impact neural differentiation of hDPSCs.

Autophagy is a main mechanism which involves self-renewal, development, and cell differentiation, and acts as a cellular cleaning process to get rid of invaded microorganisms and toxic aggregated proteins. Autophagy is initiated by the formation of double-membrane-bound vesicles, called autophagosomes, which then fuse with lysosomes to enable the degradation of autophagic cargos and the subsequent recycling of nutrients and membranes (Vessoni, Muotri, & Okamoto, 2011). Recent evidence suggests that autophagy is known as an important event for stem cell differentiation, for instance muscle stem cells and mesenchymal stem cells (Mizushima, 2007; Pantovic et al., 2013). So far, however, there has been limited exploration of the roles of autophagy during the cell reprograming using small molecules targeting Notch signaling pathway. As no definitive proof is available, it is essential to have a clear understanding on the neuronal differentiation of hDPSCs by targeting Notch signaling and the modified levels of autophagy. In this study, we induced hDPSCs toward neural lineage by DAPT and 5-Azacytidine and observed the modulation of autophagy playing roles during hDPSCs differentiation.

2. Objective

To observe the modulation of autophagy during hDPSCs differentiation and explore the impact of autophagy of neural differentiation

3. Materials and Methods

3.1. Isolation and cultivation of hDPSCs

Human dental pulp tissues were collected from healthy human third molars of an impact mandible with written informed consent from dental clinic of Suranaree University of Technology Hospital (SUTH, Nakhon Ratchasima, Thailand). The mean age of the donors was 42 year old. The protocol was approved by the ethical committee from Suranaree University of Technology. The human dental pulp tissues were isolated and cultured according to the guidelines from the previous report with some modification (Gronthos et al., 2000). Briefly, dental pulp tissue was minced into small pieces and placed in a dish with 4 mg/ml collagenase/Dispase (Roche, Germany) to digest at 37ºC for 1 hour. Digested tissues were maintained in medium containing Dulbecco's Modified Eagle Medium high glucose (DMEM/HG; Hyclone, Logan, UT, USA), supplemented with 20% (v/v) fetal bovine serum (FBS; Gibco BRL, Grand Island, NY), 1mM L-glutamine, 1 mM Minimal essential medium (MEM; Sigma-Aldrich, St. Louis, MO, USA), and 100 U/ml Penicillin, and 100 g/ml Streptomycin (Sigma-Aldrich). hDPSCs were incubated at 37oC, 5% CO2 in air for 7 to 14 days. The medium was replaced every 3 days until fibroblast-like cells migrated out from the dental pulp tissues.

3.2. Characterization of hDPSCs

hDPSCs were seeded approximately 2x104 cells on a 6-well culture plate (Nunc, Roskilde, Denmark), coated with basement membrane Geltrex (Gibco). For Osteogenic induction, hDPSCs were cultured in osteogenic induction medium, consisting of DMEM, 10% FBS, 100 nM dexamethasone, 0.2 mM L-ascorbate-2-phosphate, and 10 mM β-glycerophosphate (Sigma-Aldrich). Medium was changed

every other day for 21 days. Cells were fixed with 4% paraformaldehyde (PFA) for 15 minutes, and stained with Alizarin Red (Sigma-Aldrich) to detect the bone matrix mineralization. To induce adipogenic differentiation, cells were induced by 10 µg/ml insulin, 60 µM indomethacin, 0.5 µM hydrocortisone, and 0.5 mM isobutyl methylxanthine (IBMX) for 21 days. Cells were then fixed, and lipid droplets were stained by Oil Red O (Sigma-Aldrich). Chondrogenic differentiation, hDPSCs were induced by ITS-plus premix (BD Biosciences, San Jose,CA) at concentration of 6.25 µg/ml insulin, 6.25 µg/ml transferrin, and 6.25 ng/ml selenious acid. Additionally, 50 µg/ml ascorbate 2-phosphate, 40 µg/ml L-proline, 100 µg/ml sodium pyruvate, 100 nM dexamethasonee and 10 ng/ml TGF-β3 were also supplemented. After 21 days, cells were, and chondrocytes were stained with Alcian Blue (Sigma-Aldrich).

3.3. Flow cytometry analysis

hDPSCs were collected and washed twice in phosphate-buffered saline (PBS), and resuspended at a concentration of 1 × 106 cells/ mL. 10 µL of the detecting antibody was added to 100 µL of the cell suspension. Fluorescence-conjugated antibody against CD73, CD105, CD90, CD34, and CD45 (Biolegend, San Diego, CA, USA) were incubated with cells at 37°C for 30 minutes. Cells were analyzed using a FACS Calibur instrument and CellQuest Pro software version 3.3 (BD Biosciences, San Jose, CA, USA).

3.4. Differentiation of hDPSCs into neural-like cells

hDPSCs were seeded 2x104 cells onto a 6-well plate (Nunc), coated with Geltrex (Gibco). Neural differentiation was induced by the induction medium, containing Neurobasal (NB) medium (Gibco), DMEM/F12 (Hyclone), N2 supplement (100X, Gibco), retinoic acid 10 µM (Gibco). In addition, the neural differentiation protocol was performed by a 3-step protocol. Firstly, the induction medium was supplemented with DAPT at various concentrations (0, 5, 10 and 20 µM) for 7 days. Then, 10 µM 5-Azacytidine (Sigma-Aldrich) was added in the induction medium with 10 µM DAPT for another 7 days. Finally, either 10 µM VPA or 10 µM chloroquine was supplemented into the differentiation medium, already included 10 µM DAPT and 10µM 5-Azacytidine. Differentiated cells were collected at day 1, 5 and 7 for RT-PCR gene expression analysis, and cell morphology was observed by a phase-contrast microscope.

3.5. Immunocytochemistry

Cells were washed with 3X PBS, and fixed with 4% PFA for 15 minutes. Then, the cells were incubated in a blocking buffer, included 4% bovine serum albumin (BSA; Sigma-Aldrich) and 0.5%Triton-X 100 (Sigma-Aldrich) in PBS for 1 hour. Primary antibody was diluted in a blocking solution at followed dilutions; anti-βIII-tubulin (1:1000; Sigma-Aldrich), anti-NESTIN (1:500; MERK), anti-LC3I/II (1:000; MERK), anti-CD90-FITC (1:500; MERK), anti-Endoglin-CD105 (1:200; MERK), and anti-CD73 (1:500; MERK). Primary antibody was applied overnight at 4°C. Cells were washed 3X with washing buffer (PBS, 0.1% TritonX100), followed by the incubation of secondary antibodies conjugated with either Alexa fluorophore 488 or 593 at 1:1000 for 1 hour at room temperature. Cells were washed 3 times in PBS, and incubated with DAPI for nuclear staining (Biorad; Hercules, CA. USA).

3.6. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Cells were detached by 0.25% trypsin-EDTA (Hyclone), and centrifuged at 11,000 rpm for 5 minutes. Total RNA was extracted by RNA minikit (Nucleospin; Duran, Germany). 300 ng of total RNA sample was used for each reverse transcription with the cDNA kit (Toyobo; Osaka, Japan). Primers of PCR were obtained from Macrogen (Seoul, Korea), and listed in Table 1. GAPDH was used as an internal control gene to normalize gene expression in each sample. cDNA was amplified by a thermal cycler PCR machine (Bio-Rad).

3.7. Statistics analysis

Results from different experiments were analyzed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). All data were expressed as means ± standard deviation (SD). Statistical analysis of the data was determined by one-way ANOVA, followed by Turkey tests. P-value (P) < 0.05 denoted the presence of statistically significant result, whereas P < 0.01 was highly significant difference.

4. Results and Discussion

4.1 Derivation and Characterization of hDPSC from Human Dental Pulp Tissues

The researchers isolated hDPSCs from human dental pulp tissues of the third molar by the serial of an enzymatic technique (Figure 1 A). hDPSCs adhered onto a culture dish after 24-hour isolation, and cells migration was found after 3 days. Interestingly, hDPSCs showed a fibroblast-like structure, and reached to 100% confluent at day 14 of the isolation. hDPSCs were then harvested by 0.25% trypsin and expanded to become passage 1 (Figure 1 B). The expression of several markers cell surface protein that is a typical marker of DPSCs and MSCs indicative of the differentiation state was analyzed using immunofluorescence and flow cytometry. Immunostaining results showed that hDPSCs strongly expressed CD73, CD90 and CD 105 (Figure 1 C). Identification of MSC characteristics by flow cytometry emphasized the homogeneity of hDPSCs, regarding MSC cell surface markers. The major population of hDPSCs were positively marked by CD73, CD90 and CD105, but not hematopoietic stem cells markers, CD34 and CD45 (Figure 1 D). Finally, confirming their mesenchymal characteristics, we found that hDPSCs had an osteogenic, chondrogenic and adipogenic potential. The multipotent differentiation of hDPSCs was then examined by inducing mesodermal differentiation into osteogenic, chondrogenic, and adipogenic lineages. After 21 days of the inductions, hDPSCs possessed the typical mesenchymal stem cell differentiation propensity, and became osteocytes, chondrocytes, and adipocytes. The resulting adipocytes exhibited lipid droplet-containing cells as stained by Oil Red O, while chondrocytes and osteocytes were detected by Alcian Blue, and Alizarin Red, respectively (Figure. 1 E).

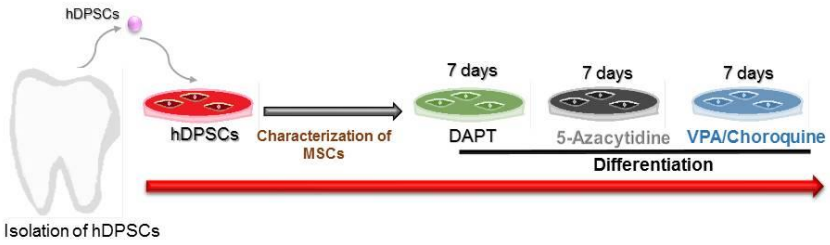

Figure 1 Isolation and characterization of human dental pulp stem cells. (A) The extraction of dental pulp tissue from human third molar. (B) The bright field images of cellular morphology of primary culture expanded from dental pulp tissues. The fibroblast-like cells of hDPSCs emigrated from the digested dental pulp tissues, and adhered to the culture dish at day 3, 5, 14 of the isolation, and hDPSCs at passage 1. (C) Immunocytochemistry of hDPSCs showed the expression of MSC surface antigens, including CD73, CD90 and CD105. (D) Major population of DPSCs was positive for CD73, CD90, and CD105, and negative for CD34 and CD45, as analyzed by flow cytometry. (E) Multipotent differentiation of hDPSCs for osteocytes, chondrocytes, and adipocytes was demonstrated by lipid droplets with Oil-Red O, mineral nodules with Alizarin red, and proteoglycan matrix of Alcian blue

4.2 Inhibition of Notch Signaling Induced Neural Differentiation and Upregulated Autophagy Genes in hDPSCs

The cause of Notch Signaling has been investigated involving in neural differentiation (Cardozo, Gómez, & Argibay, 2011; Coste, Neirinckx, Gothot, Wislet, & Rogister, 2015). The researchers aim to explore the effect of inhibition Notch signaling whether influences neural differentiation of hDPSCs. DAPT, a γ-secretase inhibitor, was used for interfering Notch signaling, and induce neural differentiation of hDPSCs. Various concentrations of DAPT (0, 5, 10 and 20 μM) were supplied into the differentiation culture for 7 days prior to assessing the expression of neural genes. At day 7, the structure of hDPSCs in 5, 10, 20 μM DAPT presented a spindle-shaped morphology (Figure 2 A). Neuronal genes, including *Nestin*, *βIII-tubulin*, and *SOX2*, were significantly upregulated when 10 and 20 μM DAPT was applied (Figure 2 B, C). Interestingly, autophagy genes, *LC3I/II* and *Beclin*, were also significantly upregulated when Notch signaling was blocked by 10 and 20 μM DAPT, compared to the undifferentiated control cells (Figure 2 D,

E), suggesting autophagy play a role in differentiation hDPSCs and thus are up-regulated during differentiation. These data exhibited that DAPT promoted neural differentiation and enhanced the activity of autophagy of hDPSCs.

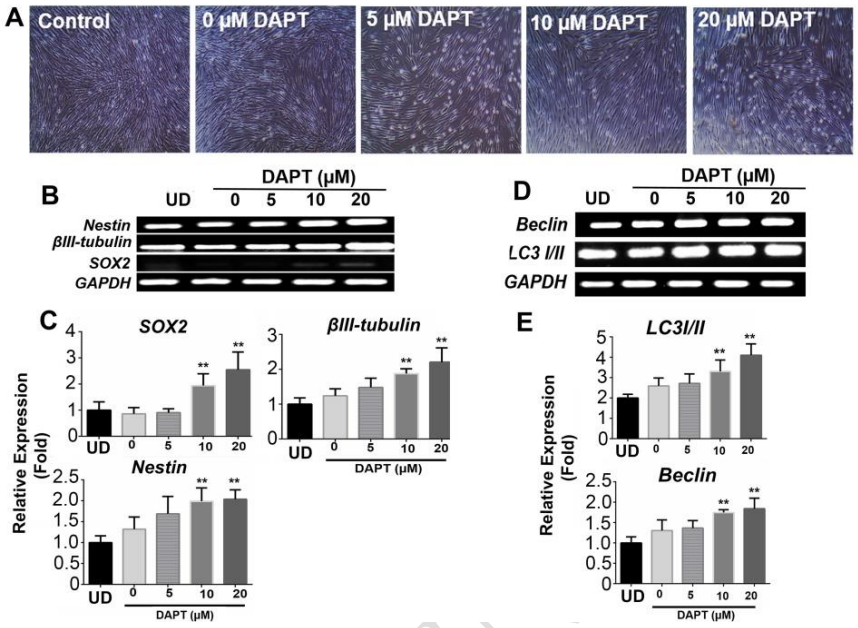

Figure 2 Inhibition of Notch signaling promoted neural differentiation of hDPSCs. (A) hDPSCs were cultured under various concentrations of DPAT (0, 5, 10, 20 μM) for 7 days, and observed their morphology under the light microscope. (B) The expression of neural genes, Nestin, βII-tubulin, and SOX2, was determined by RT-PCR. (C) The expression levels were normalized to GAPDH, and calculated the relative expression fold over the undifferentiated control cells. (D) Autophagy genes, LC3I/II and Beclin, were upregulated when hDPSCs were induced to differentiate by DAPT. (E) Quantification of autophagy genes were determined by using GAPDH as a reference gene. Values were expressed as mean ± SD (n = 3), and **p < 0.01 versus the undifferentiated control cells

4.3 5-Azacytidine Enhanced Neural Differentiation Efficiency and Activated Autophagy of hDPSCs

More recent studies examined how autophagy could enhance neural differentiation, and believed that autophagy is involved in stem cell differentiation efficiency. In addition to DNA demethylating agent, a number of studies have found 5-Azacytidine induce autophagy in various contexts (Cluzeau et al., 2011; Hsieh et al., 2016). The ability of 5-Azacytidine in enhancing neural differentiation was evaluated. hDPSCs were differentiated into neural-like cells in the differentiation media with either 10 μM DAPT or 10 μM 5-Azacytidine alone or their combination. After 7 days of the differentiation, the morphology of hDPSCs became neural phenotypes in all differentiation conditions, compared with the undifferentiated control cells (Figure 3 A). The combined treatment of DAPT and 5-Azacytidine promoted the upmost expression of neural genes, *Nestin*, and *βIII-tubulin*, and *GAD1*, a marker of glutaminergic neurons, was highly expressed when hDPSCs were simultaneously treated with DAPT and 5-Azacytidine (Figure 3 B, C).

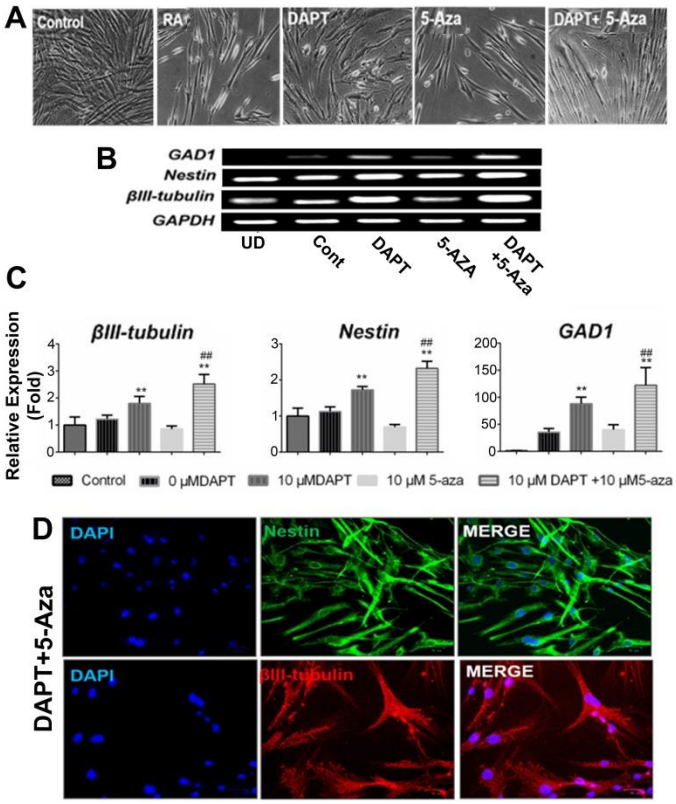

Figure 3 5-Azacytidine enhanced neural differentiation of hDPSCs. (A) Morphology of differentiated hDPSCs in various conditions at day 7. (B) The expression of neural genes, GAD1, Nestin, and βIII-tubulin, was assessed by RT-PCR. (C) The relative expression levels of neural genes were quantified by normalization with GAPDH. Values are expressed as mean ± SD (n = 3). **p < 0.01 versus the undifferentiated control cells, and ## p < 0.01 versus DAPT-treated cells. (F) The immunofluorescent images of Nestin and βII-tubulin of differentiated DPSCs with 10 μM DAPT and 10 μM 5-Azacytidine at day 7

The immunofluorescence confirmed that the combined treatment of DAPT and 5-Azacytidine induced neural markers, Nestin and βIII-tubulin, in differentiated hDPSCs (Figure 3 D). This result indicated that 5- Azacytidine could enhance the efficiency of neural differentiation of hDPSCs. Furthermore, the influence of 5- Azacytidine in autophagy was investigated during neural differentiation of hDPSCs. The expression of *LC3I/II* and *Beclin* was determined after 7 days of the differentiation in various conditions; either 10 μM DAPT or 10 μM 5-Azacytidine alone or the combined of both molecules. The combined treatment of DAPT and 5- Azacytidine significantly augmented the expression of *LC3I/II* and *Beclin* genes, compared to the undifferentiated control and DAPT treatment alone (Figure 4 A, B). In the line of LC3I/II and Beclin were also found to co-localize with βIII-tubulin in hDPSC-derived neural cells, validating the activation of autophagy during neural differentiation of hDPSCs (Figure 4 C). The evidence from this study suggests that supplying 5-Azacytidine in inductive medium could promote autophagy activity resulting in enhancing neuronal differentiation.

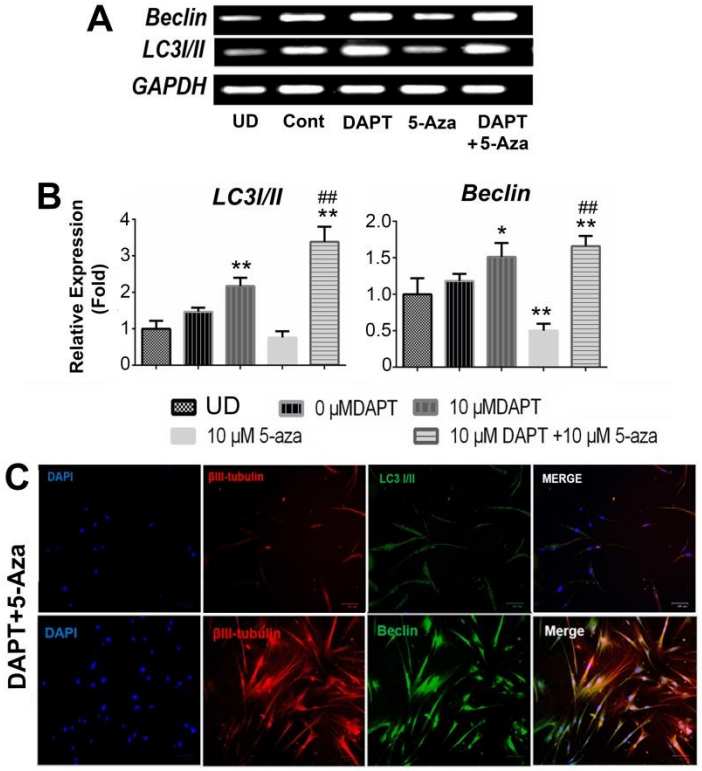

Figure 4 Autophagy was activated during neural differentiation of hDPSCs. (A) The expression of LC3I/II and Beclin was determined by RT-PCR. (B) The relative expression levels were measured by normalization with GAPDH. Values were expressed as mean ± SD (n = 3). *p < 0.05 and **p < 0.01 versus the undifferentiated control cells, and ## p < 0.01 versus DAPT-treated cells. The Immunofluorescent images of βIII-tubulin (red) and LC3I/II (green) were co-localized and merged with DAPI (blue), as well as βIII-tubulin (red) and LC3I/II (green)

4.4 Modulation of Autophagy Influenced the Efficacy of Neural Differentiation of hDPSCs

The researchers next confirmed the influence of autophagy during neural differentiation of hDPSCs. To address this, we applied the small molecules are known to modulate autophagy; chloroquine (CQ) or Valproic acid (VPA) were used to either block or enhance autophagy within cells. The changes of cellular morphology were observed at day 1, 5, and 7 of the differentiation. Compared to the differentiation control, VPA further induced spindle-like cells, while CQ caused prominently cell death (Figure 5 A). The expression of *βIII-tubulin* and *LC3I/II* was assessed at day 1, 5, and 7 of the differentiation by RT-PCR (Figure 5 B). Compared to the differentiation control, VPA significantly surged *LC3I/II* expression at day 7, while CQ did not have a notable change (Figure 5 C). *βIII-tubulin* was also significantly increased in VPA-induced hDPSCs, and it's level was clearly suppressed in CQ-supplemented condition (Figure 5 C). Consistently, immunofluorescence result showed that the treatment of chloroquine led to the reduction of βIII-tubulin-positive cells than the VPA-treated hDPSCs (Figure 5 D). These results proposed that neural differentiation of hDPSCs was modulated by autophagy within cells; the more autophagy activity, the higher neural differentiation efficiency.

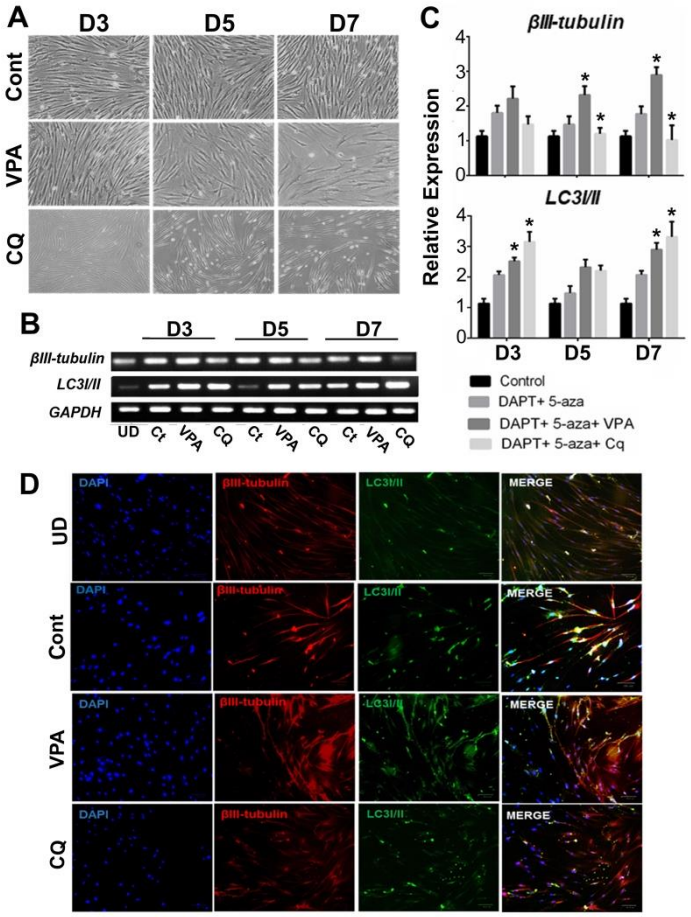

Figure 5 The modulation of autophagy altered neural differentiation of hDPSCs. (A) Morphological changes of DPSCs during neural differentiation, when either autophagy activator (Valproic acid; VPA) or antophagy inhibitor (chloroquine; CQ) was supplemented. (B) The expression of βIII-tubulin and LC3I/II were determined by RT-PCR. (C) VPA significantly enhanced the expression of LC3I/II and βIII-tubulin, compared with the differentiated control cells. Data were shown as mean ± SD, and *$p < 0.05$ versus the differentiated control cells. (E) The immunofluorescent images showed colocalization of βIII-tubulin and LC3I/II when VPA was applied to the cells

In this study, we demonstrated that hDPSCs isolated from the third tooth molar exhibit fibroblastic in appearance in primary culture and maintain hDPSCs stemness by expression of MSCs markers (CD73, 90, 105) but not HSCs (CD34, 45). However, if the isolated DPSCs were MSC-like, it has been suggested MSCs have immunoprivileged properties which would not cause an inflammatory response (Rastegar et al., 2010).Our first goal was to determine whether isolated hDPSCs maintained their multiple lineage differentiation ability. We found the capability of differentiating into adipogenic, osteogenic, and chondrocytic cell types just as normal hDPSCs have been reported to do in previous research.(Karaöz et al., 2011; Laino et al., 2006)

This study set out with the aim of assessing the differentiation efficiency, we were able to induce hDPSCs toward neurons by inhibiting Notch signaling and DNA methylation. Notch signaling plays a critical role in development and cell fate specification. Notch receptors and ligands have been found to be expressed in dental epithelium or mesenchyme in the developing tooth. Prior studies have suggested the inhibition of Notch signaling is associated with neuronal differentiation (Cardozo et al., 2011; Noisa et al., 2014). Notch signaling can be suppressed by a γ-secretase inhibitor, DAPT (Cai, Lin, Hu, & Zheng, 2008). Thus, we varied the DAPT concentrations in differentiation medium cocktail included N2 medium supplemented with RA (retinoic acid) which have ability to induce post-mitotic, neural phenotypes in

various types of stem cells (Janesick, Wu, & Blumberg, 2015; Kim, Seo, Bubien, & Oh, 2002). Upon induction, the researchers could detect increasing expression of *βIII-tubulin Nestin,* and *SOX2* in condition treated with DAPT during the neural differentiation protocol. Only the conditions treated 10 and 20 µM was found to have a clear expression of *SOX2*, so the DAPT concentration at 10 µM is more than enough for inhibition of Notch signaling and for the further experiment. The researchers observed the increase of autophagy genes expression (*LC3I/II, Beclin*) upon treatment of DAPT in dose-dependent manner. Hence, it could conceivably be hypothesized that autophagy play roles in neuronal differentiation once Notch singling is inhibited.

The researcher further examined the potential of DAPT and 5-Azacytidine whether has a capability in enhancing neuronal differentiation. Surprisingly, the result was shown to have a high expression of neural markers in condition that co-treatment DAPT and 5-Azacytidine. The high expression of both *Nestin* and *βIII-tubulin* in hDPSCs cultured in DAPT inductive media combined with 5-Azacytidine indicated that hDPSCs can differentiate into neural-like cells after DAPT treatment and a high expression after added 5-Azacytidine. hDPSCs are able to differentiate into neural precursors, but very little was found in the prior studies hDPSCs differentiate into mature neurons (Chai et al., 2000). Intriguingly, the researchers further found *GAD1*, a marker of glutaminergic neurons, was highly expressed when hDPSCs were simultaneously treated with DAPT and 5-Azacytidine. The neural markers we characterized both *Nestin* and *βIII-tubulin.* Nestin is known to be expressed within fibrous dental pulp tissue and the expression of *Nestin* was increased when cells differentiated into neurons (Arthur, Rychkov, Shi, Koblar, & Gronthos, 2008). *βIII-tubulin* is expressed after neuronal differentiation and utilized as a marker of mature neuron cells during the final stages of growth (Johansson et al., 1999; Karaöz et al., 2010). The researchers results were similar to the previous study represented that when Notch signaling was inhibited by DAPT, the differentiation of human induced pluripotent stem cells was facilitated into neural stem cell fate (Chen et al., 2014).

In addition, epigenetic modifier 5-Azacytidine has been used to enhance neural differentiation potential in this study. In 2013, Zemelko and colleagues reported that 5-Azacytidine has potential in additional differences in neurogenic potential of the MSCs cells (Zemelko et al., 2013). Another study presented that adipose-derived mesenchymal stem cells (ASCs) maintained in the medium with RA combination with 5-Azacytidine for 7 days were increased the mRNA and protein levels of *Nestin* and *βIII-tubulin* (Pavlova et al., 2012). Similar to this study, the combination 5-Azacytidine with DAPT medium promoted neural-like cell production from hDPSC-MSCs at a greater than DAPT alone. Thus, it could be suggested that only two small molecules, 5-Azacytidine cytidine and DAPT, were potentially direct hDPSC reprograming toward neuronal cells. In addition to the inhibition of Notch signaling, autophagy genes expression (*LC3I/II, Beclin*) were enhanced upon the treatment of DAPT in a dose-dependent manner. Most recent data showed the bone marrow mononucleated cells exposed to 5-Azacytidine was increased the key proteins associated with autophagy pathway (Romano et al., 2017). Hence, it could conceivably hypothesize that autophagy played roles in neuronal differentiation once Notch singling was inhibited. Corresponding to the previous observation, the expression of LC3I/II an autophagy marker was peaked after the induction, and demonstrated the same trend as neural differentiation (Lu, Yuan, Sun, & Ou, 2013). Moreover, autophagy activity was upregulated during the neuronal differentiation of N2a cells (Zeng & Zhou, 2008). In previous studies, autophagy was shown to play an important role in support and protection cells during the differentiation (Kabeya et al., 2000; Mizushima, Yamamoto, Matsui, Yoshimori, & Ohsumi, 2004). The aforementioned evidence implied that autophagy activity may be one of the most important supportive conditions in neural cells differentiation, and, in response to neural differentiation signal, autophagy was activated (Figure 6).

It was therefore convincing that such connections exist between autophagy with neural differentiation. The researchers thus validated the function autophagy by further confirmed using chemical approach either autophagy activator or inhibitor was tested. Valproic acid (VPA), a histone deacetylase inhibitors (HDACs), has been widely used for activating autophagy activity, which could then induce cell differentiation (Xia et al., 2016). Whereas, chloroquine is an anti-malarial drug, and it is general accepted as an autophagy inhibitor that led to a massive accumulation of ubiquitinated proteins LC3I/II (Torgersen, Engedal, Bøe, Hokland, & Simonsen, 2013). Besides autophagy activation, the treatment of VPA promoted neural markers, contrasting with the addition of chloroquine and the control. These data proposed that neural differentiation of hDPSCs was in conjunction with the activation of autophagy (Jeong, Ohn, Kim, & Cho, 2013; Vukicevic et al., 2015). There were more complex signaling factors involving in the regulation

of neuronal differentiation, and the mechanistic relationship between Notch signaling and autophagy required further exploration.

5. Conclusion

The combination of DAPT and 5-Azacytidine efficiently induced neural differentiation of hDPSCs, and autophagy was significantly activated upon stem cell differentiation. The levels of autophagy activity positively correlated with the efficiency of neural differentiation, implicating that autophagy might play an instructive role in regulating stem cell differentiation (Figure 6).

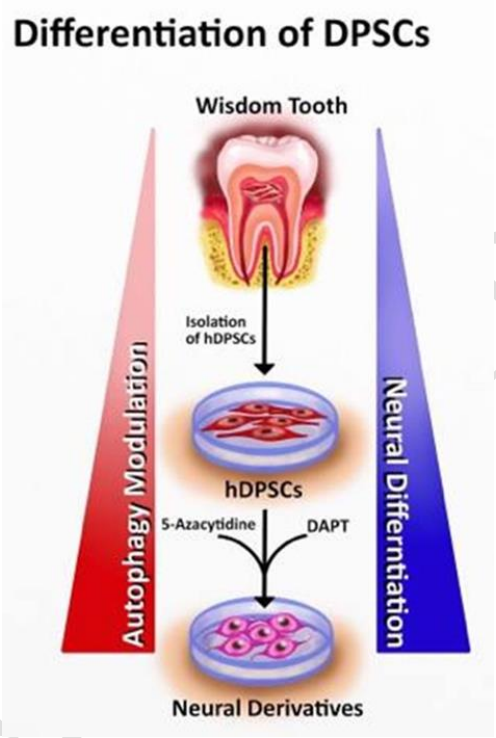

Figure 6 Graphical image depicted the involvement of autophagy during neural differentiation of hDPSCs. Autophagy was induced during neural differentiation of hDPSCs; therefore, the optimized autophagy activity should refine the efficiency of neural differentiation from hDPSCs

6. Acknowledgements

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