EFFECT OF AUTOPHAGY MODULATION DURING NEURAL DIFFERENTIATION OF HUMAN DENTAL PULP STEM CELLS INDUCED BY DAPT AND 5-AZACYTIDINE

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ผลกระทบของการเปลี่ยนแปลงออโตฟาจีระหว่างการเหนี่ยวนำเซลล์ต้นกำเนิดจากเนื้อเยื่อโพรงประสาทฟันมนุษย์ไปเป็นเซลล์ประสาทด้วยสาร DAPT และ 5-AZACYTIDINE

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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พัทธมน เมืองจันทร์ : ผลกระทบของการเปลี่ยนแปลงออโตฟาจีระหว่างการเหนี่ยวนำเซลล์ต้นกำเนิดจากเนื้อเยื่อโพรงประสาทฟันมนุษย์ไปเป็นเซลล์ประสาทด้วยสาร DAPT และ 5-AZACYTIDINE (EFFECTS OF AUTOPHAGY MODULATION DURING NEURAL DIFFERENTIATION OF HUMAN DENTAL PULP STEM CELLS INDUCED BY DAPT AND 5-AZACYTIDINE) อาจารย์ที่ปรึกษาวิทยานิพนธ์ : ผู้ช่วยศาสตราจารย์ ดร.ปริญญา น้อยสา, 96 หน้า

อันเนื่องมาจากข้อจำกัดของการนำเซลล์ต้นกำเนิดตัวอ่อนมนุษย์ (EMBRYONIC STEM CELLS, ESCS) มาใช้ในการรักษาส่งผลให้เซลล์ต้นกำเนิดมีเซนไคม์ (MESENCHYMAL STEM CELLS, MSCS) เป็นทางเลือกหนึ่งที่มีศักยภาพและเป็นความหวังสำหรับแพทย์ทางเลือกโดยเฉพาะโรคที่เกี่ยวข้องกับการเสื่อมในระบบประสาท เซลล์ต้นกำเนิดมีเซนไคม์โดยเฉพาะจากเนื้อเยื่อโพรงประสาทฟัน (DENTAL PULP-DERIVED STEM CELLS, DPSCS) เป็นเซลล์ต้นกำเนิดที่มีศักยภาพในการรักษาด้วยเซลล์บำบัด ด้วยเหตุผลในเรื่องขั้นตอนการเก็บที่ไม่ยุ่งยากและมีการบาดเจ็บไม่มาก อีกทั้งประเด็นปัญหาทางด้านจริยธรรมที่น้อย นอกจากนี้แล้วเซลล์ต้นกำเนิดมีเซนไคม์จากเนื้อเยื่อโพรงประสาทฟันยังสามารถเปลี่ยนแปลงไปเป็นเซลล์ได้หลากหลายประเภท โดยเฉพาะอย่างยิ่งเซลล์ในระบบประสาทเมื่ออยู่ภายใต้สภาวะการเหนี่ยวนำที่เหมาะสม

วัตถุประสงค์ของการศึกษานี้คือเพื่อวิจัยหาผลของสารยับยั้งการส่งสัญญาณ Notch ด้วย GSI-IX, LY-374973, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) ร่วมกับการเติมสารยับยั้งการเติมหมู่เมททิล (methyl) ลงบนสาย DNA (5-Azacytidine, 5-aza) ต่อการเปลี่ยนแปลงเซลล์ต้นกำเนิดจากเนื้อเยื่อโพรงประสาทฟันของมนุษย์ (human DENTAL PULP-DERIVED STEM CELLS, hDPSCs)ไปเป็นเซลล์ประสาทและตรวจสอบบทบาทของกระบวนการออโตฟาจี (autophagy) ในระหว่างการเหนี่ยวนำ จากผลการทดลองพบว่าหลังจากทำการคัดแยกเซลล์ต้นกำเนิดจากเนื้อเยื่อโพรงประสาทฟันของมนุษย์ เซลล์เหล่านี้มีการแสดงออกต่อโปรตีน CD-73 CD90 และ CD105 นอกจากนั้นเซลล์ต้นกำเนิดจากเนื้อเยื่อโพรงประสาทฟันมนุษย์ยังสามารถเปลี่ยนไปเป็นเซลล์กระดูก เซลล์กระดูกอ่อน และเซลล์ไขมัน

ผลการตรวจสอบการแสดงออกของยีนในเซลล์ต้นกำเนิดจากเนื้อเยื่อโพรงประสาทฟันของมนุษย์ที่เหนี่ยวนำด้วยสาร DAPT ที่ความเข้มข้น 0, 5, 10 และ 20 ไมโครโมลาร์ เพื่อหาความเข้มข้นที่เหมาะสมในการเหนี่ยวนำให้เปลี่ยนแปลงไปเป็นเซลล์ประสาท ผลการศึกษาพบว่าภายหลัง 7 วันหลังทำการเหนี่ยวนำ กลุ่มที่เติม DAPT ที่ความเข้มข้น 10 และ 20 ไมโครโมลาร์ มีระดับของยีนที่จำเพาะต่อเซลล์ที่จะพัฒนาไปเป็นเซลล์ประสาท Nestin, Sox2 และ βIII-tubulin สูงกว่ากลุ่มควบคุม

อย่างมีนัยสำคัญทางสถิติ ($p<0.01$) นอกจากนี้ยังมีการแสดงออกของ LC3I/II and Beclin-1 ซึ่งเป็นยีนที่เกี่ยวข้องกับกระบวนการออโตฟาจีสูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ ($p<0.01$)

หลังจากนั้นจึงทำการตรวจสอบการแสดงออกของยีนในเซลล์ต้นกำเนิดจากเนื้อเยื่อโพรงประสาทฟันของมนุษย์ที่เหนี่ยวนำด้วยสาร DAPT ร่วมกับการเติมสารยับยั้งการเติมหมู่ methyl ลงบนสาย DNA คือ 5-Azacytidine (5-aza) พบว่าภายหลังจากการเติม 5-aza ยีนที่จำเพาะต่อเซลล์ประสาท เช่น βIII-tubulin, Nestin และGAD1 มีระดับการแสดงออกที่สูงขึ้นกว่ากลุ่มที่ไม่ได้เติม 5-aza อย่างมีนัยสำคัญทางสถิติ ($p<0.01$) เช่นเดียวกับการแสดงออกที่สูงขึ้นของยีนในกระบวนการออโตฟาจี คือ LC3I/II และ Beclin-1 นอกจากนี้ผลการย้อมโปรตีนที่จำเพาะต่อเซลล์ประสาทคือโปรตีน βIII-tubulin รวมทั้ง LC3I/II, Beclin-1 ที่เป็นโปรตีนจำเพาะต่อการเกิดกระบวนการออโตฟาจี พบว่าเซลล์ประสาทที่ได้มาจากกลุ่มที่มีการเติมทั้ง 10 ไมโครโมลาร์ DAPT และ 5-aza มีการแสดงออกของโปรตีน βIII-tubulin ร่วมกับ LC3I/II และ βIII-tubulin และ Beclin-1 ร่วมกัน ซึ่งแสดงให้เห็นถึงความเป็นเซลล์ประสาทที่มีการกระตุ้นกระบวนการออโตฟาจีเกิดขึ้น ด้วยเหตุนี้จึงทำการยืนยันถึงความสำคัญของกระบวนการ ออโตฟาจีต่อการเปลี่ยนแปลงเซลล์ต้นกำเนิดจากโพรงประสาทฟันมนุษย์ไปเป็นเซลล์ประสาทโดยการเติม Valproic acid (VPA) ซึ่งเป็นตัวกระตุ้นกระบวนการออโตฟาจีและ Chloroquine (Cq) ซึ่งทำหน้าที่ยับยั้งกระบวนการออโตฟาจีลงในน้ำยาเหนี่ยวนำที่มี DAPT และ 5-aza โดยผลการทดลอง พบว่าในกลุ่มที่มีการเติม VPA มีการแสดงออกของยีนและโปรตีนของ βIII-tubulin และ LC3I/II สูงขึ้นเมื่อเปรียบเทียบกับกลุ่มควบคุมที่ไม่มีการเติมและกลุ่มที่มีการเติม Cq

จากผลการทดลองสามารถสรุปได้ว่าเซลล์ต้นกำเนิดจากเนื้อเยื่อโพรงประสาทฟันมนุษย์ที่ถูกยับยั้งการส่งสัญญาณ Notch และลดการเติมหมู่ methyl บนสาย DNA สามารถเปลี่ยนไปเป็นเซลล์คล้ายเซลล์ประสาทได้อย่างมีประสิทธิภาพ และเกิดการกระตุ้นกระบวนการออโตฟาจีในระหว่างกระบวนการเหนี่ยวนำ ซึ่งแสดงให้เห็นถึงความสำคัญของกระบวนการออโตฟาจีต่อการเปลี่ยนสภาพของเซลล์ต้นกำเนิดจากเนื้อเยื่อโพรงประสาทฟันมนุษย์ไปเป็นเซลล์ประสาท จากการศึกษานี้แสดงให้เห็นถึงแนวทาง และโอกาสในการรักษาผู้ป่วยอันเนื่องมาจากความเสื่อมของเซลล์ประสาทต่อไปได้

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2559 ลายมือชื่ออาจารย์ที่ปรึกษา _____

PATTAMON MUANGCHAN : EFFECT OF AUTOPHAGY MODULATION DURING NEURAL DIFFERENTIATION OF HUMAN DENTAL PULP STEM CELLS INDUCED BY DAPT AND 5-AZACYTIDINE. THESIS ADVISOR : ASST. PROF. PARINYA NOISA, PH. D., 96 PP.

HUMAN DENTAL PULP TISSUE-DERIVED STEM CELLS/AUTOPHAGY/ NEURAL DIFFERENTIATION/NOTCH SIGNALING PATHWAY/ DNA DEMETHYLATING AGENT

The limitations in utilizing human embryonic stem cells (ESCs) resulted in promoting the promise of mesenchymal stem cells (MSCs) in regenerative medicine. MSCs, especially from dental pulp tissues and called dental pulp-derived stem cells (DPSCs), serve as a potential source for cell therapy because of their high accessibility, minimal invasive collection, and the least ethical concern.

The primary aim of this study was to explore the effects of the Notch signaling inhibitor GSI-IX, LY-374973, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) and the DNA demethylating agent (5-Azacytidine, 5-aza) on neural differentiation of human dental pulp stem cells (hDPSCs) and investigate the role of autophagy during neural differentiation. It was found that after the isolation of hDPSCs, the cells could be expanded, and exhibited typical MSC property. The isolated hDPSCs were positive for CD73, CD90 and CD105. Additionally, hDPSCs were multipotent, as they were able to differentiate toward osteocytes, chondrocytes, and adipocytes.

To optimize the DAPT concentration, hDPSCs were treated under different DAPT concentrations (0, 5, 10 and 20 µM). The results showed that after 7 days of the induction, the differentiated hDPSCs with 10 and 20 µM DAPT significantly upregulated neural-specific genes *Nestin*, *Sox2* and *βIII-tubulin* ($p<0.01$). Moreover, autophagy-related genes (*LC3I/II* and *Beclin-1*) were highly upregulated during cell differentiation consistent with the neural gene expression. Gene expression analysis revealed that the addition of DAPT, together with 5-azacytidine, contributed to the highest expression level of neural-specific genes, including *βIII-tubulin*, *Nestin* and *GAD1*. Interestingly, this combined treatment of DAPT and 5-aza significantly promoted the expression of *LC3I/II* and *Beclin-1* ($p<0.01$). The significance of autophagy during neural differentiation was further confirmed by supplementing either Valproic acid (VPA; autophagy activator) or chloroquine (Cq; autophagy inhibitor). The results demonstrated that after the treatment of VPA, βIII-tubulin and LC3I/II genes and proteins were significantly upregulated, while the treatment of Cq led to the downregulation of βIII-tubulin.

Altogether, this study concluded that the inhibition of Notch signaling and DNA methylation could promote the differentiation of hDPSCs toward neural cells, and autophagy was modulated during this process. Hence, this study paved the way for an effective way to differentiate hDPSCs, and would be beneficial for clinical applications in the future.

School of Biotechnology Student's Signature Pattamon

Academic Year 2016 Advisor's Signature

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LIST OF ABBREVIATIONS

5-aza	=	5-Azacytidine
CD	=	cluster of differentiation
Cq	=	choroquine
˚C	=	degree Celsius
DAPI	=	4’, 6-diamidino-2-phenylindole
DAPT	=	N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DMEM (H/G)	=	dulbecco's Modified Eagle Medium high glucose
DMEM F12	=	dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
FBS	=	fetal bovine serum
GAPDH	=	glyceraldehyde 3-phosphate dehydrogenase
GAD1	=	glutamate decarboxylase 1
hDPSCs	=	human dental pulp stem cells
l	=	liter
LC3	=	microtubule-associated protein 1 light chain 3 alpha
MSCs	=	mesenchymal stem cells
µg	=	microgram
µl	=	microliter
µm	=	micrometer (micron)
µM	=	micromolar

LIST OF ABBREVIATIONS (Continued)

ml	=	milliliter
mm	=	millimeter
PBS	=	phosphate-buffered saline
PFA	=	paraformaldehyde
RA	=	retinoic acid
RT-PCR	=	reverse transcription polymerase chain reaction
SOX2	=	sex determining region Y-box 2
VPA	=	valproic acid

CHAPTER I

INTRODUCTION

1.1 Background

The discovery of stem cells has been developed for novel therapeutic strategies that purpose to rescue injured and diseased tissues (Vishnubhatla, Corteling, Stevanato, Hicks, and Sinden, 2014). Among many types of stem cells, human dental pulp stem cells (hDPSCs) are an interesting source of stem cells; they are neural crest-derived stem cells that harvested from human third molar. Human DPSCs are mesenchymal stem cells (MSCs); they represent one of the most convenient source of multipotent stem cells and are easily obtained, minimal pain, and no teratoma formation compared to other stem cell types (Karaöz et al., 2011; Lizier et al., 2012; Ranganathan and Lakshminarayanan, 2012; Zhang, Walboomers, Shi, Fan, and Jansen, 2006). Moreover, hDPSCs have a great potential to differentiate into various cells types, especially neural-like cells. A number of studies have been successfully shown the differentiation of hDPSCs into neural lineage under specific conditions (Chang, Chang, Tsai, Chang, and Lin, 2014; Gronthos, Mankani, Brahim, Robey, and Shi, 2000; Hei et al., 2016; Zainal Ariffin et al., 2013). Therefore, neural differentiation of hDPSCs may be a promising treatment option for neurological disorders, such as neurodegenerative diseases or peripheral nerve injury in the future. Although there are many strategies to convert hDPSCs to ward neural-like cells, the

connection of Notch signaling and autophagy in hDPSC differentiation has not been studied.

Notch signaling has a critical role for maintaining stemness and directing stem cell to differentiate into several cell types of (Boni et al., 2008; Jönsson, Xiang, Pettersson, Lardelli, and Nilsson, 2001; Ramasamy and Lenka, 2010). Notch signaling is activate when Notch receptor binds to its ligand, resulting in the cleavage of Notch intracellular domain (NICD) of the Notch receptor, which then translocate into the nucleus and initiates the transcription of Notch target genes (Engin and Lee, 2010; Løvschall, Tummers, Thesleff, Füchtbauer, and Poulsen, 2005). For the inhibition of Notch signaling pathway, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), a γ-secretase inhibitor, was shown to associate with neural differentiation in neural stem/progenitor cells (Kageyama, Ohtsuka, Shimojo, and Imayoshi, 2008; Kanungo, Zheng, Amin, and Pant, 2008; J. Wang et al., 2016) and in MSCs (Y. Wang et al., 2009). In 2007, Crawford and colleagues were success to establish neuronal-like cells by inhibiting Notch signaling and activating Sonic Hedgehog signaling pathway (Crawford and Roelink, 2007).

There is a strong correlation between epigenetic modifiers and genes expression changes during cell differentiation (Sheaffer et al., 2014). DNA methylation is an essential epigenetic modification for development and is a key for the establishment and maintenance of cellular entity (Suelves, Carrió, Núñez-Álvarez, and Peinado, 2016). DNA demethylating agents is a compound that can inhibit DNA methylation at the promoter region. The lack of methylation at gene promoters is possibly a prerequisite for the activation of transcription. 5-azacytidine (5-aza) is a DNA demethylating chemical compound that most commonly used as an epigenetic modifier. 5-aza can integrate into DNA strand and then inhibit DNA methyltransferase

(DNMT) (Holliday, 1990; Schneider-Stock et al., 2005). Previous studies proved that 5-aza can induce the differentiation of cardiomyocytes (Antonitsis, Ioannidou-Papagiannaki, Kaidoglou, and Papakonstantinou, 2007; Deng, Li, and Zhang, 2009). Thus, the combination of 5-aza with DAPT might be particularly relevant for inducing neural-like cells differentiation of hDPSCs. Commonly, there are number of cellular mechanisms that are altered during stem cell differentiation, in particular autophagy. Autophagy is a conserved lysosome degradation pathway and highly active during cell differentiation and embryo development (Mizushima, 2007). Autophagy also plays a critical role in variety of cell differentiation processes (Vessoni, Muotri, and Okamoto, 2011). In this study, the connection of autophagy activity during differentiation was assessed and autophagy activator Valproic acid (VPA) and autophagy inhibitor chloroquine (Cq) were used to address this phenomenon. VPA, a histone deacetylase inhibitors (HDACs), has been widely used to activate autophagy activity within cells (Xia et al., 2016), whereas chloroquine (Cq) is a autophagy inhibitor that led to a massive accumulation of ubiquitinated proteins that correlated with the increased cell death (Torgersen, Engedal, Bøe, Hokland, and Simonsen, 2013). Here, Notch signaling inhibitor, DAPT, was combined with DNA demethylating agent 5-azacytidine, to induce hDPSC differentiation. The autophagy response in hDPSC differentiation was also investigated. The primary aim of this study was to envisage the role of autophagy during neuronal differentiation of hDPSCs.

1.2 Research objectives

1.2.1 To isolate and characterize hDPSCs from human dental pulp tissue.

1.2.2 To evaluate the effect of γ-secretase inhibitor (DAPT) on neural differentiation of hDPSCs.

1.2.2 To investigate the effect of 5-azacytidine (DNA demethylating agent) in combination with DAPT on neural differentiation.

1.2.3 To determine the effect of autophagy activity on neural differentiation of hDPSCs.

1.2.4 To characterize neural cells derived from hDPSCs *in vitro* using immunocytochemistry and RT-PCR.

1.3 Research hypotheses

1.3.1 Under the defined culture system, hDPSCs could be isolated, established, and expanded from human dental pulp tissues and named human dental pulp stem cells (hDPSCs). hDPSCs are capable of exhibiting MSC characteristics, which are self-renew, and tri-mesodermal lineage differentiation. Besides, hDPSCs are able to show typical MSCs characteristics and can be induced toward neural-like cells.

1.3.2 Notch signaling inhibitor, DAPT, could promote neural differentiation of hDPSCs.

1.3.3 Epigenetic modifier, 5-azacytidine, could enhance the expression of neural-related genes in the presence of DAPT.

1.3.4 Autophagy activity should be altered during neural differentiation of hDPSCs.

1.4 Scope of the study

1.4.1 Under the defined culture system, hDPSCs were isolated, established, and expanded from human dental tissues. hDPSCs were examined MSC characteristics by immunotyping of MSC cell surface antigens (*e.g.* CD73, CD90 and CD105). Multipotent differentiation of hDPSCs was performed by the tri-mesodermal lineage differentiation, including osteocytes, chondrocytes, and adipocytes.

1.4.2 DAPT and 5-aza were primarily used to direct neural differentiation of hDPSCs toward neural-like cells with appropriate culture conditions. The optimized treatment was confirmed by the expression of neural genes, including *SOX2, Nestin, βIII-tubulin* and *GAD1*, and also the detection of neural protein, such as βIII-tubulin and Nestin.

1.4.3 Autophagy was examined its activity during neural differentiation by assessing the expression of autophagy genes, including *LC3I/II* and *Beclin-1,* and detecting autophagy protein by immunocytochemistry.

CHAPTER II

LITERATURE REVIEW

2.1 Neurons

Cells within the nervous system are called neurons. Neuron is a major components both in the brain and spinal cord of the central nervous system (CNS), and in the autonomic ganglia of peripheral nervous system (PNS) (Kandel, Schwartz, Jessell, Siegelbaum, and Hudspeth, 2000). These cells have a specialized cell function to transmit information to other nerve cells, muscle or gland cells. Normally, neurons exist in a number of different shapes and sizes, and can be classified by their morphology and function (Maurya, 2015).

Scientists have classified neurons into four main groups based on differences in shape included unipolar neurons, bipolar neurons that usually found in sensory organs, pseudo-unipolar neurons similar to unipolar neurons they has an axon, but no true dendrites. Multipolar neurons are the dominating neurons in vertebrates in terms of number (Fig. 2.1A) (Wichterle, Gifford, and Mazzoni, 2013).

Neurons can be classified based on their specific functions. Sensory neurons are the neurons that manage information from the different sensory source such as eyes and nose. Transmission of signals from brain to spinal cord and muscles to initiate the action are motor neuron function. Interneurons serves as connectors of neurons (Fig. 2.1B) (Lodish H and Freeman;, 2000).

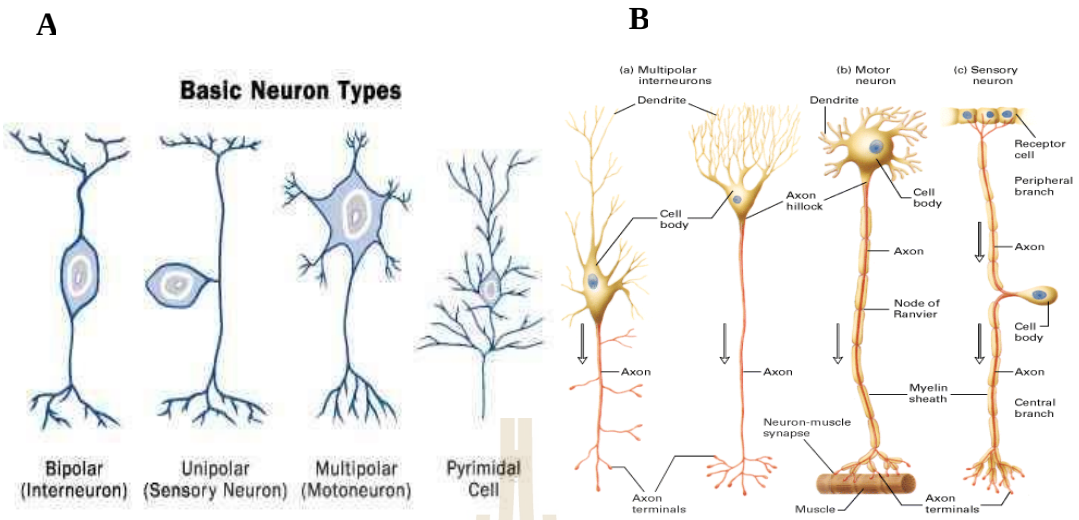

Figure 2.1 Classification of neurons according to morphology and function. (Lodish H and Freeman;, 2000; Wichterle et al., 2013)

(A) Shape of neurons

(B) Types of neurons according to function

2.2 Neurodegenerative diseases and their therapies

2.2.1 Neurodegenerative diseases

Neurodegenerative diseases are disorders that cause by the degenerating neurons in either central nervous system (CNS) or peripheral nervous system (PNS). These diseases can affect millions of people worldwide of age. The fundamental mechanism of neurodegenerative diseases is progressive loss of structure, function or decrease number of neurons, including death of neurons (Hung, Chen, Hsieh, Chiou, and Kao, 2010). To date, there are many neurodegenerative diseases including Alzheimer's disease (AD), amyotrophic lateral sclerosis, Parkinson's disease (PD), and Huntington's disease that cause by neurodegenerative processes. Degenerative diseases can seriously affect patient life-threatening (Gitler, Dhillon, and

Shorter, 2017). At present, there is no cure and method to inhibit neurodegeneration. Treatment may help in pain reliable. However, in some cases scientists believe that brain damage can be reversed by replacing lost cell with a new cell which can mature or differentiate to nerve cells, called neural stem cells (Panchision, 2006).

2.2.2 Stem cells therapy

Stem cells is un-functional cells type that have the capacity to proliferate and differentiate into multiple cell lineages. Stem-cell therapy is the use of stem cells to treat or prevent a disease or condition. Many researchers attempt to develop various stem cell sources to apply the stem-cell for treatments of neurodegenerative diseases. To understand the capacity of stem cells, there are growing in public hope that stem cell therapies may useful for treatments of neurodegenerative diseases (Lunn, Sakowski, Hur, and Feldman, 2011).

To date, stem cell therapy provide promising hope for almost of neurodegenerative diseases including Parkinson's disease (PD) (Abeliovich and Gitler, 2016), Alzheimer's disease (AD) (Canter, Penney, and Tsai, 2016), amyotrophic lateral sclerosis (ALS) (Wyss-Coray, 2016), and Huntington's disease (HD). The contribution of stem cells to cure in neurodegenerative diseases has been explored extensively over the past few years (Gitler et al., 2017). There are different classifications of stem cells such as the possible cell types that they can produce and the ways in the stem cells are derived. These cells are categorized as embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and adult stem cells. Mesenchymal stem cells (MSCs) are adult stem cells which can be isolated from human and animal sources.

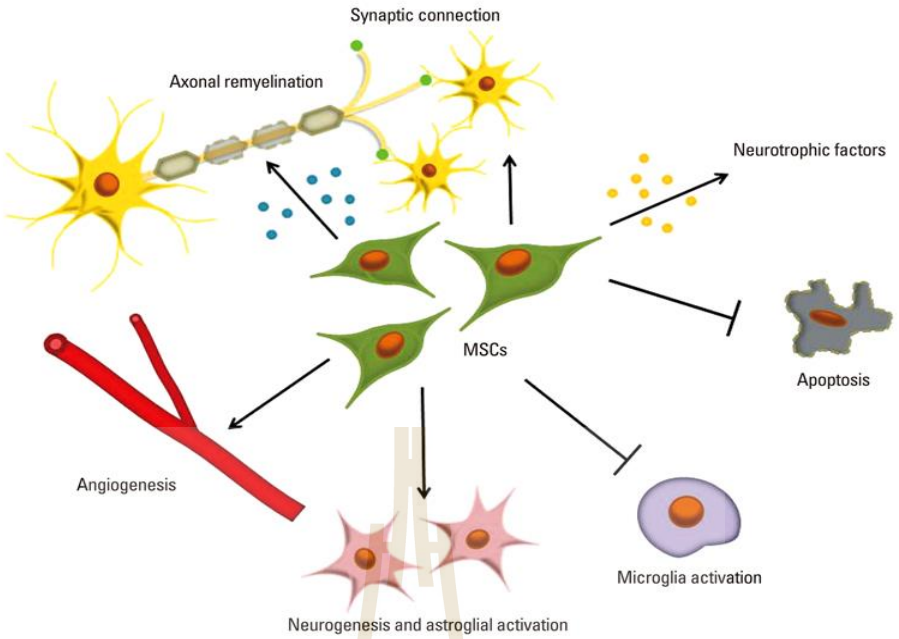

Figure 2.2 Potential therapeutic mechanisms of neuro-restoration using mesenchymal stem cells (Seo and Cho, 2012).

2.3 Mesenchymal Stem Cells (MSCs)

Friedenstein was first identified mesenchymal stem cells (MSCs) in 1961 (Friedenstein, 1961). MSCs is the stem cell that derived from the mesoderm and can differentiate into neural lineage and connective tissue. Currently, MSCs could be isolated from many tissues including bone marrow, wharton's jelly, adipose tissue, umbilical cord blood and dental pulp tissue (Gronthos, Mankani, Brahim, Robey, and Shi, 2000; Pittenger et al., 1999; Van Phuc, Nhung, Loan, Chung, and Ngoc, 2011; H. S. Wang et al., 2004; Zuk et al., 2001). Several sources of MSCs exhibited different properties of stemness and expansion capacity, and multilinage differentiation (Bonab et al., 2006). In 2006, The International Society for Cellular Therapy (ISCT) definite the properties of MSCs which are adherent to plasticity when maintained in standard culture conditions, have a fibroblast-like morphology and highly express a specific

surface antigens i.e. CD105, CD73 and CD90, and low expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules of hematopotic stem cells (Dominici et al., 2006). MSCs can secrete neurotrophic factors, promote endogenous neurogenesis and angiogenesis, encourage synaptic connection and remyelination of damaged axons. Accordingly, MSCs may prevail as a promising cell source for cell-based therapy in neurological diseases (Seo and Cho, 2012).

2.4 Human dental pulp stem cells (hDPSCs)

The presence of stem cells in dental pulp tissue have been reported primarily in 1985 by Yamamura (Yamamura, 1985). In 2000, Gronthos and his coworkers isolated human dental pulp stem cells (hDPSCs) from adult human dental tissue (Gronthos et al., 2000). The discovery of stem cells in the pulp of human third molar have high attention for future therapies due to hDPSCs are easily accessible and minimal invasive with higher proliferation capacity than bone marrow-derived MSCs (G.-J. Huang, Gronthos, and Shi, 2009). It is known that these stem cells have the potential to differentiate into several cell types, including odontoblasts, osteoblasts, chondrocytes, adipocytes and neural progenitors (Estrela, Alencar, Kitten, Vencio, and Gava, 2011). More importantly, these hDPSCs are originate from the neural crest (Janebodin and Reyes, 2012).

The characteristics of MSCs from human dental pulp are similar to stem cells derived from bone marrow (Shi, Robey, and Gronthos, 2001). Moreover, hDPSCs can be expressed markers of MSCs including STRO-1, CD146, SSEA4, CD90, CD73, CD 105, CD106 and CD 166 while CD34 and CD31 are not express (Young, Sloan,

and Song, 2013). In addition, hDPSCs can express the neural markers and differentiate into functionally active neurons (Arthur, Rychkov, Shi, Koblar, and Gronthos, 2008), suggesting their potential as therapy-based treatments of neuronal disorders and injury (Nör, 2006).

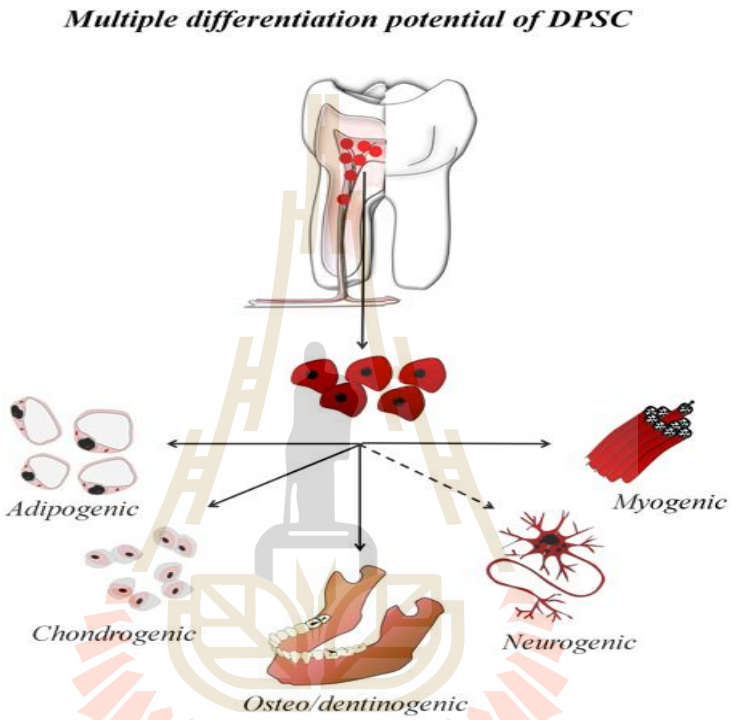

Figure 2.3 Multiple differentiation potential of hDPSCs (Aurrekoetxea et al., 2015)

2.5 Neural differentiation property of MSCs and hDPSCs

Several *in vitro* and *in vivo* studies reported the differentiation of human bone marrow derived mesenchymel stem cells to neural lineages (Bossolasco et al., 2005; Jiang et al., 2002; Sanchez-Ramos et al., 2000), especially, neuron-like cells (Muñoz-Elías, Woodbury, and Black, 2003; Neuhuber et al., 2004; Shi et al., 2001). In the

other hand, hDPSCs can differentiate into multi-lineage cells (Gandia et al., 2008; Sumita, Tsuchiya, Asahina, Kagami, and Honda, 2009) including neuron-like cells (Gronthos et al., 2000). Although neural progenitor stage derived-hDPSC could be produced, these cells was not matured further into functional neuron cells (Aanismaa, Hautala, Vuorinen, Miettinen, and Narkilahti, 2012). On the other hand, some report claim that hDPSC can even differentiate to functionally active adult neurons (Arthur et al., 2008; Gervois et al., 2014).

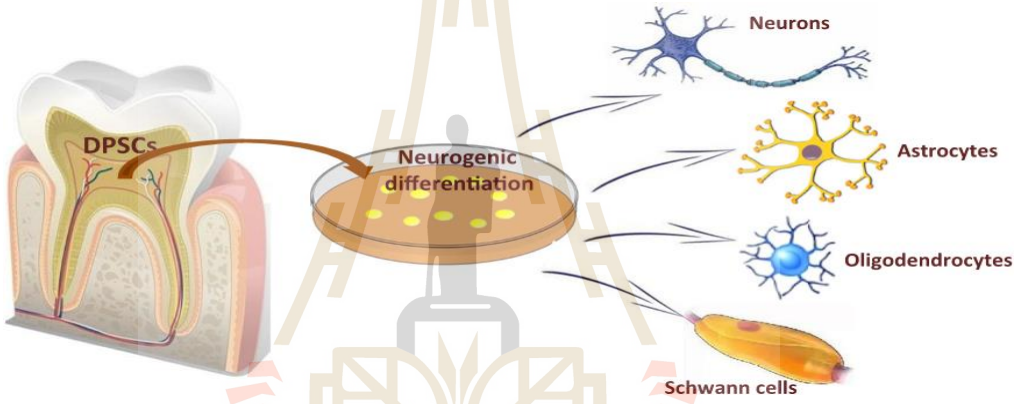

Figure 2.4 Neural-lineage differentiation of hDPSCs (Nuti, Corallo, Chan, Ferrari, and Gerami-Naini, 2016)

The hDPSCs present some striking similarities with neural stem cells which is positively stained for the neural stem cell marker Nestin (Bonnamain et al., 2013; Ibarretxe et al., 2012; Xiao and Tsutsui, 2013). For the capacity of hDPSCs to differentiate to neurons and other neural cell lineages, these cells could be a very interesting choice for repair injured nerve tissues as a result of their active secretion of neurotrophic and immune-modulatory factors (Nosrat, Smith, Mullally, Olson, and Nosrat, 2004; Pierdomenico et al., 2005). Although stem cells can be differentiation

under appropriate conditions *in vitro*, they may also induced by a mixture of different growth factors, signaling molecules and/or genetic manipulation as well.

2.6 Notch signaling on neural differentiation

In 1917, Thomas Hunt Morgan was the first who discovered the Notch gene in *Drosophila melanogaster* by observed that wings loss of function of this gene that show in Notch at the wing (Morgan, 1917). Notch signaling pathway is important for cell-cell communication and involves in neuronal differentiation of ESCs (Lowell, Benchoua, Heavey, and Smith, 2006). Notch signaling is activated on direct cell-to-cell contact and started through the interaction of extracellular ligands (Jagged 1, Jagged 2, Delta-like 1, Delta-like 3, and Delta-like 4) with Notch receptors (Notch1, 2, 3 and 4). The intracellular domain of Notch (NICD) translocate to the nucleus and interacts with RBP-Jk (also called CBF1) and activates the transcription of specific target genes, such as Hes1, Hes5, and Hey family genes (Fiúza and Arias, 2007).

The Notch signaling pathway was mainly use for neural progenitor cell (NPC) maintenance and for cells self-renewal. Recently, Notch signaling has been found to trigger the differentiation of several types of glial cells, including radial glia (Grandbarbe et al., 2003), Müller cells in retina (Furukawa, Mukherjee, Bao, Morrow, and Cepko, 2000), and astrocytes (Morrison et al., 2000). Activation of the Notch pathway is sufficient to maintain proliferating state, whereas loss-of-function and abnormal mutations in the critical components of the pathway cause neuronal differentiation and NPC depletion. Especially, Notch signaling pathway have been reported to be involved in neuronal differentiation of ESCs (Morrison et al., 2000).

Notch signaling was inhibited neuronal modulators of the Notch signal with by Numb protein, resulting in the cell cycle and differentiation of NPCs. So, Notch signaling controls NPC self-renewal as well as cell fate specification (Hoe and Rebeck, 2005; E. J. Huang et al., 2005).

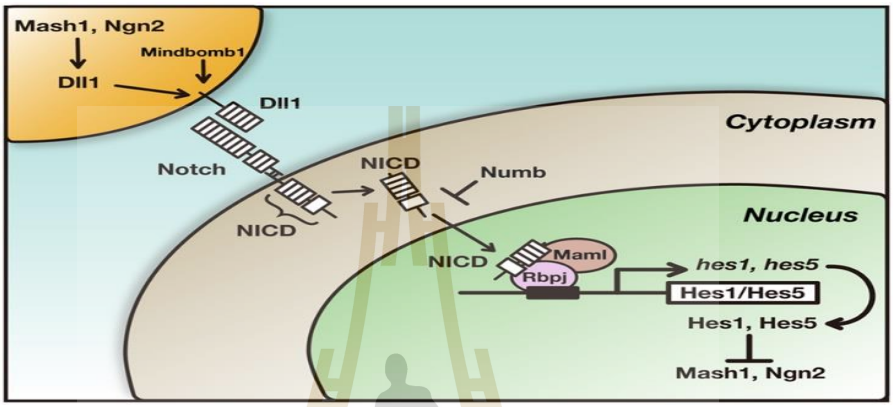

Figure 2.5 A schematic illustrating the effects of Notch signal activation to block neural differentiation in vertebrate nervous system (Hoe and Rebeck, 2005).

Notch signaling has been shown to inhibit neuronal differentiation in both invertebrate and vertebrate systems (Dorsky, Chang, Rapaport, and Harris, 1997; Fortini, Rebay, Caron, and Artavanis-Tsakonas, 1993; Henrique et al., 1997; Struhl, Fitzgerald, and Greenwald, 1993). Notch signaling affect differentiation of several neural precursors as revealed by both loss and gain of function. The process of neurons differentiation can be inhibited by Notch signaling activation. By contrast, a glial progenitor derived from a stem cell differentiates into an astrocyte with the help of Notch signals. Finally, oligodendrocyte precursors derived from glial progenitors fail to differentiate into mature oligodendrocytes in the presence of active Notch signals as shown in fig. 2.5 (Louvi and Artavanis-Tsakonas, 2006).

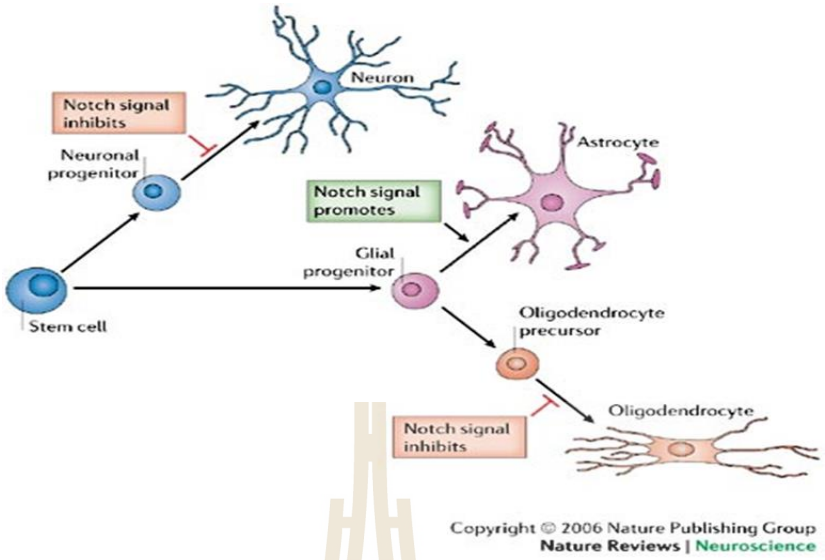

Figure 2.6 A diagram summarizing of the effects of Notch signal activation on cell fate decisions in the vertebrate nervous system (Louvi and Artavanis-Tsakonas, 2006).

2.6.1 DAPT, Notch signaling inhibitor

DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-Sphenylglycinet-butyl ester) is an inhibitor of the γ-secretase complex. Notch signaling is a key target of γ-secretase, therefore DAPT indirectly inhibits the Notch pathway and causes neural cells to commit to neuronal differentiation. For the inhibition of Notch signaling pathway by DAPT γ-secretase inhibitor was shown to associate with neural differentiation in neural stem/progenitor cells (Kageyama, Ohtsuka, Shimojo, and Imayoshi, 2008; Kanungo, Zheng, Amin, and Pant, 2008; J. Wang et al., 2016) and also in MSCs (Y. Wang et al., 2009). In 2007, Crawford and colleges were successfully to establish neuronal-like cells by inhibition of Notch signaling by

response the activation of the Sonic Hedgehog signaling (Crawford and Roelink, 2007).

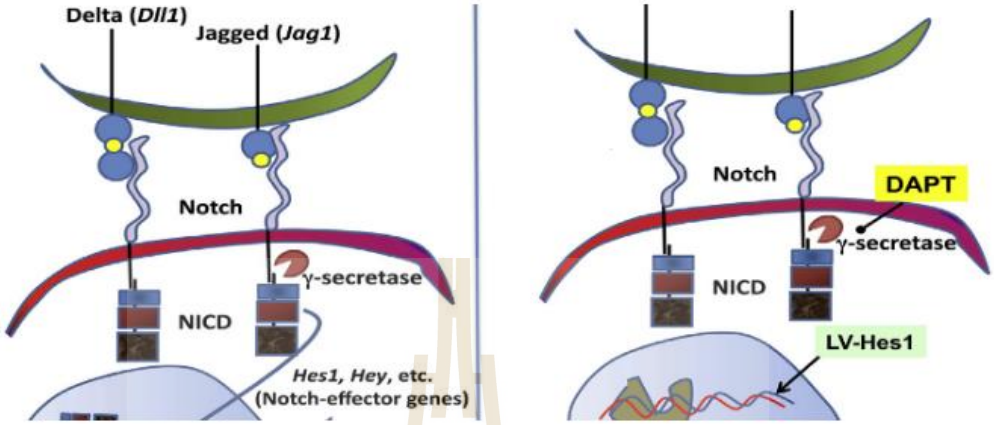

Figure 2.7 A summarizing schematic of inhibiting Notch signaling by suppressing the activity of γ -secretase by using DAPT (Dias et al., 2014).

2.7 Epigenetic modification

Epigenetic is term of alteration of gene activity without changing in DNA sequence, and leads to modifications that can be transmitted to daughter cells (Russo, Martienssen, and Riggs, 1996). In 2008, at a Cold Spring Harbor meeting was formulated consensus the definition of the concept of epigenetic trait as "stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" (Berger, Kouzarides, Shiekhattar, and Shilatifard, 2009). Epigenetic modifiers have been used extensively in stem cell research concerning the control of stem cell fate and modulating developmental pathway (Ding and Schultz, 2004). There are Many types of epigenetic processes have been identified included methylation, acetylation, phosphorylation, ubiquitylation, and sumolyation (Weinhold, 2006). Normally, epigenetic modification of the genome occurs during early development of

embryos and DNA methylation of cytosine residues is a major cause of the modification (Jones and Takai, 2001; Lei et al., 1996; Santos, Hendrich, Reik, and Dean, 2002). Epigenetic mechanisms regulate multiple aspects of normal development such as stem cell maintenance and differentiation. Gene expression can be controlled by the repressor proteins that attach to silencer regions of the DNA. (Bird, 2007).

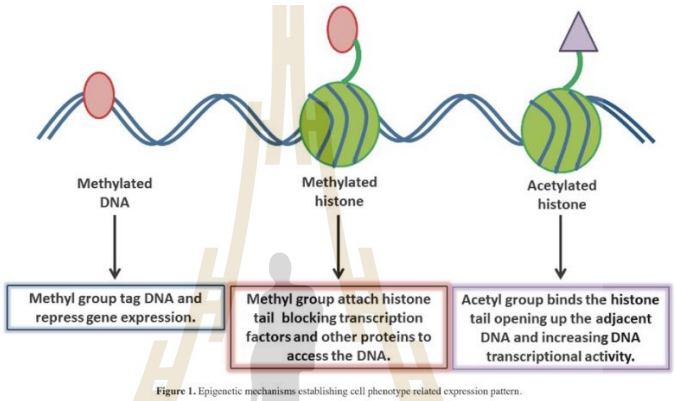

Figure 2.8 Lineage specification of epigenetic modifier (Russo et al., 1996).

2.7.1 5-azacytidine DNA methylating agent

5-azacytidine (5-aza) is a DNA demethylating agent involed in epigenetic modifier. Prior to 1980, there were a number of clues which suggested that methylation might play a role in the regulation of gene expression.(Phillips, 2008). 5-azacytidine can effects the inhibiting methylation on gene expression, 5-aza is one of many chemical analogs for the nucleoside cytidine. When 5-aza are integrated into growing DNA strands and inhibited the action of the DNA methyltransferase enzymes that normally methylated DNA in gene expression and cellular differentiation (Jones and Taylor, 1980). Generally, this molecule is well known to be a direct inhibitor of methyltransferase activity as well as of methylation in DNA. For its ability, it has been

already used to alter gene expression and reactivate the transcription of previously silent genes (Jones, 1985), as well as to modify differentiation states and phenotypes of eukaryotic cells (Glover, Coyle-Morris, Pearce-Birge, Berger, and Gemmill, 1986; Taylor and Jones, 1979). There are many research that use 5-aza on cells differentiation, 5-aza could induce muscle cell differentiation from fibroblast cell lines (Taylor and Jones, 1979) and cardiomyogenic cell differentiation from mesenchymal stem cells (Fukuda, 2001). Moreover, 5-aza can reverses differentiation state of ES cells through its DNA demethylating activity to differentiation related genes (Tsuji-Takayama et al., 2004). On the other hand, it is also evident that the combination of 5-aza with different induction protocols may drive cells towards different tissue commitments (Pennarossa, Maffei, Gandolfi, and Brevini, 2013).

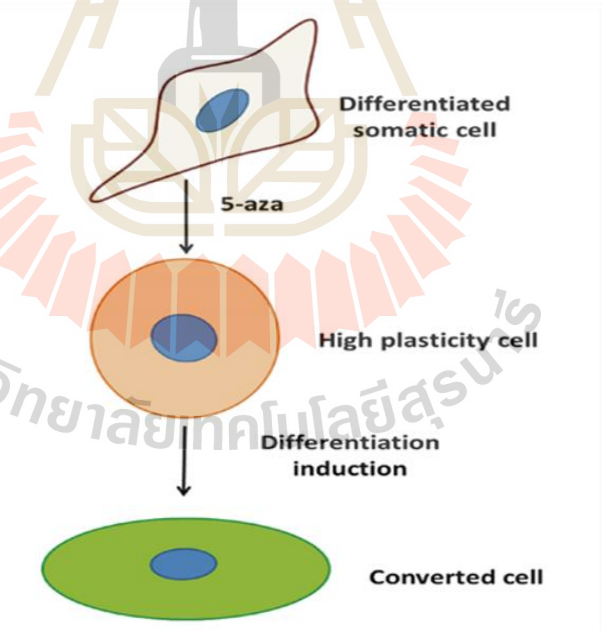

Figure 2.9 Epigenetic convert cells are generated through exposure of 5-aza (Pennarossa et al., 2013).

2.8 Autophagy and cells differentiation

Autophagy is a conserved lysosomal degradation pathway, was also shown to participate in promotes morphological and structural changes and highly active during differentiation and development (Lafontan, 2008). Autophagy also plays a critical role in stem cell maintenance and in a variety of cell differentiation processes (Vessoni, Muotri, and Okamoto, 2011). The autophagy pathway can drive the rapid cellular changes necessary for proper differentiation and/or development. For MSCs, autophagy is a well-known process by mobilizing autophagic degradation to induce recycling of components for protein production and other key energy factors. Some study indicated that autophagy plays an essential role in the differentiation of human bone marrow MSCs into both osteocytes and adipocytes by altering autophagic phenotype early in the process that allow normal autophagy to occur, and able to undergo a more efficient differentiation (Nuschke et al., 2014).

Autophagy has been proposed to play a protective role against the development of a number of neurodegenerative diseases (Mizushima and Klionsky, 2007; Rubinsztein, 2006). For neural differentiation, increasing an autophagy was also observed in neuronal differentiation in cultured NSC/progenitors *in vitro*, autophagy promote neuronal differentiation of NSCs (Guan et al., 2013). However, the level of autophagic activity is tightly regulated through a number of signaling pathways (Lafontan, 2008). In undifferentiated state, MSCs present an arrest in autophagy prior to autophagosome degradation by the lysosome. Most importantly, the autophagic phenotype is lost during cellular differentiation. Given the high concentration of autophagosomes in MSCs, the key role in differentiation, and the general lack of autophagic turnover at a basal state (Nuschke et al., 2014).

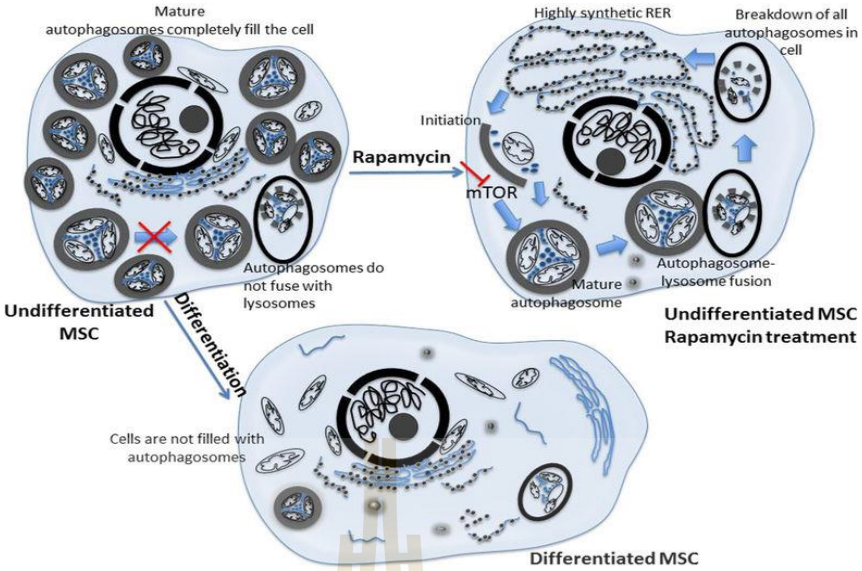

Figure 2.10 Arrest and utilization of autophagy in normal mesenchymal stem cells Function (Nuschke et al., 2014).

CHAPTER III

MATERIALS AND METHODS

3.1 Isolation and cultivation of hDPSCs

Stem cells from human dental pulp tissues were collected from third molars of healthy human with written informed consent from donors undergoing tooth extraction for orthodontic or therapeutic reasons at the dental clinic in Suranaree University of Technology Hospital (SUTH, Nakhon Ratchasima, Thailand). The protocol was approved from the ethical committee of Suranaree University of Technology. The human dental pulp tissues were isolated and cultured according to the guidelines from established protocols with some modifications (Gronthos, Mankani, Brahim, Robey, and Shi, 2000). Briefly, dental pulp tissue was minced into small pieces and placed in a 4 mg/ml collagenase/dispase culture dish (Roche, Germany), and digested at 37°C for 1 hour. Cells were maintained in MSC 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), 1 mM L-glutamine, 1 mM minimal essential medium (MEM; Sigma-Aldrish, St. Louis, MO, USA), and 100 units/ml penicillin, and 100 µg/ml Streptomycin (Sigma-Aldrich). The isolated hDPSCs were incubated at 37°C with saturated humidity containing 5% CO_2 in air for 7-14 days. The medium was replaced every 3 days until founded fibroblast-like cells migrated out from the dental pulp tissue.

3.2 Characterization of hDPSCs for MSCs phenotype

3.2.1 Multipotency assay

Cells at passage 3 to 5 of the confluent approximately $2x10^4$ cells were seeded on a 6-well culture plate (Nunc, Roskilde, Denmark), coated with the basement membrane matrix Geltrex (Gibco). For osteogenic induction, hDPSCs were cultured in osteogenic induction medium, consisting of MSC medium, 10% FBS, 100 nM dexamethasone, 0.2 mM L-ascorbate-2-phosphate, and 10 mM β-glycerophosphate (Sigma-Aldrich) (Kern, Eichler, Stoeve, Klüter, and Bieback, 2006). Medium was changed every three day for 21 days. Subsequently, cells were fixed cells with 4% paraformaldehyde (PFA) for 15-20 minutes. The bone matrix-mineralization was evaluated by Alizarin red staining (Sigma-Aldrich). Images were taken with the bright field microscope (20X magnification).

To induce adipogenic differentiation, cells were maintained in MSC medium containing 10% FBS, 10 µg/ml insulin, 60 µM indomethacin, 0.5 µM hydrocortisone, and 0.5 mM isobutyl methylxanthine (IBMX). Medium was replaced every two days for 21 days. At the end of the differentiation, cells were fixed with 4% PFA for 15-20 minutes and lipid droplets were detected by Oil Red O (Sigma-Aldrich). Images were taken by a bright field microscope (20X magnification).

For chondrogenic differentiation, hDPSCs were maintained in MSC medium supplemented with ITS-plus premix (BD Biosciences, San Jose,CA) to a final 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 dexamethasone, and 10 ng/ml TGF-β3. Medium was

replaced every two days for 21 days. After induction, cells were washed with PBS and fixed with 4% PFA, and chondrocytes were stained with Alcian blue (Sigma-Aldrich).

3.2.1 Immunocytochemistry assay

Cells were washed 3 times with phosphate-buffered saline (PBS), and fixed with 4% PFA for 15-20 minutes. Then, the cells were incubated with blocking solution, consisting of 4% bovine serum albumin (BSA; Sigma-Aldrich) in PBS for 1 hour. Prior to the incubation of primary antibody, blocking solution was removed and primary antibody were diluted in the blocking solution with appropriate dilution: Anti-CD90-FITC (1:500; (MERK-Millipore), Anti-Endoglin-CD105 (1:200; MERK-Millipore) and Anti-CD73 (1:500; MERK-Millipore). Primary antibody was applied for overnight at 4°C. Then, the cells were washed 3 times with PBS, followed by the incubation of secondary antibodies fluorophore conjugated. The secondary antibody was diluted at 1:1000 in the blocking buffer and applied for 1 hour at room temperature. The cells were then washed three times in PBS. Cells were incubated in 10 µg/ml DAPI for 10 minutes for nuclear counter staining, and fluorescent images were visualized by fluorescent microscope (Biorad; Hercules, CA. USA).

3.3 Differentiation of hDPSCs into neural-like cells

At passage 4 to 5, a 6-wells plate culture (Nunc) were coated with Geltrex (Gibco), and cells were seeded at approximately $2x10^4$ cells in each wells. For the control treatment, cells were maintained in MSC medium. Neural differentiation medium included Neurobasal (NB) medium (Gibco) and DMEM/F12 (Hyclone), supplemented with N2 (100X) (Gibco) with RA (Retinoic acid) 10 µM. (Gibco). The neural differentiation protocol was performed by a 3-step protocol. First, to optimize

DAPT concentration, the neural differentiation medium was supplemented with small DAPT to block Notch signaling at various concentrations (0, 5, 10, and 20 µM) for 7 days. Next, the neural differentiation medium was supplemented with 10 µM 5-azacytidine (Sigma-Aldrich), combined with 10 µM DAPT for 7 days. In the last step, 10 µM VPA was used to activate autophagy and 10 µM chloroquine was added for inhibiting autophagy. These two molecules were supplemented in the neural differentiation medium that included 10 µM DAPT and 10 µM 5-aza, and changed every 2 days for 7 days (Fig. 3.1). The differentiated cells were collected at day 1, 5, and 7, for further characterization. Cell morphology was monitored by using bright field microscope.

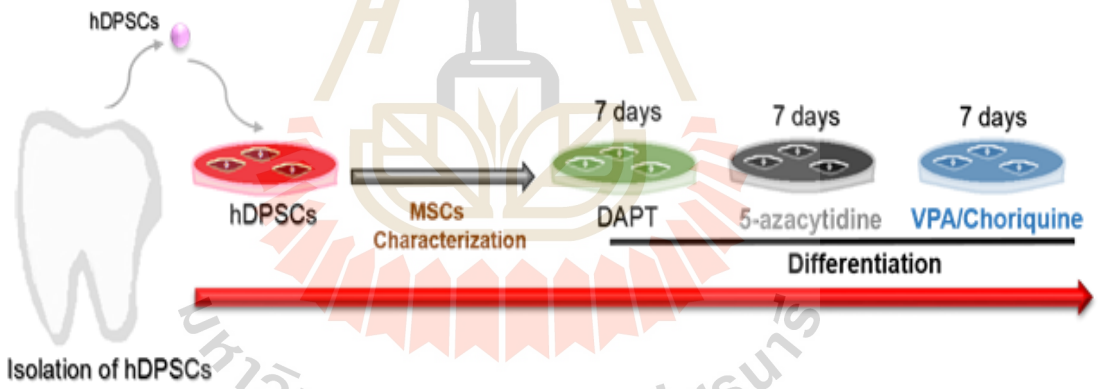

Figure 3.1 Schematic diagram of neural differentiation protocol of hDPSCs

3.4 Immunocytochemistry

Cells were washed 3 times with phosphate-buffered saline (PBS), and fixed with 4% PFA for 15-20 minutes. Then, the cells were incubated with blocking solution, consisting of 4% bovine serum albumin (BSA; Sigma-Aldrich) and 0.5%triton-X100 (Sigma-Aldrich) in PBS for 1 hour. Prior to the incubation of primary antibody, blocking solution was removed and primary antibody were diluted in the blocking solution with appropriate dilution: mouse anti-βIII-tubilin (1:1000; Sigma-Aldrich), rabbit-anti-NESTIN (1:500; (MERK-Millipore, Massachusetts, USA), rabbit-anti-LC3I/II (1:1000; (MERK-Millipore), rabbit-anti-Beclin (1:000; MERK-Millipore), and Anti-CD73(1:500; MERK-Millipore). Primary antibody was applied for overnight at 4°C. Then, the cells were washed 3 times with washing buffer (PBS, 0.1% TritonX100), followed by the incubation of secondary antibodies fluorophore conjugated. The secondary antibody was diluted at 1:1000 in the blocking buffer and applied for 1 hour at room temperature. The cells were then washed three times in PBS. Cells were incubated in 10 µg/ml DAPI for 10 minutes for nuclear counter staining, and fluorescent images were visualized by fluorescent microscope (Biorad; Hercules, CA. USA).

3.5 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Cells were detached by 0.25% (v/v) trypsin-EDTA (Hyclone) and centrifuged at 11,000 rpm for 5 minutes. Total RNA was extracted by RNA minikit (Nucleospin; Duran, Germany), according to the manufacturer's protocol. Total RNA was assessed the purity by microplate reader (Spectro star nano; BMG labtech, Ortenberg, Germany) at 260 and 280 nm, with an $A_{260} : A_{280}$ ratio of 1.8–2.0 considered

acceptable. For complementary DNA (cDNA) synthesis, 300 ng of total RNA sample was used for each RT-PCR reaction by using cDNA kit (TOYOBO; OSAKA, JAPAN), according to the manufacturer's protocol. PCR-primers were obtained from Macrogen (Seoul, Korea). Primers used in this study were described in Table 1. The expression level of *GAPDH* was used as a housekeeping gene to normalize the relative expression level in each sample. cDNA was amplified by using thermal cycle machine (Bio-rad).

Table 3.1 Primers used for RT-PCR analysis.

Gene	primer sequence (5'→ 3')	Product size (bp)
GAPDH	Forward: 5'- TCACCACCACGGCCGAGCG -3'	351
	Reverse 5'- TCTCCTTCTGCATCCTGTCG-3'	
βIII-tubulin	Forward: 5'-GCTCAGGGGCCTTTGGACATCTCTT-3'	148
	Reverse 5'-TTTTCACACTCCTTCCGCACCACATC-3'	
NESTIN	Forward: 5'- CAGCTGGCGCACCTCAAGATG-3'	209
	Reverse 5'- AGGGAAGTTGGGCTCAGGACTGG-3'	
SOX2	Forward: 5'- CCCCCGGCGGCAATAGCA-3'	448
	Reverse 5'- TCGGCGCCGGGGAGATACAT -3'	
LC3I/II	Forward: 5'- CTTCGCCGACCGCTGTAA -3'	261
	Reverse 5'- GGTGCCTACGTTCTGATCTGT G -3'	
Beclin-1	Forward: 5'- GCT CAG TACCAGCGAGAATA -3'	350
	Reverse 5'- GTC AGGACTCC AGA TAC GA -3'	
GAD1	Forward: 5'- GTCGAGGACTCTGGACAGTA -3'	357
	Reverse 5'- GGAAGCAGATCTCTAGCAAA -3'	

3.6 Statistical analysis

Data were assessed by one-way ANOVA followed by *Turkey* tests SPSS program version 16.0. Differences with p-value < 0.05 were considered statistically significant. Obtained data were presented as average (mean ± SD; standard deviation) from three independent experiments (n=3). Data were analyzed by GraphPad Prism 5.0 (GraphPad Software, San Diego, CA).

CHAPTER IV

RESULTS

4.1 Isolation of human dental pulp stem cells

Human dental pulp stem cells (hDPSCs) were successfully isolated from human dental pulp tissues of human third molar by enzymatically method (Fig. 4.1 A-B). Cells adhered to plastic culture plates after 24 hours and migrating stem cells were observed after 3 days with fibroblast-like morphology. Approximately day 21, hDPSCs reached to 100% confluent, and cells were harvested and expanded by 0.25% trypsin in PBS for digestion and passage for cell expansion to become hDPSCs passage 1 (Fig. 4.1 C).

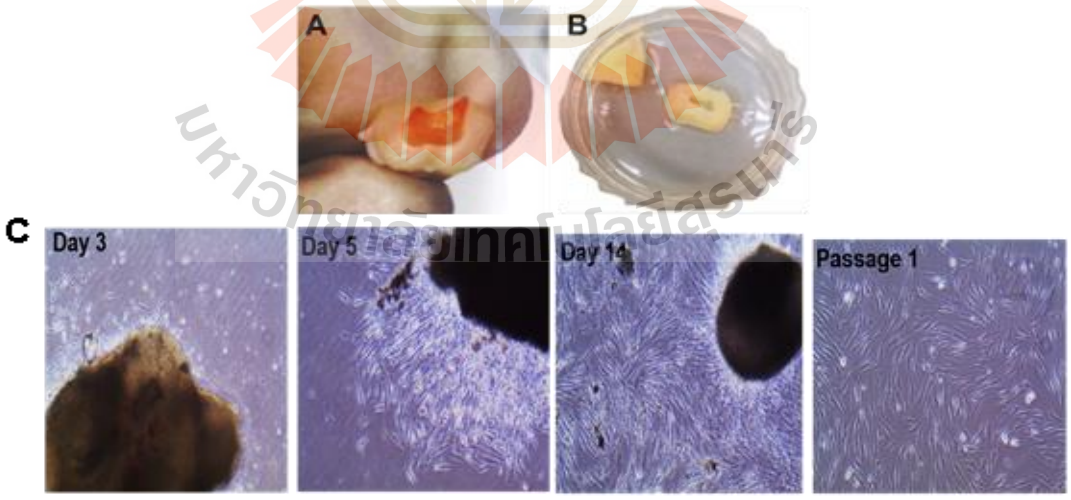

Figure 4.1 The isolation of hDPSCs from human third molar tissues. (A-B) Human dental pulp was excised in order to access the dental tissue. Human third molar with dental pulp tissue was rinsed and washed in

PBS. **(C)** Morphology of hDPSCs with typical fibroblast-like morphology. A bright field images of hDPSCs, expanded form human dental pulp tissue at day 3, 5, 14 and termed them as passage1.

4.2 Characterization of mesenchymal stem cells characteristics from human dental pulp stem cells

The MSC phenotypes from human dental pulp tissues were characterized at the third passage by immunocytochemistry, flow cytometry, and multipotency assays. The expression of specific surface antigens of MSC markers were tested by immunocytochemistry and found that hDPSCs expressed CD73, CD90, and CD105. This result indicated that hDPSCs exhibited typical MSC characteristics (Fig 4.2A). To determine their multipotency, hDPSCs were cultured in tri-mesodermal induction medium, including osteogenic, chondrogenic, and adipogenic induction mediums for 21 days. Then, after 21 days of the induction hDPSCs were induced toward osteocytes, adipocytes, and chondrocytes. Osteocytes, chondrocytes, and adipocytes were examined by Alizarin-red, Alcian blue and Oil Red-O staining respectively (Fig 4.2C).

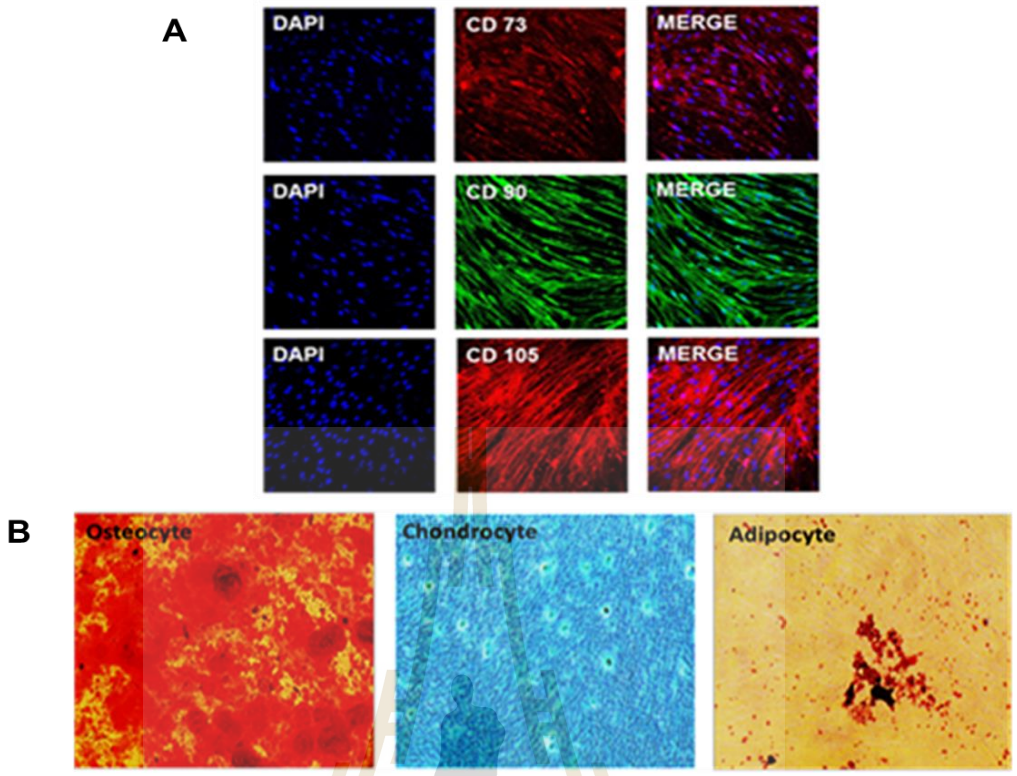

Figure 4.2 Characterization of hDPSCs. (A) Representative images of immunophenotype of hDPSCs CD73, CD90 and CD105. **(B)** Multilineage differentiation potential of hDPSCs after 21 days, evaluated by Alizarin Red (osteocyte), Alcian Blue (chondrocyte) and Oil Red O (adipocyte) staining.

4.3 Effect of DAPT on neural-like cells differentiation and up-regulated autophagy activity in hDPSCs

DAPT, a γ-secretase inhibitor, was used to interfering Notch signaling during neural differentiation of hDPSCs. Firstly, the optimal concentration of DAPT on neural differentiation of hDPSCs was tested by inducing cells with various concentration of DAPT (0, 5, 10, and 20 µM) for 7 day. The morphology of hDPSCs was shown as a spindle-shaped morphology when 5, 10 and 20 µM DAPT was applied

and no morphological change was found in the control cells (Fig 4.3A). The expression of neural genes was analyzed by RT-PCR and shown that neural stem cell markers, *SOX2* and *Nestin*, were significantly upregulated when 10 and 20 µM DAPT was applied compared with the control cells (Fig 4.3C). Nestin was also found to express in undifferentiated hDPSCs, confirming the progenitor characteristics of hDPSCs (Fig 4.3D). The expression of *βIII-tubulin*, the early neural marker, were found to be upregulated as a dose-dependent manner, and it was significantly increased at 10 and 20 µM DAPT (Fig 4.3E). Immunocytochemistry for βIII-tubulin protein presented its high and homogeneous expression in hDPSC-derived neurons when a high concentration of DAPT was supplemented (Fig 4.5F). These data proposed that DAPT can promote neural differentiation of hDPSCs, and 10 µM DAPT was selected as an optimal concentration to induce neural differentiation. Further, 10 µM DAPT was combined with epigenetic modifier, 5-azacytidine (5-aza), which is a DNA demethylating agent for the next differentiation step.

The modulation of autophagy activity in hDPSCs during neural differentiation was determined by assessing the expression of autophagy genes, *LC3I/II* and *Beclin-1*. Compared with the control, *LC3I/II* and *Beclin-1* were significantly upregulated in differentiated hDPSCs (Fig. 4.4 A). After treated with 10 and 20 µM DAPT, hDPSC-derived neurons were significantly enhanced the expression of *LC3I/II* and *Beclin* (Fig 4.4 B, C). This result suggested that the autophagy was activated during neural differentiation of hDPSCs.

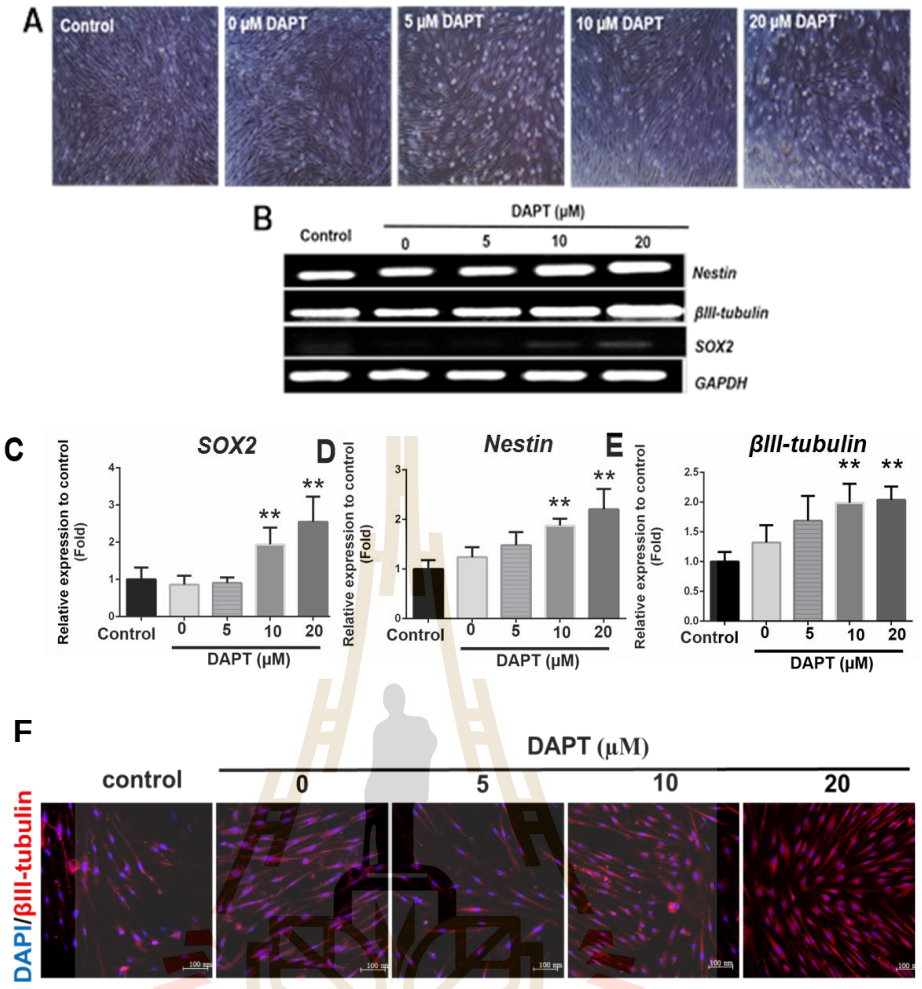

Figure 4.3 **Effect of DAPT on neural differentiation of hDPSC. (A)** The morphology of hDPSCs after treated with 0, 5, 10, 20 μM DAPT for 7 days. **(B)** Neural genes were assessed their expression by RT-PCR for **(C)** *SOX2*, **(D)** *βIII-tubulin*, and **(E)** *Nestin*. Gene expressions were normalized to *GAPDH* and calculated their relative fold expression compared to the undifferentiated cells. The experiments were performed in triplicates. Data were shown as mean ± SD, $^{**}p < 0.01$. **(F)** Representative immunofluorescent images of βIII-tubulin after induced neural differentiation for 7 days. Scale bar represented 100 μm.

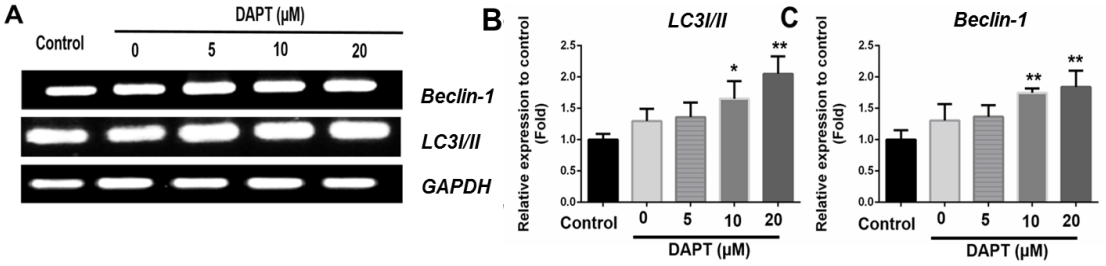

Figure 4.4 Autophagy was modulated during neural differentiation of hDPSCs. **(A)** The expression of *LC3I/II* and *Beclin* was evaluated by RT-PCR. (B, C) Relative expression of autophagy genes, **(B)** *LC3I/II* and **(C)** *Beclin-1*, was determined by using GAPDH as a reference gene. Values were expressed as mean ± SD (n = 3), $*p < 0.05$ and $**p < 0.01$.

4.4 DNA demethylating agent 5-azacytidine enhanced neural differentiation of hDPSCs and altered autophagy activity

To investigate the effect of 5-azacytidine (5-aza), a DNA demethylating agent, on neural differentiation, hDPSCs were differentiated into neural-like cells in the differentiation condition of 10 µM DAPT, including 10 µM 5-aza. After 7 days of the induction, the morphology of hDPSCs in the combined condition of 10 µM DAPT and 10 µM 5-aza presented neuronal-like cells phenotype with a bipolar shape and shown clear neural elongation (Fig 4.5).

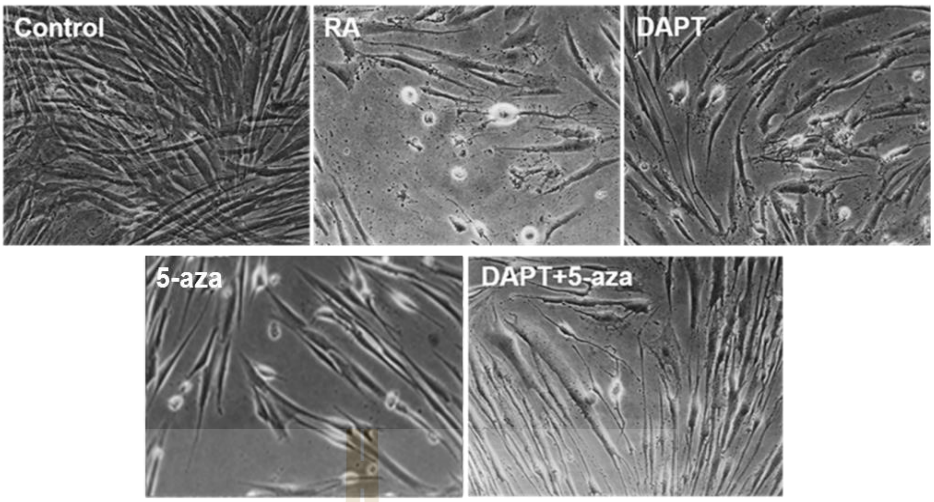

Figure 4.5 Effect of 5-azacytidine on neural differentiation of hDPSCs. The morphology of differentiated hDPSCs in 5 conditions, including undifferentiated hDPSCs, 0 µM DAPT (designated as RA), 10 µM DAPT, 10 µM 5aza (5-aza), and 10 µM DAPT with 10 µM 5-aza. hDPSCs were induced in the mentioned conditions for 7 days prior to microscopic imaging.

Noteworthy, the combined condition of DAPT and 5-aza showed the best neural differentiation efficiency as measured by RT-PCR analysis of neural genes (Fig 4.6A). *Nestin* and *βIII-tubulin* were significantly upregulated when compared to the differentiated control, 5-aza and DAPT treatment alone respectively ($p<0.01$) (Fig 4.6 B, C). Interestingly, *GAD1*, a marker of mature glutaminergic neurons, was highly enhanced in differentiated hDPSCs, induced by the combined DAPT and 5-aza ($p<0.01$) (Fig 4.6D). Moreover, immunofluorescence staining highlighted that the combination of DAPT and 5-aza triggered the presence of neural protein specific Nestin and βIII-tubulin (Fig 4.6E). This result indicated that DNA demethylating agent enhanced neural differentiation efficiency of hDPSCs.

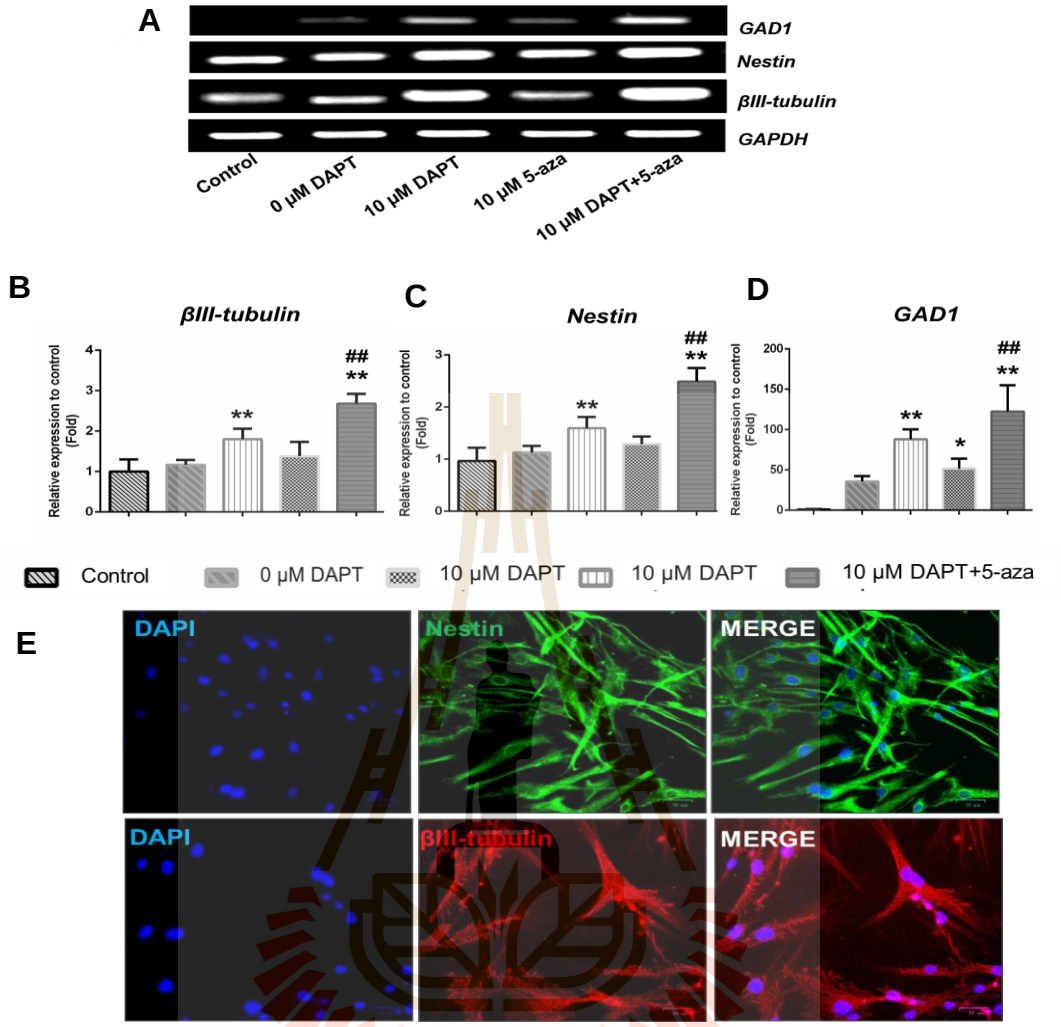

Figure 4.6 Effect of 5-azacytidine enhanced neural differentiation of hDPSCs. **(A)** The expression of neural gene expression was assessed by RT-PCR. **(B)** *βIII-tubulin*, **(C)** *Nestin*, and **(D)** *GAD1* was determined by using *GAPDH* as a reference gene. Values were expressed as mean ± SD (n = 3), $*p < 0.05$ and $**p < 0.01$ versus control cells, and $\#\# p < 0.01$ versus DAPT-treated cells. (E) The immunofluorescent images of hDPSCs-derived neurons induced by the combination of 10 μM DAPT and 10 μM 5-aza. βIII-tubulin (red) and Nestin (green) marked for neural cells and the merged images with DAPI (blue). Scale bar was 100 μm.

To investigate whether the combination of 5-aza could alter autophagy in hDPSCs during neural differentiation, the expression of *LC3I/II* and *Beclin-1* was determined after treated with following treatments; undifferentiated hDPSCs, 0 µM DAPT, 10 µM DAPT, 10 µM 5-aza, and 10 µM DAPT combined with 10 µM 5-aza for 7 days. Gene expression was measured by RT-PCR and indicated that the combined 10µM DAPT and 5-aza resulted in the significant increase of *LC3I/II* and *Beclin-1* genes, compared to the differentiated control and DAPT-treated cells (Fig. 4.7 A, B). Immunocytochemistry was used to ensure the modulation of autophagy, and it indicated that βIII-tubulin with LC3I/II and βIII-tubulin with Beclin-1 were co-localized within hDPSC-derived neural cells (Fig 4.7D).

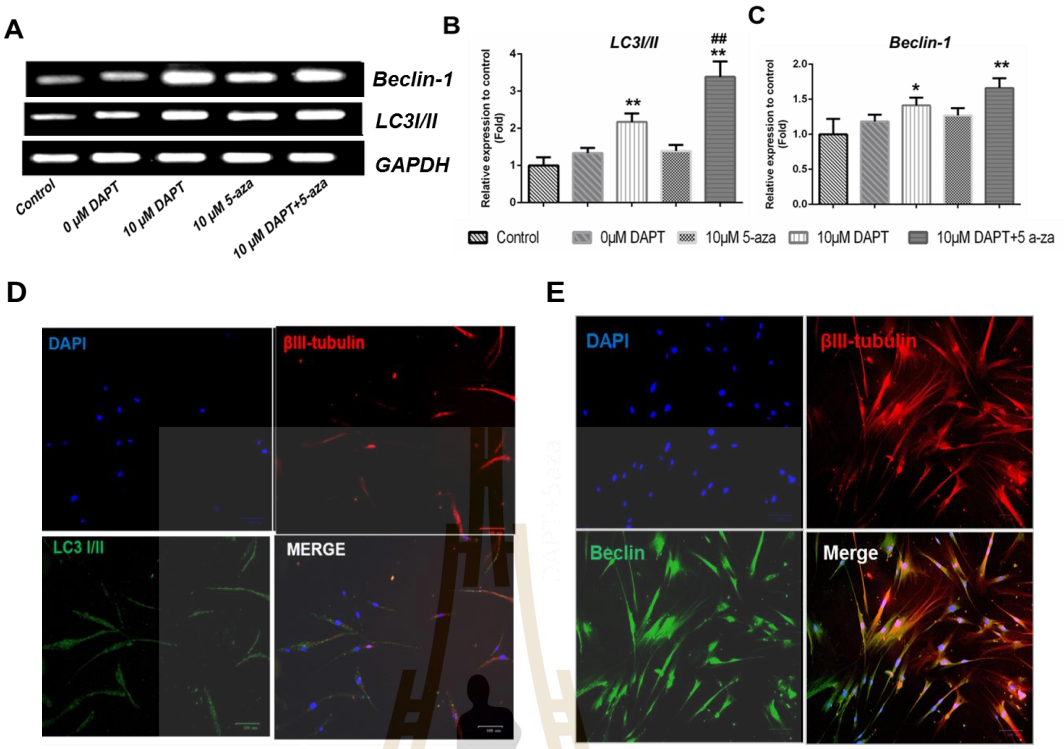

Figure 4.7 **Autophagy was activated during 5-aza treatment to enhance neural differentiation of hDPSCs. (A)** The expression of autophagy genes were measured by RT-PCR analysis after induced differentiation in various conditions for 7 days. **(B, C)** The relative expression values of autophagy gene *LC3I/II* **(B)** and *Beclin* **(C)** was determined by using *GAPDH* as a reference gene. Values were expressed as mean ± SD (n = 3), ***p < 0.01* versus the control cells, and *## p<0.01* versus DAPT-treated cells. **(D)** The immunofluorescent images of βIII-tubulin (red) as neural markers and LC3I/II **(D)** and Beclin-1 **(E)** (green) as autophagy markers were found to co-localize and were merged with DAPI (blue). Scale bar was 100 μm.

4.5 Modulations of autophagy activity by VPA and chloroquine altered neural differentiation of hDPSCs

To confirm the role of autophagy in neural differentiation of hDPSCs, either chloroquine (CQ; autophagy inhibitor) or Valproic acid (VPA, autophagy activator) were supplemented into the differentiation culture. The morphology of hDPSCs was observed at day 1, 5 and 7, and shown in Fig. 4.8 A. Cells were collected at different time points (day 1, 5 and 7), and the markers of neural cells and autophagy were determined by RT-PCR analysis (Fig 4.8 B). It was found that *LC3I/II* was upregulated and peaked at day 7 in the presence of VPA, compared with CQ supplement and the differentiated control (Fig 4.8 C). Similarly, *βIII-tubulin* gene was also upregulated when VPA was applied, and downregulated under CQ treatment (Fig 4.8 D). As βIII-tubulin and LC3I/II was previously found to co-localize within neural-derived hDPSCs, this evidence were clearly enhanced in VPA-treated cells. These results suggested that the activation of autophagy could improve neural differentiation capacity of hDPSCs (Fig 4.9).

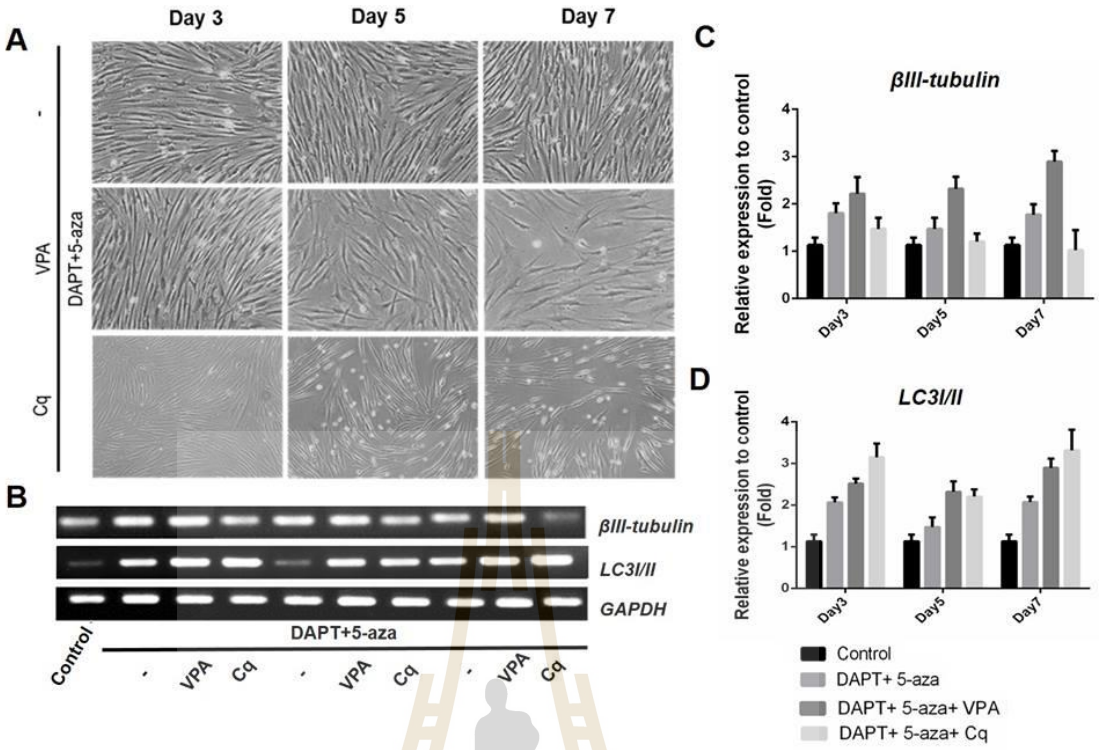

Figure 4.8 Modulation of autophagy altered neural differentiation of hDPSCs. (A) The morphological changes of hDPSCs were presented at different time points and in the presence of wither VPA or Cq. (B) Gene expression was examined by RT-PCR analysis for neural and autophagy genes, including *LC3I/II and βIII-tubulin*. (C, D) The relative expression of *LC3I/II* and *βIII-tubulin* in the condition with VPA and Cq (D) was determined by using GAPDH as a reference gene. Data were shown as mean ± SD.

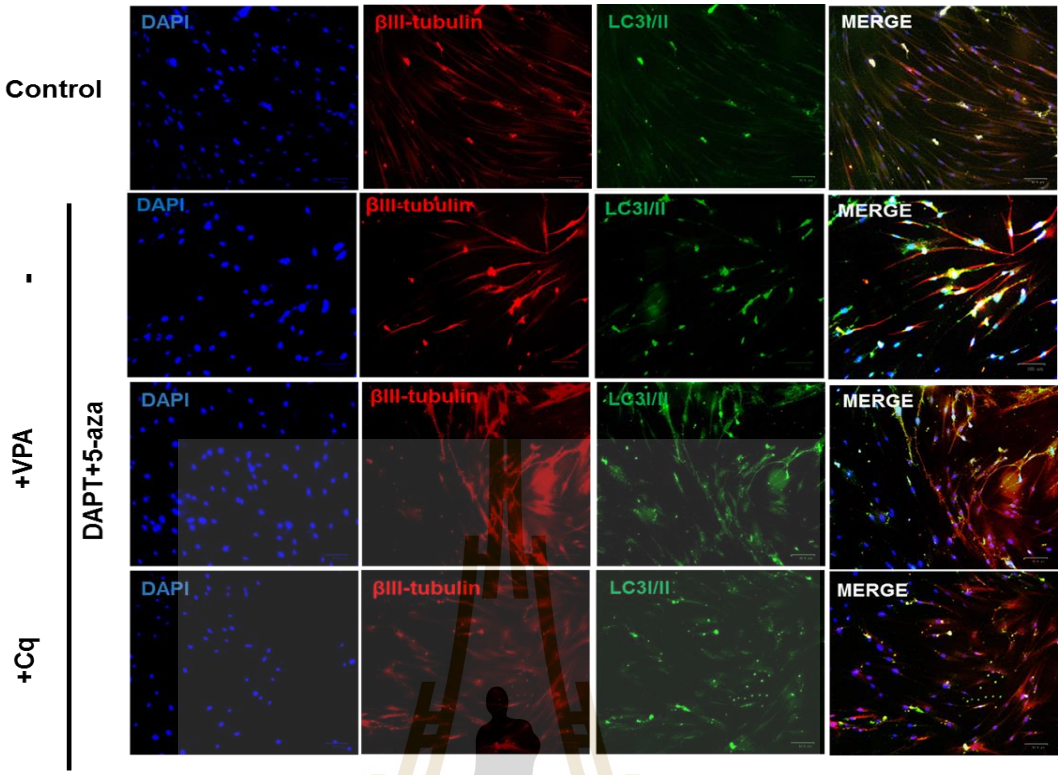

Figure 4.9 Immunocytochemistry detecting modulations of autophagy during differentiation. hDPSC-derived neurons were positively stained for LC3I/II in condition with VPA and Cq. *βIII-tubulin* was found to colocalize with LC3I/II in the presence of VPA. Scale bar was 100 μm.

CHAPTER V

DISCUSSION AND CONCLUSION

Nowadays, most of the drugs used for treated of neurodegenerative diseases are palliative and do not cure progression of diseases, and stem cell is a treatment option (Björklund and Lindvall, 2000). Due to the complicationof stem cells, including potential immune rejection, teratoma formation, and ethical concerns, researchers have paid serious attention to utilize mesenchymal stem cells (MSCs) as a possible cell source for transplantation (Huang, Gronthos, and Shi, 2009). Numbers of pre-clinical and clinical studies showed the promising results of bone marrow-derived MSCs (BM-MSCs) in treating neuronal and non-neuronal diseases (Bae et al., 2007; Derubeis and Cancedda, 2004; S. Wang, Qu, and Zhao, 2012). However, BM-MSCs need invasive surgical procedure with less yields of MSCs, low proliferation, and limited differentiation capacity (Stenderup, Justesen, Clausen, and Kassem, 2003). Thus, human dental pulp stem cells (hDPSCs) hold several advantages over BM-MSCs, in particular least invasive isolation, superior *ex vivo* proliferation, and inherent propensity to differentiate into neurons and glia (Shamir, Venugopal, and Dhanushkodi, 2015). hDPSCs appear to possess beneficial effects on the treatment of neurodegenerative diseases, and might be viewed as an alternative resource for cell therapy. Here, we investigated the influence of Notch inhibition and DNA demethylation on neural differentiation of hDPSCs and the consequence of autophagy modulation.

hDPSCs are the derivatives of neural crest cells and contain high proliferation rate (Chai et al., 2000). The population of hDPSCs in this study was obtained from human dental pulp tissues by enzymatic method according to the previously published procedure with some modification (Gronthos, Mankani, Brahim, Robey, and Shi, 2000). hDPSCs isolated in this study possessed the MSC characteristics, following *the International Society for Cellular Therapy* (ISCT) criteria (Dominici et al., 2006). hDPSCs positively expressed typical MSC markers, CD73, CD90 and CD105, but did not expressed hematopoietic markers, CD34 and CD 45. Moreover, hDPSCs were multipotent of tri-mesodermal lineage, being able to differentiate into osteoblasts, chondrocytes, and adipocytes *in vitro* (Figure 4.2).

Notch signaling is shown to inhibit neuronal differentiation in both invertebrate and vertebrate systems (Dorsky, Chang, Rapaport, and Harris, 1997; Fortini, Rebay, Caron, and Artavanis-Tsakonas, 1993; Ohtsuka et al., 1999). In the other hand, the inhibition of Notch signaling by γ-secretase inhibitor like DAPT was demonstrated to promote neural differentiation of stem cells (Kageyama, Ohtsuka, Shimojo, and Imayoshi, 2008; Kanungo, Zheng, Amin, and Pant, 2008; J. Wang et al., 2016). In 2008, Cai and collegues determined the effect of γ-secretase inhibitor (DAPT) on the proliferation and differentiation of neural stem cells, and the results showed that Notch signaling was downregulated while neuronal differentiation was enhanced when DAPT was added to the cells (Cai, Lin, Hu, and Zheng, 2008). In consistent with the previous study, the current study revealed the mechanisms by which DAPT suppressed Notch signaling and promoted neural differentiation of hDPSCs. To assess the influence of Notch signaling in neural differentiation, DAPT was varied its concentrations in differentiation medium. This medium included N2 supplement and retinoic acid (RA), which is known to have ability to induce neural phenotypes in

various types of stem cells (Janesick, Wu, and Blumberg, 2015; Kim, Seo, Bubien, and Oh, 2002). Upon the induction, hDPSCs were able to differentiate into neural-like cells as examined by morphological changes and gene expression. The expression of neuronal genes, including *βIII-tubulin* and *Nestin*, was found to upregulate in a dose-dependent manner of DAPT. Several studies showed that 10 μM DAPT effectively blocked Notch signaling (Chigurupati et al., 2007; Hoe and Rebeck, 2005; Olivier, Lauret, Gonin, and Galy, 2006). Similarly, this study showed that 10 and 20 μM DAPT significantly upregulated neural genes, compared to the control treatment; thus, 10 μM DAPT was then chosen for the following experiments. *Nestin* is known to express within the fibrous dental pulp tissues, and the expression of *Nestin* was increased when cells differentiated into neurons (Arthur, Rychkov, Shi, Koblar, and Gronthos, 2008). *βIII-tubulin* is expressed after neuronal differentiation, and utilized as a marker of mature neurons during the final stages of neural development (Johansson et al., 1999; Karaöz et al., 2010).

Autophagy activity was determined by detecting *LC3I/II* and *Beclin-1* genes, and the results showed that during neural differentiation, autophagy gens were significantly upregulated upon the addition of 10 and 20 μM DAPT (Figure 4.4). Next, the epigenetic modifier, 5-azacytidine (5-aza), was added in the differentiation condition in order to investigate the effect of DNA demethylation on neural differentiation of hDPSCs. The combined treatment of DAPT and 5-aza in could drive the expression of neural-related genes to be greater than that of the treatment of DAPT alone. Surprisingly, the result was shown the high upregulation of neural markers in the condition that supplemented with 5-aza. Nevertheless, the combination of DAPT and 5-aza could promote neural differentiation greater than that of the treatment either DAPT or 5-aza alone. The high expression of both *Nestin* and *βIII-tubulin* induced by

the combination of DAPT and 5-aza indicated that these two molecules switched on the activity of neural genes, and promoted neural differentiation. In addition, DNA demethylating agent, 5-aza, could enhance neural differentiation efficiency in this study. In 2013, Zemelko and colleagues presented that 5-aza has neurogenic potential in differentiating MSCs (Zemelko et al., 2013). In accordance with the previous research, adipose-derived mesenchymal stem cells (ASCs), maintained in the medium with RA and 5- aza for 7 days, increased the mRNA and protein levels of *Nestin* and *βIII-tubulin* (Pavlova et al., 2012). Similar to our study, we illustrated that the combination 5-aza cytidine with DAPT could promote neural differentiation of hDPSCs greater than that of the treatment of either DAPT or 5-aza alone.

Again, we could detect the increase of autophagy genes, *LC3I/II* and *Beclin-1*, in hDPSCs during neural differentiation. It was shown the significant upregulation of both *LC3I/II* and *Beclin* in the combined condition of DAPT and 5-aza. This result indicated that the addition of DNA demethylating agent significant upregulated both neural and autophagy genes. It was previously shown that autophagy was upregulated during neuronal differentiation of N2a cells (Zeng and Zhou, 2008). Moreover, some studies proved that autophagy played an important role in support and protection of cells during differentiation process (Kabeya et al., 2000; Mizushima, Yamamoto, Matsui, Yoshimori, and Ohsumi, 2004). The aforementioned statement suggested that autophagy activity should be one of the important supportive conditions during the differentiation of neural cells.

The crucial role of autophagy activity on neural differentiation of hDPSCs in this study was evaluated by the activation and inhibition of autophagy by the specific molecules. The correlation of autophagy activity during neural differentiation was confirmed by the treatment of either Valproic acid (autophagy activator) or

chloroquine (autophagy inhibitor) in neural differentiation system, containing 10 µM DAPT and 10 µM 5-aza. Valproic acid (VPA), a histone deacetylase inhibitors (HDACs), has been widely used to activate autophagy activity in cell culture (Xia et al., 2016). Chloroquine (Cq) has been used to inhibit autophagy, which leads to inhibition of both fusion of autophagosome with lysosome and lysosomal protein degradation (Shintani and Klionsky, 2004). The results in this study showed that after the treatment of VPA, both neural and autophagy genes were highly upregulated at day 5 and 7, when compared with the control and Cq-treated cells. Besides autophagy activation, VPA also functions in inducing cellular differentiation, growth arrest, and apoptosis in gliomas and other types of cancers cells (Michaelis, Doerr, and Cinatl, 2007). On the other hands, Cq is an autophagy inhibitor that leads to a massive accumulation of ubiquitinated proteins and correlated with the increased cell death (Torgersen, Engedal, Bøe, Hokland, and Simonsen, 2013). The treatment of Cq in this study resulted in the downregulation of neural genes. Immunocytochemistry results showed the colocalization of βIII-tubulin and LC3I/II in VPA-treated cells, but small number cells populations in control and Cq-treated cells. These data demonstrated that neural differentiation of hDPSCs induced the upregulation of neural gene along with the enhanced activity of autophagy. Recently, there has been interested study focus on identifying the role of the autophagy in neurodegeneration (Martinez-Vicente and Cuervo, 2007). Because of many evidence revealed that neuronal autophagy is essential for the healthy aging of neurons and play the major process for the degradation of an abnormal protein accumulation, which is the major cause of most neurodegenerative diseases (Nah, Yuan, and Jung, 2015). For this reason, when pathways controlling autophagy process increases, that likely to be able to develop ever more selective therapies that were upregulate autophagy in a manner that were

optimal for the specific disease to be treated (Frake, Ricketts, Menzies, and Rubinsztein, 2015). Thus, therapeutic strategies aim to upregulate of autophagy appear as a promising tools to cure neurodegenerative diseases.

Our results suggested that there were more complex signaling factors involved in the regulation of neural differentiation, and the mechanistic relationship between the Notch signaling, DNA demethylation, and autophagy required further exploration and investigation.

In conclusion, the inhibition of Notch signaling by a γ-secretase inhibitors (DAPT) could promote neural differentiation of hDPSCs, while DNA demethylating agent, 5-aza, could enhance neural differentiation efficiency. The activity of autophagy correlated with neural differentiation of hDPSCs. This study also suggested that DAPT and 5-aza could be a potential candidate for further *in vivo* therapeutic trials, and would be of great importance for neurological regeneration.

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APPENDIX

Table 1 The relative expression fold of neural-related genes compared to the undifferentiated control cells (hDPSCs) at day 7 of the differentiation (N=3).

Genes / **Conditions**	***βIII-tubulin***	***Nestin***	***SOX2***
hDPSCs (Control)	1±0.18	1±0.16	1±0.31
Basic neurogenic medium	1.23±0.20	1.32±0.18	0.86±0.23
Neurogenic medium 5 µM DAPT	1.48±0.26	1.68±0.49	0.90±0.14
Neurogenic medium 10 µM DAPT	1.87±0.13	1.98±0.32	1.94±0.45
Neurogenic medium 20 µM DAPT	2.20±0.49	2.20±0.37	2.54±0.68

Table 2 The relative expression fold of autophagy-related genes compared to the undifferentiated control cells (hDPSCs) at day 7 of differentiation (N=3)

Genes / **Conditions**	***LC3I/II***	***Beclin-1***
hDPSCs (Control)	1±0.23	1±0.26
Basic neurogenic medium	1.29±0.66	1.30±0.26
Neurogenic medium +5 µM DAPT	1.35±0.23	1.36±0.18
Neurogenic medium +10 µM DAPT	1.65±0.27	1.74±0.20
Neurogenic medium +20 µM DAPT	2.05±0.27	1.84±0.25

Table 3 The relative fold expression of neural-related genes compared to the undifferentiated control cells (hDPSCs) at day 7 of differentiation (N=3).

Genes / **Conditions**	***βIII-tubulin***	***Nestin***	***GAD1***
hDPSCs (Control)	1±0.29	1±0.21	1±0.67
Basic neurogenic medium	1.29±0.07	1.12±0.26	35.0±0.26
Neurogenic medium +10 µM DAPT	1.85±0.23	1.72±0.18	88.0±12.16
Neurogenic medium +10 µM 5-aza	1.31±0.27	1.15±0.20	33.7±0.20
Neurogenic medium +10 µM DAPT+10 µM 5-aza	2.51±0.35	2.32±0.25	122.4±32.5

Table 4 The relative expression fold of autophagy-related genes compared to the undifferentiated control cells (hDPSCs) at day 7 of differentiation (N=3).

Genes / **Conditions**	***LC3I/II***	***Beclin-1***
hDPSCs (Control)	1±0.21	1±0.21
Neurogenic medium	1.46±0.11	1.18±0.26
Neurogenic medium +10 µM DAPT	2.17±0.22	1.41±0.20
Neurogenic medium +10 µM 5-aza	1.76±0.27	1.54±0.20
Neurogenic medium +10 µM DAPT+10 µM 5-aza	3.38±0.41	1.66±0.13

Table 5 The relative expression fold of neural and autophagy genes compared to the undifferentiated control cells (N=3).

Genes / Conditions	βIII-tubulin			LC3I/II		
	Day 1	**Day 5**	**Day 7**	**Day 1**	**Day 5**	**Day 7**
hDPSC (negative control)	1±0.28			1±0.36		
Neurogenic medium 10 µM DAPT+10 µM 5-aza	1.96±0.56	2.45±0.28	2.89±0.26	2.31±1.0	1.58±0.26	2.43±0.78
Neurogenic medium 10 µM DAPT+10 µM 5-aza+VPA	2.21±0.56	2.38±0.28	2.89±0.26	2.28±1.0	1.91±0.26	2.59±0.78
Neurogenic medium 10 µM DAPT+10 µM 5-aza+CQ	1.80±0.20	1.91±0.28	2.28±0.56	1.57±0.78	1.29±0.81	2.91±0.61

BIOGRAPHY

Pattamon Muangchan was born in Nakhonphanom province, Thailand, on October 8th, 1990. She finished her high school from Nakhonphanom Wittayakhom School in Nakhonphanom. In 2012, she received a Bachelor Degree (BS.) in Agriculture from Khonkaen University, Khonkaen, Thailand. She then was interested in stem cell research, and decided to continue her Master of Science Degree (M.Sc.) study in Biotechnology at Suranaree University of Technology, Nakhon Ratchasima, Thailand. Her M.Sc. study was supported by SUT master degree grant and One Research One Grant (OROG) scholarship under the supervision of Assist. Prof. Dr. Parinya Noisa. Her M.Sc.