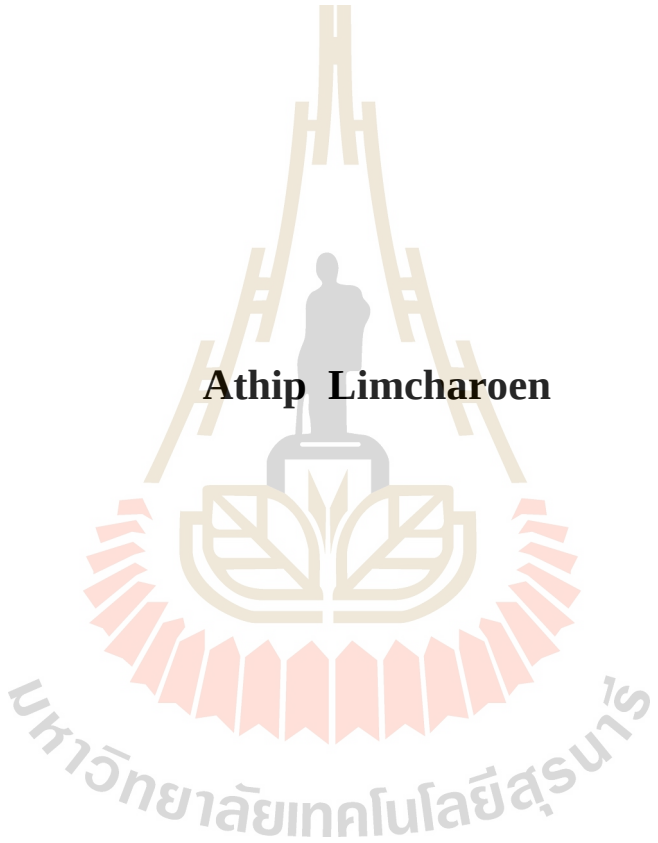
EFFECT OF 8 BROMO-CYCLIC GMP ON NEURAL TRANSDIFFERENTIATION OF HUMAN ADIPOSE STEM CELLS

Athip Limcharoen



A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science in Biotechnology

Suranaree University of Technology

Academic Year 2014

ผลของ 8 Bromo-cyclic GMP ต่อการเหนี่ยวนำเซลล์ต้นกำเนิดจากเนื้อเยื่อไขมันของมนุษย์เป็นเซลล์ระบบประสาท

นายอธิป หลิมเจริญ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

สาขาวิชาเทคโนโลยีชีวภาพ

มหาวิทยาลัยเทคโนโลยีสุรนารี

ปีการศึกษา 2557

EFFECT OF 8 BROMO-CYCLIC GMP ON NEURAL TRANSDIFFERENTIATION OF HUMAN ADIPOSE STEM CELLS

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

Thesis Examining Committee

(Assoc. Prof. Dr. Mariena Ketudat-Cairns)
Chairperson

(Assoc. Prof. Dr. Rangsun Parnpai)
Member (Thesis Advisor)

(Dr. Sanong Suksaweang)
Member

(Prof. Dr. Sukit Limpijumnong)
Vice Rector for Academic Affairs and Innovation

(Asst. Prof. Dr. Suwayd Ningsanond)
Dean of Institute of Agricultural Technology

อธิป หลิมเจริญ : ผลของ 8 Bromo-cyclic GMP ต่อการเหนี่ยวนำเซลล์ต้นกำเนิดจากเนื้อเยื่อไขมันของมนุษย์เป็นเซลล์ระบบประสาท (EFFECT OF 8 BROMO-CYCLIC GMP ON NEURAL TRANSDIFFERENTIATION OF HUMAN ADIPOSE STEM CELLS) อาจารย์ที่ปรึกษาวิทยานิพนธ์ : รองศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 121 หน้า.

การปลูกถ่ายเซลล์ต้นกำเนิดเป็นหนึ่งในวิธีการที่น่าจะมีประสิทธิภาพในการนำมาใช้รักษาโรคที่เกี่ยวข้องกับการเสื่อมของระบบประสาท แต่เนื่องจากข้อจำกัดของการนำเซลล์ต้นกำเนิดตัวอ่อนมนุษย์และเซลล์ตั้งต้นระบบประสาทมาใช้ในการรักษา เซลล์ต้นกำเนิดมีเซนไคม์ โดยเฉพาะจากเนื้อเยื่อไขมันจึงเป็นอีกทางเลือกหนึ่งที่มีศักยภาพ การเก็บเซลล์ต้นกำเนิดมีเซนไคม์จากเนื้อเยื่อไขมันเป็นวิธีการที่ง่าย มีเป็นจำนวนมากในร่างกาย และยังสามารถเลี้ยงในห้องปฏิบัติการได้เป็นระยะเวลาหลายเดือนอีกด้วย นอกจากนี้แล้วเซลล์เหล่านี้ยังสามารถเปลี่ยนแปลงไปเป็นเซลล์ในระบบประสาทได้เมื่ออยู่ภายใต้ภาวะการเหนี่ยวนำที่เหมาะสม ดังนั้นการหาวิธีที่มีประสิทธิภาพในการเหนี่ยวนำเซลล์เหล่านี้ให้กลายไปเป็นเซลล์ระบบประสาทจึงเป็นประโยชน์ต่อการวิจัยทางการแพทย์และอาจนำไปสู่การนำไปใช้ในทางคลินิกในอนาคต

วัตถุประสงค์การศึกษานี้คือ เพื่อหาผลของ 8 Bromo-cyclic GMP (8Br-cGMP) ต่อการเปลี่ยนแปลงเซลล์ต้นกำเนิดจากเนื้อเยื่อไขมันของมนุษย์ไปเป็นเซลล์ระบบประสาท ผลการทดลองพบว่าหลังจากทำการคัดแยกเซลล์ต้นกำเนิดจากเนื้อเยื่อไขมันของมนุษย์ เซลล์เหล่านั้นถูกนำไปเพาะเลี้ยงเพิ่มจำนวน และนำไปตรวจสอบคุณสมบัติของเซลล์ต้นกำเนิดมีเซนไคม์ด้วยวิธีการย้อมโปรตีนที่จับบนผิวเซลล์และการเหนี่ยวนำไปเป็นเซลล์ชนิดต่างๆ เซลล์ต้นกำเนิดจากเนื้อเยื่อไขมันของมนุษย์ที่คัดแยกได้มีการแสดงออกโปรตีน CD73 CD90 CD105 และ vimentin แต่ไม่แสดงออกโปรตีน CD34 และ CD45 นอกจากนั้นเซลล์ต้นกำเนิดจากเนื้อเยื่อไขมันมนุษย์ยังสามารถเปลี่ยนแปลงไปเป็นเซลล์กระดูก เซลล์กระดูกอ่อน และเซลล์ไขมัน ซึ่งสามารถยืนยันได้ว่าเซลล์ต้นกำเนิดจากเนื้อเยื่อไขมันมนุษย์ที่คัดแยกมาในการวิจัยครั้งนี้มีคุณสมบัติของเซลล์ต้นกำเนิดมีเซนไคม์ หลังจากนั้น จึงทำการตรวจสอบฟีโนไทป์และการแสดงออกของยีนในเซลล์ต้นกำเนิดจากเนื้อเยื่อไขมันมนุษย์ที่เหนี่ยวนำด้วย 8Br-cGMP ที่ความเข้มข้น 0μM 10μM และ100μM เพื่อหาความเข้มข้นที่เหมาะสมในการเหนี่ยวนำให้เปลี่ยนแปลงไปเป็นเซลล์ประสาท ผลการศึกษาพบว่าหนึ่งสัปดาห์หลังทำการเหนี่ยวนำ ประชากรเซลล์ส่วนใหญ่ที่เติม 8Br-cGMP ความเข้มข้น 10μM มีรูปร่างเรียว ยาว มีขนาดเล็กลง และพบเซลล์ที่มีลักษณะคล้ายเซลล์ประสาทที่มี bipolar และ multipolar ซึ่งมีแขนงคล้ายแอกซอนยื่นออกมาจากตัวเซลล์อีกด้วย จำนวนสัดส่วนเซลล์ที่แสดงออก Nestin Sox2 TUJ1 และ NF-L หลังการเหนี่ยวนำด้วย 10μM 8Br-cGMP สูงกว่าความเข้มข้นอื่นอย่างมีนัยยะสำคัญทางสถิติ ($p<0.001$) ผลการตรวจสอบการแสดงออกของยีนในเซลล์

ต้นกำเนิดจากเนื้อเยื่อไขมันมนุษย์หลังการเหนี่ยวนำด้วย 10μM 8Br-cGMP มีระดับของยีนที่จำเพาะต่อเซลล์ที่จะพัฒนาไปเป็นเซลล์นิวรอน (*MASH1 GAP43 TUJ1 NF-L* และ *MAP2*) สูงกว่ากลุ่มความเข้มข้นอื่นๆอย่างมีนัยยะสำคัญทางสถิติ ในการศึกษา neurite outgrowth พบว่าเซลล์หลังการเหนี่ยวนำด้วย 10μM 8Br-cGMP มีสัดส่วนประชากรเซลล์ที่แสดงออกของโปรตีน TUJ1 ร่วมกับมี neutire สูงที่สุดอย่างมีนัยยะสำคัญทางสถิติ (73.10%±7.40%, $p<0.001$) และ มีการแสดงออกของยีน *GAP43* ที่สูงขึ้นภายในเซลล์เหล่านั้น ซึ่งมีบทบาทในการพัฒนาของ neurite และการสร้าง synapse ของเซลล์ประสาท ในการนำเซลล์ต้นกำเนิดจากเนื้อเยื่อไขมันมนุษย์จากการเหนี่ยวนำด้วย 10μM 8Br-cGMP ให้เปลี่ยนแปลงไปเป็นเซลล์ประสาทนิวรอนและค้ำจุนเต็มวัย พบว่าเซลล์เหล่านั้นสามารถกลายไปเป็นเซลล์ประสาทนิวรอนและค้ำจุนเต็มวัยได้ ซึ่งยืนยันด้วยการตรวจสอบการแสดงออกของยีนด้วยวิธี RT-qPCR และการย้อมเซลล์ด้วยวิธี Immunocytochemistry จากผลการทดลองสามารถสรุปได้ว่า เซลล์ต้นกำเนิดจากเนื้อเยื่อไขมันมนุษย์ที่ถูกเหนี่ยวนำด้วย 10μM 8Br-cGMP สามารถเปลี่ยนแปลงไปเป็นเซลล์ตั้งต้นของเซลล์ประสาทนิวรอน และยังสามารถเปลี่ยนแปลงไปเป็นเซลล์ประสาทนิวรอนและเซลล์ค้ำจุนเต็มวัยภายใต้ภาวะที่เหมาะสมได้อีกด้วย

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2557

ลายมือชื่อนักศึกษา _____
ลายมือชื่ออาจารย์ที่ปรึกษา _____

ATHIP LIMCHAROEN : EFFECT OF 8 BROMO-CYCLIC GMP ON NEURAL TRANSDIFFERENTIATION OF HUMAN ADIPOSE STEM CELLS. THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 121 PP.

ADIPOSE STEM CELLS/NEURAL STEM CELLS/NEURAL DIFFERENTIATION/8 BROMO-CYCLIC GMP/NO-CGMP SINGLAING PATHWAY

Stem cell transplantation is a promising tool in neurodegenerative diseases treatment. Due to limitations to utilize human embryonic stem cells (ESCs) and neural stem cells (NSCs), the mesenchymal stem cells (MSCs), especially from adipose tissue can serve as a potential alternative. They are easily accessible, abundantly available throughout the body and can be cultured for several months *in vitro*. Furthermore, they are also able to differentiate toward cells in neuroectodermal lineage under appropriate conditions. Therefore, finding the most efficient way to differentiate these cells would be beneficial for medical research and will be used in clinical applications.

The aim of this study was to find the effect of 8 Bromo-cyclic GMP (8Br-cGMP) on neural differentiation of human adipose stem cells (hASCs). The results found that after hASCs were isolated from the adipose tissue, they were expanded and characterized by immunophenotypical and multipotency to be several cell types. The isolated hASCs were positive for CD73, CD90, CD105 and Vimentin and were negative for CD34 and CD45. Additionally, hASCs could differentiate toward

osteocytes, chondrocytes and adipocytes implying that hASCs isolated from adipose tissue displayed the mesenchymal stem cell properties. To optimize 8Br-cGMP concentration, hASCs under different 8Br-cGMP concentration (0μM, 10μM, 100μM) were investigated using phenotypical and gene expression analyses. The results showed that after one-week induction, the majority of neural induced hASCs (NI-hASCs) under 10μM 8Br-cGMP condition appears as smaller, elongated bi- or multipolar cells with primary and secondary processes similar to axon. The proportion of positive cells for Nestin, Sox2, TUJ1 and NF-L was significantly ($p<0.001$) higher than other conditions. Gene expression analysis revealed that NI-hASCs under 10μM 8Br-cGMP condition had significant higher level of genes which is specific to cells differentiated to be neuron cells (*MASH1*, *GAP43*, *TUJ1*, *NF-L*, *MAP2*). NI-hASCs under 10μM 8Br-cGMP condition displayed significant highest of TUJ1 protein population with neurite (73.10%±7.40%, $p<0.001$) and significant higher expression level of *GAP43* gene, which is involved in neurite outgrowth and synaptic formation. Upon further differentiation into mature neuron and glial cells, NI-hASCs from 10μM 8Br-cGMP condition could differentiate toward neuronal and glia cells as confirmed by RT-qPCR and immunocytochemistry results. This study concluded that hASCs after induction with 10μM 8Br-cGMP could be differentiated to be neuronal progenitor cells and also differentiated to be neuronal and glial cells under suitable conditions.

School of Biotechnology

Academic Year 2014

Student's Signature Athip Lin

Advisor's Signature

ACKNOWLEDGMENTS

Thank you my wonderful counselor and personal savior, Jesus Christ. My journey with You at Suranaree University of Technology has been incredibly precious as You have taught me valuable lessons through Your promise: “And we know that in all things God works for the good of those who love Him, who have been called according to His purpose.” [Romans 8:28, NIV]. Thank you Father God and Holy Spirit for Your comfort and unconditional love. You always reminded me of who I am in You and never give up on me through all those mistakes I have caused and always be by my side through all hardships I have faced.

I would like to express my deepest gratitude to my supervisor, Assoc. Prof. Dr. Rangsun Parnpai, for his guidance and the scholarship he granted me even though my background was unrelated to Biotechnology. This opportunity he gave has been strongly significance for my experience in science research. My deep appreciation is also to Assoc. Prof. Dr. Mariena Ketudat-Cairns for her advices on the experiments, her warm wishes and encouragement, and her hospitality on the banana cake she usually baked for me. You are my role model and the kind of teacher that I want to be like. I am profoundly indebted to my thesis committee, Dr. Sanong Suksaweang, for his valuable comments on my thesis and the experiments. I am very thankful for all the lecturers of School of Biotechnology who have taught me how to obtain and contribute strong scientific knowledge.

I would like to express my sincere gratitude to all Embryo and Stem Cell Research Center members for their support and advices. To be specific, thank you Ms.

Sujitra Khampang, Mr. Wachira Panta, Mr. Prapot Thiantisong and Ms. Suthatip Jirundon for their helps on figure layout. Thank you Mr. Ton Yoi sungnern and Ms. Darawan Ruamkusol for their helps on qPCR analysis. The neurite outgrowth analysis couldn't have been done without the help of Dr. Wanchana Seubwai and Ms. Suphachira Srijangwang from Research Instrument Center, Khonkean University. The statistical analyses of this study were from the help of Dr. Ashit Kumar Paul and Ms. Natnicha Nisaisuk. Special thanks go to Ms. Kanokwan Srirattana, Mr. Sumeth Imsoonthornruksa, Dr. Anawat Sangmalee and Dr. Yuan-Yuan Liang for being such a wonderful academic and professional mentor. It was a really valuable experience working with you. I am thankful for support from decent friends: Mr. Theesit Juanpanich, Ms. Tayita Suttirojpatana, Ms. Kanchana Panyawai and Ms. Nucharin Sripanya. I also appreciate all the help and advice that Ms. Prangkhow Prukhett has given me throughout my study.

I own a million thanks to Pastor Virat and Sopa Prommanok and brothers and sisters at the Vision of Christ Church, Nakhon Ratchasima for their prayer supports. You have been a big impact on my spiritual life to grow stronger in Christ, to walk a worship lifestyle, and to do my best to honor Him. May God bless you abundantly in return with His favor.

I am deeply indebted to my parent and my brother who always offer the best for my future with everything they could do. Thank you for understanding and taking care of me when I underwent the surgery during my study. Without their support and encouragement, none of this would have been possible.

Athip Limcharoen

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

8Br-cGMP	=	8 Bromo-cyclic GMP
DAPI	=	4',6-diamidino-2-phenylindole
ASCs	=	adipose stem cells
bFGF	=	basic fibroblast growth factor
BMP	=	bone morphogenetic protein
BHA	=	butylated hydroxyanisole
BMSCs	=	bone marrow stem cells
CD	=	cluster of differentiation
°C	=	degree Celsius
cGMP	=	cyclic GMP
d	=	day
DMSO	=	dimethyl sulfoxide
EGF	=	epidermal growth factor
ESCs	=	embryonic stem cells
FBS	=	fetal bovine serum
IBMX	=	3-isobutyl-1-methylxanthine
l	=	liter
LDL	=	low-density lipoprotein
MSCs	=	mesenchymal stem cells
µg	=	microgram

LIST OF ABBREVIATIONS (Continued)

µl	=	microliter
µm	=	micrometer (micron)
μm^2	=	micrometer square
µM	=	micromolar
ml	=	millilter
min	=	minute
ng	=	nanogram
NSCs	=	neural stem cells
NI-hASCs	=	neural-induced human adipose stem cells
NGF	=	nerve growth factor
NO	=	Nitric Oxide
PDGF	=	platelet derived growth factor
RA	=	retinoic acid
SCID	=	severe combined immunodeficiency

CHAPTER I

INTRODUCTION

1.1 Background

Neurodegenerative disease is caused by the progressive loss of structure or function of neurons. In most case, the disease is triggered for unknown reasons and progress continuously. Major diseases of neurodegenration are Alzheimer's disease (AD) and Parkinson's disease (PD). Additionally, not only AD and PD can be found among the nation more frequent with increasing lifespan, but stroke and traumatic brain injury are also a result of a decline in neuronal function. Furthermore, in chronic inflammatory CNS diseases, such as multiple sclerosis (MS), a loss function of neuroectodermal cells occurs as well. The prevalence of these diseases is commonly seen worldwide with increasing cases reported each year (National Institute of Neurological Disorders and Stroke, 2004). Therefore, a need for therapeutic approaches to restore the loss of neural cells and their physiological functions is urgently required.

Cell-based therapy for the neural regeneration offers a paradigm shift that may lead to alternative therapeutic solutions. Stem cells have been considered as a promising tool in cell-based therapy. Stem cell therapy aims to induce the neuro-restorative process that is essential to support recovery after a loss of function. Among many applications, neurological diseases are interesting potential target, since the adult nervous system has a limited regenerative capacity.

Stem cells are immature tissue precursor cells that are able to self-renew and differentiate into multiple cell lineages. Stem cells are defined by its capacity to generate more stem cells and differentiated daughters, rather than by generating itself and a differentiated daughter at each division, also known as asymmetric cell division (Morrison and Kimble, 2006). Stem cells from embryonic and somatic origins could be distinguished by their origin and their differentiation potential. Embryonic stem cell (ESCs) could be derived from the inner cell mass of early blastocyst embryo and could give rise to cell types of the three germ layers (Schuldiner et al, 2000). Somatic stem cells are isolated from fetal embryo (after gastrulation) or adult tissues. These cells are normally committed to only produce cell types that belong to the tissue which they originate from. At the present, Somatic stem cells have been isolated from many different organs or tissues, including bone marrow (Wolfe et al., 2008), brain (Reynolds and Weiss, 1992), adipose tissue (Fraser et al., 2008), dental pulp (Liu et al., 2009), blood (Domen and Weissman, 1999), epidermis (Gandarillas and Watt, 1997) and skeletal muscle (Seale and Rudnicki, 2000), umbilical cord blood (Phuc et al., 2011) and Wharton's jelly (Wang et al., 2004).

Stem cells are believed to maintain tissue homeostasis by replacing damaged cells. However, while a great number of organs or tissues contains somatic stem cells, it appears that these cells are mostly quiescent or rarely active and are unable to efficiently repair the damaged tissue. This is especially true for neural stem cells (NSCs) which are found in the sub-ependymal layer of the 5 ventricular zone and the dentate gyrus of the hippocampus in adult mammals (Gage, 2002), within a cellular niche (Rossi and Cattaneo, 2002). Even though those cells are able to proliferate and differentiate in vitro into neurons, astrocytes and oligodendrocytes (McKay, 1997),

they do not guarantee a successful brain homeostasis after transplantation. In recent years, many researchers have focused on the therapeutic potential of stem cells for damaged central nervous system (McKay et al, 1997; Gage et al, 2000; Isacson et al, 2003; Dietrich and Kampermann, 2006).

Among many types of cells that are proposed to be a candidate for neurodegenerative treatment, human ESCs have seemed to be the most powerful tool for cell therapy in animal models, but several challenges need to be resolved before ESCs application in humans. ESCs are genetically and epigenetically unstable, and consequently human ESCs lines can vary substantially with regard to differentiation potential. Moreover, most differentiation procedures sequentially require growth factor addition until the desired phenotype is obtained. Undifferentiated ESC in such procedure can cause teratoma formation in the transplant recipients. Another risk factor for tumor formation is the protracted proliferation and differentiation times which leads to continuous cell proliferation after transplantation, resulting in neural overgrowth. These limitations of ESCs emphasize the need for more basic research of their control proliferation and differentiation before use in clinical applications.

Neural stem cells (NSCs) are alternative source that seems to rise up patients' hope due to their characteristics. The unlimited differentiation potential *in vitro* and a potential of producing all kinds of brain-specific cell types make them as a feasible candidate in therapy (Bain et al, 1995; Lee et al, 2000). However, due to their accessible difficulty and limited source of cells, very small numbers of donors would not represent a sufficient pool to allow immunological matches. Approximately only 70% of recipients could match immunologically to those donor cells. To overcome this challenge, it would be impossible to establish a NSCs bank to approach this level

of matching ability in the business since it is extremely laborious, expensive and time-consuming. However, some NSCs are likely to have a weak immune rejection potential, and when implanted directly into the brain without having to get through the blood brain barrier, the transplanted NSCs might evade the immune system altogether (Schwartz, 2006).

Thus, other sources of adult stem cells have been investigated and mesenchymal stem cells (MSCs) have been recently brought up against those cells because of little ethical controversy surrounding them. These cells hold many features which are easily collect, abundant availability, extensively expansion *in vitro*, and the ability to repair the tissue by differentiating itself to replace injured cells or by creating an environment favorable for the tissue repair by endogenous cells (Izadpanah et al, 2005). Moreover, increasing evidence on the neuroectodermal lineage commitment of mesenchymal stem cells (MSCs) has also raised a good appeal of MSCs in cell-based therapies (Deng et al, 2001; Jiang et al, 2002; Sanchez-Ramos et al, 2000, Woodbury et al, 2002). Among many sources of MSCs, adipose tissue seems to be promising source of MSCs since they are available in large quantities (~2 $x10^8$ nucleated cells per 100 ml of lipoaspirate, yielding around 4 x 10^6 ASCs) and are easily isolated and can be cultured for several months *in vitro* with low levels of senescence (Zuk et al, 2001; Zuk et al, 2002). Investigating the utilization and application of these cells would be beneficial in cell-based therapy.

Nitric oxide (NO) is a short lived diatomic free radical species that is synthesized by nitric oxide synthases (NOS). At low nanomolar concentrations of NO, activation of soluble guanylyl cyclase (sGC: the major NO receptor) triggers intracellular cyclic GMP level elevation (Arnold et al, 1977; Katsuki et al, 1977). This

signaling pathway, also known as NO-cGMP signaling pathway, is involved in diverse physiological processes, such as smooth muscle relaxation, neurotransmission, blood pressure regulation, inhibition of platelet aggregation and immunomodulation. Its roles at cellular level are to regulate cell growth, survival, differentiation, proliferation, migration, axon guidance and many other processes through a variety of downstream signaling cascades depending on cell type specific regulation. cGMP, as the mediator, helps support NO regulate cell survival, differentiation and neuroprotection (Thippeswamy and Morris, 1997, 2001; Ciani et al, 2002). Nitric oxide (NO) donor, 8 bromo cyclic GMP (8Br-cGMP), has been used in neural differentiation of many types of cell, but the evidence of applying this agent in hMSCs is rarely seen. The effect of this NO donor on nearal differentiation of hASCs remained questionable. In this study, the effect of 8Br-cGMP was investigated whether they could differentiate hASCs into neural-like cells that hold both of neural phenotypic and genotypic profiles.

1.2 Research objectives

1.2.1 To isolate and characterize hASCs from human adipose tissue *in vitro*

1.2.2 To identify the effect of 8Br-cGMP on neural differentiation of hASCs *in vitro*

1.2.3 To characterize neural-like progenitor derived from hASCs *in vitro* using immunocytochemistry, RT-qPCR , neurite outgrowth and multipotency analyses

1.3 Research hypotheses

1.3.1 Under defined culture system, hASCs could be isolated and expanded from human adipose tissue.

1.3.2 8Br-cGMP could efficiently differentiate hASCs intro neural-like cells *in vitro***.**

1.3.3 Neural-induced hASCs (NI-hASCs) displayed neural phenotypic and gene expression profiles. They could further differentiate toward mature neurons and glia cells.

1.4 Scope of the study

1.4.1 Optimal culture condition of hASCs isolated from human adipose tissue was assessed by different % fetal bovine serum at early (P2) and intermediate (P5) passage. Standard MSCs characterization methods including colony forming units-fibroblast (CFU-F), expression profile of surface antigens (CD45, CD73, CD90 and CD105) multipotent property were performed to verified hASCs as hMSCs.

1.4.2 hASCs was transdifferentiate into neural-like cells. Effects of 8Br-cGMP on neural transdifferentiation were assessed by morphology, gene expression and immunocytochemistry analyses.

1.5 References

Aggarwal, S., and Pittenger, M.F. (2005). Human mesenchymal stem cells modulate allogeneic immune cell responses. **Blood**, *105*(4): 1815-1822.

Ami, D., Neri, T., Natalello, A., Mereghetti, P., Doglia, S.M., Zanoni, M., Zuccotti,

M., Garagna, S., and Redi, C.A. (2008). Embryonic stem cell differentiation studied by ft-ir spectroscopy. **Biochimica et Biophysica Acta (BBA) - Molecular Cell Research**, *1783*(1): 98-106.

Arnold, W.P., Mittal, C.K., Katsuki, S. and Murad, F. (1977). Nitric oxide activates guanylate cyclase and increases guanosine 3′-5′-cyclic monophosphate levels in various tissue preparations. **Proc Natl Acad Sci** 74:3203–3207.

Bain, G., Kitchens, D., Yao, M., Huettner, J.E., Gottlieb, D.I. (1995). Embryonic Stem Cells Express Neuronal Properties in Vitro. **Developmental Biology** 168:342-357.

Bieback, K., Hecker, A., Kocaömer, A., Lannert, H., Schallmoser, K., Strunk, D., and Klüter, H. (2009). Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow. **Stem cells**, *27*(9): 2331-2341.

Chan, J.W., and Lieu, D.K. (2009). Label-free biochemical characterization of stem cells using vibrational spectroscopy. **Journal of Biophotonics**, *2*(11): 656-668.

Chao, K.C., Chao, K.F., Fu, Y.S., and Liu, S.H. (2008). Islet-like clusters derived from mesenchymal stem cells in wharton's jelly of the human umbilical cord for transplantation to control type 1 diabetes. **PLoS ONE**, *3*(1): e1451.

Ciani, E., Guidi, S., Della Valle, G., Perini, G., Bartesaghi, R., Contestabile, A. (2002). Nitric oxide protects neuroblastoma cells from apoptosis induced by serum deprivation through cAMP-response element-binding protein (CREB) activation. **J. Biol. Chem.** 277:49896–49902.

Deng, W., Obrocka, M., Fischer, I., Prockop, D.J. (2001). In Vitro Differentiation of Human Marrow Stromal Cells into Early Progenitors of Neural Cells by Conditions That Increase Intracellular Cyclic AMP. **Biochemical and Biophysical Research Communications**. 282:148-152.

Dietrich, J. and Kempermann, G. (2006) Role of Endogenous Neural Stem Cells in Neurological Disease and Brain Repair, in: M. Bähr (Ed.), **Brain Repair** Springer US. pp. 191-220.

Domen, J. and Weissman, I.L. (1999) Self-renewal, differentiation or death: regulation and manipulation of hematopoietic stem cell fate. **Mol Med Today** 5: 201-208.

Fraser, J.K., Zhu, M., Wulur, I., and Alfonso, Z. (2008). Adipose-derived stem cells **Methods Mol Biol.** 449:59-67.

Fu, Y.S., Cheng, Y.C., Lin, M.Y.A., Cheng, H., Chu, P.M., Chou, S.C., Shih, Y.H., Ko, M.H., and Sung, M.S. (2006). Conversion of human umbilical cord mesenchymal stem cells in wharton's jelly to dopaminergic neurons in vitro: Potential therapeutic application for parkinsonism. **Stem cells**, 24: 115–124.

Gandarillas, A. and Watt, F.M. (1997). c-Myc promotes differentiation of human epidermal stem cells. **Genes Dev** 11:2869-82.

Gage, F.H. (2000). Mammalian Neural Stem Cells. **Science**. 287:1433-1438. DOI: 10.1126/science.287.5457.1433.

Gage, F.H. (2002). Neurogenesis in the adult brain. **J Neurosci** 22:612-13.

Hartmann, I., Hollweck, T., Haffner, S., Krebs, M., Meiser, B., Reichart, B., and Eissner, G. (2010). Umbilical cord tissue-derived mesenchymal stem cells grow best under gmp-compliant culture conditions and maintain their phenotypic and functional properties. **Journal of Immunological Methods** 363(1): 80-89.

Isacson, O., Bjorklund, L.M., Schumacher, J.M. (2003). Toward full restoration of synaptic and terminal function of the dopaminergic system in Parkinson's disease by stem cells. **Annals of Neurology** 53:S135-S148. DOI: 10.1002/ana.10482.

Izadpanah, R., Joswig, T., Tsien, F. et al. (2005) Characterization of multipotent mesenchymalstem cells from the bone marrow of rhesus macaques. **Stem Cells Dev** 14(4):440-51.

Jiang, Y., Jahagirdar, B.N., Reinhardt, R.L., Schwartz, R.E., Keene, C.D., Ortiz-Gonzalez, X.R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M., Du, J., Aldrich, S., Lisberg, A., Low, W.C., Largaespada, D.A., Verfaillie, C.M. (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. **Nature**. 418:41-49.

Jung, J., Moon, N., Ahn, J.-Y., Oh, E.-J., Kim, M., Cho, C.-S., Shin, J.-C., and Oh, I.-H. (2009). Mesenchymal stromal cells expanded in human allogenic cord blood serum display higher self-renewal and enhanced osteogenic potential. **Stem Cells and Development**, 18(4): 559-572.

Katsuki, S., Arnold, W., Mittal, C. and Murad, F. (1977). Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. **J Cyclic Nucleotide Res** 3:23–35.

Lee, S.-H., Lumelsky, N., Studer, L., Auerbach, J.M., McKay, R.D. (2000) Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. **Nature Biotechnology** 18:675-679.

Liu, H., Gronthos, S., and Shi, S. (2009). Dental pulp stem cells. In R. Lanza & I. Klimanskaya (Eds.), **Essential Stem Cell Method** (1 ed., pp. 73-86). Oxford:

Elsevier Inc.

Ma, H.-Y., Yao, L., Yu, Y.-q., Li, L., Ma, L., Wei, W.-j., Lu, X.-m., Du, L.-l., and Jin, Y.-n. (2012). An effective and safe supplement for stem cells expansion ex vivo : Cord blood serum. **Cell Transplantation** 21(5): 857-869.

Majore, I., Moretti, P., Stahl, F., Hass, R., and Kasper, C. (2011). Growth and differentiation properties of mesenchymal stromal cell populations derived from whole human umbilical cord. **Stem Cell Reviews and Reports** *7*(1): 17-31.

McKay, R. (1997). Stem Cells in the Central Nervous System. **Science**. 276:66-71. DOI: 10.1126/science.276.5309.66.

Meuleman, N., Tondreau, T., Delforge, A., Dejeneffe, M., Massy, M., Libertalis, M., Bron, D., and Lagneaux, L. (2006). Human marrow mesenchymal stem cell culture: Serum-free medium allows better expansion than classical α-mem medium. **European Journal of Haematology** 76(4): 309-316.

Mitchell, K.E., Weiss, M.L., Mitchell, B.M., Martin, P., Davis, D., Morales, L., Helwig, B., Beerenstrauch, M., Abou-Easa, K., Hildreth, T., and Troyer, D. (2003). Matrix cells from wharton's jelly form neurons and glia. **Stem cells** 21(1): 50-60.

Morrison, S.J. and Kimble, J. (2006) Asymmetric and symmetric stem-cell divisions in development and cancer. **Nature** 441: 1068-74.

National Institute of Neurological Disorders and Stroke. (2004). Parkinson’s disease: challenges, progresses and promises. **National Institute of Health** 5(5585):1-41.

Phadnis, S., Joglekar, M., Venkateshan, V., Ghaskadbi, S., Hardikar, A., and Bhonde,

R. (2006). Human umbilical cord blood serum promotes growth, proliferation, as well as differentiation of human bone marrow-derived progenitor cells. **In Vitro Cellular & Developmental Biology - Animal** 42(10): 283-286.

Phuc, P., Nhung, T., Loan, D., Chung, D., and Ngoc, P. (2011). Differentiating of banked human umbilical cord blood-derived mesenchymal stem cells into insulin-secreting cells. **In Vitro Cellular & Developmental Biology - Animal** 47(1): 54-63.

Reynolds, B.A. and Weiss, S. (1992). Generation of neuron and astrocytes from isolated cells of the adult mammalian central nervous system. **Science** 255: 1707-10.

Rossi, F. and Cattaneo, E. (2002). NSC therapy for neurological diseases: dreams and reality. **Nat Rev** 3:401-408.

Sanchez-Ramos, J., Song, S., Cardozo-Pelaez, F., Hazzi, C., Stedeford, T., Willing, A., Freeman, T.B., Saporta, S., Janssen, W., Patel, N., Cooper, D.R., Sanberg, P.R. (2000). Adult Bone Marrow Stromal Cells Differentiate into Neural Cells in Vitro. **Experimental Neurology**. 164:247-256.

Schuldiner, M., Yanuka, O., Itskovitz-Eldor, J. et al. (2000) Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. **Proc Natl Acad Sci USA** 97: 11307-12.

Schwartz, PH. (2006). The potential of stem cells for therapies for neurological disease. **Exp Rev Neurotherapeutics** 6(2):153-61.

Seale, P. and Rudnicki, M.A. (2000). A new look at the origin, function, and “stem-cell” status of muscle satellite cells. **Dev Biol** 218:115-24.

Shetty, P., Bharucha, K., and Tanavde, V. (2007). Human umbilical cord blood serum can replace fetal bovine serum in the culture of mesenchymal stem cells. **Cell**

Biology International 31(3): 293-298.

Stute, N., Holtz, K., Bubenheim, M., Lange, C., Blake, F., and Zander, A.R. (2004). Autologous serum for isolation and expansion of human mesenchymal stem cells for clinical use. **Experimental Hematology** 32(12): 1212-1225.

Thippeswamy, T., Morris, R., (1997). Cyclic guanosine 3',5'-monophosphate-mediated neuroprotection by nitric oxide in dissociated cultures of rat dorsal root ganglion neurones. **Brain Res.** 774:116–122.

Thippeswamy, T., Morris, R., (2001). Evidence that nitric oxide-induced synthesis of cGMP occurs in a paracrine but not an autocrine fashion and that the site of its release can be regulated: studies in dorsal root ganglia in vivo and in vitro. **Nitric Oxide** 5:105–115.

Troyer, D.L., and Weiss, M.L. (2008). Concise review: Wharton's jelly-derived cells are a primitive stromal cell population. **Stem cells** 26(3): 591-599.

Wang, H.-S., Shyu, J.-F., Shen, W.-S., Hsu, H.-C., Chi, T.-C., Chen, C.-P., Huang, S.-W., Shyr, Y.-M., Tang, K.-T., and Chen, T.-H. (2011). Transplantation of insulin-producing cells derived from umbilical cord stromal mesenchymal stem cells to treat nod mice. **Cell Transplantation** 20(3): 455-466.

Wang, H.S., Hung, S.C., Peng, S.T., Huang, C.C., Wei, H.M., Guo, Y.J., Fu, Y.S., Lai, M.C., and Chen, C.C. (2004). Mesenchymal stem cells in the wharton's jelly of the human umbilical cord. **Stem cells** 22(7):1330-1337.

Wolfe, M., Pochampally, R., Swaney, W., and Reger, R.L. (2008). Isolation and culture of bone marrow-derived human multipotent stromal cells (hmscs) **Methods Mol Biol.** 449:3-25.

Woodbury, D., Reynolds, K., Black, I.B. (2002). Adult bone marrow stromal stem

cells express germline, ectodermal, endodermal, and mesodermal genes prior to neurogenesis. **Journal of Neuroscience Research.** 69:908-917.

Wu, L.-F., Wang, N.-N., Liu, Y.-S., and Wei, X. (2009). Differentiation of wharton's jelly primitive stromal cells into insulin-producing cells in comparison with bone marrow mesenchymal stem cells. **Tissue Engineering Part A** 15(10): 2865-2873.

Zhang, H.-T., Fan, J., Cai, Y.-Q., Zhao, S.-J., Xue, S., Lin, J.-H., Jiang, X.-D., and Xu, R.-X. (2010). Human wharton's jelly cells can be induced to differentiate into growth factor-secreting oligodendrocyte progenitor-like cells. **Differentiation** 79(1):15-20.

Zhou, C., Yang, B., Tian, Y., Jiao, H., Zheng, W., Wang, J., and Guan, F. (2011). Immunomodulatory effect of human umbilical cord wharton’s jelly-derived mesenchymal stem cells on lymphocytes. **Cellular Immunology** 272(1):33-38.

Zuk, P.A., Zhu, M., Mizuno, H., et al. (2001). Multilineage cells from human adipose tissue: implication for cell-based therapies. **Tissue Eng** 7:211-28.

Zuk, P.A., Zhu, M., Ashjian, P., et al. (2002). Human adipose tissue is a source of multipotent stem cells. **Mol Biol Cell** 13:4279-95.

CHAPTER II

REVIEW OF LITERATURES

Neurodegenerative disease is caused by the progressive loss of structure of function of neurons. In most case, the disease is triggered for unknown reasons and progress continuously. Major diseases of neurodegenration are Alzheimer's disease (AD) and Parkinson's disease (PD). Additionally, not only AD and PD can be found among the nation more frequent with increasing lifespan, but stroke and traumatic brain injury are also a result of a decline in neuronal function. Furthermore, in chronic inflammatory CNS diseases, such as multiple sclerosis (MS), a loss function of neuroectodermal cells occurs as well. The prevalence of these diseases is commonly seen worldwide with increasing cases reported each year (National Institute of Neurological Disorders and Stroke, 2004). Therefore, a need for therapeutic approaches to restore the loss of neural cells and their physiological functions is urgently required.

Cell-based therapy for the neural regeneration offers a paradigm shift that may lead to alternative therapeutic solutions. Stem cells have been considered as a promising tool in cell-based therapy. Stem cells are immature tissue precursor cells that are able to self-renew and differentiate into multiple cell lineages. In recent years, many researchers have focused on the therapeutic potential therapeutic of stem cells for damaged central nervous system (McKay et al, 1997; Gage et al, 2000; Isacson et

al, 2003). Among many types of cells that are proposed to be a candidate for neurodegenerative treatment, human embryonic stem cells (hESCs) and neural stem cells (NSCs) seem to rise up the patient's hope due to their characteristics. The unlimited differentiation potential both *in vitro* and *in vivo* of hESCs and a potential of producing all kinds of brain-specific cell types of NSCs make them as a feasible candidate in the therapy (Bain et al, 1995; Lee et al, 2000). However, difficult accessible of NSCs in human combined with ethical issues, limited source of cells and potential problems of cell regulation of ESCs have limited their applications in clinical treatment. Adult stem cells have been recently brought up against those cells because of little ethical controversy surrounding them. Moreover, increasing evidence on the neuroectodermal lineage commitment of mesenchymal stem cells (MSCs) has also raised a good appeal of MSCs in cell-based therapies (Deng et al, 2001; Jiang et al, 2002; Sanchez-Ramos et al, 2000, Woodbury et al, 2002).

2.1 Human mesenchymal stem cells (hMSCs)

hMSCs was discovered in 1968 by Friedenstein and co-workers. They reported a small number of adherent cells obtained from rat bone marrow were observed in culture condition. They were heterogeneous in appearance and began to multiply rapidly. The morphology of those cells is uniformly spindle-shaped even after several passages. Furthermore, the hold the ability to differentiate into colonies that are similar to small deposits of bone or cartilage (Friedenstein et al, 1968). After this initial investigation, those cells were further investigated in the 1980s by two groups

of research team, Piersma and colleagues and Owen and his team, with similar findings (Piersma et al, 1985; Owen etl a., 1988).

The term mesenchymal stem cell was popularized later in the early 1990s by Caplan (Caplan, 1991), but was not referred as stem cells by some investigators when publishing pre-clinical (Pereira et al, 1995; Pereira et al, 1998) or clinical (Horwitz et al, 1999; Horwitz et al, 2001; Horwitz et al, 2002; Keating et al, 1998) studies. They were later labeled by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) which proposed a uniform nomenclature in two position statement papers in the mid 2000s (Dominici et al, 2006; Horwitz et al, 2005). They proposed that the plastic-adherent cells currently described as mesenchymal stem cells should be termed multipotent mesenchymal stromal cells, while the term mesenchymal stem cell should be reserved for a subset of these cells that demonstrate stem cell activity by clearly stated criteria. To define MSCs, some minimal criteria were suggested by the ISCT for in vitro demonstrations of long-term survival with self-renewal capacity and tissue repopulation with multilineage differentiation. Firstly, MSCs have been characterized by their plastic adherent growth and subsequent expansion under appropriate culture conditions. Secondly, they must express surface markers CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules. Thirdly, they must differentiate into osteoblasts, adipocytes and chondroblasts *in vitro* under appropriate culture conditions (Zuk et al, 2002).

MSCs are capable of differentiating into a large variety of specialized mesenchymal tissues including bone, cartilage, muscle, marrow stroma, tendon, ligament, fat and a range of other connective tissues (Brinchmann, 2008; Caplan and

Bruder, 2001; Pittenger et al, 1999). Furthermore, MSCs reside in various locations throughout the body, such as in bone marrow (Hermann et al, 2006), around blood vessels (as pericytes), in adipose tissue, skin, muscle and other locations (Caplan and Bruder, 2001; Pittenger et al, 1999). In addition to their potential to differentiate into cells of different lineages, MSCs have also been shown to possess some level of plasticity. Until recently, it was believed that tissue-specific stem cells only differentiated into mature phenotypes within their restricted lineages. This novel notion of stem cell plasticity is perhaps not surprising, since within the mesenchymal cell lineages, plasticity of mature cells was proposed several decades ago by showing that chondrocytes could transdifferentiate into osteoblasts (Kahn and Simmons, 1977), and that adipocytes could switch their phenotype to that of osteoblasts (Bennett et al, 1991). In summary, the application of MSCs hold a great impact in cell based therapy by controlling the body's capacity to naturally remodel, repair, and rejuvenate various tissues.

2.2 Human adipose stem cells (hASCs)

Although MSCs derived from bone marrow continue have been applied in research and clinical investigation, there are drawbacks for its application. Harvesting bone marrow is a painful procedure with possible donor site morbidity as a result (Zuk, 2002). Secondly, although MSCs grow well under standard tissue culture conditions, ex vivo expansion is necessary due to relatively low numbers of MSCs present in the harvested marrow (Zuk, 2002). To overcome these drawbacks, adipose tissue has become a promising alternative since subcutaneous adipose depots are

easily accessible and abundant. Therefore, these benefits bring up adipose tissue as a potential adult stem cell reservoir for each individual.

Adipose tissue is comprised of adipocytes and heterogeneous cell populations surrounding adipocytes as a supporter. This composition is called the stromal vascular fraction (SVF) upon the isolation (Fig 1.1). ASCs reside as a part of SVF and they have the ability to differentiate into cells of several lineages such as adipocytes osteoblasts, chondrocytes, myocytes, endothelial cells, hematopoietic cells, hepatocytes and neuronal cells (Gimble and Guilak, 2003; Gimble et al, 2007; Huang et al, 2004; Planat-Benard et al, 2004; Safford et al, 2002; Seo et al, 2005; Timper et al, 2006; Winter et al, 2003; Zuk et al, 2001; Zuk et al, 2002). Additionally, the SVF contains cells from the microvasculature, such as vascular endothelial cells and their progenitors, vascular smooth muscle cells and also cells with hematopoietic progenitor activity (Daher et al, 2008; Fraser et al, 2006). Leukocytes are also present in the SVF in the parenchyma of adipose tissue (Daher et al, 2008). As ASCs are among other heterogeneous population, isolation technique is required to isolate relatively homogeneous cell populations expressing a stromal immunopenotype out of SVF.

Adipose tissue has been considered a metabolic reservoir for energy substrates in the forms of triglycerides and cholesterol as well as lipid-soluble vitamins storing, and later was found to be involved in the metabolism of sex steroids hormone (Rada et al, 2009). At the present, adipose tissue is known as stem cells' abundant source. Martin Rodbell was the first to successfully isolate mature adipocytes and progenitors from rat fat tissue (Rodbell, 1964). In his protocol, the tissue was minced into small fragments, digested at 37°C with type I collagenase, and the cellular components were separated by centrifugation. Following centrifugation, the supernatant contained the

mature adipocytes, which floated due to their high lipid content, and the pellet contained the SVF components, including the presumptive adipocyte progenitor cells in addition to cells of the hematopoietic lineages. The protocol to isolate human adipocyte progenitors was later modified by Van, Roncari, Deslex, Hauner and others (Deslex et al, 1987; Hauner et al, 1989; Van et al, 1976), who found that when the SVF components are cultured in the presence of inductive factors, the cells accumulated lipid vacuoles and expressed the adipogenic enzymes (Gimble and Guilak, 2003).

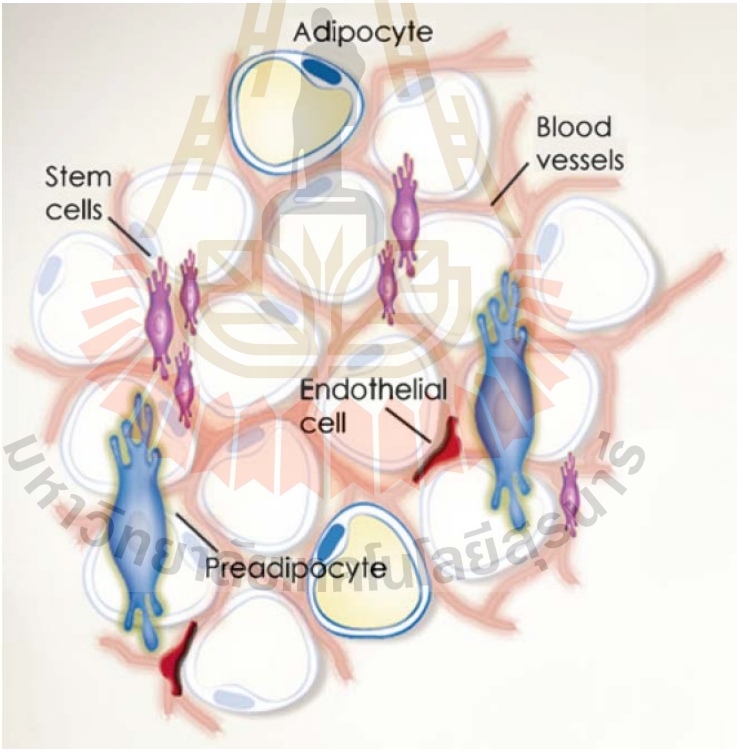


Figure 2.1 The component of adipose tissue modified from Wellen and Hotamisligil, 2003.

Katz and Zuk & his colleagues were the first two research teams to show that the SVF fraction isolated from human lipoaspirates in fact contained cells with multilineage potential and termed these cells processed lipoaspirate (PLA) cells (Katz et al, 1999; Zuk et al, 2001; Zuk et al, 2002). Since then, several groups working independently have developed and refined procedures of isolating and characterizing adipose stem cells (Boquest et al, 2006; Gimble and Guilak, 2003; Gronthos et al, 2001; Katz et al, 2001; Katz et al, 2005).

Many terms have been used to describe the plastic adherent cell population isolated from collagenase digests of adipose tissue, such as lipoblast, pericyte, preadipocyte, processed lipoaspirate (PLA) cells, adipose stem/stromal cells (ASCs), adipose-derived adult stem(ADAS) cells, adipose-derived adult stromal cells, adipose-derived stromal cells (ADSCs), adipose stromal cells (ASCs), multipotent adipose-derived stem cells (hMADS) and adipose mesenchymal stem cells (AdMSCs). To unify this term, the International Fat Applied Technology Society (IFATS) proposed a standardized nomenclature during the 2004 IFATS meeting in Pittsburgh by adopting the term adipose-derived stem cells or adipose stem cells (ASCs) to identify the isolated, plastic-adherent, multipotent cell population (Daher et al, 2008). As for MSCs, the use of the term stem cell may be questioned and thus it is widely accepted that investigators may use the acronym ASCs to mean adipose-derived stromal cells (Gimble et al, 2007).

2.3 Characterization of human adipose stem cells

Adipose stem cells are commonly characterized by the same methods used for MSCs derived from bone marrow which are by their immunophenotype in the

undifferentiated state and by their differentiation potential towards the adipogenic, osteogenic, and chondrogenic lineages in the presence of lineage-specific induction factors (Gimble and Guilak, 2003; Pittenger et al, 1999).

2.3.1 ASCs immunophenoytpe

Unlike embryonic stem cells (Thomson et al, 1998), undifferentiated ASCs cannot be identified by a single surface marker or a few surface markers. Many markers are needed to identify this population. Many reports have been published suggesting markers for identifying the mesenchymal stem cell population, such as STRO-1 (Simmons and Torok-Storb, 1991), CD271 (Quirici et al, 2002), STRO-3 (Gronthos et al, 2007) and MSCA-$1^{+}$$CD56^{+}$ (Battula et al, 2009).

BMSCs and ASCs display very resemble surface marker expression patterns for MSCs (De Ugarte et al, 2003; Garcia-Castro et al, 2008; Strem et al, 2005; Tapp et al, 2009). The pattern is under the criteria set by ISCT for MSCs (Dominici et al, 2006). However, some minor difference existed between BMSCs and AScs. First of all, BMSCs lack expression of CD49d, which is strongly expressed on ASCs, while ASCs lack expression of CD106, which is expressed on BMSCs (Strem et al, 2005). This pattern is surprising because CD106 is the cognate receptor of CD49d and both molecules are involved in hematopoietic stem and progenitor cell homing to and mobilization from the bone marrow (De Ugarte et al, 2003; Simmons et al, 1992; Sudhoff and Sohngen, 2002).

Other CD markers consistently reported highly expressed in ASCs are CD13, CD29, CD44, CD73, CD90, CD105, CD166 and MHC I. In contrast, markers of the hematopoietic and angiogenic lineages, such as CD31, CD45 and CD133, have been reported to show low or lack of expression on ASCs. Moreover, MHC II has also

been found to be absent on ASCs. Moderate expression (>50%) has been reported for markers CD9, CD34, CD49d, CD106, CD146 and STRO-1. The expression of of STRO-1 is particularly controversial because it could be detected by Zuk and co-workers (Zuk et al, 2002) while Gronthos and co-workers reported absence of this marker in ASC cultures (Gronthos et al, 2001). Similar controversies are seen for CD34 and CD106, where Gronthos and co-workers (Gronthos et al, 2001) reported detection of these markers in ASCs, while and co-workers (Zuk et al, 2002) and Katz and co-workers (Katz et al, 2005) reported their absence or expression on a small population of cells. Evidences have found that the expression pattern has changed through passages. For example, the expression level of CD29, CD44, CD73, CD90 and CD166 rise up from the SVF to passage 2 (P2) where they stabilize at a high expression level (Mitchell et al, 2006). In contrast, the expression level of CD11, CD14, CD34 and CD45 (hematopoietic cell markers), expressed on cells in the SVF and decrease with increasing passage number overtime, suggesting that adherence to plastic and subsequent expansion will select for a relatively homogeneous cell population compared with the SVF (McIntosh et al, 2006; Tapp et al, 2009). Moreover, ASCs expression profile reported elsewhere were not consistently and were unique for each line of ASCs, similarly to that of BMSCs (Gronthos et al, 2001). Due to the difference of marker antibodies source, sensitivity, proliferative stages, donor heterogeneity, it might be impossible to unify the protocols of surface marker characterization. However, some minimal criteria for characterization of ASCs as suggested by ISCT could be useful to identify these cells.

2.3.2 ASCs Multipotency

2.3.2.1 Adipogenic differentiation

Adipocytes are derived from multipotent stem cell population residing in the vascular stroma of adipose tissue. They stay silent as adipogenic progenitors but do not yet express markers of terminal differentiation. Subsequent differentiation occurs by activation of several transcription factors resulting in then adipocyte phenotype factors secreted by cells within the stromal vascular population and/or adipocytes undergoing hypertrophy. Factors relating to this phenomenal have been identified, but the molecular mechanisms remain undetermined still (Otto and Lane, 2005).

With suitable differentiation condition, hASCs could differentiate toward adipogenic lineage. The first adipogenic induction media reported was a chemically defined serum-free (SF) media containing insulin or IGF-1, triiodothyronine and transferrin (Deslex et al, 1987), with serum only used briefly for cell attachment. The addition of isobutylmethylxanthine (IBMX; a phosphodiesterase inhibitor resulting in elevated cyclic AMP levels), hydrocortisone or dexamethasone (glucocorticoid receptor agonist), indomethacin or thiazolidinedione (PPAR γ ligand), pantothenate, biotin and serum has been applied since then to enhance the differentiation efficiency (Hauner et al, 1989; Sen et al, 2001; Zuk et al, 2001; Zuk et al, 2002). After a week of induction, neutral lipid containing vacuoles accumulate in Lipid containing vacuoles accumulated in the ASCs can be detected by Oil Red O or Nile red staining. In PCR anaylsis, ASCs and the production of adipogenic mRNAs, such as lipoprotein lipase (LPL), PPAR γ , C/EBP α / β / δ , followed by FABP4/aP2 and leptin, can be detected (Christodoulides et al, 2009; Gregoire et al, 1998; Hauner

et al, 1989; Moldes et al, 2003; Sen et al, 2001; Won Park et al, 2008; Zuk et al, 2001; Zuk et al, 2002).

2.3.2.2 Osteogenic differentiation

Osteogenic differentiation has been more extensively examined in BMSCs (Zhou et al, 2004). The mechanism underlying hASCs is still unclear. However, it is now commonly believed that osteogenic cells arise from multipotential mesenchymal cells found in bone marrow or adipose tissue that could give rise to progeny with more committed lineage (Aubin et al, 1995; Aubin, 2001; Liu et al, 2007; Takada et al, 2009). In the presence of ascorbate, β -glycerophosphate, dexamethasone and/or 1,25 vitamin D3, ASCs differentiate into osteoblast-like cells in vitro (Gimble and Guilak, 2003; Halvorsen et al, 2000; Halvorsen et al, 2001). Moreover, it has been demonstrated that ASCs cultured in the presence of these factors express ALP, RUNX2, BMP-2, BMP-4, BMP receptors I and II, and PTH receptor genes characteristic of osteoblast-like cells (Halvorsen et al, 2001; Rada et al, 2009; Strem et al, 2005; Tapp et al, 2009; Zuk et al, 2002). Upon differentiae in suitable condition, ASCs start to produce calcium phosphate mineral within their extracellular matrix that can be detected by Alizarin Red or von Kossa staining, and begin to express osteogenic genes and proteins (Halvorsen et al, 2001; Zuk et al, 2001; Zuk et al, 2002). They also hold more osteogenic potential in male than female in vitro (Aksu et al, 2008), and the potential appears to decrease with increasing age (Zhu et al, 2009). The explanation of this phenomenal is likely due to are likely due to the different steroid functions in males and females with hormone levels varying at different phases of life. Furthermore, some transcription factors such as PPAR γ and RUNX2 have been suggested to play a critical role in the commitment of bipotent

stem cells with the capacity to differentiate to the osteoblastic or adipocyte phenotype (Rada et al, 2009).

2.3.2.3 Chondrogenic differentiation

Similarly to adipocytes and osteoblasts, chondrocytes likely develop from multipotent mesenchymal cells that give rise to progeny with more limited capacities (Aubin et al, 1995). It is still questionable whether bone and cartilage forming cells arise from a common bipotential progenitor, or whether the two cell types arise from two separate monopotential precursors (Aubin et al, 1995). Evidences suggested that, in BMSCs and ASCs, osteogenesis and adipogenesis appear to be linked in a differentiation branch separate from chondrogenesis (Liu et al, 2007). Still, osteogenic and chondrogenic lineages is somehow related as hypertrophic chondrocytes can transdifferentiate into osteoblast-like cells, suggesting their origin from bipotential progenitor (Aubin et al, 1995; Johnstone et al, 1998).

For chondrogenic differentiation, ASCs are routinely cultured in micro mass culture or pellet culture systems (Denker et al, 1995; Johnstone et al, 1998). The micro mass or pellet culture model mimics pre-cartilage condensation during embryonic development, which increases the cell-to-cell interaction and leads to the production of a cartilage-like matrix (Wei et al, 2007). The suspension of cells in hydrogel scaffolds (Awad et al, 2004) has been done in the attempt to mimic the composition of native cartilage. Ascorbate-2-phosphate, dexamethasone, L-proline and TGF-β1 are involved in chondrogenic differentiation. BMP family factors have also been investigated for chondrogenic induction of ASCs. For example, while BMP-6 promotes chondrogenic differentiation, BMP-7 induces chondrogenic differentiation only at high doses (Diekman et al, 2009; Estes et al, 2006; Knippenberg et al, 2006).

With the addition of chondrogenic induction factors and when maintained in an appropriate 3D environment in vitro, ASCs will start to secrete the extracellular matrix proteins of cartilage, including COLL II, COLL VI and aggrecan (Erickson et al, 2002; Koga et al, 2009).

The expression profiles of human ASCs and are similar in monolayer (Winter et al, 2003). When both cell sources are cultured as chondrogenic micromass pellets, the BMSCs exhibited greater chondrogenic differentiation capacity than ASCs (Huang et al, 2004; Huang et al, 2005; Im et al, 2005; Liu et al, 2007; Winter et al, 2003). However, addition of TGF-β2 and IGF-I at high dose could enhance chondrogenesis of ASCs (Kim and Im, 2009). Still, more investigation is needed to establish the optimal culture conditions of ASCs chondrogenic potential in vitro.

2.4 ASCs plasticity

Apart from osteogenesis, adipogenesis and chondrogenesis, ASCs have shown function in various other differentiation processes with potential clinical applicability. In vitro expanded ASCs contain progenitor cells that have the ability to differentiate into mature endothelial cells and participate in blood vessel formation (De Francesco et al, 2009; Madonna et al, 2009; Miranville et al, 2004; Planat-Benard et al, 2004) although the capacity may be limited (Boquest et al, 2007). ASC induced vessel formation and growth may be related to the secretion of proangiogenic factors (Rehman et al, 2004) or through perivascular functions of the ASCs (Madonna et al, 2009) or perhaps both. Together with the angiogenic inductive features, human ASCs

also have potential to differentiate along the cardiomyocyte pathway (van Dijk et al, 2008). Nevertheless, in vitro studies on human ASCs are limited, and in most studies only low percentages (<2%) of differentiation have been described (Bai et al, 2007; Song et al, 2007; Valina et al, 2007). Still, the efficiency of differentiation capacity was enhanced by treatment of cells with 2-deoxy- 5-azacytidin (van Dijk et al, 2008). Regarding in vivo study, ASCs improved cardiac function and perfusion via angiogenesis when injected intracoronarily in a porcine myocardial infarction model (Valina et al, 2007). These preliminary reports demonstrated the potential of ASCs on regenerating cardiac tissue damaged through infarctions or ischemic injury (Gimble and Guilak, 2003).

ASCs cells also demonstrate in vitro evidence for differentiation along the skeletal myocyte pathway (Goudenege et al, 2009; Mizuno et al, 2002; Zuk et al, 2001). Under appropriate induction conditions, ASCs express myoD and myogenin, transcription factors regulating skeletal muscle differentiation (Mizuno et al, 2002; Zuk et al, 2001; Zuk et al, 2002). The cells fuse, form multi-nucleated myotubes, and express protein markers of the skeletal myocyte lineage, such as myosin light chain kinase. This suggests that ASCs may have applicability in the repair of damaged skeletal muscle in tissue engineering applications.

There is some evidence to suggest that human ASCs can differentiate into cells of ectodermal origin, such as hepatocytes (Safford et al, 2002; Seo et al, 2005; Talens-Visconti et al, 2006). Upon induction to hepatocyte, their morphology displays hepatocyte-like cells, although the mechanisms are not yet clear. The cells expressed albumin and α -fetoprotein and showed LDL uptake and production of urea.

Additionally, when transplanted into a SCID mouse model, with a carbon tetrachloride induced hepatic injury, the transplanted cells were able to express albumin in vivo.

2.5 Neural differentiation of MSCs and ASCs

Even though MSCs could only undergo the mesodermal differentiation, they could differentiate beyond this barrier toward neuroectodermal lineage under vary appropriate conditions. Evidences of neural differentiation from MSCs have been extensively revealed worldwide in recent decades (Mitchelle et al, 2003; Hermann et al, 2006; Kiraly et al, 2009; Zhang et al, 2010; Jang et al, 2010) . The majority source of cells is primarily from bone marrow (Hermann et al, 2006), whereas a few has been investigated in MSCs from other sources, including, adipose tissue (Tholpady et al, 2003; Safford et al, 2004; Jang et al, 2010), dental pulp (Kiraly et al, 2009), and Wharton's jelly (Mitchelle et al, 2003; Yang et al, 2008; Salgado et al, 2010, Zhang et al, 2010).

Two first reports of neural differentiation *in vitro* in MSCs derived from bone morrow were done by the US research groups (Sanchez-Ramos et al, 2000; Woodberry et al, 2000). Both groups developed two independent neural induction protocols that resulted in typical neural morphology and neuroectodermal protein expression. After these studies, there were many studies reported a single-step differentiation of MSCs from many primary tissues using different approaches, including a combination of growth factors and cytokines, glial feeder cell layers and/or conditioned media known to induce neuroectodermal differentiation in ESCs or NSCs (Sanchez-Ramos et al, 2000; Sanchez-Ramos, 2002; Padovan et al, 2003; Tao et al, 2005). Additionally, an increase of intracellular cAMP levels as well as chemical-

defined media with various compounds, including antioxidants such as β-mercaptoethanol (BME), butylated hydroxyanisole (BHA), sodium ferulate (SF), dimethylsulfoxide (DMSO) and tricyclodecane-9-yl-xanthogenate (D609) were reported in these studies (Woodberry et al, 2000; Woodburry et al, 2002; Hermann et al, 2006; Wenisch et al, 2006). MSCs in these studies were able to express properties of neuroectodermal cells *in vitro* and *in vivo* after transplantation into the brain and spinal cord. However, the results were not consistent as the neuroectodermal expression in some studies is weak while others could go up to 60 – 80% in a short period of time (Wang et al, 2004).

A few years after the first publication of neural induction of MSCs, an advance approach for the neuroectodermal conversion of MSCs is presented. The approach is known as two-step technology which is an initial conversion of MSCs into neural progenitor-like cells, and subsequent terminal differentiation into mature neuronal and glial cell types (Lee et al, 2000; Hermann et al, 2004; Bossolasco et al, 2005; Hermann et al, 2006). Each step is independent from each other and required characterization to demonstrate their similarity to brain-derived NSCs. This step could be more appropriate for neuroregenerative strategies as the cells could differentiate into various types of mature neural cells. The process of this novel approach is much involved with two major growth factors which are epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF; also known as FGF2). Additional growth factors found in protocol includes leukemia inhibitory factor (LIF), sonic hedgehog (SHH), fibroblast growth factor 8 (FGF8), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and *platelet-derived growth factor* (*PDGF*). Moreover, most of the protocol are under the condition of serum free, which is suggested by previous

evidence of the ability to induce hMSCs to differentiate into neural-like cell *in vitro* (Wang et al, 2005). Chemical compounds and small molecules are used as supplementation to subsequent terminal differentiation as well. The expression of NSC marker (Nestin, Musashi-1) could be detected in the cell after the initial conversion. When cultured those cells in the low-attachment culture dish, they could form neurosphere-like structure (Lee et al, 2003) and Nestin expression can be seen more than 90% of the cell population (Hermann et al, 2004; Hermann et al, 2006). After the sequential treatment with different growth factors or conditions, the expression of mature neural cell types (*GFAP, MBP, TUBB4/III, SNCA* and *TH*) could be seen, yet it is very low. Moreover, the mesodermal differentiation capacity of the converted cells was dramatically decreased during the conversion step (Hermann et al, 2004). In the second step, those cells were induced to be terminal mature neurons or glia cells by plating the cells on specific surfaces, withdrawal of mitogens and incubation with various different factors. The general profile of the cells after terminal differentiation has found to be similar to differentiated fetal NSCs (Lee et al, 2003). However, only gene expression data is available and less functional data present in the public

Among MSCs, neural differentiation potential of ASCs has captured the attention of scientists recently. Compared to BMSCs, ASCs show a higher tendency to differentiate into the neural line as they produce larger amount of BDNF and react to NB and B27 media expressing higher level of nestin and MAP-2. Moreover, in basal condition they express low levels of nestin, Neu-N, Ca++ α_1 channel, sinapsyn I (Kang et al, 2003; Kang et al, 2004; Ashjian et al, 2003; Safford et al, 2004; Yang et al, 2004).

Safford and colleagues were the first to induce neural differentiation by ASCs in vitro, by treatment with EGF and bFGF, followed by BHA and forskolin: the cells readily change in morphology and phenotype, expressing at the same time early and some mature neuronal (nestin, Neu-N), glial markers (GFAP) and neurotransmettitor receptors (glutamate and GABA receptors) and lacking the expression of tyrosine hydroxylase (TH), dopamine-decarboxylase and 5-hydroxytryptophan. Moreover, there was a massive cell death at the 5th day of culture. Such differentiation was unspecific, incomplete and short-lasting (Safford et al, 2002; Safford et al, 2004). Other chemical treatments with BME induced more selective neuronal morphology and phenotype, with no data about the functional properties (Zuk et al, 2002; Tholpady et al, 2003; Romanov et al, 2005). Only Ashjian et al. *in vitro* showed K+ outward rectified currents in modified hASCs exposed to IBMX and indomethacin for 14 days. This data was not selective for neuronal differentiation (Ashjian et al, 2003). The ability of hASCs to form neurosphere-like is not well studied. hASCs treated by NB and B27 after neurosphere-step express at the same time neuronal and glial markers (nestin, MAP-2, GFAP), with increasing percentage of differentiated cells after adding BDNF (Kang et al, 2004).

2.6 Neural regulation of NO-cGMP pathway

Nitric oxide (NO) is a short lived diatomic free radical species that is synthesized by nitric oxide synthases (NOS). NOS catalyze the oxidation of L-argininesulting in the formation of NO and L-citruline. Three major isoforms of nitric oxide synthase are known. These are nNOS or NOS1, iNOS or NOS II and eNOS or NOSIII. nNOS and eNOS are constitutively expressed enzymes and are

regulated in a $Ca2^{+}$ dependent manner. From the moment it is synthesized, NO takes part in a number of physiological processes such as smooth muscle relaxation, blood pressure and volume regulation, immunomodulation, axon outgrowth and guidance platelet aggregation, neurotransmission mechanisms as well as cellular growth, survival, apoptosis, proliferation and differentiation. NO is produced as needed and is not stored as other messengers. However, NO complexes may exist as stored precursors to release NO.

NO is highly reactive and its local concentrations range from low nanomolar to low micromolar. The downstream targets and physiological actions depend on the local concentration and the availability of target molecules. However, at low nanomolar concentrations, activation of soluble guanylyl cyclase (sGC: the major NO receptor) triggers intracellular cyclic GMP level elevation (Arnold et al, 1977; Katsuki et al, 1977). This incidence leads to diverse physiological processes, such as smooth muscle relaxation, neurotransmission, blood pressure regulation, inhibition of platelet aggregation and immunomodulation. Its roles at cellular level are to regulate cell growth, survival, differentiation, proliferation, migration, axon guidance and many other processes through a variety of downstream signaling cascades depending on cell type specific regulation.

Evidences have revealed that cGMP might help support NO to regulate cell survival and neuroprotective (Fig 2.2). The study of NO role dorsal root ganglia (DRG) neurons survival in adult rats confirmed this finding. NGF-devrived DRG neurons that underwent apoptosis was rescued by the inclusion in the culture medium of the NO substrate, l-arginine, or of the cGMP analogue, 8Br-cGMP (Thippeswamy and Morris, 1997, 2001; Ciani et al, 2002). Their role in neuronal differentiation was

convinced by the evidence results done by Ciani and colleagues. They observed that nNOS overexpressing clones responded more quickly to differentiation than in the parental cells upon adding retinoic acid by slowing down proliferation and speeding up differentiation as well as a larger and faster decrease of N-Myc expression in NO overexpressing neuroblastoma (Ciani et al, 2002). N-Myc expression, an oncogene functioning on cell proliferation and tumor expansion, downregulates progressively during retinoic acid-induced cytostasis and neuronal differentiation. The assumption of this incidence relies on NO-cGMP system as the proliferation rate of parental cells were decreasing by NO donor or cGMP analog. Moreover, NO/cGMP singling also regulate GAP43 mRNA level which play a vital roles in neurite outgrowth and synaptogenesis as well (Chen et al, 2004; López-Jiménez et al, 2009).

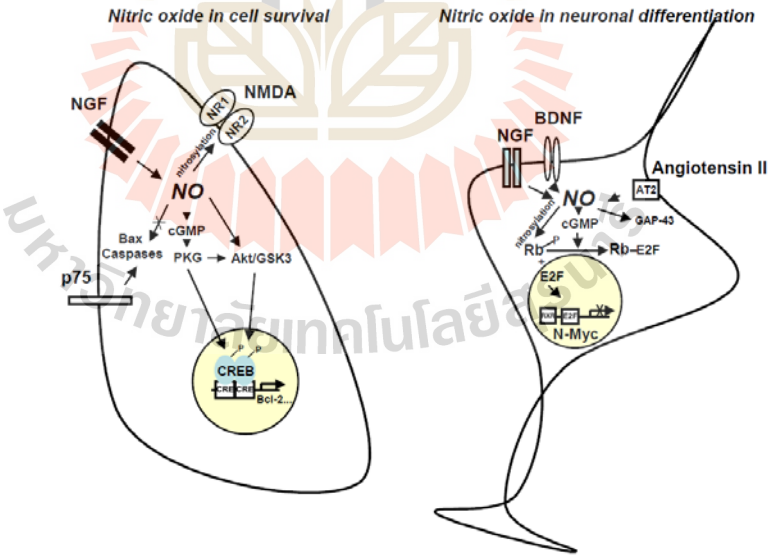


Figure 2.2 NO-cGMP is one of key pathways involved in survival promoting or neuroprotective and neurodifferentiative effects mediated by NO (Contestabile and Ciani, 2004)

2.7 References

Aksu, A.E., Rubin, J.P., Dudas, J.R., Marra, K.G. (2008): Role of gender and anatomical region on induction of osteogenic differentiation of human adipose-derived stem cells. **Ann Plast Surg** 60: 306-322.

Arnold, W.P., Mittal, C.K., Katsuki, S. and Murad, F. (1977) Nitric oxide activates guanylate cyclase and increases guanosine 3′-5′-cyclic monophosphate levels in various tissue preparations. **Proc Natl Acad Sci** 74:3203–3207.

Ashjian, P.H., Elbarbary, A.S., Edmonds, B., DeUgarte, D., Zhu, M., Zuk, P.A., et al. (2003). In vitro differentiation of human processed lipoaspirate cells into early neural progenitors. **Plast Reconstr Surg** 111(6):1922-1931.

Aubin, J.E., Liu, F., Malaval, L., Gupta, A.K. (1995). Osteoblast and chondroblast differentiation. **Bone** 17: 77S-83S.

Aubin, J.E. (1998). Bone stem cells. **J Cell Biochem Suppl** 30-31: 73-82.

Aubin, J.E. (2001). Regulation of osteoblast formation and function. **Rev EndocrMetab Disord** 2: 81-94.

Awad, H,A,,Wickham, M.Q., Leddy, H.A., Gimble, J.M., Guilak, F. (2004). Chondrogenic differentiationof adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. **Biomaterials** 25: 3211-3222.

Bai, X., Pinkernell, K., Song, Y.H., Nabzdyk, C., Reiser, J., Alt, E. (2007). Genetically selected stem cells from human adipose tissue express cardiac markers. **Biochem Biophys Res Commun** 353: 665-671.

Bain, G., Kitchens D., Yao, M., Huettner, J.E., Gottlieb, D.I. (1995) Embryonic Stem Cells Express Neuronal Properties in Vitro. **Developmental Biology.** 168:342-357.

Battula, V.L., Treml, S., Bareiss, P.M.,Gieseke, F., Roelofs, H, de Zwart, P., Muller, I., Schewe, B., Skutella, T., Fibbe, W.E., Kanz, L., Buhring, H.J. (2009). Isolation of functionally distinctmesenchymal stem cell subsets using antibodies against CD56, CD271, and mesenchymal stem cell antigen-1. **Haematologica** 94: 173-184.

Bauer, H.-C., Tempfer, H., Bernroider, G., Bauer, H. (2006) Neuronal stem cells in adults. **Experimental Gerontology.** 41:111-116.

Boquest, A.C., Noer, A., Sorensen, A.L., Vekterud, K., Collas, P. (2007). CpG methylation profiles of endothelial cell-specific gene promoter regions in adipose tissue stem cells suggest limited differentiation potential toward the endothelial cell lineage. **Stem Cells** 25: 852-861.

Bossolasco, P., Cova, L., Calzarossa, C., Rimoldi, S.G., Borsotti, C., Deliliers, G.L., Silani, V., Soligo, D., Polli E. (2005) Neuro-glial differentiation of human bone marrow stem cells in vitro. **Experimental Neurology.** 193:312-325.

Chen, T.J., Huang, C.W., Wang, D.C., Chen, S.S. (2004) Co-induction of growth-associated protein GAP-43 and neuronal nitric oxide synthase in the cochlear nucleus following cochleotomy. **Exp Brain Res 158**:151–162.

Christodoulides, C., Lagathu, C., Sethi, J.K., Vidal-Puig, A. (2009): Adipogenesis and WNT signalling. **Trends Endocrinol Metab** 20: 16-24.

Ciani, E., Guidi, S., Della Valle, G., Perini, G., Bartesaghi, R., Contestabile, A. (2002). Nitric oxide protects neuroblastoma cells from apoptosis induced by serum deprivation through cAMP-response element-binding protein (CREB) activation. **J. Biol. Chem.** 277:49896–49902.

Contestabile, A. and Ciani, E. (2004). Role of nitric oxide in the regulation of neuronal proliferation, survival and differentiation. **Neurochemistry International** 45: 903-914.

Daher, S.R., Johnstone, B.H., Phinney, D.G., March, K.L. (2008). Adipose stromal/stem cells: basic band translational advances: the IFATS collection. **Stem Cells** 26: 2664-2665.

De Francesco, F., Tirino, V., Desiderio, V., Ferraro, G., D' Andrea, F., Giuliano, M., Libondi, G., Pirozzi, G., De Rosa, A., Papaccio, G. (2009). Human CD34+/CD90+ ASCs Are Capable of Growing as Sphere Clusters, Producing High Levels of VEGF and Forming Capillaries. **PLoS ONE** 4:e6537.

De Ugarte, D.A., Alfonso, Z., Zuk, P.A., Elbarbary, A., Zhu, M., Ashjian, P., Benhaim, P., Hedrick, M.H., Fraser, J.K. (2003). Differential expression of stem cell mobilization-associated molecules on multi-lineage cells from adipose tissue and bone marrow. **Immunol Lett** 89: 267-270.

Deng, W., Obrocka, M., Fischer, I., Prockop, D.J. (2001) In Vitro Differentiation of Human Marrow Stromal Cells into Early Progenitors of Neural Cells by Conditions That Increase Intracellular Cyclic AMP. **Biochemical and Biophysical Research Communications**. 282:148-152.

Denker, A.E., Nicoll, S.B., Tuan, R.S. (1995): Formation of cartilage-like spheroids by micromass cultures of murine C3H10T1/2 cells upon treatment with transforming growth factor-beta 1. **Differentiation** 59: 25-34.

Deslex, S., Negrel, R., Vannier, C., Etienne, J., Ailhaud, G. (1987). Differentiation of human adipocyte precursors in a chemically defined serum-free medium. **Int J Obes** 11: 19-27.

Diekman, B.O., Estes, B.T., Guilak, F. (2009) The effects of BMP6 overexpression on adipose stem cell chondrogenesis: Interactions with dexamethasone and exogenous growth factors. **J Biomed Mater Res A.** 93(3):994-1003.

Dietrich, J., Kempermann, G. (2006) Role of Endogenous Neural Stem Cells in Neurological Disease and Brain Repair, in: M. Bähr (Ed.), **Brain Repair**, Springer US. pp. 191-220.

Ding, S., Schultz, P.G. (2004) A role for chemistry in stem cell biology. **Nature Biotechnology**. 22:833-840.

Erickson, G.R., Gimble, J.M., Franklin, D.M., Rice, H.E., Awad, H., Guilak, F. (2002). Chondrogenic potential of adipose tissue-derived stromal cells in vitro and in vivo. **Biochem Biophys Res Commun** 290: 763-769.

Estephane, D., Anctil, M. (2010) Retinoic acid and nitric oxide promote cell proliferation and differentially induce neuronal differentiation in vitro in the cnidarian Renilla koellikeri. **Developmental Neurobiology**. 70:842-852. DOI: 10.1002/dneu.20824.

Estes, B.T., Wu, A.W., Guilak, F (2006)Potent induction of chondrocytic differentiation of human adipose-derived adult stem cells by bone morphogenetic protein 6. **Arthritis Rheum** 54: 1222-1232.

Fraser, J.K.,Wulur, I., Alfonso, Z., Hedrick, M.H. (2006): Fat tissue: an underappreciated source of stem cells for biotechnology. **Trends Biotechnol** 24: 150-154.

Gage, F.H. (2000) Mammalian Neural Stem Cells. **Science**. 287:1433-1438. DOI: 10.1126/science.287.5457.1433.

Garcia-Castro, J., Trigueros, C., Madrenas, J., Perez-Simon, J.A., Rodriguez, R., Menendez P (2008). Mesenchymal stem cells and their use as cell replacement therapy and disease modelling tool. **J Cell Mol Med** 12: 2552-2565.

Gimble, J.M., Guilak F. (2003) Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. **Cytotherapy**. 5:362-369. DOI: 10.1080/14653240310003026.

Gimble, J.M., Katz, A.J., Bunnell, B.A. (2007). Adipose-derived stem cells for regenerative medicine. **Circ Res** 100: 1249-1260.

Goudenege, S., Pisani, D.F., Wdziekonski, B., Di Santo, J.P., Bagnis, C., Dani, C., Dechesne, C.A. (2009). Enhancement of myogenic and muscle repair capacities of human adipose-derived stem cells with forced expression of MyoD. **Mol Ther** 17: 1064-1072.

Gregoire, F.M., Smas, C.M., Sul, H.S. (1998): Understanding adipocyte differentiation. **Physiol Rev** 78: 783-809.

Gronthos, S., Mankani M., Brahim J., Robey P.G., Shi S. (2000) Postnatal human dental pulp stem cells (DPSCs) in vitro and invivo. **Proceedings of the National Academy of Sciences of the United States of America**. 97:13625-13630.

Gronthos, S. et al. (2001). Surface protein characterization of human adipose tissue-derived stromal cells. **J Cell Physiol** 189:54.

Guilak, F., Lott, K.E., Awad, H.A., Cao, Q., Hicok, K.C., Fermor, B., Gimble, J.M. (2006). Clonal analysis of the differentiation potential of human adipose-derived adult stem cells**. Journal of Cellular Physiology** 206(1): 229–237**.**

Halvorsen, Y.C., Wilkison, W.O., Gimble, J.M. (2000). Adipose-derived stromal cells-their utility and potential in bone formation. **Int J Obes Relat Metab Disord** 24 Suppl 4: S41-44.

Halvorsen, Y.D., Franklin, D., Bond, A.L., Hitt, D.C., Auchter, C., Boskey, A.L., Paschalis, E.P., Wilkison, W.O., Gimble JM (2001): Extracellular matrix mineralization and osteoblast gene expression by human adipose tissue-derived stromal cells. **Tissue Eng** 7: 729-741.

Hauner, H., Entenmann, G., Wabitsch, M., Gaillard, D., Ailhaud, G., Negrel, R., Pfeiffer, E.F. (1989). Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium. **J Clin Invest** 84: 1663-1670.

Hermann, A., Gastl, R., Liebau, S., Popa, M.O., Fiedler, J., Boehm, B.O., Maisel, M., Lerche, H., Schwarz, J., Brenner, R., Storch, A. (2004) Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells. **Journal of Cell Science**. jcs.01307.

Hermann, A., Maisel, M., Storch, A. (2006) Epigenetic conversion of human adult bone mesodermal stromal cells into neuroectodermal cell types for replacement therapy of neurodegenerative disorders. **Expert Opinion on Biological Therapy**. 6:653-670. DOI: 10.1517/14712598.6.7.653.

Huang, J.I., Zuk, P.A., Jones, N.F., Zhu, M., Lorenz, H.P., Hedrick, M.H., Benhaim, P. (2004). Chondrogenic potential of multipotential cells from human adipose tissue. Plast Reconstr Surg 113:585-594.

Hwang, N.S., Varghese, S., Elisseeff, J. (2008) Controlled differentiation of stem cells. **Advanced Drug Delivery Reviews**. 60:199-214.

Isacson, O., Bjorklund, L.M., Schumacher, J.M. (2003) Toward full restoration of synaptic and terminal function of the dopaminergic system in Parkinson's disease by stem cells. **Annals of Neurology**. 53:S135-S148. DOI: 10.1002/ana.10482.

Jang, S., Cho, H.-H., Cho, Y.-B., Park, J.-S., Jeong, H.-S. (2010) Functional neural differentiation of human adipose tissue-derived stem cells using bFGF and forskolin. **BMC Cell Biology**. 11:25.

Jiang, Y., Jahagirdar B.N., Reinhardt, R.L., Schwartz, R.E., Keene, C.D., Ortiz-Gonzalez, X.R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M., Du, J., Aldrich, S., Lisberg, A., Low, W.C., Largaespada, D.A., Verfaillie, C.M. (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. **Nature**. 418:41-49..

Johnstone, B., Hering, T.M., Caplan, A.I., Goldberg, V.M., Yoo, J.U. (1998). In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. **Exp Cell Res** 238: 265-272.

Kang, S.K., Lee, D.H., Bae, Y.C. et al. (2003). Improvement of neurological deficits by intracerebral transplant of human adipose-derived stem cells after ischemia in rats. **Exp Neurol** 183: 355-66.

Kang, S.K., Putnam, L.A., Ylostalo, J. et al. (2004). Neurogenesis of rhesus adipose stromal cells. **J Cell Sci** 117(18): 4289-99.

Katsuki, S., Arnold, W., Mittal, C. and Murad, F. (1977) Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. **J Cyclic Nucleotide Res** 3:23–35.

Katz, A.J., Llullm, R., Hedrick, M.H., Futrell, J.W. (1999). Emerging approaches to the tissue engineeringof fat. **Clin Plast Surg** 26: 587-603, viii.

Katz, A.J., Hedrick, M.H., Llull, R., Futrell, J.W. (2001). A novel device for the simple and efficient refinement of liposuctioned tissue. **Plast Reconstr Surg** 107: 595-597.

Katz, A.J., Tholpady, A., Tholpady, S.S., Shang, H. & Ogle, R.C. (2005). Cell surface and transcriptional characterization of human adipose-derived adherent stromal (hADAS) cells. **Stem Cells** 23:412.

Kim, H.J. and Im, G.I. (2009). Chondrogenic differentiation of adipose tissue-derived mesenchymal stem cells: greater doses of growth factor are necessary. **J Orthop Res** 27: 612-619.

Király, M., Porcsalmy, B., Pataki, Á., Kádár, K., Jelitai, M., Molnár, B., Hermann, P., Gera, I., Grimm, W.-D., Ganss, B., Zsembery, Á., Varga, G. (2009) Simultaneous PKC and cAMP activation induces differentiation of human dental pulp stem cells into functionally active neurons. **Neurochemistry International**. 55:323-332.

Knippenberg, M., Helder, M.N., Zandieh-Doulabi, B., Wuisman, P.I., Klein-Nulend, J. (2006) Osteogenesis versus chondrogenesis by BMP-2 and BMP-7 in adipose stem cells. **Biochem Biophys Res Commun** 342: 902-908.

Koga., H., Engebretsen, L., Brinchmann, J.E., Muneta, T., Sekiya, I. (2009). Mesenchymal stem cell-based therapy for cartilage repair: a review. **Knee Surg Sports Traumatol Arthrosc.** 17(11):1289-97.

Kohyama, J., Abe, H., Shimazaki, T., Koizumi, A., Nakashima, K., Gojo, S., Taga, T., Okano, H., Hata, J.-i., Umezawa, A. (2001) Brain from bone: Efficient "meta-

differentiation" of marrow stroma-derived mature osteoblasts to neurons with Noggin or a demethylating agent. **Differentiation**. 68:235-244.

Lee, J., Elkahloun, A.G., Messina, S.A., Ferrari, N., Xi D., Smith, C.L., Cooper, R., Albert, P.S., Fine, H.A. (2003) Cellular and Genetic Characterization of Human Adult Bone Marrow-Derived Neural Stem-Like Cells. **Cancer Research**. 63:8877-8889.

Lee, S.-H., Lumelsky, N., Studer, L., Auerbach, J.M., McKay, R.D. (2000) Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. **Nature Biotechnology**. 18:675-679.

Lee, T.-H., Yoon, J.-G. (2008) Intracerebral transplantation of human adipose tissue stromal cells after middle cerebral artery occlusion in rats. **Journal of Clinical Neuroscience**. 15:907-912.

Li, J.T., Somasundaram, C., Bian, K., Xiong, W., Mahmooduddin, F., Nath, R.K., Murad, F. (2010) Nitric Oxide Signaling and Neural Stem Cell Differentiation in Peripheral Nerve Regeneration. **Journal of Plastic Surgery**. 10:343-353.

Liu, T.M., Martina, M., Hutmacher, D.W., Hui, J.H., Lee, E.H., Lim, B. (2007) Identification of common pathways mediating differentiation of bone marrow- and adipose tissue-derived human mesenchymal stem cells into three mesenchymal lineages. **Stem Cells** 25: 750-760.

López-Jiménez, M.E., Bartolomé-Martín, D., Sánchez-Prieto, J. and Torres, M. (2009). Suppression of guanylyl cyclase (β1 subunit) expression impairs neurite outgrowth and synapse maturation in cultured cerebellar granule cells. **Cell Death and Differentiation 16:**1266–1278.

Madonna, R., Geng, Y.J., De Caterina, R. (2009): Adipose Tissue-Derived Stem Cells. Characterization and Potential for Cardiovascular Repair. **Arterioscler Thromb Vasc Biol.** 29(11):1723-9.

McIntosh, K. et al. (2006). The immunogenicity of human adiposederived cells: temporal changes in vitro. **Stem Cells 24**:1246.

McKay, R. (1997) Stem Cells in the Central Nervous System. **Science**. 276:66-71. DOI: 10.1126/science.276.5309.66.

Miranville, A., Heeschen, C., Sengenes, C., Curat, C.A., Busse, R., Bouloumie, A. (2004). Improvement of postnatal neovascularization by human adipose tissue-derived stem cells. **Circulation** 110: 349-355.

Mitchell, K.E., Weiss, M.L., Mitchell, B.M., Martin, P., Davis, D., Morales, L., Helwig, B., Beerenstrauch, M., Abou-Easa, K., Hildreth, T., Troyer, D. (2003) Matrix Cells from Wharton's Jelly Form Neurons and Glia. **STEM CELLS**. 21:50-60. DOI: 10.1634/stemcells.21-1-50.

Mizuno, H., Zuk, P.A., Zhu, M., Lorenz, H.P., Benhaim, P., Hedrick, M.H. (2002): Myogenic differentiation by human processed lipoaspirate cells. **Plast Reconstr Surg** 109: 199-209.

Moe, M.C., Westerlund, U., Varghese, M., Berg-Johnsen, J., Svensson, M., Langmoen, I.A. (2005) Development of Neuronal Networks from Single Stem Cells Harvested from the Adult Human Brain. **Neurosurgery**. 56.

Moldes, M., Zuo, Y., Morrison, R.F., Silva, D., Park, B.H., Liu, J., Farmer, S.R. (2003) Peroxisomeproliferator- activated receptor gamma suppressesWnt/beta-catenin signalling during adipogenesis. **Biochem J** 376: 607-613.

Otto, T.C. and Lane, M.D. (2005). Adipose development: from stem cell to adipocyte. **Crit Rev Biochem Mol Biol** 40: 229-242.

Padovan, C., Jahn, K., Birnbaum, T., Reich, P., Sostak, P., Strupp, M., Straube, A. (2003) Expression of Neuronal Markers in Differentiated Marrow Stromal Cells and CD133+ Stem-Like Cells. **Cell Transplantation**. 12:839-848.

Pittenger, M.F.,Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas. R., Mosca, J.D., Moorman, M.A., Simonetti D.W., Craig, S., Marshak, D.R. (1999). Multilineage potential of adult human mesenchymal stem cells. **Science** 284: 143-147.

Planat-Benard, V., Silvestre, J.S., Cousin, B., Andre, M., Nibbelink, M., Tamarat, R., Clergue, M., Manneville, C., Saillan-Barreau, C., Duriez, M., Tedgui, A., Levy, B., Penicaud, L., Casteilla, L. (2004). Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. **Circulation** 109: 656-663.

Quirici, N., Soligo, D., Bossolasco, P., Servida, F., Lumini, C., Deliliers, G.L. (2002). Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. **Exp Hematol** 30: 783-791.

Rada, T., Reis, R.L., Gomes, M.E. (2009). Adipose Tissue-Derived Stem Cells and Their Application in Bone and Cartilage Tissue Engineering. **Tissue Eng** Part B Rev.

Rehman, J., Traktuev, D., Li, J., Merfeld-Clauss, S., Temm-Grove, C.J., Bovenkerk, J.E., Pell, C.L., Johnstone, B.H., Considine, R.V., March, K.L. (2004). Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. **Circulation** 109: 1292-1298.

Rodbell, M. (1964): Metabolism of Isolated Fat Cells. I. Effects of Hormones on Glucose Metabolism and Lipolysis. **J Biol Chem** 239: 375-380.

Romanov, Y.A., Darevskaya, A.N., Merzlikina, N.V. et al. (2005). Mesenchymal stem cells from human bone marrow and adipose tissue: isolation, characterization and differentiation potentials. **Cell Tecn Biol Medicine** 3: 138-43.

Safford, K.M., Hicok, K.C., Safford, S.D., Halvorsen, Y.-D.C., Wilkison, W.O., Gimble, J.M., Rice, H.E. (2002) Neurogenic differentiation of murine and human adipose-derived stromal cells. **Biochemical and Biophysical Research Communications**. 294:371-379.

Salgado J.A., Fraga J.S., Mesquita, A.R., Neves, M.N., Reis, R.L. and Sousa, N. **(2010).** Role of Human Umbilical Cord Mesenchymal Progenitors Conditioned Media in Neuronal/Glial Cell Densities, Viability, and Proliferation. **Stem Cells and Development** 19(7): 1067-1074.

Sanchez-Ramos, J., Song, S., Cardozo-Pelaez, F., Hazzi, C., Stedeford, T., Willing, A., Freeman, T.B., Saporta, S., Janssen, W., Patel, N., Cooper, D.R., Sanberg, P.R. (2000) Adult Bone Marrow Stromal Cells Differentiate into Neural Cells in Vitro. **Experimental Neurology**. 164:247-256.

Sanchez-Ramos, J.R. (2002) Neural cells derived from adult bone marrow and umbilical cord blood. **Journal of Neuroscience Research**. 69:880-893. DOI: 10.1002/jnr.10337.

Sen, A., Lea-Currie, Y.R., Sujkowska, D., Franklin, D.M., Wilkison, W.O., Halvorsen, Y.D., Gimble, J.M. (2001) Adipogenic potential of human adipose derived stromal cells from multiple donors is heterogeneous**. J Cell Biochem** 81: 312-319.

Seo, B.M., Miura, M., Gronthos, S., Bartold, P.M., Batouli, S., Brahim, J., Young, M., Robey, P.G.,Wang, C.Y., Shi, S. (2004). Investigation of multipotent postnatal stem cells from human periodontal ligament. **Lancet** 364: 149-155.

Shi, Y., Sun, G., Zhao, C., Stewart, R. (2008) Neural stem cell self-renewal. **Critical Reviews in Oncology/Hematology**. 65:43-53.

Simmons, P.J. and Torok-Storb, B (1991): Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. **Blood** 78: 55-62.

Skene, J.H. (1989). Axonal growth-associated proteins. **Annu Rev Neurosci 12**:127–156.

Strem, B.M., Hicok, K.C., Zhu, M., Wulur, I., Alfonso, Z., Schreiber, R.E., Fraser, J.K., Hedrick, M.H. (2005). Multipotential differentiation of adipose tissuederived stem cells. **Keio J Med** 54:132 - 41.

Song, Y.H., Gehmert, S., Sadat, S., Pinkernell, K., Bai, X., Matthias, N., Alt, E. (2007). VEGF is critical for spontaneous differentiation of stem cells into cardiomyocytes. **Biochem Biophys Res Commun** 354: 999-1003.

Strem, B.M., Hicok, K.C., Zhu, M., Wulur, I., Alfonso, Z., Schreiber, R.E., Fraser, J.K., Hedrick, M.H. (2005). Multipotential differentiation of adipose tissue-derived stem cells. **Keio J Med** 54:132-141.

Sudhoff, T. and Sohngen, D. (2002). Circulating endothelial adhesion molecules (sE-selectin, sVCAM-1 and sICAM-1) during rHuG-CSF-stimulated stem cell mobilization. **J Hematother Stem Cell Res** 11: 147-151.

Sulz, L., Astorga, G., Bellette, B., Iturriaga, R., Mackay-Sim, A., Bacigalupo, J. (2009). Nitric oxide regulates neurogenesis in adult olfactory epithelium in vitro. **Nitric Oxide** 20:238–252.

Takada, I., Kouzmenko, A.P., Kato, S. (2009): Molecular switching of osteoblastogenesis versus adipogenesis: implications for targeted therapies. **Expert Opin Ther Targets** 13: 593-603.

Talens-Visconti, R., Bonora, A., Jover, R., Mirabet, V., Carbonell, F., Castell, J.V., Gomez-Lechon, M.J. (2006). Hepatogenic differentiation of human mesenchymal stem cells from adipose tissue in comparison with bone marrow mesenchymal stem cells. **World J Gastroenterol** 12: 5834-5845.

Tao, H., Rao, R., Ma, D.D.F. (2005) Cytokine-induced stable neuronal differentiation of human bone marrow mesenchymal stem cells in a serum/feeder cell-free condition. **Development, Growth & Differentiation**. 47:423-433. DOI: 10.1111/j.1440-169X.2005.00810.x.

Tapp, H., Hanley, E.N., Jr., Patt, J.C., Gruber, H.E. (2009). Adipose-derived stem cells: characterization and current application in orthopaedic tissue repair. Exp Biol Med (Maywood) 234:1-9.

Thippeswamy, T., Morris, R., (1997). Cyclic guanosine 3',5'-monophosphate-mediated neuroprotection by nitric oxide in dissociated cultures of rat dorsal root ganglion neurones. **Brain Res.** 774:116–122.

Thippeswamy, T., Morris, R., (2001). Evidence that nitric oxide-induced synthesis of cGMP occurs in a paracrine but not an autocrine fashion and that the site of its release can be regulated: studies in dorsal root ganglia in vivo and in vitro. **Nitric Oxide** 5:105–115.

Thomson, M., Liu, S.J., Zou, L.N., Smith, Z., Meissner, A., Ramanathan, S. (2011). Pluripotency factors in embryonic stem cells regulate differentiation into germ layers. **Cell** 145, 875–889.

Tholpady, S.S., Katz, A.J., Ogle, R.C. (2003). Mesenchymal stem cells from rat visceral fat exhibit multipotential differentiation in vitro. **Anat Rec A Discov Mol Cell Evol Biol.** 272(1):398-402.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., Jones, J.M. (1998). Embryonic stem cell lines derived from human blastocysts. Science 282: 1145-1147

Timper, K., Seboek, D., Eberhardt, M., Linscheid, P., Christ-Crain, M., Keller, U.,Muller, B., Zulewski, H. (2006). Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin, and glucagon expressing cells. **Biochem Biophys Res Commun** 341: 1135-1140.

Tio, M., Tan, K.H., Lee, W., Wang T.T., Udolph, G. (2006). Roles of db-cAMP, IBMX and RA in aspects of neural differentiation of cord blood derived mesenchymal-like stem cells. **PLoS One** 5(2):e9398.

Valina, C., Pinkernell, K., Song, Y.H., Bai, X., Sadat, S., Campeau, R.J., Le Jemtel, T.H., Alt, E. (2007). Intracoronary administration of autologous adipose tissue-derived stem cells improves left ventricular function, perfusion, and remodelling after acutemyocardial infarction. **Eur Heart J** 28: 2667-2677.

van Dijk, A., Niessen, H.W., Zandieh Doulabi, B., Visser, F.C., van Milligen, F.J. (2008). Differentiation of human adipose-derived stem cells towards cardiomyocytes is facilitated by laminin. **Cell Tissue Res** 334: 457-467.

Van, R.L., Bayliss, C.E., Roncari, D.A. (1976). Cytological and enzymological characterization of adult human adipocyte precursors in culture. **J Clin Invest 58**:699-704.

Wang, N., Du, C.Q., Wang, S.S., Xie, K., Zhang, S.L., Miao, J.Y. (2004) D609 induces vascular endothelial cells and marrow stromal cells differentiation into neuron-like cells. **Acta Pharmacologica Sinica**. 25:422-6.

Wang, Y., Deng, Z., Lai, X., Tu, W. (2005) Differentiation of human bone marrow stromal cells into neural-like cells induced by sodium ferulate in vitro. **Cellular and Mollecular Immunology**. 2:225-9.

Wei, Y., Sun, X., Wang, W., Hu, Y. (2007). Adipose-derived stem cells and chondrogenesis. **Cytotherapy** 9: 712-716.

Wenisch, S., Trinkaus, K., Hild, A., Hose, D., Heiss, C., Alt, V., Klisch, C., Meissl, H., Schnettler, R. (2006) Immunochemical, ultrastructural and electrophysiological investigations of bone-derived stem cells in the course of neuronal differentiation. **Bone**. 38:911-921.

Winter, A., Breit, S., Parsch, D., Benz, K., Steck, E., Hauner, H., Weber, R.M., Ewerbeck, V., Richter, W. (2003). Cartilage-like gene expression in differentiated human stem cell spheroids: a comparison of bone marrow-derived and adipose tissue-derived stromal cells. **Arthritis Rheum** 48: 418-429.

Wislet-Gendebien, S., Wautier, F., Leprince, P., Rogister, B. (2005a) Astrocytic and neuronal fate of mesenchymal stem cells expressing nestin. **Brain Research Bulletin**. 68:95-102.

Wislet-Gendebien, S., Hans G., Leprince, P., Rigo, J.-M., Moonen, G., Rogister, B. (2005b) Plasticity of Cultured Mesenchymal Stem Cells: Switch from Nestin-Positive to Excitable Neuron-Like Phenotype. **STEM CELLS**. 23:392-402.

Woodbury, D., Reynolds, K., Black, I.B. (2002) Adult bone marrow stromal stem cells express germline, ectodermal, endodermal, and mesodermal genes prior to neurogenesis. **Journal of Neuroscience Research.** 69:908-917. DOI: 10.1002/jnr.10365.

Woodbury, D., Schwarz, E.J., Prockop, D.J., Black, I.B. (2000) Adult rat and human bone marrow stromal cells differentiate into neurons. **Journal of Neuroscience Research**. 61:364-370.

Yang, C-C., Shih, Y-H., Ko, M-H., Hsu, S-Y., Cheng, H., et l. (2008). Transplantation of Human Umbilical Mesenchymal Stem Cells from Wharton's Jelly after Complete Transection of the Rat Spinal Cord. **PLoS ONE** 3(10): e3336.

Zhang, H.-T., Fan, J., Cai, Y.-Q., Zhao, S.-J., Xue, S., Lin, J.-H., Jiang, X.-D., Xu, R.-X. (2010) Human Wharton's jelly cells can be induced to differentiate into growth factor-secreting oligodendrocyte progenitor-like cells. **Differentiation**. 79:15-20.

Zhou, S., Eid, K., Glowacki, J. (2004): Cooperation between TGF-beta andWnt pathways during chondrocyte and adipocyte differentiation of human marrow stromal cells. **J Bone Miner Res** 19: 463-470.

Zuk, P.A., Zhu, M., Mizuno, H., Huang, J., Futrell, J.W., Katz, A.J., Benhaim, P., Lorenz, H.P., Hedrick, M.H. (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. **Tissue Engineering**. 7:211-28.

Zuk, P.A., Zhu, M., Ashjian, P., et al. (2002). Human adipose tissue is a source of multipotent stem cells. **Mol Biol Cell** 13:4279-95.

CHAPTER III

EFFECT OF 8 BROMO-CYCLIC GMP ON NEURAL TRANSDIFFERENTIATION OF HUMAN ADIPOSE STEM CELLS

3.1 Abstract

Stem cell transplantation is one of the most promising tools for neurogenerative diseases treatment. Due to limitations and challenges to utilize human embryonic stem cells (ESCs) and neural stem cells (NSCs), mesenchymal stem cells (MSCs), especially from adipose tissue serve a potential alternative. They are easily accessible, abundantly available throughout the body and can be cultured for several months *in vitro* with low levels of senescence. They are also able to differentiate toward cells in neuroectodermal lineage under appropriate condition. Therefore, finding the most efficient way to differentiate these cells would be beneficial for clinical research.

Nitric oxide (NO), a well-characterized inter- and intra-cellular messenger synthesized by nitric oxide synthase (NOS), has been demonstrated to play roles in both embryonic neural tissue formation and adult neurogenesis. NO together with soluble guanylyl cyclase (sCG) reguluates intracellular cyclic GMP (cGMP) level elevation through NO-cGMP signaling pathway. This pathway is involved in diverse cellular regulations such as cell growth, survival, differentiation, proliferation,

migration, axon guidance and many other processes through a variety of downstream signaling cascades. Moreover, cGMP, as the mediator, help support NO regulate neuronal cell survival, differentiation and neuroprotective as well. The aim of this study was to find the effect of 8Br-cGMP on neural differentiation of hASCs.

After hASCs were isolated from the adipose tissue using standard protocol, they were expanded and characterized by immunophenotypical and multipotency analyses. They were positive for CD73, CD90, CD105 and Vimentin and were negative for CD34 and CD45. hASCs upon induction could differentiate toward adipocytes, chondrocytes and osteocytes, implying that hASCs isolated from adipose tissue displayed the mesenchymal stem cell properties. To optimize 8Br-cGMP concentration, hASCs under different 8br-cGMP concentration (0µM, 10µM, 100µM) were investigated using phenotypical and genotypical analyses. After induction for one week, the majority of neural induced hASCs (NI-hASCs) under 10µM 8Br-cGMP condition appears as sharp, elongated bi- or multipolar cells with primary and secondary processes. They were positive for Nestin, Sox2, TUJ1 and NF-L, significantly ($p<0.001$) higher than other conditions. Gene expression analysis revealed that NI-hASCs under 10µM 8Br-cGMP condition were neuronal committed as significant higher level of MASH1, GAP43, TUJ1, NF-L, MAP2 mRNAs were expressed in 10µM 8Br-cGMP. Nestin and Sox2 expression level of all treated 8Br-cGMP conditions were relatively similar, but significantly higher than the controls. NI-hASCs under 10µM 8Br-cGMP condition displayed significant highest population of $TUJ1^+$ NI-hASCs (73.10%±7.40%, $p<0.001$) with neurite. The GAP43 expression level of $TUJ1^+$ NI-hASCs under 10µM 8Br-cGMP condition was consistent with neurite outgrowth analysis results as they showed higher total neurite length and

number of neurite per cell ($p<0.05$). Upon further differentiation into mature neuron and glial cells, NI-hASCs from 10μM 8Br-cGMP condition could differentiate toward neuronal and glia cells as confirmed by RT-qPCR and immunocytochemistry results. Our findings highlight that NI-hASCs displayed neuronal progenitor profile, yet remained their plasticity as they can differentiate toward glial cells after exposed to PDGF-AA. Future studies employing *in vivo* transplantation models and their mechanisms to investigate the effectiveness of NI-hASCs are warranted.

3.2 Introduction

Neurodegenerative disease is caused by the progressive loss of structure of function of neurons. In most case, the disease is triggered for unknown reasons and progress continuously. Major diseases of neurodegenration are Alzheimer's disease (AD) and Parkinson's disease (PD). Additionally, not only AD and PD can be found among the nation more frequent with increasing lifespan, but stroke and traumatic brain injury are also a result of a decline in neuronal function. Furthermore, in chronic inflammatory CNS diseases, such as multiple sclerosis (MS), a loss function of neuroectodermal cells occurs as well. The prevalence of these diseases is commonly seen worldwide with increasing cases reported each year (National Institute of Neurological Disorders and Stroke, 2004). Therefore, a need for therapeutic approaches to restore the loss of neural cells and their physiological functions is urgently required.

Cell-based therapy for the neural regeneration offers a paradigm shift that may lead to alternative therapeutic solutions. Stem cells have been considered as a promising tool in cell-based therapy. Stem cell therapy aims to induce the neuro-

restorative process that is essential to support recovery after a loss of function. Among many applications, neurological diseases are interesting potential target, since the adult nervous system has a limited regenerative capacity.

Among many types of cells that are proposed to be a candidate for neurodegenerative treatment, human ESCs have seemed to be the most powerful tool for cell therapy in animal models, but several challenges need to be resolved before translate ESCs application in humans. ESCs are genetically and epigenetically unstable which could cause teratoma formation upon transplantation. Another alternative source is neural stem cells (NSCs) that seem to rise up the patient's hope due to their advantageous characteristics. The unlimited differentiation potential *in vitro* and a potential of producing all kinds of brain-specific cell types make them as a feasible candidate in the therapy (Bain et al, 1995; Lee et al, 2000). However, due to their accessible difficulty, limited source of cells, and weak immune rejection potential, utilizing these cells in cell-based therapy would be challenging.

Mesenchymal stem cells (MSCs) have been recently brought up against those cells because of little ethical controversy surrounding them. These cells hold many features which are easily collect, abundant availability, extensively expansion *in vitro*, and the ability to repair the tissue by differentiating itself to replace injured cells or by creating an environment favorable for the tissue repair by endogenous cells (Izadpanah et al, 2005). Moreover, increasing evidence on the neuroectodermal lineage commitment of mesenchymal stem cells (MSCs) has also raised a good appeal of MSCs in cell-based therapies (Deng et al, 2001; Jiang et al, 2002; Sanchez-Ramos et al, 2000, Woodbury et al, 2002). Among many sources of MSCs, adipose tissue seems to be promising source of MSCs since they are available in large quantities

(over hundreds of million cells per individual) and are easily isolated and can be cultured for several months *in vitro* with low levels of senescence (Zuk et al, 2001; Zuk et al, 2002). Investigating the utilization and application of these cells would be beneficial in cell-based therapy.

Nitric oxide (NO) is a short lived diatomic free radical species that is synthesized by nitric oxide synthases (NOS). At low nanomolar concentrations of NO, activation of soluble guanylyl cyclase (sGC: the major NO receptor) triggers intracellular cyclic GMP level elevation (Arnold et al, 1977; Katsuki et al, 1977). This signaling pathway, also known as NO-cGMP signaling pathway, is involved in diverse physiological processes, such as smooth muscle relaxation, neurotransmission, blood pressure regulation, inhibition of platelet aggregation and immunomodulation. Its roles at cellular level are to regulate cell growth, survival, differentiation, proliferation, migration, axon guidance and many other processes through a variety of downstream signaling cascades depending on cell type specific regulation. cGMP, as the mediator, help support NO regulate cell survival, differentiation and neuroprotective (Thippeswamy and Morris, 1997, 2001; Ciani et al, 2002). 8 bromo cyclic GMP (8Br-cGMP), NO donor, has been extensively investigated in neural differentiation of many types of cell, but the evidence of applying this agent in hMSCs is rarely seen. The effect of this NO donor on neural differentiation of hASCs remained questionable. In this study, the effect of 8 bromo cyclic GMP was investigated whether they could induce the differention of hASCs into neural-like cells that hold both of neural phenotypic and genotypic profiles.

3.3 Materials and Methods

3.3.1 Isolation and expansion of hASCs

Isolation of human adipose stem cells was carried out using mechanical and enzymatic isolation procedures described previously (Gimble and Guilak, 2003; Zuk et al., 2001). Briefly, adipose tissue (N=1) was obtained from female patient (42 years of age) that underwent a surgery at Maharaj hospital, Nakhon Ratchasima. Patient was initially informed and the sample was collected after the inform consent was voluntarily signed by the patient. This work has been approved by Ethics Committee for Researches Involving Haman Subjects, Suranaree University of Technology and Maharat Nakorn Ratchasima Hospital Institutional Review Board (MNRH IRB). Adipose Tissue was stored in 4°C sterile phosphate buffered saline without calcium and magnesium (PBS). Then, adipose tissue was transferred to Embryo Technology and Stem Cell Research Center (ESRC), Suranaree University of Technology. Once adipose tissue was immersed in 75% ethanol for 30 seconds under sterile manner, the tissue was then transferred to PBS at 37˚C and manually minced into small fragments using surgical blade and scissors. After that, the tissue was digested by collagenase type I with stirring magnetic bar in a water bath at 37˚C for 60 min. This cell suspension was then centrifuged at 400 g for 5 min and the supernatant was discarded. The cells were seeded and expanded in media containing alpha modification of Eagle's medium (α-MEM; GIBCO-BRL) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen), and then incubated at 37°C under humidified atmosphere of 5% CO_2 in air. The medium was changed three times a week to remove cell debris. Upon 70-80% confluency, hMSCs cultured in media supplemented with FBS was

detached using trypsin, an animal-derived serine protease. The cells were expanded until passage 1 (P1) then either experimented directly or cryopreserved with 10% dimethyl sulfoxide (DMSO; Sigma -Aldrich) and stored in liquid nitrogen.

3.3.2 Characterization of hASCs by standard methods

3.3.2.1 Colony forming unit - fibroblast (CFU-F) assay.

To optimize culture condition of hASCs, CFU-F assay was performed. Briefly, 100 cells of P2 and P5 hASCs were independently cultured in 100 mm culture dish (SPL life sciences) with culture medium supplemented with 5%, 10% and 15% fetal bovine serum (FBS, Hyclone) for two weeks. The medium was changed every three days. After 14 days of seeding, the cells were fixed in 4% paraformaldehyde (PFA, Sigma) for 20 minutes, followed by staining with 3% crystal violet (Sigma). Colony that contains ≥50 cells was counted. The number of the colony was calculated in the following equation. Each condition was performed in triplicate.

$$\%\, CFU = \frac{total\ number\ of\ colony}{initial\ cells\ seeded} \times 100$$

3.3.2.2 Immunocytochemistry analysis

Passage 4 of hASCs were plated into 4-well tissue culture dish (Nunc, Roskilde, *Denmark*) and cultured under their original condition until reaching 70% confluence. The cells were then washed with PBS for 3 times and fixed by 4% PFA (Sigma) at room temperature for 15 minutes. The fixed cell were then washed 3 more times with PBS and incubated with blocking buffer consisting of 10% normal goat serum at room temperature for 2 hours. After that, the cells in each well were

incubated at 4°C overnight with primary rabbit antibodies raised against CD45, CD73, CD90 and CD105; with primary mouse antibodies raised again CD34 and Vimentin (1:200; all from Santa Cruz Biotechnology, California, *USA*). Next, the samples were washed 3 times with PBS and incubated with secondary antibody, Alexa fluor® 488 goat anti rabbit IgG (1:1000; Invitrogen), for 2 hours at room temperature. After 6 times washing with PBS, cells were then counterstained with 1 mg/ml 40, 6-diamino-2-phenylindole (DAPI; Sigma) for 10 minutes before 2 times washed with PBS. Finally, the cells were observed and photographed under a fluorescent microscope (Olympus, model BH2-RFL-T3).

3.3.2.3 Multipotency analysis

Isolated cells were induced to differentiate into mesodermal lineages which are osteoblasts, adipocytes and chondrocytes.

i) Osteogenic differentiation

hASCs at the density of $5x10^3$ cells/cm^2 were plated onto 0.1% gelatin (Sigma) coated 35 mm diameter tissue culture dish (SPL life sciences) under MSC culture medium until reaching complete confluence. After that, the hMSCs culture medium without any fetal bovine serum was supplemented with 100 nM dexamethasone, 0.2 mM L-ascorbate-2-phosphate, 2 mM L-glutamine, 10 mM β-glycerophosphate, 100 units/ml penicillin and 100 µg/ml streptomycin (all from Sigma –Aldrich). The medium was subsequently changed every other day for three weeks. The culture was then fixed in 4% PFA followed by Alizarin Red S (Sigma) staining for 30 minutes. Then, cells were washed with DI water until no background

was observed. Morphology and stained cells were observed under inverted microscope (Olympus, nodel BH2-RFL-T3).

ii) Adipogenic differentiation

Cells were prepared as described for osteogenic differentiation until reaching 80% confluence. Afterwards, the medium without fetal bovine serum was supplemented with 10 µg/ml insulin, 100 µM indomethacin, 1 µM dexamethasone, 0.5 mM isobutyl methylxanthine (IBMX), 100 units/ml penicillin and 100 µg/ml streptomycin. Medium were subsequently replaced every 3 days for 3 weeks. Then, cells were fixed in 4% PFA (Sigma) for 30 minutes and washed 3 times by PBS. The evidence of fat globules formation was confirmed by Oil Red O (Sigma) staining for 30 minutes. Then, cells were washed 3 times by PBS and stained cells were observed under inverted microscope (Olympus, model BH2-RFL-T3).

iii) Chondrogenic differentiation

To induce chondrogenic differentiation, the cells were prepared as described for osteogenic differentiation until reaching 80% confluence. Then, the medium were reduced to 2% serum content and supplemented with 0.05 mM ascorbate 2-phosphate, 40 µg/ml L-proline, 1 mM sodium pyruvate, 100 nM dexamethasone, 100 units/ml penicillin and 100 µg/ml streptomycin (all from Sigma –Aldrich). Additionally, 10 ng/ml TGF-β3 (R&D Systems, Minnesota, USA) and 1% ITS-X (Gibco®, Invitrogen) were also supplemented. Medium were replaced every 3 days for 3 weeks. After that, cells were fixed with 4% PFA (Sigma) for 30 minutes and washed 3 times by PBS. In order to detected extracellular matrix produced by chondrocyte, cells were stained by Alcian blue 8GX (Sigma) for 30 minutes. Then,

cells were washed 3 times by PBS and stained cells were observed under inverted microscope (Olympus, model BH2-RFL-T3).

3.3.3 Neural transdifferentiation of hASCs

3.3.3.1 8Br-cGMP concentration optimization

Neural induction was performed according to a modified protocol from Johe et al. (Johe et al, 1996). Passage 5 of hASCs were cultured in 35 mm culture dish (SPL LIFE Science) coated with 10µg/ml Poly-L-Lysine (Cultrex) and 5µg/ml mouse laminin (Cultrex). Upon reaching confluence of 70%, basal medium was replaced with N2B27 basal medium which is a mixture of DMEM/F12 (Gibco) and Neurobasal (Gibco) medium (1:1) supplemented with 1%N2 (v/v) and 2%B27 (v/v) supplementation (Gibco). 8Br-cGMP (Sigma) was added to each independent hASCs induction medium at different concentration (0µM, 10µM and 100µM) for 14 days. Additional supplementations which are 20ng/ml epidermal growth factor (EGF, Sigma), 20ng/ml basic fibroblast growth factor (bFGF, Sigma), 100ng/ml human noggin (Prospec), 10µM SB431542 (Calbiochem), 10µM retinoic acid (Sigma), 5U/ml Heparin (Sigma), 100U/ml penicillin and 100µg/ml streptomycin (Invitrogen) were added in every conditions mentioned earlier. Induction medium was changed in every two days for 14 days. hASCs under N2B27 basal medium supplemented with 0.5% DMSO (v/v, Sigma) and DMEM/F12 alone conditions, serving as controls in this study, were cultured along with 8Br-cGMP treated groups. hASCs at Summary of experimental groups are listed below:

1) N2B27 basal medium with supplementations+ 0µM 8Br-cGMP
2) N2B27 basal medium with supplementations+ 10µM 8Br-cGMP
3) N2B27 basal medium with supplementations+ 100µM 8Br-cGMP

4) N2B27basal medium with 0.5% DMSO

5) DMEM/F12 medium

hASCs at D7 and D14 were examined in different analyses. Optimal condition was selected for terminally differentiate to mature neurons and glia using NeuroCult™ NS-A differentiation kit for human cells (Stem Cell, USA) for 10 days. Neural like cells were analyzed by immunocytochemistry and RT-qPCR analyses. Every experiment was done in triplicate independently.

3.3.4 Characterization of neural induced hASCs (NI-hASCs)

3.3.4.1 Immunocytochemistry analysis

NI-hASCs at d7, d14 and mature neural cells cultured on 8-well cell culture chamber slide (SPL Life Sciences) were washed with PBS for three times and fixed in 4% PFA, permeabilized with 0.2% Triton X-100 (Sigma, USA) in PBS at room temperature for 10 min, and blocked with 10% normal goat serum (Zymed, USA) at room temperature for 60 min. Cells were incubated at 4˚C overnight with the following primary antibodies: mouse a-Nestin (Abcam, 1:200), mouse a-Sox2 (R&D systems, 1:50), rabbit a-Glial-Fibrillary-Acidic-Protein (GFAP, Abcam, 1:200), mouse a-Beta III tubulin (TUJ1, Abcam, 1:200), mousea-Neurofilament-Light (NF-L, Abcam, 1:200), mouse a-Olig2 (Abcam, 1:100) and goat a-Choline acetyltransferase (ChAT, R&D systems, 1:100). Next, the samples were washed 3 times with PBS and incubated with three different secondary antibody which are Alexa fluor® 488 goat anti rabbit IgG (1:1000; Invitrogen), Alexa fluor® 488 goat anti mouse IgG (1:1000; Invitrogen) Alexa fluor® 488 donkey anti goat IgG (1:1000; Invitrogen) based on primary antibody reactive species for 2 hours at room temperature. After 3 times

washing with PBS, cells were then counterstained with 1 mg/ml 40, 6-diamino-2-phenylindole (DAPI; Sigma) for 10 minutes before 2 times washed with PBS. Finally, the cells were observed and photographed under a fluorescent microscope (Nikon, model Eclipse-Ti-S1 DRi-UJ-D).

3.3.4.2 RT-qPCR Analysis

The Total RNA extraction mini kit (RBC Bioscience) was used for the isolation of total RNA from the cell pellets of hASCs, NI-hASCs and mature neural cells. DNaseI set (amplification grade, life technologies) was used for the removal of genomic DNA in the RNA sample. Then, RNA was reverse-transcribed in the presence of oligo-dT primer using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad). Primers that are specific to different types of neural cells were used (See Appendix.) The optimum annealing temperature for each primer pair was determined experimentally. The KAPA SYBR® Fast qPCR Kit (KAPA Biosystems) was used for all RT-PCRs as per the manufacturer's instructions with the addition of cDNA or RNase-free water (negative control). Biorad CFB3240G Chromo4 Thermocycle was used for all reactions. The cycling parameters were as follows: a reverse transcription step (42°C, 30 min), RT inactivation step (85°C, 5 min), KAPA Enzyme activation step (95°C, 3 min), followed by 40 cycles of denaturation (95°C, 3 sec) and annealing/extension (20 s at different temperature, see Appendix) followed by fluorescence plate read at the temperature obtained from melting curve analysis. Once the thermal cycling was complete, the mRNA values for each gene were normalized to that of the housekeeping gene *β-actin*. The Ct value was calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schimittgen, 2001).

3.3.4.3 Neurite analysis of NI-hASCs

The morphology of NI-hASCs treated with various 8Br-cGMP concentrations for 7 days were analyzed using In Cell Analyzer 1000 Cellular Imaging and Analysis System (GE Healthcare Life Science). Neurite outgrowth automated scoring was performed on the samples immunostainned with TUJ1 (essential tubulin highly expressed in neural cells) together with DAPI as previously described by Ramm et al., 2003 with some modification. Neurite widths and lengths from a few typical images were measured at 100X magnification. Prior entry of these values was required to minimize errors in this system. Minimum neurite width was specified as 0.8µm, representing a width of 1 pixel using a 10X objective, and maximum width was specified as 10µm. Minimum neurite length was specified as greater than 20µm.

To separate cell body out of neurites and debris, mean equivalent cell area (MECA) was calculated for each condition based on DAPI staining. Total cell area was then divided by MECA to estimate the number of cells in each field. The mean of the 6 measurements was recorded as the MECA value. Differentiated cells that were positive for TUJ1/DAPI were selected based on these criteria. Well that contains more than 2,000 cells was selected and cells were analyzed in 12 different fields (N=12).

The primary parameters of the automated scoring are total neurite length, total cell body area and number of neuritis. Then, these values were derived into mean neurite length, mean neurite length/cell and total neurite length/cell by following equations:

$$Mean\ neurite\ length = \frac{total\ neurite\ length}{number\ of\ neurites}$$

$$Total\ neurtie\ length/Cell\ = \frac{total\ neurite\ length}{number\ of\ cells}$$

3.3.5 Neuronal and glial differentiation of 10μM 8Br-cGMP induced NI-hASCs

10μM 8Br-cGMP induced NI-hASCs were further differentiated using NeuroCult™ NS-A Differentiation Kit Human (STEMCELL™ Technologies) for another 10 days as recommended by the instruction manual. To differentiate NI-hASCs into neurons, 10ng/ml Nerve Growth Factor (NGF) was added into the differentiation medium as well as 10ng/ml Platelet Derived Growth Factor-AA (PDGF-AA) for glial differentiation. NI-hASCs under NGF, PDGF, and normal NS-A differentiation medium were used to examine their gene expression pattern and phenotype by immunocytochemistry and RT-qPCR analyses as previously mentioned earlier. hASCs from DMEM condition were also differentiated under NS-A differentiation medium to justify the spontaneous differentiation of hASCs and the effect of 10μM 8Br-cGMP. Neural cells derived from human embryonic carcinoma (NPC-EC) according to Peter W. Andrews' protocol were used as a positive control (Andrews, 1984).

3.3.6 Statistical analysis

All experiment was performed in triplicate. Neurite outgrowth analysis was measured in 6 replications. For RT-qPCR analysis, three independent samples

were replicate in the analysis. Statistical analysis was performed using SPSS 17.0 (SPSS, Inc., USA). CFU-F and RT-qPCR analyses were determined using a two-way ANOVA, with the level of significance set at $p < 0.05$. Immunopositive cell and neurite outgrowth analyses were determined using a one-way ANOVA, with the level of significance set at $p < 0.05$. *Post hoc* comparisons between all groups were performed using the Turkey multiple comparison test. Data are represented as mean ± SD. In every analysis, Difference were considered significant at $*p< 0.05$, statistically significant at $**p< 0.01$ and statistically significant at $***p<0.001$

3.4 Results and discussions

Recently, stem cell technology has become a promising tool regenerative medicine practice. Among other types of stem cell, hMSCs are considered as an outstanding candidate because of their availability, home and engraft ability, immunosuppressive properties as well as ability to differentiate into various specific cell types (Brooke et al., 2007). Apart from that, they hold a potential to differentiate toward neural cells as evidenced from many reports elsewhere (Mitchelle et al, 2003; Hermann et al, 2004; Kiraly et al, 2009; Zhang et al, 2010; Jang et al, 2010). As NO/cGMP signaling pathway plays a vital role in neuronal cell survival and differentiation, many reports have found that nitric oxide donor could differentiate many type of cells into neural-like cells (Feng et al, 2002; Estephane and Anctil, 2009; Sulz et al, 2009; Li et al, 2010; Muller et al, 2010). However, only a few evidences of using this agent were found in hMSCs, and most of them were done in MSCs from bone marrow. In this study, genotype and phenotype analyses of neural

differentiation of hASCs using 8Br-cGMP were performed to establish another efficient method of neural differentiation in hASCs.

3.4.1 Optimization of cell passage and fetal bovine serum concentration in culture condition

hASCs isolated from human adipose tissue obtained from the hospital was successfully established in this study (Fig. 3.1). At primary passage (P0), cells growth and survival were observed during 7 days of culture. As shown in Fig 3.1 A, hASCs at P0 had the fibroblast-like morphology. Upon reaching their confluence, hASCs were expanded in their originate condition. They grew as monolayer of adherent fibroblast like cells (Fig 3.1 B). The morphology of hASCs in this study had no remarkable differences in cell morphology observed among different culture systems in other studies (Gimble and Guilak, 2003; Zuk et al., 2001).

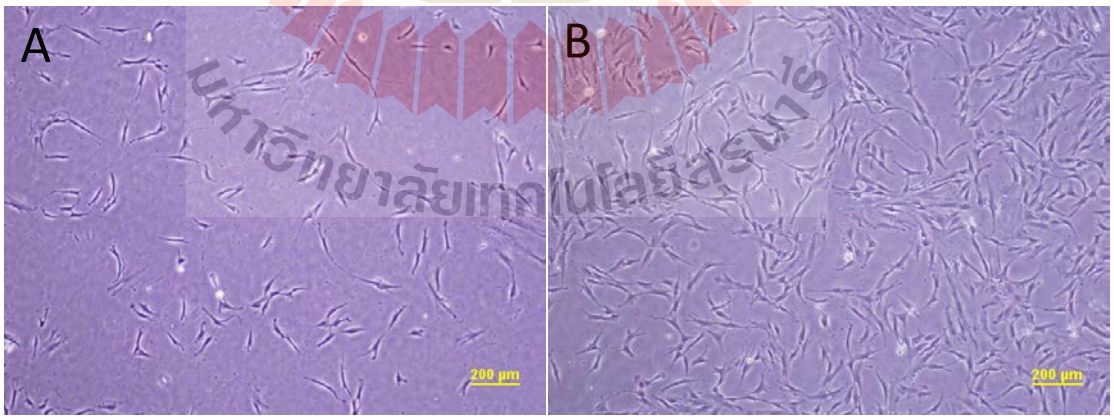


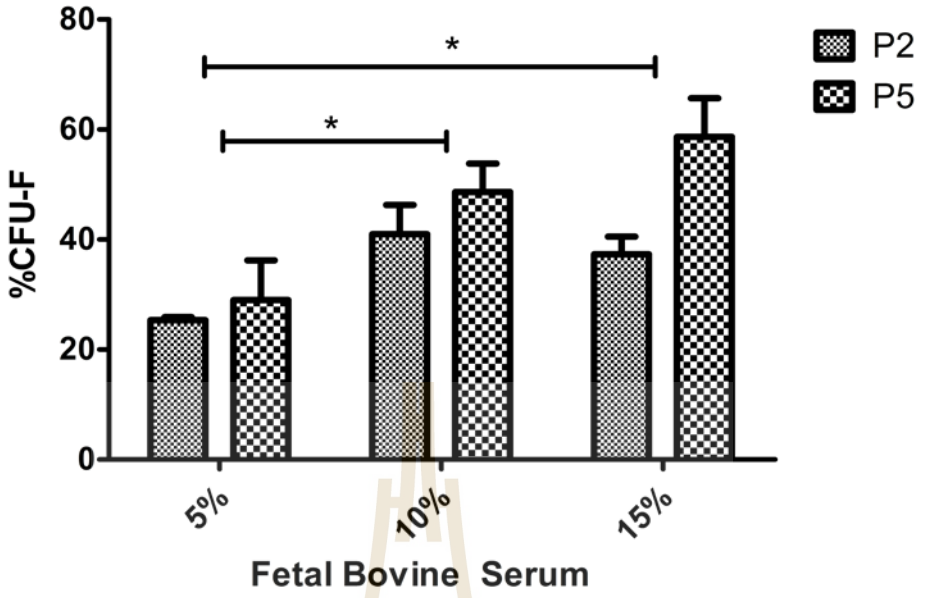
Figure 3.1 Morphology of day 1 hASCs at P0 and P1 culture. Representative phase contrast images of hASCs at (A) primary passage at d1 and (B) passage 1 up on reaching confluence in standard culture systems. hASCs displayed typical fibroblast-like morphology. Scale bar: 200 µm.

To assess optimal culture condition, colony forming unit-fibroblast assay was used in this study to define suitable cell passage and optimal fetal bovine serum concentration. Three FBS concentrations (5%, 10%, 15%) in the culture media was optimized with culture media supplemented with 10% FBS as reference in hASCs at P2 and P5 as shown in Fig. 3.2. We found no significance difference between hASCs at P2 and P5 in every FBS concentration. Regarding FBS concentration, hASCs cultured in media with 5% FBS showed significantly lower number of cell colony than other concentrations ($p<0.05$), whereas no statistical difference was found between 10% FBS and 15% FBS (Fig 3.2). Based on the results, the optimal culture condition of hASCs isolated in this study was at 10% FBS and hASCs at P5 showed no significance difference to P2 in CFU-F. The implication of this finding suggests that hASCs at P5 could be used for differentiation and further analyses which is in consistent with Guilak and his colleagues' findings that hASCs could also differentiate to more than two lineage specific pathway even at passage 4 with 10 fold greater differentiation capacity than the earlier passage (Guilak etl al, 2006).

3.4.2 Characterization of hASCs by standard MSCs method

Standard characterization methods prescribed for identification of MSCs (Dominici et al, 2006), including surface immunoantigen and multipotency analyses, were used in this study.

Surface marker analysis was performed using immunocytochemistry (ICC) method. Overall, hASCs showed positive expression of adhesion molecules CD73, CD105, extracellular matrix protein CD90 and Vimentin (Fig. 3.3).



CFU-F Assay (%)	*FBS Concentration*		
	5%	**10%***	**15%***
P2	25.33±0.58***	41±5.29	37.33±3.21
P5	29±7.21	48.67±5.13	58.67±7.02

Figure 3.2 CFU-F analysis of hASCs at P2 and P5 with various FBS concentrations. Comparison of colony forming unit of hASCs at P2 and P5 with three different FBS concentrations. Mean ± SD from three independent experiments is shown in the table below the figure. Statistical significance is indicated (two-way ANOVA, *post hoc* comparisons between all groups were performed using the Turkey test, *p<0.05, **p<0.01, ***p<0.001).

All cells lacked expression of CD34 and CD45 (Fig.3.3), suggesting lack of cells of hematopoietic and angiogenic lineages. Altogether, the ICC results show that hASCs in this study analyzed display similar mesenchymal stem cell phenotypes based on their surface marker expression.

All cell sources expressed stem cell markers reported existing in other human MSC sources which are DPSCs, ASCs and BMSCs (Pittenger et al, 1999; Zuk et al, 2002; Gimble and Guilak, 2003; Katz et al, 2005; Strem et al, 2005; Kocaoemer et al, 2007; Bieback et al., 2009). These markers include mesenchymal cell markers CD73, CD90, CD105 which are the least markers required to identify human mesenchymal stem cells (Gronthoset al., 2001; Dominici et al, 2006; Ikeda et al, 2006).

Multipotency potential of hMSCs was evaluated by the induction of mesodermal lineage differentiation. After 21 days of induction, isolated hASCs from were able to differentiate toward osteoblasts, adipocytes, and chondrocytes, as evidenced by calcium minerization, extracellular matrix proteoglycan accumulation and lipid droplets formation, respectively (Fig 3.4). These results could identify that hASCs isolated in this study were stromal multipotent cells that could differentiate in the mesodermal lineage pathway.

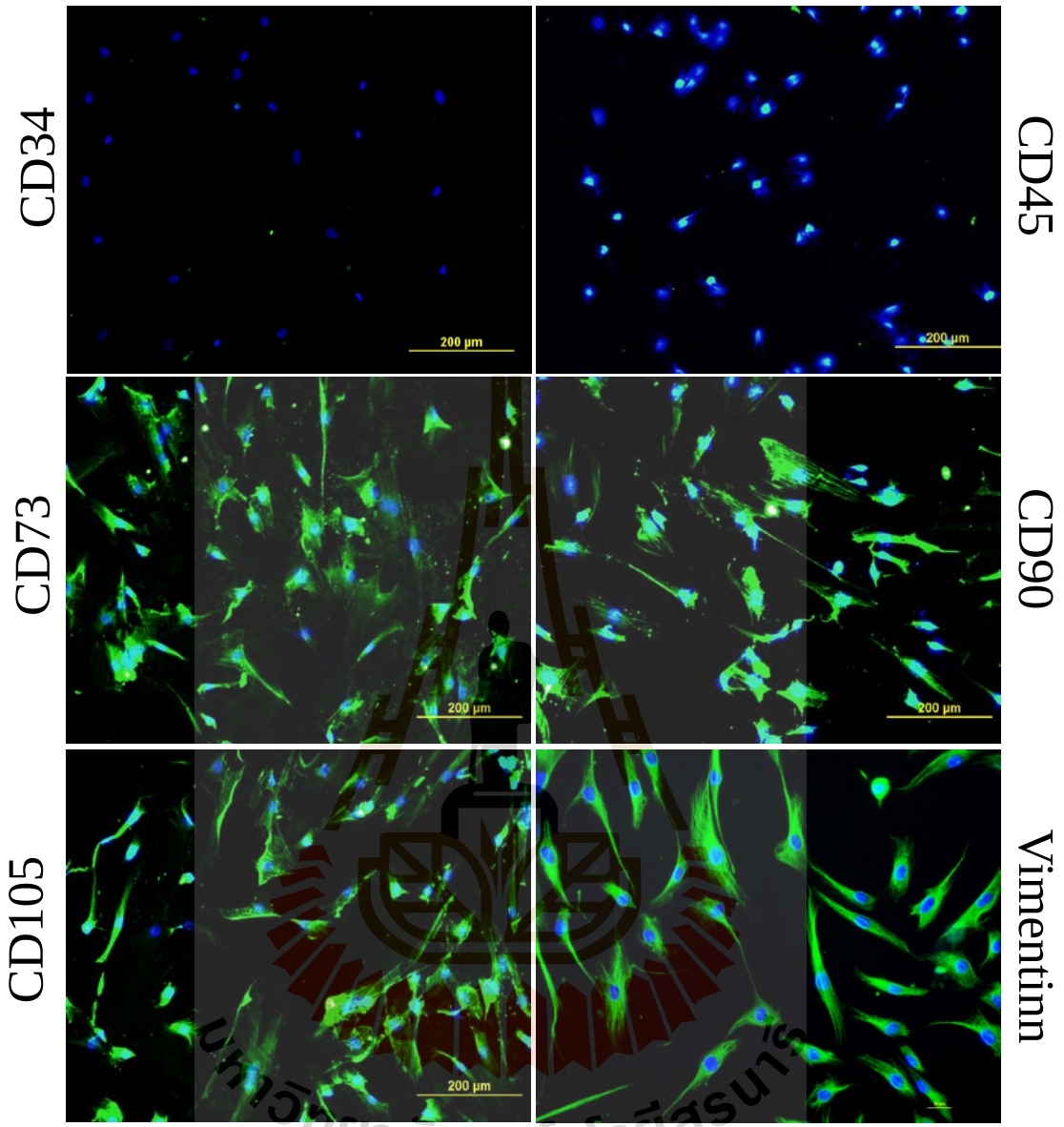


Figure 3.3 Immunocytochemical staining of surface antigen expression pattern. Representative images of immunophenotype of hASCs. Cells were positive for CD73, CD90, CD105 and Vimentin but negative for CD34 and CD45. Scale bar: 200 µm and 100 µm (Vimentin)

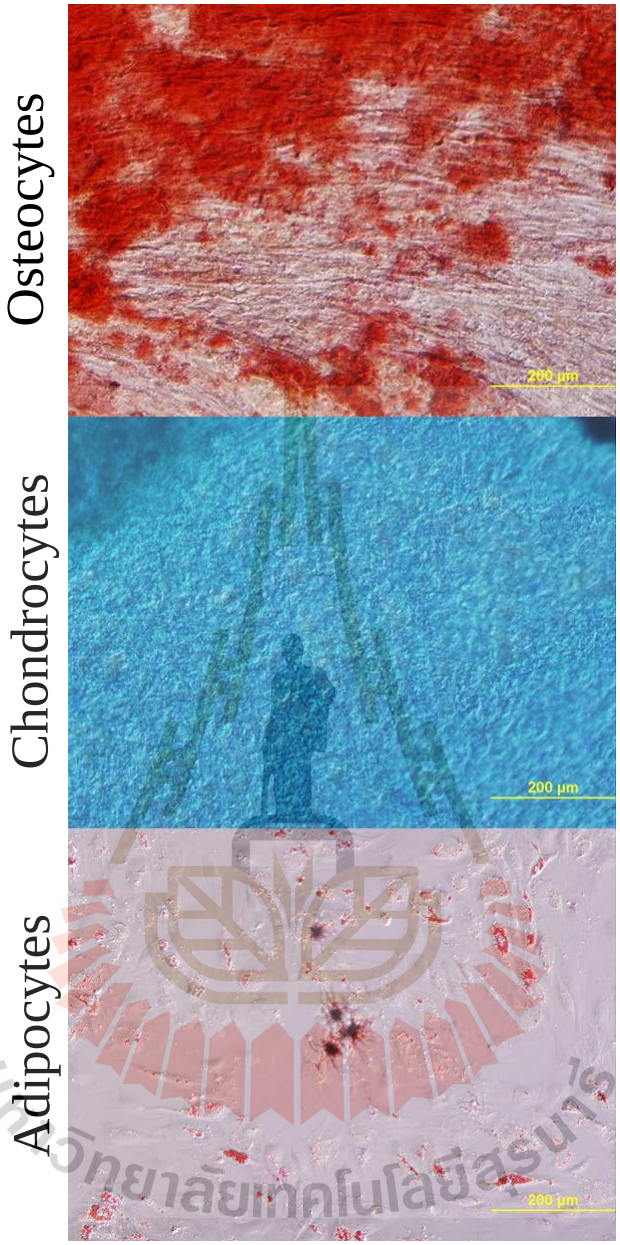


Figure 3.4 Mesodermal lineage differentiation potentials of hASCs. Passage 5 of hASCs was subjected to differentiate toward osteoblasts, chondrocyte and adipocyte. Representative images showed the detectable of calcium minerization, extracellular matrix proteoglycan accumulation and lipid droplets formation stained with Alizarin Red, Alcian blue and Oil red O, respectively. Scale bar: 200 µm

3.4.3 Phenotypic evaluation of neural induction of NI-hASCs

In order to assess neural differentiation potential of 8Br-cGMP, the phenotype of NI-hASCs were analyzed by inverted microscope and immunocytochemistry analysis. In this study, hASCs isolated from adipose tissue were neurally induced by 8Br-cGMP supplementation at three various concentrations. NI-hASCs from 3 replicate experiments were analyzed. After exposure to neural induction medium for a few days, NI-hASCs exhibit very rapid morphological changes: most cells retract their cytoplasm and emit cellular protrusions; some cells form spherical cell body; some cells remain large and flat just like normal hASCs (Fig 3.6 hASCs). Fig 3.5 represents the morphology of NI-hASCs after exposure to neural induction medium supplemented with 8Br-cGMP for 7 days. We can identify three morphologically distinct subsets of NI-hASCs: the majority of NI-hASCs, especially in 10μM (Fig 3.5 10μM) appears as sharp, elongated bi- or multipolar cells with primary and secondary processes (Fig 3.5). The remaining hASCs show no apparent response to the differentiation protocol and remain large and flat, with abundant cytoplasm and do not or faintly expressed neural markers. These finding were consistent with previous reports of hASCs neural differentiation (AshiJan et al., 2003; Jang et al., 2010; Zavan et al., 2010). In contrast, the morphology of hASCs induced by DMSO (Fig 3.6 DMSO) and cultured in DMEM/F12 (Fig 3.6 DMEM) as controls were slightly different when compared to hASCs under normal culture condition (Fig. 3.6 hASCs). The nucleus of cells under DMSO and DMEM conditions were enlarged together with radiated cytoplasm in most of the cells in DMSO group.

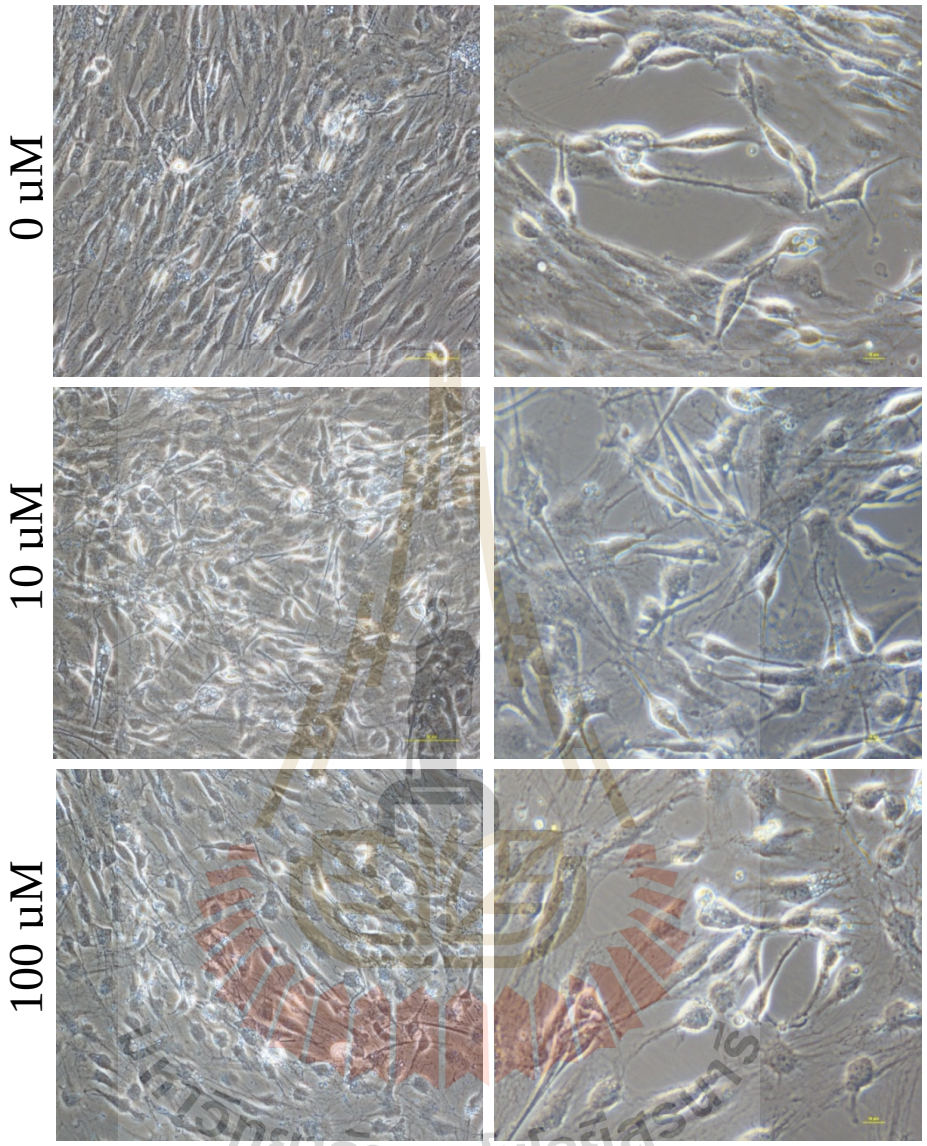


Figure 3.5 Morphology of 8Br-cGMP induced hASCs displayed at d7. Dramatic changes after the exposure to neural differentiation medium in terms of morphology were observed on hASCs; the majority of hASCs assumed an astrocytic/neuronal morphology with bi- and tripolar shape with primary and secondary processes. Scale bar: 50 µm and 10 µm

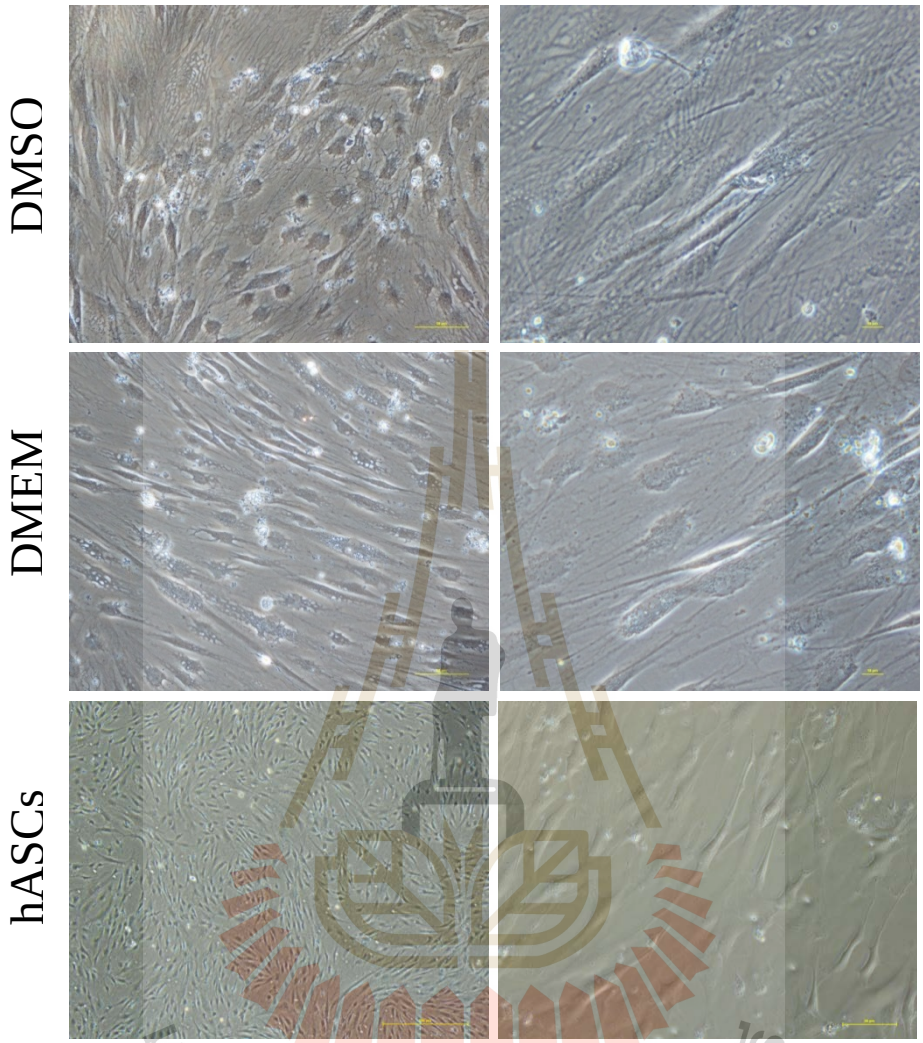


Figure 3.6 Morphology of control conditions of hASCs displayed at d7. The majority of hASCs in DMSO and DMEM treatment remain unchanged. Insignificant changes of morphology were observed in this study. Scale bar: 50 µm and 10 µm (DMSO and DMEM), 100 µm and 50 µm (hASCs)

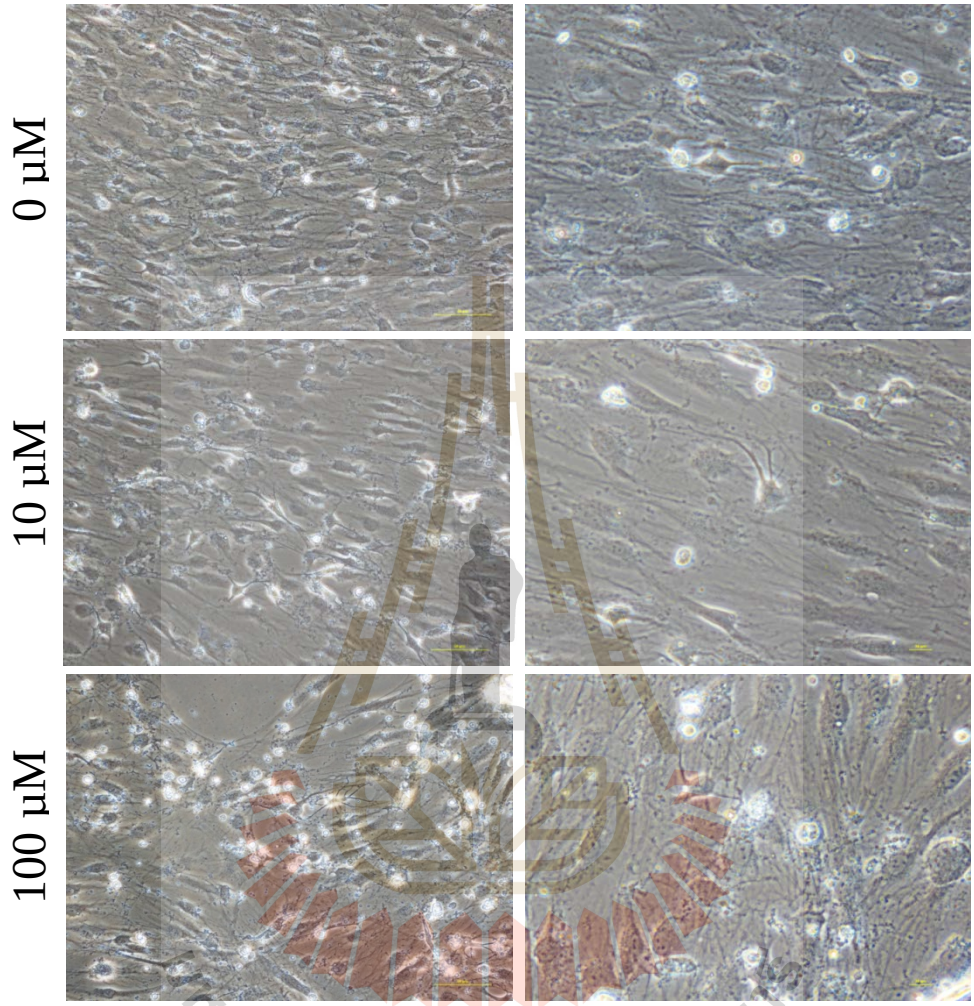


Figure 3.7 Morphology of 8Br-cGMP induced hASCs displayed at d14. The morphology of differentiated hASCs is similar to those at d7. Increasing cell deaths were observed at this period of time. Scale bar: 50 µm and 10 µm

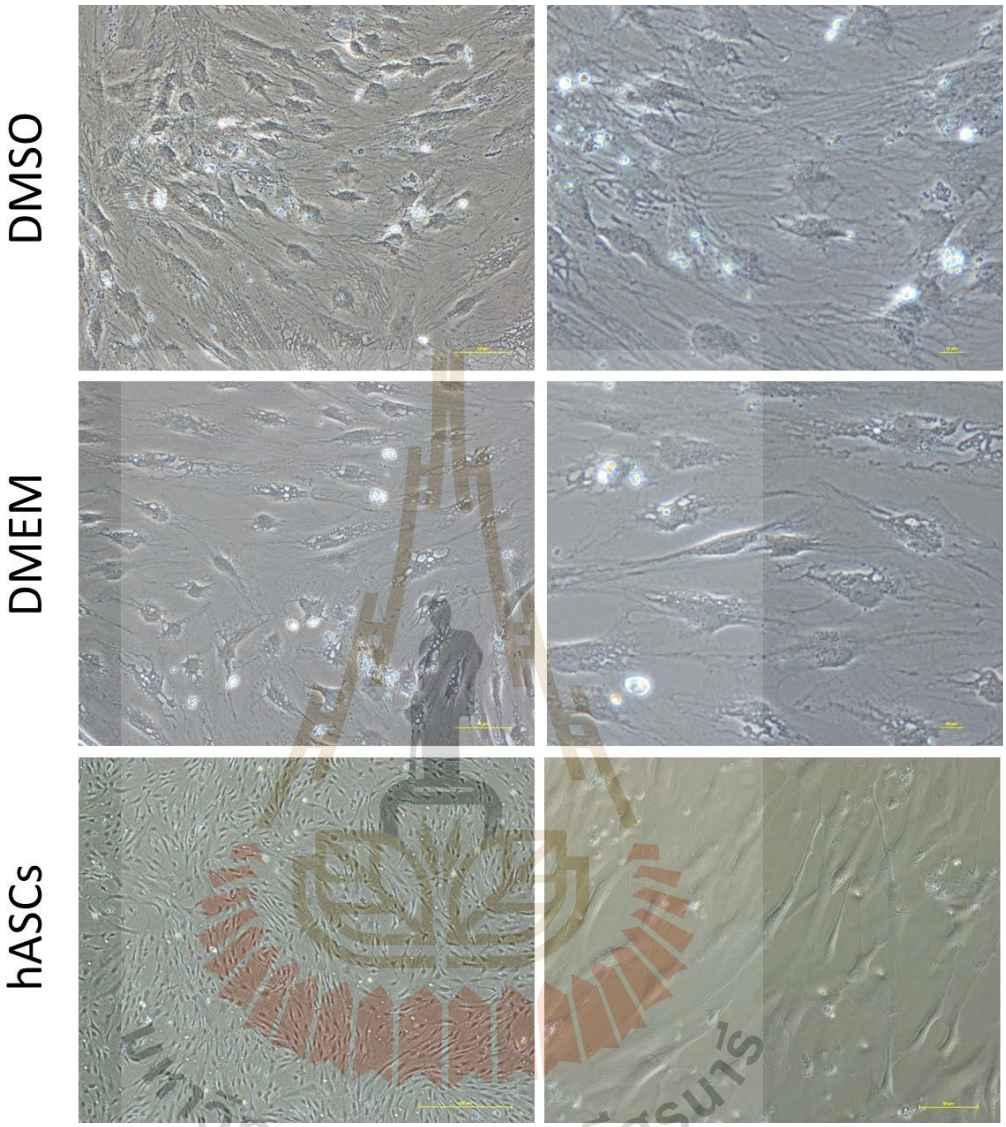


Figure 3.8 Morphology of control conditions of hASCs displayed at d14.

The majority of hASCs in DMSO remain similar to hASCs whereas hASCs in DMEM treatment were flattened, assuming cell aging overtime. Scale bar: 50 µm and 10 µm (DMSO and DMEM), 100 µm and 50 µm (hASCs)

Upon reaching 14 days of differentiation, NI-hASCs population number under 8Br-cGMP conditions was roughly decreasing (Fig 3.7). However, their morphology remains similarly to those at d7 (Fig 3.5). Large number of cell detachment could be observed in all treatments. This incidence might be because of induced apoptosis of undifferentiated cells which also occurred in previous study of neural differentiation in hMSCs from elsewhere (Sanchez-Ramos et al., 2000; Sanchez-Ramos, 2002; Jin et al., 2003; Padovan et al., 2003; Wislet-Gendebien et al., 2003; Zuk et al., 2003; Hermann et al., 2004; Kang et al., 2004; Wislet-Gendebien et al., 2005;). In DMSO and DMEM treatment, their morphology was not different to those at d7.

To assess other features of NI-hASCs, immunocytochemistry and neurite outgrowth analyses were used in this study to clarify whether they hold neural cells properties. Nestin, Sox2, TUJ1 and NF-L staining were used to determine the stage of neural cells induced from hASCs. Analyses (Fig 3.9A) showed that hASCs in normal culture condition were weakly positive for Nestin, Sox2 and TUJ1 (Fig 3.11C, F, I), 10.41%±1.67% of hASCs for Nestin, 34.52%±7.66% for Sox2 and 18.71%±9.99% for TUJ1. Many previous reports also found that hASCs (Ashijan et al, 2003; Mareschi et al, 2006; Jang et al, 2010; Zavan et al, 2010) and hMSCs from other sources (Tondreau et al., 2004; Bertani et al, 2005; Croft and Przyborski, 2009; Wetzig et al, 2013) both in rat and human were positive for these markers. Nestin and Sox2 are typically highly expressed in neural stem cells and has been used as neural progenitor markers. However, Zuk et al. (2002) reported that myogenic cells, endothelial cells and hepatic cells also expressed Nestin which is consistent with our finding that low level of Nestin expression was detected in non-differentiated hASCs.

hASCs in this study also expressed TUJ1 which is the putative neuronal marker. TUJ1 expression in mouse is highly associated with neuronal phenotypes, but the expression is broader distributed in non-neural tissue in human. This incidence reflects the pattern of the transcriptional activity of the gene *tubb3* (Pontius et al., 2003). hASCs also expressed low level of Fibronectin, MAP2, GFAP and S100 (Bertani et al., 2005; Jang et al., 2010), indicating a native neural differentiation potential of these cells. However, in our study, NI-hASCs were not positive for NF-L (Fig 3.11L) and GFAP (data not shown).

Many comparative studies of ASCs and MSCs from other sources found that ASCs held a better potential of neural differentiation as revealed by the immunophenotype results (Kwan et al, 2011; Mostafavi et al, 2014). In this study, NI-hASCs in all experimental groups were positive for Nestin, Sox2, TUJ1 and NF-L after one week of 8Br-cGMP exposure (Fig 3.9). Among all treatment, % positive cells of Sox2 and TUJ1 in 10µM 8Br-cGMP condition were found distinctively higher than other conditions (79.08%±11.31% and 51.98%±18.16%, respectively). NI-hASCs in all 8Br-cGMP induced conditions were highly positive for Nestin and NF-L. The highest % Nestin positive cells was in 10µM 8Br-cGMP condition (83.42%±3.42%), but was not significantly higher than other 8Br-cGMP treated groups. The highest % NF-L positive cells was in 10µM 8Br-cGMP condition (98.34%±0.76%) which was significantly higher than 0µM 8Br-cGMP (77.23%±6.99%), but was not significantly different to 100µM 8Br-cGMP (86.49%±3.92%). It is surprising that NF-L positive cells of 10µM 8Br-cGMP condition were almost a hundred percent. However, GFAP staining was very faint and mostly could not be detected in any experimental group (data not shown).

Immunophenotype of NI-hASCs profiles likely indicated that they committed to neuronal differentiation. This might be because of the complete medium were favorable for neuronal differentiation. It is well known that the role of bFGF in neural regulation is to generate neural precursor cells with a greater capacity for neuronal differentiation whereas EGF and specific neurotrophic factor are reported to restrict astrocyte lineages (Romero-Ramos et al, 2002; Schultz and Lucas, 2006; Xu et al, 2008, 2009). Laminin has differential action in the proliferation, survival, and differentiation of cultured neuronal cells (Romero-Ramos et al, 2002; Schultz and Lucas, 2006). Retinoic acid (RA) is known to be involved in brain development. RA demonstrated to act on the up-regulation of NF-L expression in MSCs derived from the umbilical cord blood (Tia et al, 2010), which is consistent with our findings. Tia and colleagues highlighted the role of RA and cAMP/PKA pathways associated in motor neurons (TH^+ neuronal cells, tyrosine hydroxylase) produced from UCBMSCs. Moreover, study of Scintu and his teams reported NSE, TUJ1, GFAP and NF-L positive cells could be observed in both two different protocols used to differentiate BM-MSCs into neuronal cells. The first one was carried out by activating the cAMP and PKC pathway (with forskolin, TPA and IBMX), whereas the second one consisted of only RA (Scintu et al, 2006)

For the control conditions, DMSO, DMEM and hASCs overall displayed significant lower % positive cells of all markers. However, Nestin (53.15%±6.59%) and NF-L (18.62%±14.06%) positive cells ratio in DMSO condition were significantly higher than other controls. Other studies also found that DMSO treated cells were lowly positive for neural markers (Lu et al., 2004; Neuhuber et al., 2004; Bertani et al., 2005; Tao et al., 2005). The morphology of hMSCs after exposed to

DMSO changed. Changes in morphology and increase in immunoreactivity for neural markers following chemical induction might be due to related cytoskeletal changes caused by cellular toxicity. Therefore, the results might not represent a neural differentiation process.

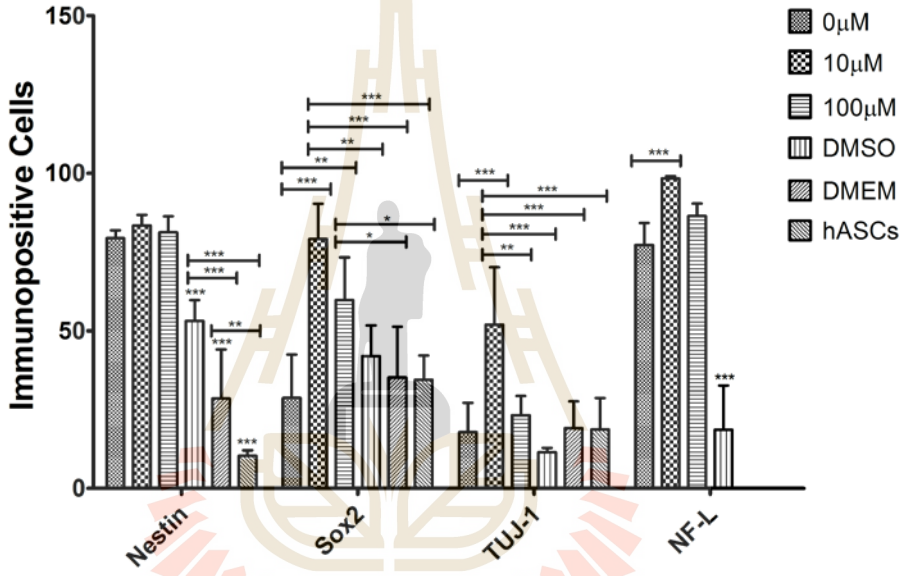


Figure 3.9 Immunopositive cells ratio of NI-hASCs in 8Br-cGMP induction groups and the controls at d7. Overall, 10μM 8Br-cGMP condition display significantly higher immunopositive cells in all neural markers than other conditions. Nestin and Sox2 represent neural stem cells markers. TUJ-1 and NF-L represent neuronal progenitors. All treatments were counted in twelve different fields (N=12). Statistical significance is indicated (one-way ANOVA, *post hoc* comparisons between all groups were performed using the Turkey test, *p<0.05, **p<0.01, ***p<0.001).

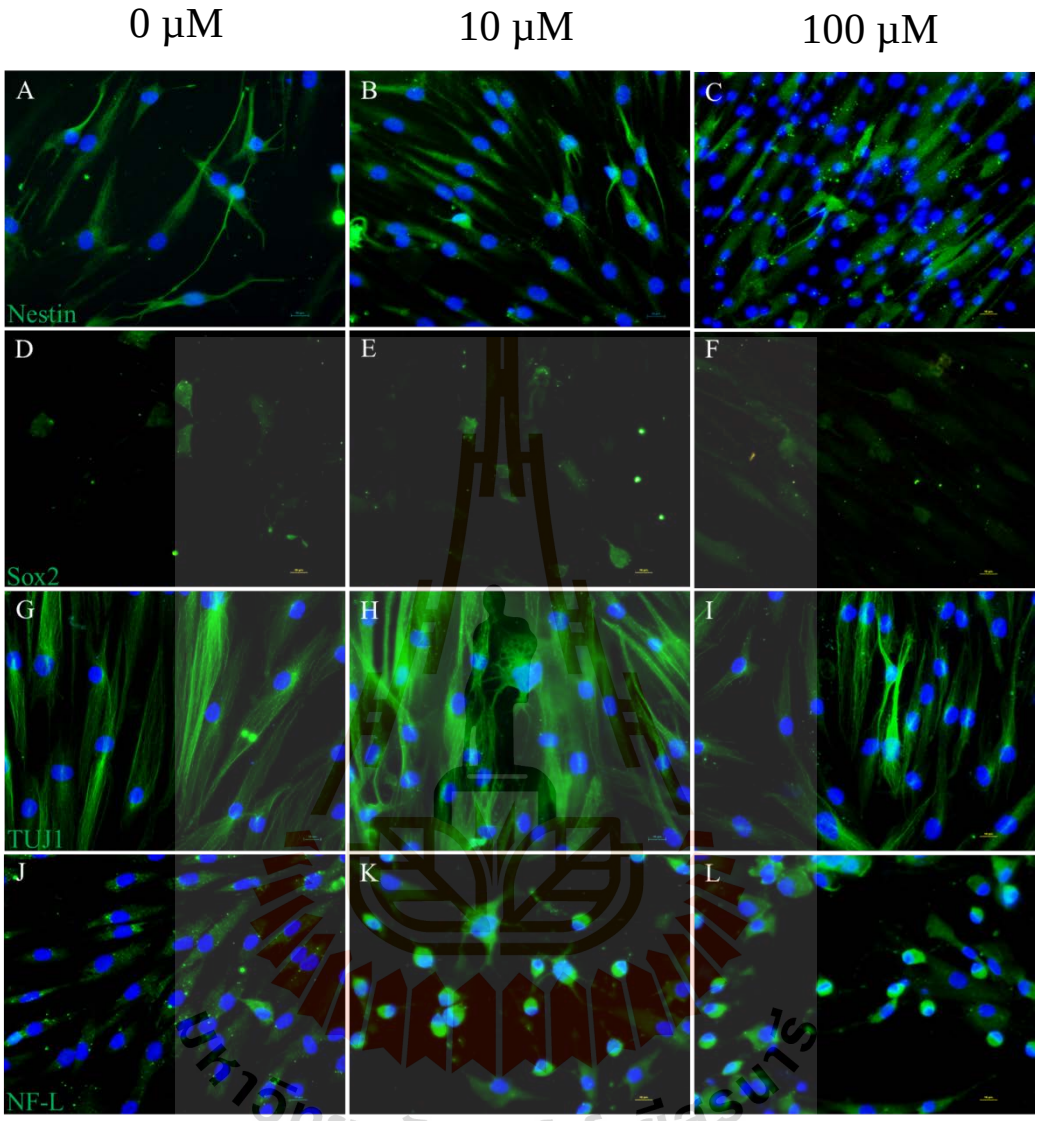


Figure 3.10 Immunocytochemical staining of NI-hASCs in the experimental conditions at d7. Representative images of immunopositive NI-hASCs for Nestin (A-C), Sox2 (D-F), TUJ1 (G-I) and NF-L (J-L). Scale bar: 10µm

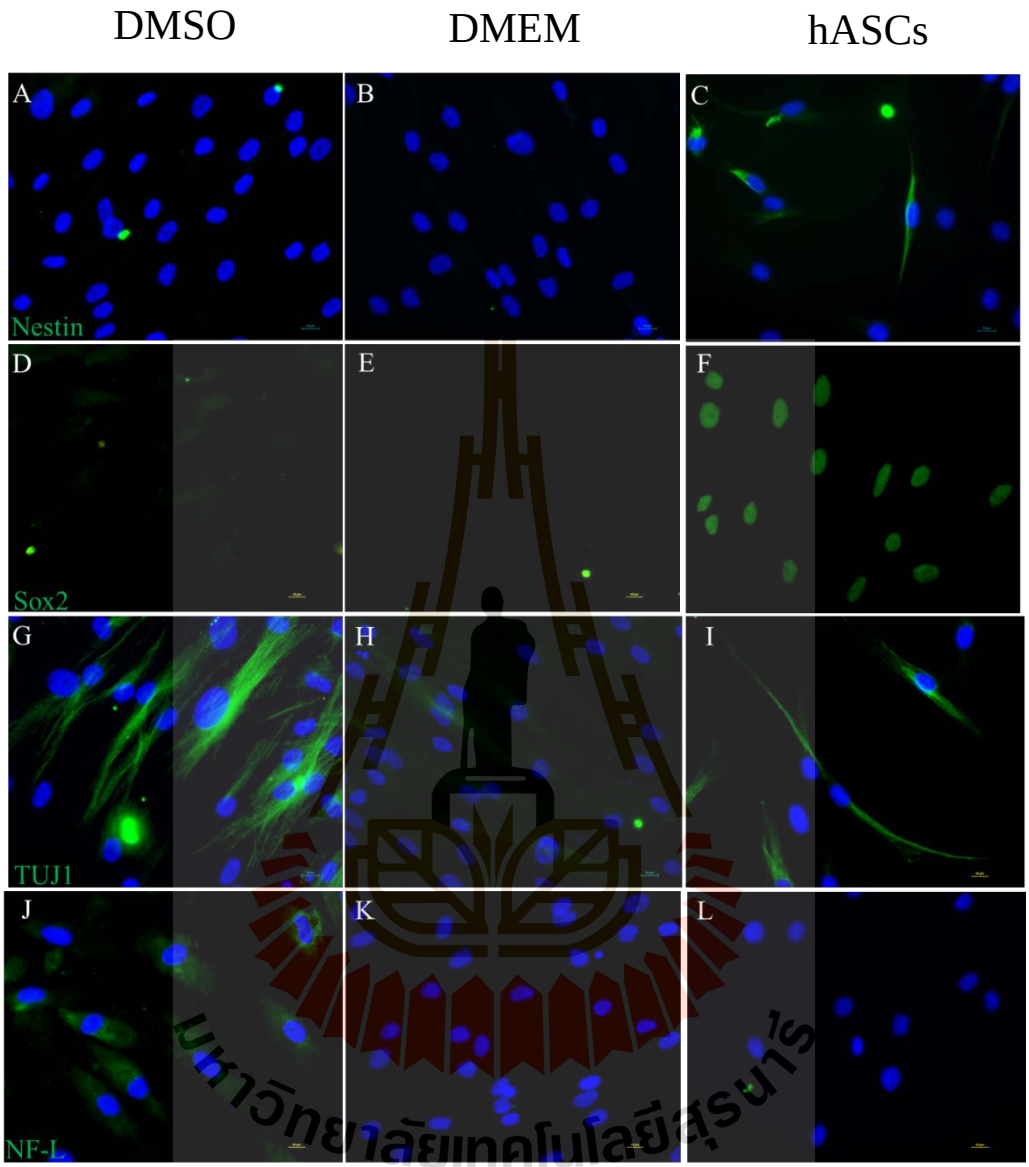


Figure 3.11 Immunocytochemical staining of the control conditions at d7. Representative images of immunopositive NI-hASCs for Nestin (A-C), Sox2 (D-F), TUJ1 (G-I) and NF-L (J-L). Scale bar: 10µm

3.4.4 Neurite outgrowth analysis of NI-hASCs

To get rid of NI-hASCs negative for TUJ1 with neurite protrusion which could mislead the result interpretation, NI-hASCs which were positive for TUJ1 and fell under specific criteria (see section 3.3.4.3) were selected for neurite outgrowth analysis. Out of about 20% of $TUJ1^+$ in DMEM conditions, there weren't any cells with neurite under defined criteria presented (Fig 3.9). This analysis was performed using In Cell Analyzer 1000 Cellular Imaging and Analysis System (GE Healthcare Life Science). Among $TUJ1^+$ NI-hASCs population in ever conditions, 10µM 8Br-cGMP showed highest number of $TUJ1^+$ NI-hASCs with neurites and significantly greater ($p<0.001$) than other conditions (Fig 3.12A). Their cell body area and number of neurite/cell were significantly higher ($p<0.05$) than the control (Fig 3.12B and 3.12D). Total neurite length of untreated and 10µM 8Br-cGMP was about at 50µm with 10µM 8Br-cGMP slightly greater than the untreated (Fig 3.12C). Their lengths were higher than the 100µM 8Br-cGMP treated group and the control but not statistically significant. Total neurite length per cell and mean neurite length were found to be not statistically different across all conditions (Fig 3.12E and 3.12F).

3.4.5 RT-qPCR analysis of NI-hASCs

NI-hASCs of each condition was analyzed by qPCR analysis at d7 and d14 to examine the most optimal condition for further differentiation. One week after induction, most of neural gene expression were increased in 10µM 8Br-cGMP condition (Fig 3.13A), and started to downregulate overtime. Surprisingly, the only gene that was upregulated at d14 was *TUJ1* (Fig 3.13B). *SOX2* mRNA level in each

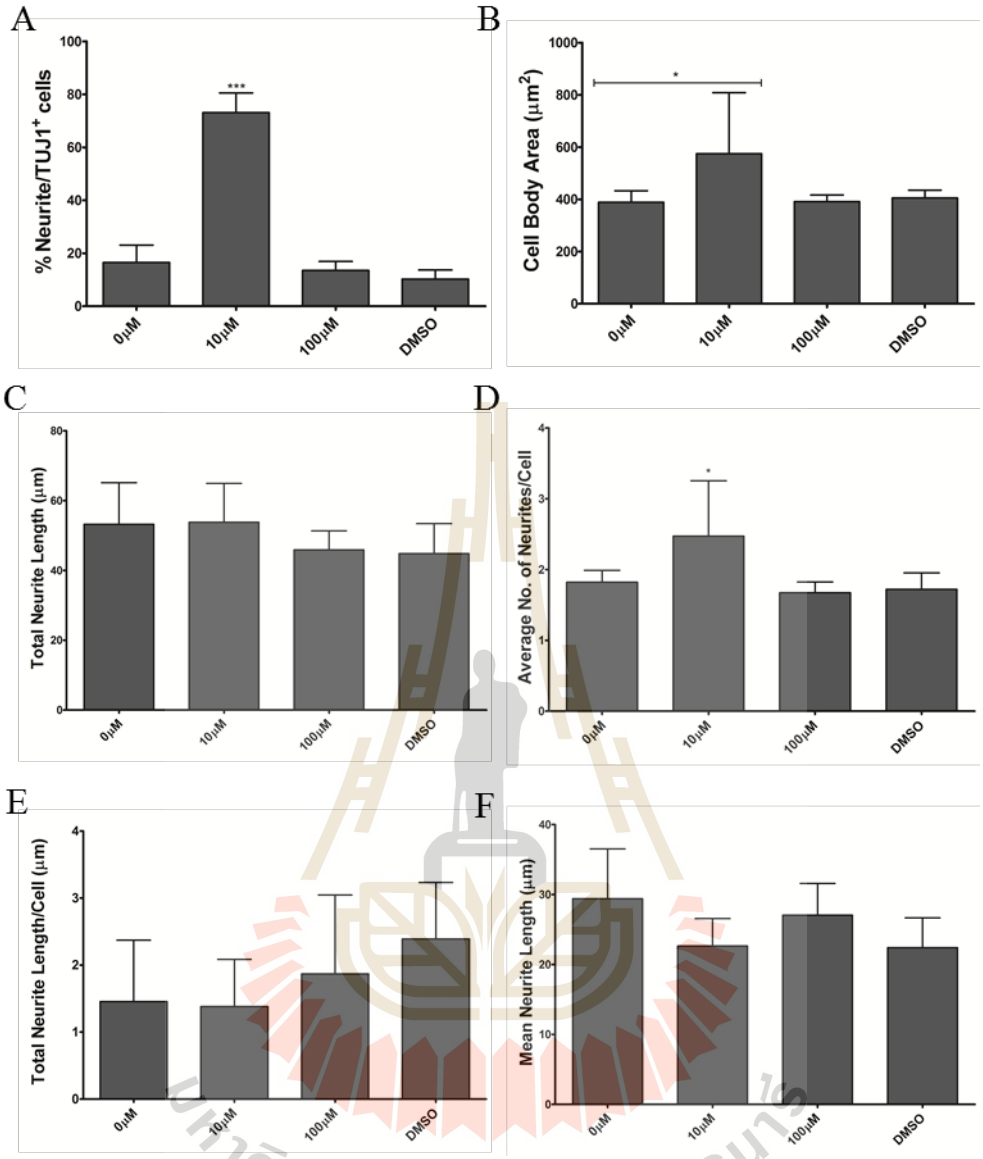


Figure 3.12 Neurite outgrowth analysis of NI-hASCs in the experimental conditions at d7. Majority of TUJ^+ NI-hASCs populations in 10μM 8Br-cGMP condition fell under specific neurite outgrowth criteria (A) and their cell body area (B) and average number of neurite/cell (C) were significantly higher than the control. All treatments were counted in twelve different fields (N=12). Statistical significance is indicated (one-way ANOVA, *post hoc* comparisons between all groups were performed using the Turkey test, *p<0.05, **p<0.01, ***p<0.001).

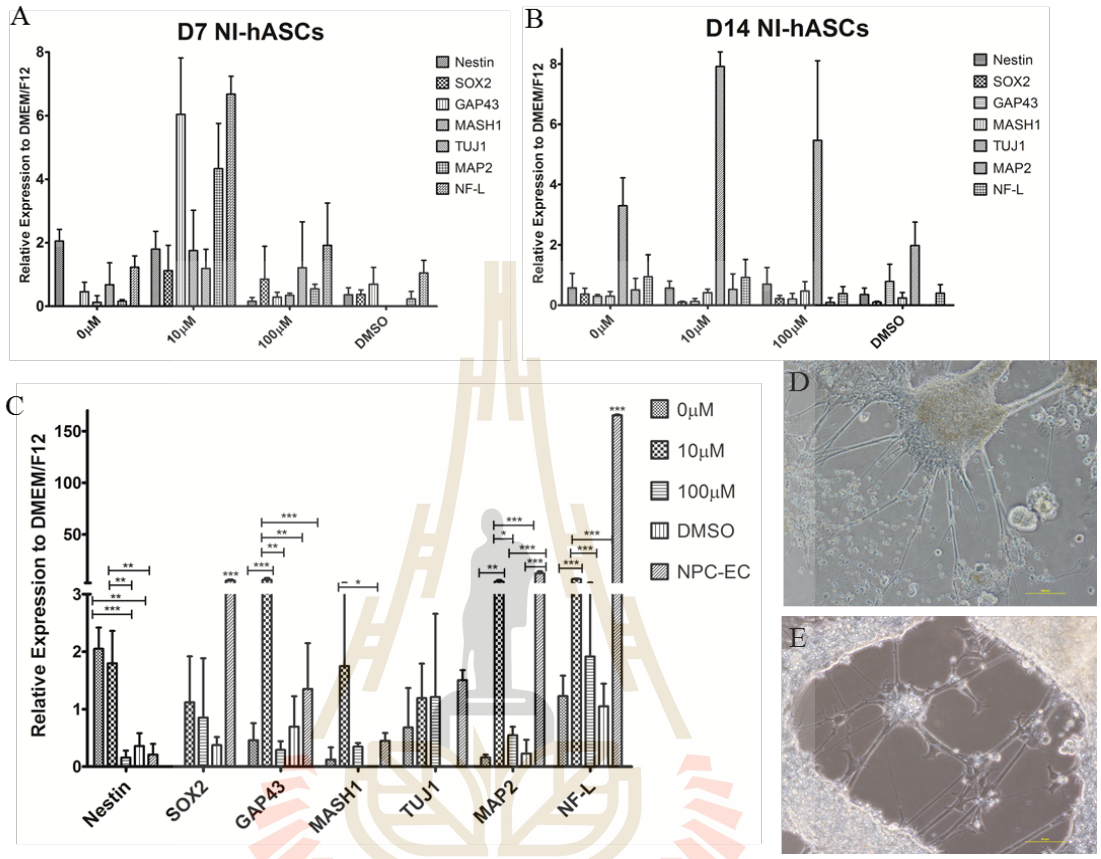


Figure 3.13 Relative gene expression of NI-hASCs to DMEM condition at d7 and d14. Each of 3 independent samples in all treatments was performed in replicate. Statistical significance is indicated (two-way ANOVA, *post hoc* comparisons between all groups were performed using the Turkey test, *p<0.05, **p<0.01, ***p<0.001). Neural cell derived from human EC cells were used as a positive control (D and E). Scale bar: 100 μm (D) and 50 μm (E)

condition was not significantly different, still the highest expression level was found in the 10µM 8Br-cGMP treated group. *SOX2* is neural stem cells and pluripotent marker. The Upregulation of *SOX2* could affect the pluripotency of stem cells, stimulate differentiation to the ectoderm lineage, and improve induction efficiency (Thomson et al., 2011; Wang et al., 2012). Therefore, *SOX2* upregulation in each condition suggested that each condition were under the stage of differentiation.

Among any other condition, *MASH1*, *GAP43*, *MAP2*, *TUJ1* and *NF-L* mRNAs level of 10µM 8Br-cGMP treated NI-hASCs were significantly higher (Fig 3.13C). These markers are associated with neuronal maturation and neurite outgrowth. Among these gene, the relationship between cGMP pathway and *GAP43* expression has been demonstrated, as 8Br-cGMP fully prevented *GAP43* and *synapsin I* reduction in cells that were depleted neurite outgrown related gene. They suggested that their regulation levels are cGMP independent (Chen et al, 2004; López-Jiménez et al, 2009). *GAP43* an integral membrane protein associated with the cytoplasmic surface of axonal growth cones in developing neurons, and it is generally considered an intrinsic determinant of neurite outgrowth and plasticity (Skene, 1989; Lalli et al, 2005). When this gene is altered, neurite outgrowth and axonal path finding are affected both during development and in cultured cells. The significance uprising of this mRNA level is consistent with the highest total length of neurite (Fig 3.12C) and the most significant highest number of neurite (Fig 3.12D) presented in 10µM 8Br-cGMP. Even though most of neural markers were suppressed and significantly lower in 100µM 8Br-cGMP, about 20% of $TUJ1^{+}$ NI-hASCs with slightly lower total neurite length existed. The mechanism under this finding is questionable. We assume that the underlying mechanism is involved with the expression or activity of RhoA

and GTPase controlled by cGMP/cGK signaling in cell shape control and neurite outgrowth (Sauzeau et al, 2003). Nestin mRNAs level of the 10µM 8Br-cGMP was significantly higher than the controls but not significantly different when compared to other 8Br-cGMP conditions.

We then compared each condition to the positive control which is neural cells derived from human embryonic carcinoma cells or NPC-EC differentiated as previously reported by Peter W. Andrews in 1984. The morphology of NPC-EC was distinctly different from the experimental group (Fig 3.13D and 3.13E) but displayed similar neural profile to previous study (Andrews, 1984). NPC-EC significantly expressed *MAP2* and *NF-L*, suggesting a commit to neurons. However, no significant different were found in *MAP2* expression between NPC-EC and the 10µM 8Br-cGMP treated group.

In conclusion, the most optimal condition was NI-hASCs treated wtih 10µM 8Br-cGMP which display neuronal progenitor profiles, yet remain their plasticity as indicated by expression level of Nestin and SOX2. Therefore, treatment of 10µM 8Br-cGMP was selected for the NI-hASCs differentiation into mature neurons and glia cell in next experiment.

3.4.6 Neuronal and glial differentiation of NI-hASCs

To test their progenitor properties, NI-hASCs under 10µM 8Br-cGMP condition were differentiated toward neuronal and glial cells. In this study, NeuroCult^{TM} NS-A Differentiation Kit was used to differentiate NI-hASCs as a standard differentiation. To support specific differentiation, NGF and PDGF were added in the medium for neuronal and glial differentiation, respectively.

The morphology of NI-hASCs induced by the induction medium display clearly different morphology when compared to hASCs from DMEM condition cultured under NS-A medium (Fig 3.14G-H). The morphology of NI-hASCs under NGF condition consisted of larger soma and longer axon with neural ends on each neuronal-like cell (Fig 3.14B). They were immunopositive for TUJ1 (Fig3.15A) and ChAT (Fig 3.15B). *TUJ1* mRNA levels were significantly higher than other condition and NPC-EC (Fig 3.16A), also roughly 50 folds greater than NI-hASCs before treated with NGF. Apart from that, *S100B* mRNA levels was almost a hundred folds higher ($p<0.01$) than NS-A and NPC-EC condition and about 50 folds higher than PDGF condition. This gene plays a vital role in neurite extension and axon proliferation. *MAP2*, a marker exclusively expressed in mature postmitotic neurons, was expressed in terminally differentiated neuronal cells as well. However, *NF-L* mRNAs level of NGF condition was slightly downregulating, but not significant, when compared to NI-hASCs and significantly lower than that of NPC-EC (Fig 3.16A).

NI-hASCs under PDGF and normal NS-A condition display smaller cell body with a shorter axonal protrusion (Fig 3.14C-F). Large number of glial-like cells could be observed in PDGF condition (Fig 3.14C-D). Those population were positive for glia cells markers, GFAP (Fig 3.15C) and Olig2 (Fig 3.15D). These results were confirmed by the significant upregulating of *GFAP* (Fig 13.6 C) and *CNPase* (Fig 13.6A) mRNA levels, suggesting that those populations could be glial and oligo progenitor cells. The lack of *MAP2* and *TUJ1* expression in PDGF condition also confirmed that these cells are committed to glia lineage. Neuronal-like cells with a shorter neurite extension in the normal NS-A condition were immunopositive for TUJ1 (Fig 3.15E) but not positive for GFAP (Fig 3.15F) and olig2 (data not shown).

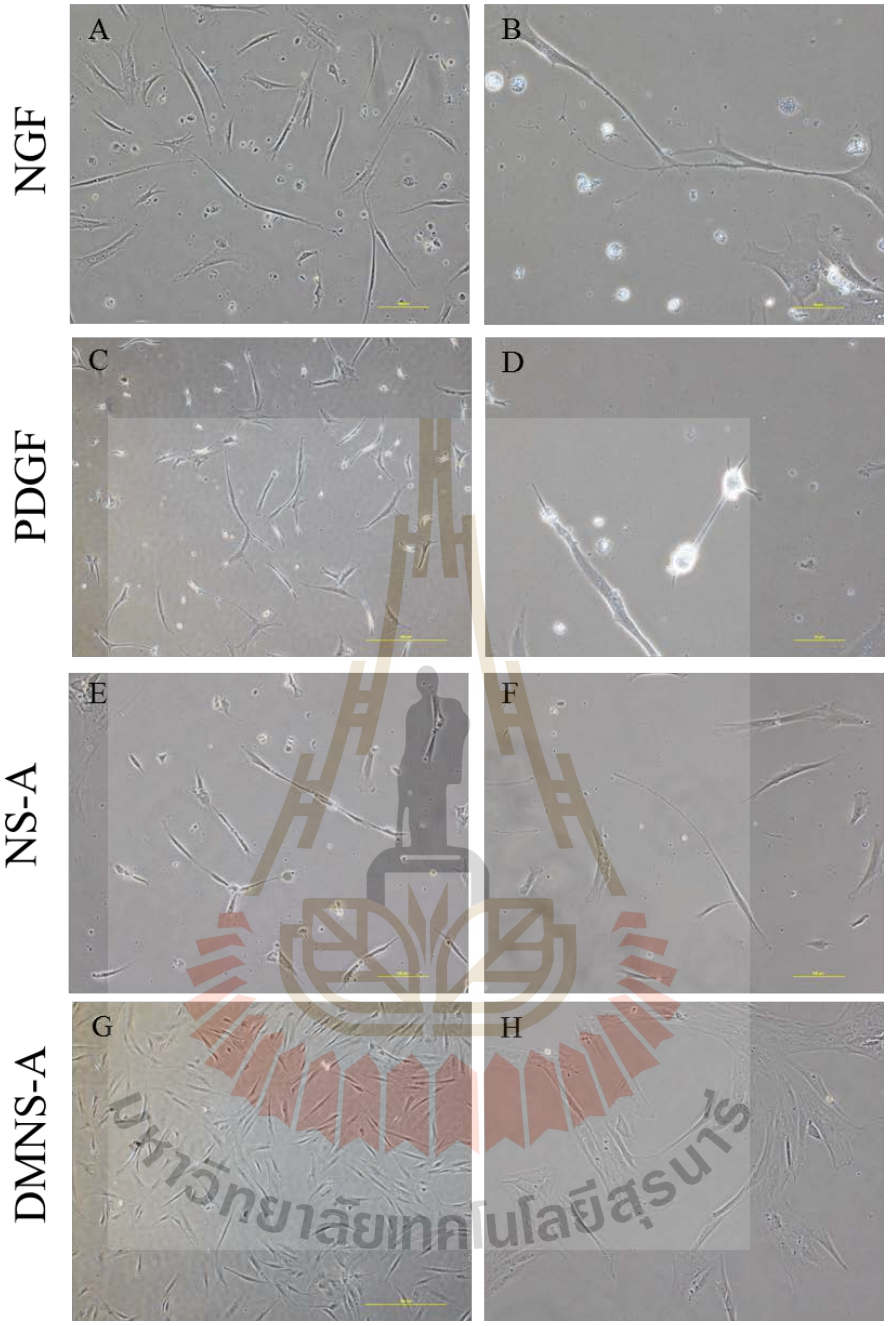


Figure 3.14 Morphology of NI-hASCs under neuronal differentiation medium (A-B), glial differentiation medium (C-D), normal NS-A differentiation medium (E-F), and hASCs from DMEM/F12 culture medium under NS-A differentiation medium (G-H) at d10. The morphology of NI-hASCs under neuronal, glial, NS-A differentiation

medium (A-F) were distinct from DMNS-A condition (G-H). Scale bar: 100µm, 50 µm, and 10 µm

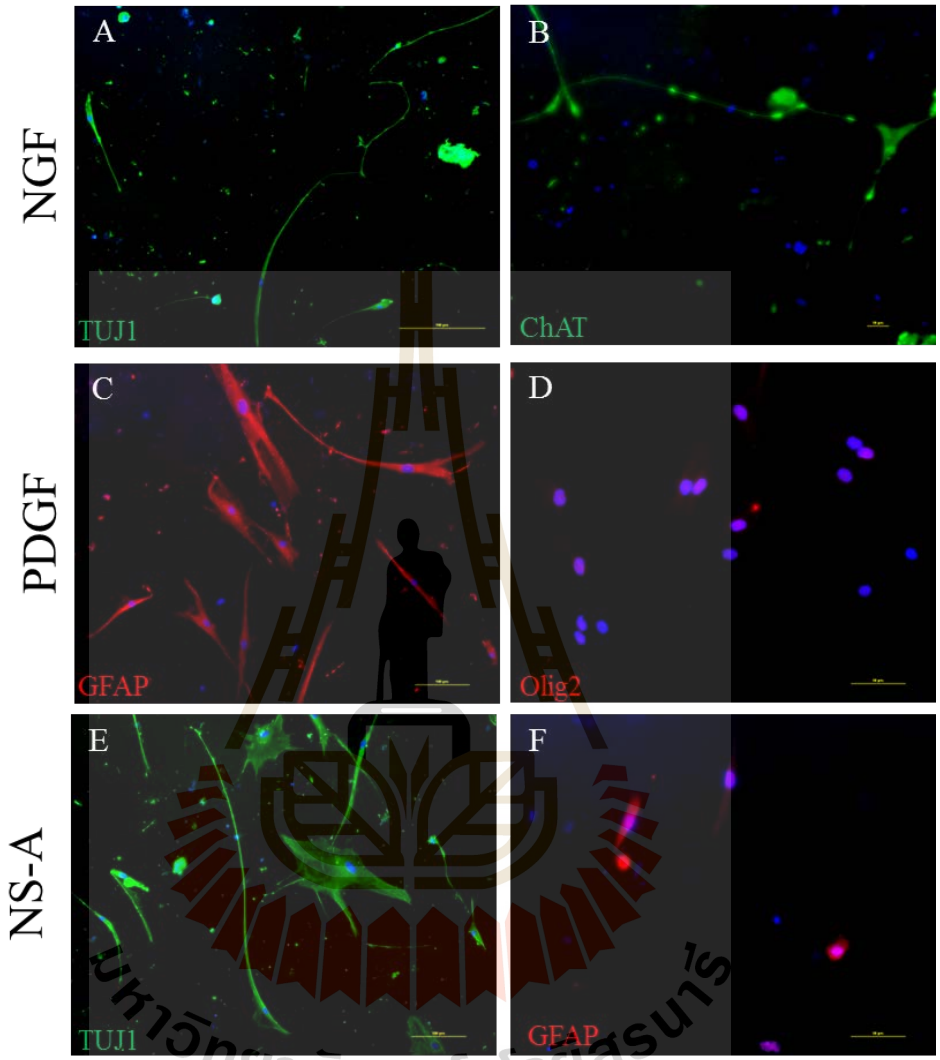


Figure 3.15 Representative figures of immunopositive NI-hASCs under neuronal differentiation medium (A-B), glial differentiation medium (C-D), and normal NS-A differentiation medium (E-F). Cholinergic neurons (B) and glia (C-D) were present in specific differentiation conditions. Most of the populations in NS-A condition were $TUJ1^{+}$ cells (E). Scale bar: 50 µm, and 10 µm

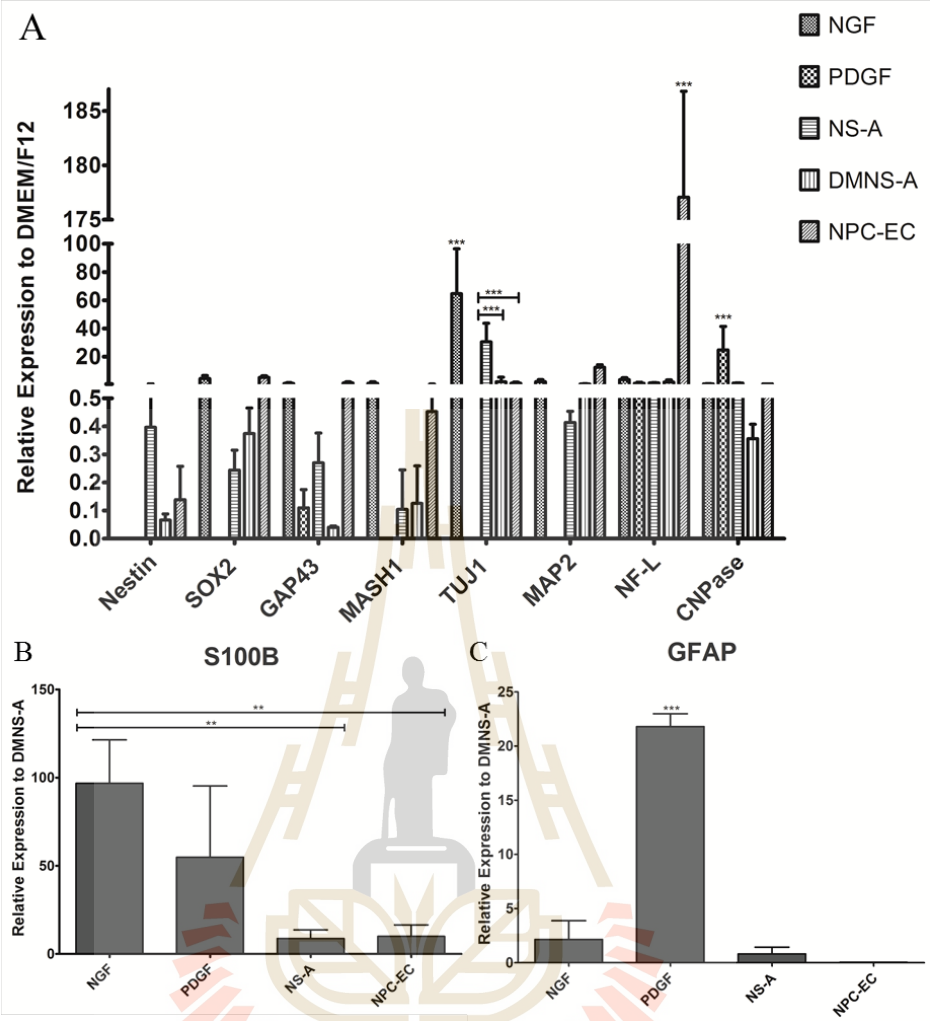


Figure 3.16 Relative gene expression of NI-hASCs under neuronal, glail and NS-A differentiation conditions to DMEM condition (A) and to DMNS-A condition (B-C) at d10. *S100B* (B) and *GFAP* (C) mRNAs of DMEM condition could not be detected. Each of 3 independent samples in all treatments was performed in replicate. Statistical significance is indicated (two-way ANOVA, *post hoc* comparisons between all groups were performed using the Turkey test, *p<0.05, **p<0.01, ***p<0.001).

3.5 Conclusions

This study aimed to elucidate the effect of 8Br-cGMP on neural differentiation of hASCs. hASCs was successfully isolated from human adipose tissue. These cells after expansions display mesenchymal stem cells properties. They were positive for CD73, CD90, CD105 and Vimentin, but were negative for CD34 and CD45. NI-hASCs under 10μM 8Br-cGMP treatment display neuronal progenitor committed profiles, yet remains their plasticity toward other neural fates. They could differentiate toward cholinergic neurons and oligoglial cell upon induction in defined medium. The findings of this study were to provide an efficient alternative way of differentiating hASCs into neural progenitor cells which could be beneficial for basic and clinical research in cell-based therapy in the future.

3.6 References

Andrews, P.W. (1984). Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line in vitro. **Dev Biol.** 103(2):285-293.

Arnold, W.P., Mittal, C.K., Katsuki, S. and Murad, F. (1977). Nitric oxide activates guanylate cyclase and increases guanosine 3·-5·-cyclic monophosphate levels in various tissue preparations. **Proc Natl Acad Sci** 74:3203–3207.

Ashjian, P.H., Elbarbary, A.S., Edmonds, B., DeUgarte, D., Zhu, M., Zuk, P.A., et al. (2003). In vitro differentiation of human processed lipoaspirate cells into early neural progenitors. **Plast Reconstr Surg** 111(6):1922-1931.

Bain, G., Kitchens, D., Yao, M., Huettner, J.E., Gottlieb, D.I. (1995). Embryonic Stem Cells Express Neuronal Properties in Vitro. **Developmental Biology** 168:342-357.

Bieback, K., Hecker, A., Kocaomer, A., Lannert, H., Schallmoser, K., Strunk, D., Kluter, H. (2009). Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow. **Stem Cells** 27:2331-2341.

Bertani, N., Malatesta, P., Volpi, G., Sonego, P., Perris, R. (2005). Neurogenic potential of human mesenchymal stem cells revisited: analysis by immunostaining, time-lapse video and microarray. **J Cell Sci** 118:3925–3936.

Brooke, G., Cook, M., Blair, C., Han, R., Heazlewood, C., Jones, B., Kambouris, M., Kollar, K., McTaggart, S., Pelekanos, R., Rice, A., Rossetti, T., and Atkinson, K. (2007). Therapeutic applications of mesenchymal stromal cells. **Seminars in cell & developmental biology**, 18(6):846-858.

Chen, T.J., Huang, C.W., Wang, D.C., Chen, S.S. (2004) Co-induction of growth-associated protein GAP-43 and neuronal nitric oxide synthase in the cochlear nucleus following cochleotomy. **Exp Brain Res 158**:151–162.

Ciani, E., Guidi, S., Della Valle, G., Perini, G., Bartesaghi, R., Contestabile, A. (2002). Nitric oxide protects neuroblastoma cells from apoptosis induced by serum deprivation through cAMP-response element-binding protein (CREB) activation. **J. Biol. Chem.** 277:49896–49902.

Croft, A.P. and Przyborski, S.A. (2006). Formation of neurons by non-neural adult stem cells: potential mechanism implicates an artifact of growth in culture. **Stem Cells** 24(8)1841–1851.

Deng, W., Obrocka, M., Fischer, I., Prockop, D.J. (2001). In Vitro Differentiation of Human Marrow Stromal Cells into Early Progenitors of Neural Cells by Conditions That Increase Intracellular Cyclic AMP. **Biochemical and Biophysical Research Communications**. 282:148-152.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., and Horwitz, E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. **Cytotherapy** 8(4):315-317.

Estephane, D. and Anctil, M. (2010). Retinoic Acid and Nitric Oxide Promote Cell Proliferation and Differentially Induce Neuronal Differentiation In Vitro in the Cnidarian *Renilla koellikeri*. **Develop Neurobiol** 70: 842–852

Feng, Z., Li, L., Ng, P.Y., Porter, A.G. (2002). Neuronal Differentiation and Protection from Nitric Oxide-Induced Apoptosis Require c-Jun-Dependent Expression of NCAM140. **Molecular and Cellular Biology** 22(15): 5357–5366

Gimble J.M. and Guilak F. (2003). Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. **Cytotherapy** 5:362-369.

Gronthos, S. et al. (2001). Surface protein characterization of human adipose tissue-derived stromal cells. **J Cell Physiol** 189:54.

Guilak, F., Lott, K.E., Awad, H.A., Cao, Q., Hicok, K.C., Fermor, B., Gimble, J.M. (2006). Clonal analysis of the differentiation potential of human adipose-derived adult stem cells**. Journal of Cellular Physiology** 206(1): 229–237**.**

Hermann, A., Gastl, R., Liebau, S., Popa, M. O., Fiedler, J., Boehm, B. O., Maisel, M., Lerche, H., Schwarz, J., Brenner, R., Storch, A. (2004). Efficient generation of

neural stem cell-like cells from adult human bone marrow stromal cells. **J. Cell. Sci. 117**:4411-4422.

Ikeda, E., Hirose, M., Kotobuki, N., Shimaoka, H., Tadokoro, M., Maeda, M., Hayashi, Y., Kirita, T., Ohgushi, H. (2006). Osteogenic differentiation of human dental papilla mesenchymal cells. **Biochem Biophys Res Commun** 342:1257-1262.

Izadpanah, R., Joswig, T., Tsien, F. et al. (2005). Characterization of multipotent mesenchymalstem cells from the bone marrow of rhesus macaques. **Stem Cells Dev** 14(4):440-451.

Jang S., Cho H.-H., Cho Y.-B., Park J.-S., Jeong H.-S. (2010). Functional neural differentiation of human adipose tissue-derived stem cells using bFGF and forskolin. **BMC Cell Biology**. 11:25-38.

Jiang, Y., Jahagirdar, B.N., Reinhardt, R.L., Schwartz, R.E., Keene, C.D., Ortiz-Gonzalez, X.R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M., Du, J., Aldrich, S., Lisberg, A., Low, W.C., Largaespada, D.A., Verfaillie, C.M. (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. **Nature**. 418:41-49.

Jin, K., Mao, X.O., Batteur, S., Sun, Y. and Greenberg, D.A. (2003). Induction of neuronal markers in bone marrow cells: differential effects of growth factors and patterns of intracellular expression. **Exp. Neurol. 184**:78-89.

Johe, K.K., Hazel, T.G., Muller, T., Dugich-Djordjevic, M.M., McKay, R.D. (1996). Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. **Genes Dev** 10:3129 –3140.

Kang, S. K., Putnam, L. A., Ylostalo, J., Popescu, I. R., Dufour, J., Belousov, A. and Bunnell, B. A. (2004). Neurogenesis of Rhesus adipose stromal cells. **J. Cell. Sci. 117**:4289-4299.

Katsuki, S., Arnold, W., Mittal, C. and Murad, F. (1977). Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. **J Cyclic Nucleotide Res** 3:23–35.

Katz, A.J., Tholpady, A., Tholpady, S.S., Shang, H. & Ogle, R.C. (2005). Cell surface and transcriptional characterization of human adipose-derived adherent stromal (hADAS) cells. **Stem Cells** 23:412-423.

Király M., Porcsalmy B., Pataki Á., Kádár K., Jelitai M., Molnár B., Hermann P., Gera I., Grimm W.-D., Ganss B., Zsembery Á., Varga G. (2009). Simultaneous PKC and cAMP activation induces differentiation of human dental pulp stem cells into functionally active neurons. **Neurochemistry International** 55:323-332.

Kocaoemer, A., Kern, S., Kluter, H. & Bieback, K. (2007). Human AB serum and thrombin-activated platelet-rich plasma are suitable alternatives to fetal calf serum for the expansion of mesenchymal stem cells from adipose tissue. **Stem Cells** 25: 1270-1278

Kwon, E.B., Lee, J.Y., Piao, S., Kim, I.G., Ra, J.C., Lee, J.Y. (2011). Comparison of human muscle-derived stem cells and human adipose-derived stem cells in neurogenic trans-differentiation. **Korean J Urol.** 52(12):852-857.

Lalli, G., Hall, A., Ral. (2005) GTPases regulate neurite branching through GAP-43 and the exocyst complex. **J Cell Biol** 171: 857–869.

Lee, S.-H., Lumelsky, N., Studer, L., Auerbach, J.M., McKay, R.D. (2000) Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. **Nature Biotechnology** 18:675-679.

Li, T.J., Somasundaram, C., Bian, K., Xiong, W., Mahmooduddin, F., Nath, R.K., Murad, F. (2010). Nitric Oxide Signaling and Neural Stem Cell Differentiation in Peripheral Nerve Regeneration. **Journal of Plastic Surgery** 10: 343-353.

Livak, K.J. and Schmittgen, T.D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. **Methods** 25:402-408.

López-Jiménez, M.E., Bartolomé-Martín, D., Sánchez-Prieto, J. and Torres, M. (2009). Suppression of guanylyl cyclase (β1 subunit) expression impairs neurite outgrowth and synapse maturation in cultured cerebellar granule cells. **Cell Death and Differentiation 16:**1266–1278.

Lu, P., Blesch, A., Tuszynski, M.H. (2004). Induction of bone marrow stromal cells to neurons: differentiation, transdifferentiation, or artifact? **J Neurosci Res.** 77:174–191.

Mareschi, K., Novara, M., Rustichelli, D., Ferrero, I., Guido, D., Carbone, E., et al. (2006). Neural differentiation of human mesenchymal stem cells: evidence for expression of neural markers and eag K+ channel types. **Exp Hematol.** 34:1563–1572.

Mitchell, K.E., Weiss, M.L., Mitchell, B.M., Martin, P., Davis, D., Morales, L., Helwig, B., Beerenstrauch, M., Abou-Easa, K., Hildreth, T., and Troyer, D. (2003). Matrix cells from wharton's jelly form neurons and glia. **Stem cells** 21(1): 50-60.

Mostafavi, F.S., Razavi, S., Mardani. M., Esfandiari, E., Esfahani, H.Z., Kazemi, M. (2014). Comparative Study of Microtubule-associated Protein-2 and Glial Fibrillary Acidic Proteins during NeuralInduction of Human Bone Marrow Mesenchymal Stem Cells and Adipose-Derived Stem Cells. **Int J Prev Med** 5(5):584-95.

Muller, D., Greenland, K.J., Speth, R.C., Middendorff, R. (2010). Neuronal differentiation of NG108-15 cells has impact on nitric oxide- and membrane (natriuretic peptide receptor-A) cyclic GMP-generating proteins. **Molecular and Cellular Endocrinology** 320:118–127.

National Institute of Neurological Disorders and Stroke. (2004). Parkinson's disease: challenges, progresses and promises. **National Institute of Health** 5(5585):1-41.

Neuhuber, B., Gallo, G., Howard, L., Kostura, L., Mackay, A., Fischer, I. (2004). Reevaluation of in vitro differentiation protocols for bone marrow stromal cells: disruption of actin cytoskeleton induces rapid morphological changes and mimics neuronal phenotype. **J Neurosci Res** 77:192–204.

Padovan, C. S., Jahn, K., Birnbaum, T., Reich, P., Sostak, P., Strupp, M. and Straube, A. (2003). Expression of neuronal markers in differentiated marrow stromal cells and CD133+ stem-like cells. **Cell Transplant.** 12:839-848.

Pittenger, M.F.,Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas. R., Mosca, J.D., Moorman, M.A., Simonetti D.W., Craig, S., Marshak, D.R. (1999). Multilineage potential of adult human mesenchymal stem cells. **Science** 284: 143-147.

Pontius, J.U., Wagner, L. and Schuler, G.D. (2003). UniGene: a unified view of the transcriptome. In *The NCBI Handbook*. Bethesda MD: National Center for Biotechnology Information.

Ramm, P., Alexandrov, Y., Cholewinski, A., Cybuch, Y., Nadon, R., Soltys, B.J. (2003). Automated screening of neurite outgrowth**. J Biomol Screen.** (1):7-18.

Romero-Ramos, M., Vourc'h, P., Young, H.E., Lucas, P.A., Wu, Y., Chivatakarn, O., et al. (2002). Neuronal differentiation of stem cells isolated from adult muscle. **J Neurosci Res** 69:894-907.

Sanchez-Ramos, J., Song, S., Cardozo-Pelaez, F., Hazzi, C., Stedeford, T., Willing, A., Freeman, T.B., Saporta, S., Janssen, W., Patel, N., Cooper, D.R., Sanberg, P.R. (2000). Adult Bone Marrow Stromal Cells Differentiate into Neural Cells in Vitro. **Experimental Neurology**. 164:247-256.

Sanchez-Ramos, J.R. (2002). Neural cells derived from adult bone marroe and umbilical cord blood. **J. Neurosci. Res**. **69**:880-893.

Sauzeau, V., Rolli-Derkinderen, M., Marionneau, C., Loirand, G., Pacaud, P. RhoA expression is controlled by nitric oxide through cGMP-dependent protein kinase activation. **J Biol Chem** 2003; **278**: 9472–9480.

Schultz, S.S., Lucas, P.A. (2006). Human stem cells isolated from adult skeletal muscle differentiate into neural phenotypes. **J Neurosci Methods** 152:144-55.

Scintu, F., Reali, C., Pillai, R., Badiali, M., Sanna, M.A., Argiolu, F., Ristaldi, M.S., Sogos, V. (2006). Differentiation of human bone marrow stem cells into cells with a neural phenotype: diverse effects of two specific treatments. **BMC Neurosci** 7:14-26.

Skene, J.H. (1989). Axonal growth-associated proteins. **Annu Rev Neurosci 12**:127–156.

Strem, B.M., Hicok, K.C., Zhu, M., Wulur, I., Alfonso, Z., Schreiber, R.E., Fraser, J.K., Hedrick, M.H. (2005). Multipotential differentiation of adipose tissuederived stem cells. **Keio J Med** 54:132‐41.

Sulz, L., Astorga, G., Bellette, B., Iturriaga, R., Mackay-Sim, A., Bacigalupo, J. (2009). Nitric oxide regulates neurogenesis in adult olfactory epithelium in vitro. **Nitric Oxide** 20:238–252.

Tao, H., Rao, R., Ma, D.D. (2005). Cytokine-induced stable neuronal differentiation of human bone marrow mesenchymal stem cells in a serum/feeder cell-free condition. **Dev Growth Differ** 47:423–433.

Thippeswamy, T., Morris, R., (1997). Cyclic guanosine 3',5'-monophosphate-mediated neuroprotection by nitric oxide in dissociated cultures of rat dorsal root ganglion neurones. **Brain Res.** 774:116–122.

Thippeswamy, T., Morris, R., (2001). Evidence that nitric oxide-induced synthesis of cGMP occurs in a paracrine but not an autocrine fashion and that the site of its release can be regulated: studies in dorsal root ganglia in vivo and in vitro. **Nitric Oxide** 5:105–115.

Thomson, M., Liu, S.J., Zou, L.N., Smith, Z., Meissner, A., Ramanathan, S. (2011). Pluripotency factors in embryonic stem cells regulate differentiation into germ layers. **Cell** 145, 875–889.

Tio, M., Tan, K.H., Lee, W., Wang T.T., Udolph, G. (2006). Roles of db-cAMP, IBMX and RA in aspects of neural differentiation of cord blood derived mesenchymal-like stem cells. **PLoS One** 5(2):e9398.

Tondreau, T., Lagneaux, L., Dejeneffe, M., Massy, M., Mortier, C., Delforge, A. and Bron, D. (2004). Bone marrow-derived mesenchymal stem cells already express specific neural proteins before any differentiation. **Differentiation** 72:319-326.

Wang, Z., Oron, E., Nelson, B., et al. (2012). Distinct lineage specification roles for NANOG, OCT4, and SOX2 in human embryonic stem cells. **Cell Stem Cell** 10, 440–454.

Wetzig, A., Alaiya, A., Al-Alwan, M., Pradez, C.B., Pulicat, M.S., Al-Mazrou, A., Shinwari, Z., Sleiman, G.M., Ghebeh, H., Al-Humaidan, H., Gaafar, A., Kanaan, I., Adra, C. (2013). Differential marker expression by cultures rich in mesenchymal stem cells. **BMC Cell Biol.** 5(14):54-66.

Wislet-Gendebien, S., Leprince, P., Moonen, G. and Rogister, B. (2003). Regulation of neural markers nestin and GFAP expression by cultivated bone marrow stromal cells. **J. Cell Sci.** 116: 3295-3302.

Wislet-Gendebien, S., Hans, G., Leprince, P., Rigo, J. M., Moonen, G. and Rogister, B. (2005). Plasticity of cultured mesenchymal stem cells: switch from nestin-positive to excitable neuron-like phenotype. **Stem Cells** 23:392-402.

Woodbury, D., Reynolds, K., Black, I.B. (2002). Adult bone marrow stromal stem cells express germline, ectodermal, endodermal, and mesodermal genes prior to neurogenesis. **Journal of Neuroscience Research.** 69:908-917.

Xu, Y., Liu, L., Li, Y., Zhou, C., Xiong, F., Liu, Z., et al. (2008). Myelin-forming ability of schwann cell-like cells induced from rat adipose-derived stem cells in vitro. **Brain Res** 1239:49-55.

Xu, Y., Liu, Z., Liu, L., Zhao, C., Xiong, F., Zhou, C., et al. (2008). Neurospheres from rat adipose-derived stem cells could be induced into functional Schwann cell-like cells in vitro. **BMC Neurosci** 9:21-31.

Zavan, B., Michelotto, L., Lancerotto, L., Della-Puppa, A., D'Avella, D., Abatangelo, G., et al. (2010) Neural potential of a stem cell population in the adipose and cutaneous tissues. **Neurol Res** 32:47-54.

Zhang H.-T., Fan J., Cai Y.-Q., Zhao S.-J., Xue S., Lin J.-H., Jiang X.-D., Xu R.-X. (2010). Human Wharton's jelly cells can be induced to differentiate into growth factor-secreting oligodendrocyte progenitor-like cells. **Differentiation** 79:15-20.

Zuk, P.A., Zhu, M., Mizuno, H., et al. (2001). Multilineage cells from human adipose tissue: implication for cell-based therapies. **Tissue Eng** 7:211-228.

Zuk, P.A., Zhu, M., Ashjian, P., et al. (2002). Human adipose tissue is a source of multipotent stem cells. **Mol Biol Cell** 13:4279-4295.

APPENDIX

Table 1. Primer Sequence used for RT-qPCR in this study.

Gene	Accession number	Primer sequence 5'-3'	Product size (bp)	Annealing Temperature (°C)	References
Nestin	NM006617.1	F-CTGTTGGCAGCCTCCAGGCC R-GCGGCATTCCTTGCCCCACT	381	60	This study
SOX2	BC013923.2	F-CCCCCGGCGGCAATAGCA R-TCGGCGCCGGGGAGATACAT	448	60	Henderson et al. Stem cells 2002, 20:329-337.
MASH1 (ASCL1)	NM004316	F-TCGCACAACCTGCATCTTTA R-CTTTTGCACACAAGCTGCAT	278	60	This study
GAP43	NM002045.3	F-TCCTGAGCCCTGTCTCTCCCT R-GCCACACTGTTTGACTTGGG	175	60	Jand et al. BMC Cell Biology 2010, 11:25
MAP2	XM006712533.1	F-TCAGAGGCAATGACCTTACC R-GTGGTAGGCTCTTGGTCTTT	320	55	Ahmadi et al. Tissue and Cell 2002, 44:87-94

Table 1. Primer Sequence used for RT-qPCR in this study (Continued).

Gene	Accession number	Primer sequence 5'-3'	Product size (bp)	Annealing Temperature (°C)	References
TUJ1	NM006086.3	F- GGGCCAAGTTCTGGGAAGTC R- ATCCGCTCCAGCTGCAAGT	91	60	Arthur et al. Stem Cells 2008, 26:1787–1795
NF-L	BC013923.2	F-CCCCCGGCGGCAATAGCA R-TCGGCGCCGGGGAGATACAT	280	60	Jand et al. BMC Cell Biology 2010, 11:25
GFAP	NM002055	F-GTCCATGTGGAGCTTGACG R-CATTGAGCAGGTCCTGGTAC	406	58	Brohlin et al. Neurosci Res 2009, 64:41-49
S100	BC001766	F-GGAAATCAACGAGCAGGAGGT R-ATTAGCTACAACACGGCTGGA	408	60	Brohlin et al. Neurosci Res 2009, 64:41-49
CNPase	NM033133.4	F-GGTGCCTTTGGAGAGCATGG R-GGAACTCCATCTTCGAGGCT	302	60	Jand et al. BMC Cell Biology 2010, 11:25

Table 1. Primer Sequence used for RT-qPCR in this study (Continued).

Gene	Accession number	Primer sequence 5'-3'	Product size (bp)	Annealing Temperature (°C)	References
β-actin	X00351	F-TCACCACCACGGCCGAGCG R-TCTCCTTCTGCATCCTGTCG	350	60	This study

Table 2. Ratio of Immunopositive cells to total cells of the untreated and 8Br-cGMP treated NI-hASCs at d7 (N=5).

Condition	Nestin	Sox2	TUJ1	NF-L
0µM	79.44±2.49	28.76±13.74	17.85±9.31	77.23±6.99
10µM	83.42±3.42	79.08±11.31	51.98±18.16	98.34±0.76
100µM	81.25±5.16	59.74±13.59	23.20±6.16	86.49±3.92
DMSO	53.15±6.59	42.04±9.70	11.52±1.31	18.92±14.06
DMEM	28.54±15.58	35.19±16.12	19.12±8.49	N/A
hASCs	10.41±1.67	34.52±7.66	18.71±9.99	N/A

Table 3. Neurite outgrowth analysis results of the untreated and 8Br-cGMP treated NI-hASCs at d7 (N=12).

Criteria	0µM	10µM	100µM	DMSO
$TUJ1^{+}$/neurite (%)	16.54±6.53	71.4±7.28	13.55±3.35	10.29±3.39
Cell Body Area (μm^2)	388.6±43.98	574.7±82.67	391.5±25.12	405.3±29.71
No. of neurites	1.82±0.16	2.47±0.78	1.67±0.15	1.72±0.23
Total Neurite Length (µm)	53.23±11.93	53.84±11.11	45.93±5.40	44.83±8.59
Total Neurite Length/Cell (µm)	1.45±0.91	1.38±0.70	1.86±1.17	2.39±0.84
Mean Neurite Length (µm)	29.41±7.11	22.66±3.89	27.05±4.53	22.42±4.24

Table 4. Relative expression fold change of the untreated and 8Br-cGMP treated NI-hASCs at d7 compared to DMEM/F12 (N=3).

Gene	0µM	10µM	100µM	DMSO
Nestin	2.05±0.36	1.79±0.56	0.15±0.12	0.36±0.22
SOX2	0.0026±0.0017	1.12±0.79	0.85±1.03	0.37±0.14
GAP43	0.46±0.29	5.47±2.41	1.23±1.71	0.69±0.52
MASH1	0.12±0.23	1.75±1.27	0.34±0.06	N/A
TUJ1	0.68±0.68	1.19±0.59	3.90±4.12	N/A
MAP2	0.15±0.48	4.33±1.42	1.66±2.02	0.22±0.24
NFL	1.22±0.35	6.68±0.56	1.91±1.33	1.05±0.39

Table 5. Relative expression fold change of NGF, PDGF, NS-A, DMNS-A and NPC-EC condition compared to DMEM/F12 (N=3).

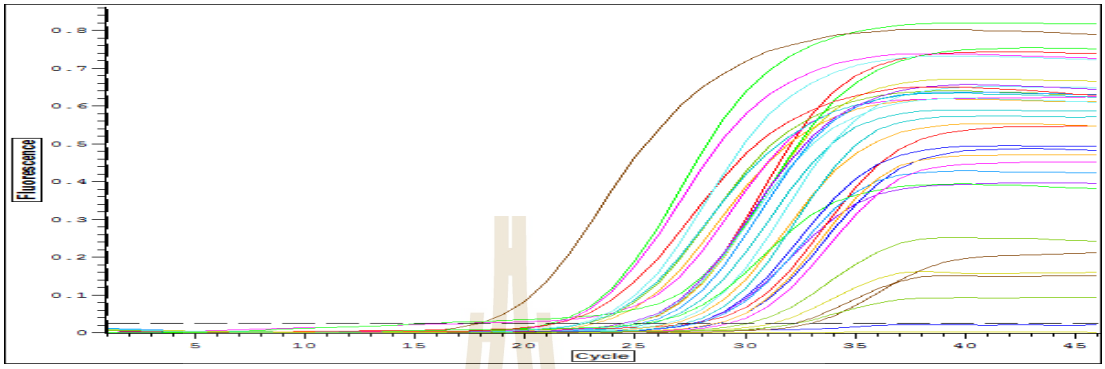
Gene	NGF	PDGF	NS-A	DMNS-A	NPC-EC
Nestin	N/A	N/A	0.39±0.26	0.066±0.022	0.13±0.11
SOX2	4.40±2.16	N/A	0.24±0.07	0.37±0.09	5.21±1.15
GAP43	0.92±0.57	0.11±0.06	0.27±0.10	0.04±0.01	1.35±0.79
MASH1	1.07±1.05	N/A	0.10±0.14	0.12±0.13	0.45±0.13
TUJ1	64.66±31.78	N/A	30.45±13.18	2.31±3.23	1.14±0.80
MAP2	2.22±1.47	N/A	0.41±0.04	0.54±0.19	12.36±1.88
NFL	3.66±1.36	1.04±0.71	1.37±0.18	1.87±1.57	177.06±9.75
CNPase	0.68±0.17	24.69±16.73	1.21±0.18	0.35±0.05	0.63±0.03

Table 6. Relative expression fold change of NGF, PDGF, NS-A, and NPC-EC condition compared to DMNS-A (N=3).

Gene	NGF	PDGF	NS-A	NPC-EC
S100B	96.79±24.64	54.88±40.34	8.77±4.87	9.98±6.46
GFAP	2.15±1.73	21.80±1.19	0.81±0.61	0.03±0.002

Figure 1. A representative validation curve of real time PCR for *β -actin* gene.

(A) The amplification curve for the *β -actin* gene.



(B) The dissociation curve of PCR products of *β-actin* gene.

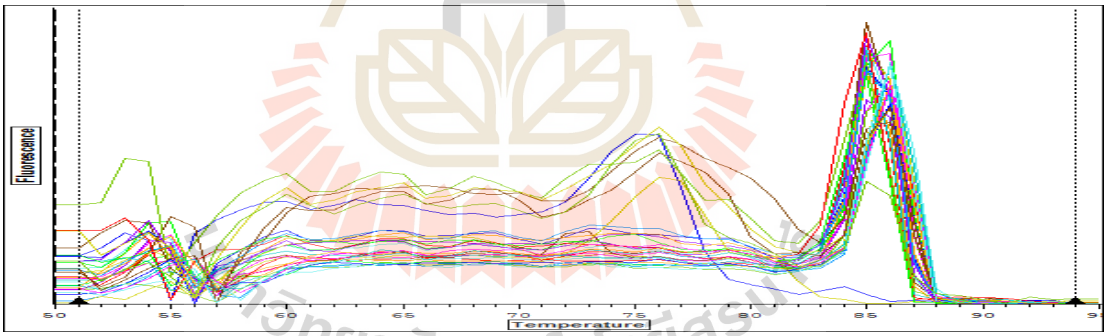
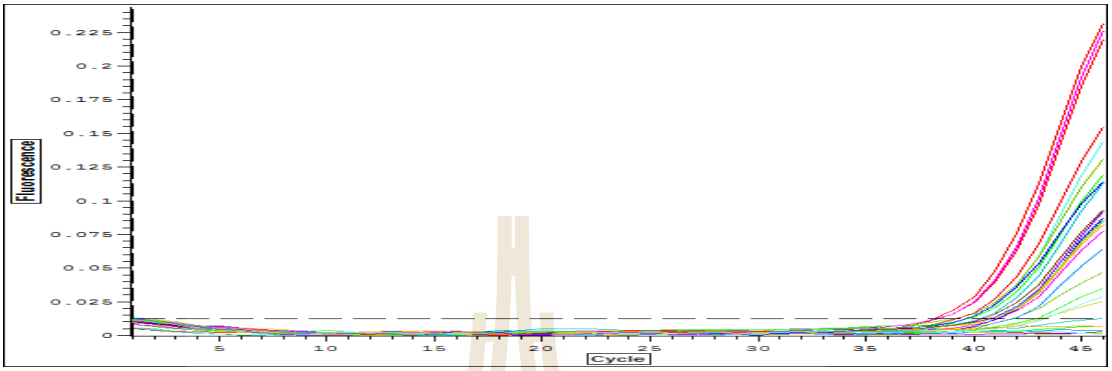


Figure 2. A representative validation curve of real time PCR for *Nestin* gene.

(A) The amplification curve for the *Nestin* gene.



(B) The dissociation curve of PCR products of *Nestin* gene.

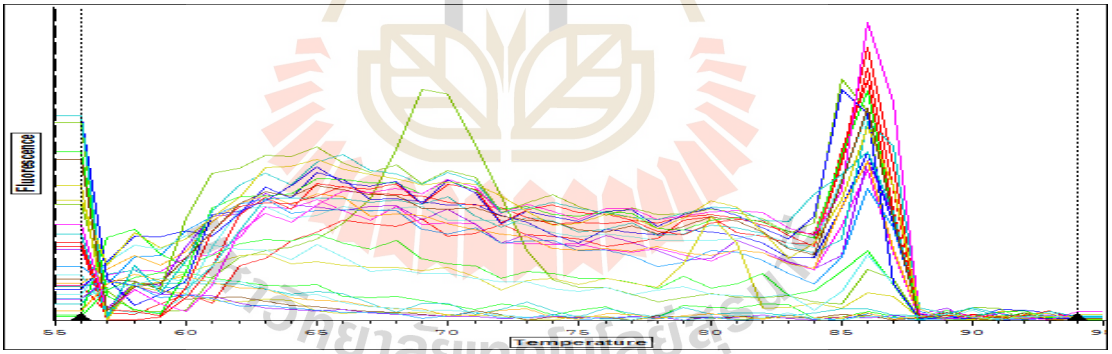
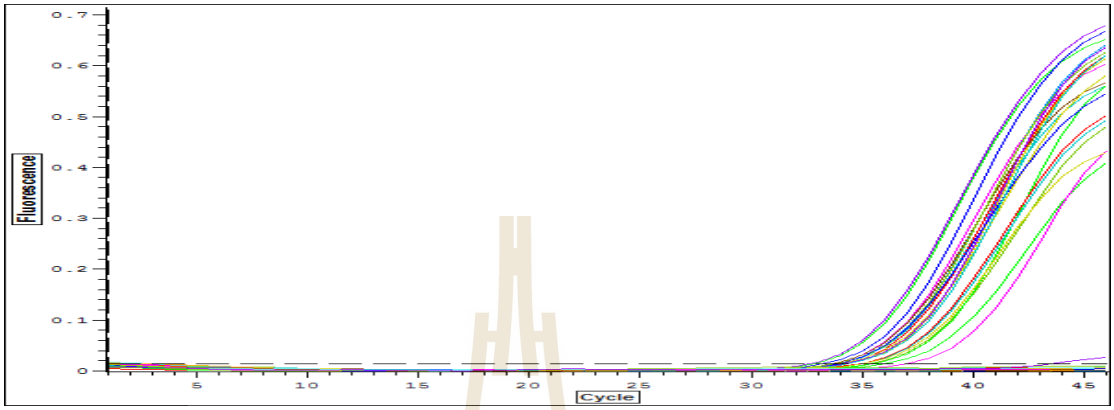


Figure 3. A representative validation curve of real time PCR for *SOX2* gene.

(A) The amplification curve for the *SOX2* gene.



(B) The dissociation curve of PCR products of *SOX2* gene.

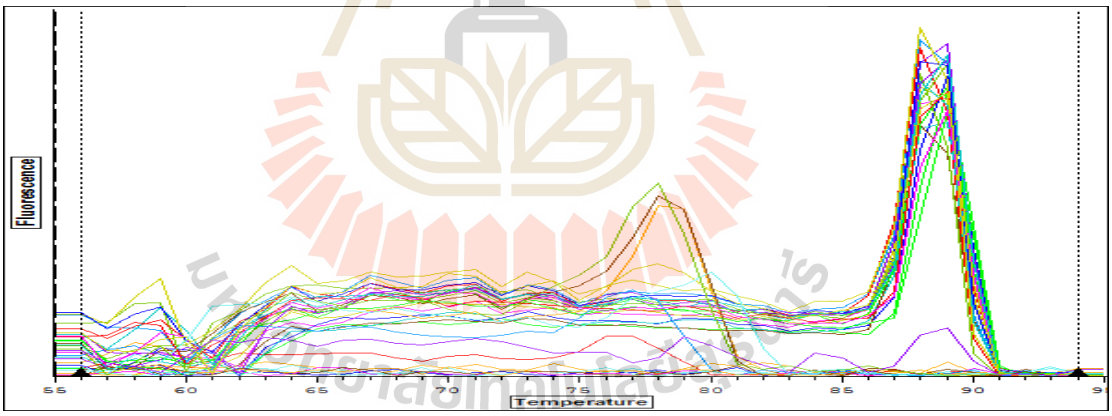
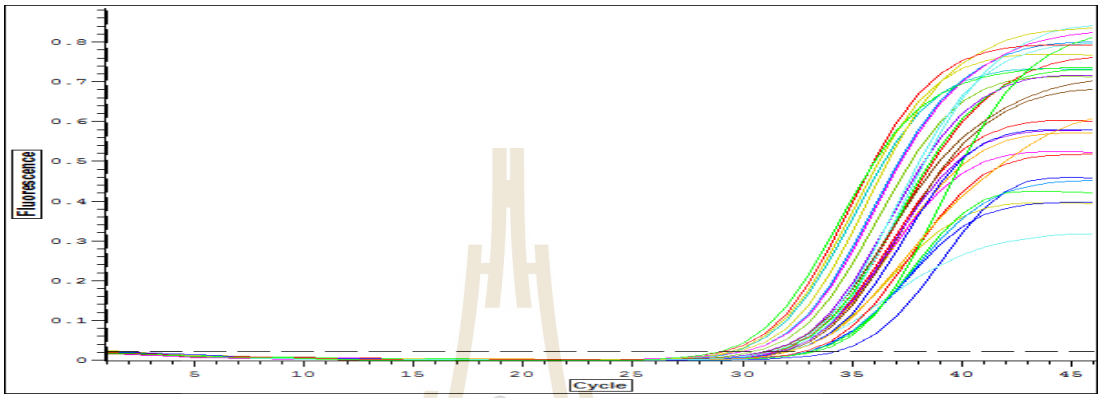


Figure 4. A representative validation curve of real time PCR for *GAP43* gene.

(A) The amplification curve for the *GAP43* gene.



(B) The dissociation curve of PCR products of *GAP43* gene.

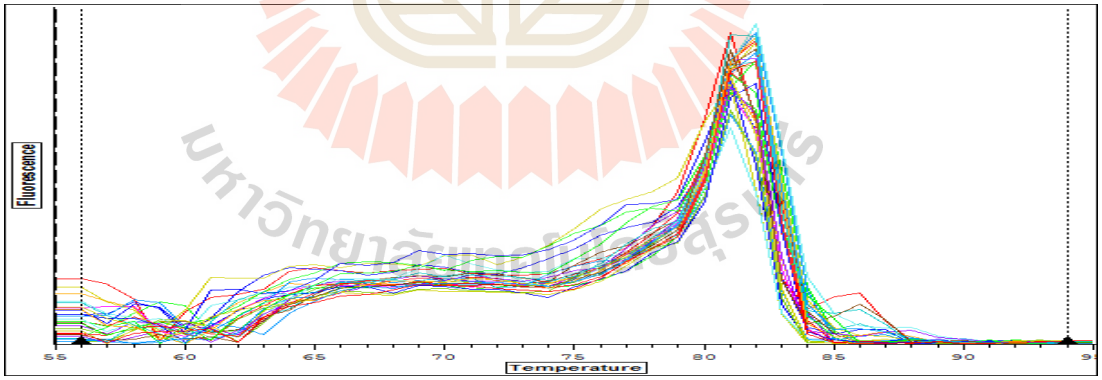
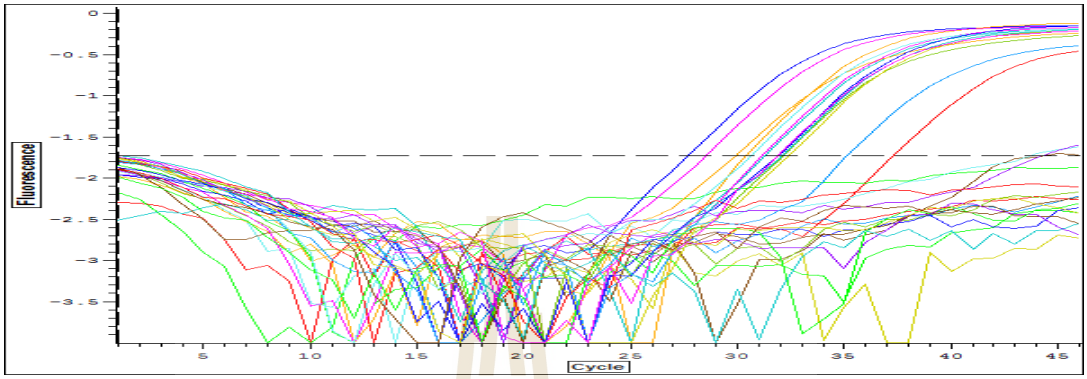


Figure 5. A representative validation curve of real time PCR for *MASH1* gene.

(A) The amplification curve for the *MASH1* gene.



(B) The dissociation curve of PCR products of *MASH1* gene.

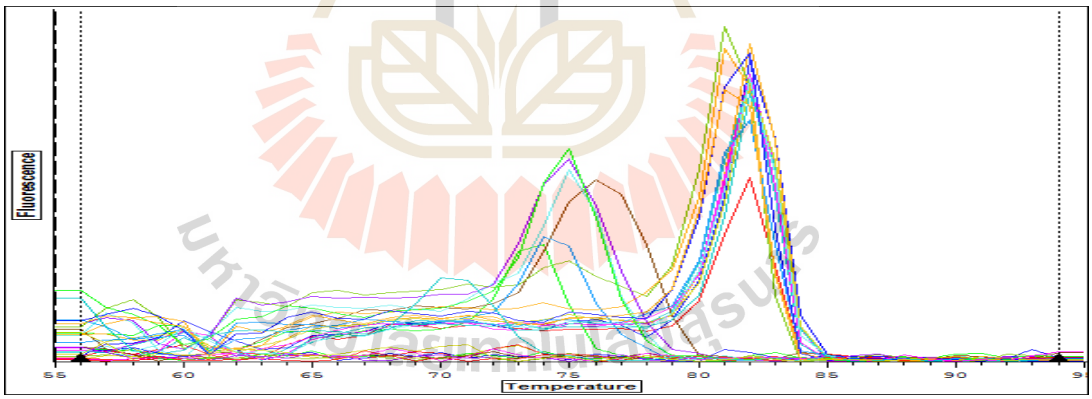
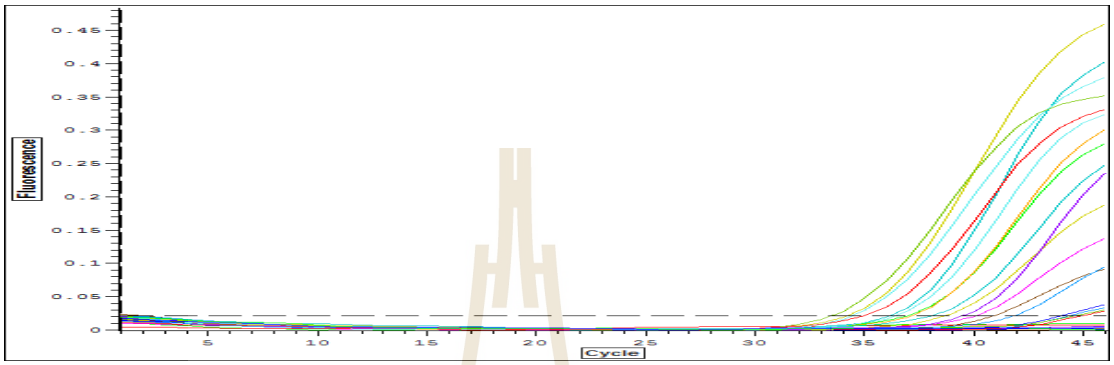


Figure 6. A representative validation curve of real time PCR for *TUJ1* gene.

(A) The amplification curve for the *TUJ1* gene.



(B) The dissociation curve of PCR products of *TUJ1* gene.

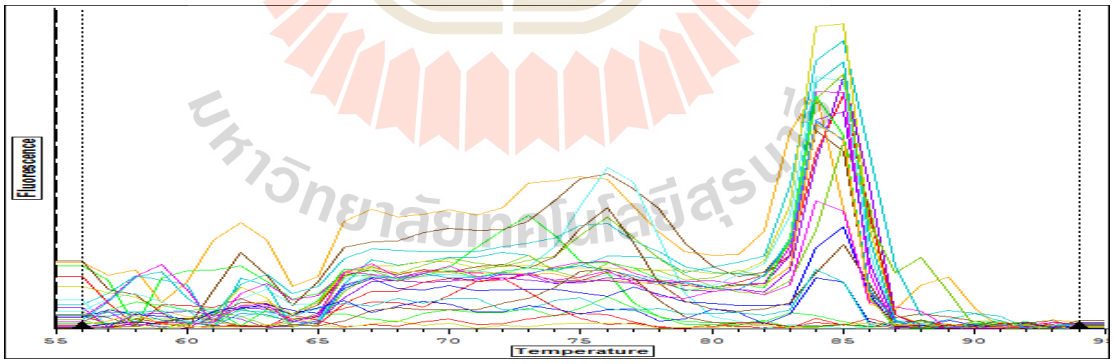
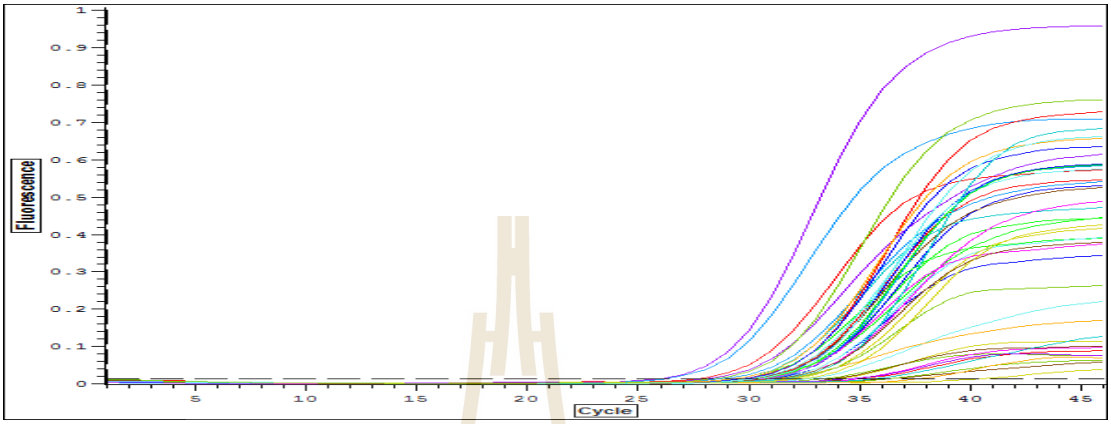


Figure 7. A representative validation curve of real time PCR for *MAP2* gene.

(A) The amplification curve for the *MAP2* gene.



(B) The dissociation curve of PCR products of *MAP2* gene.

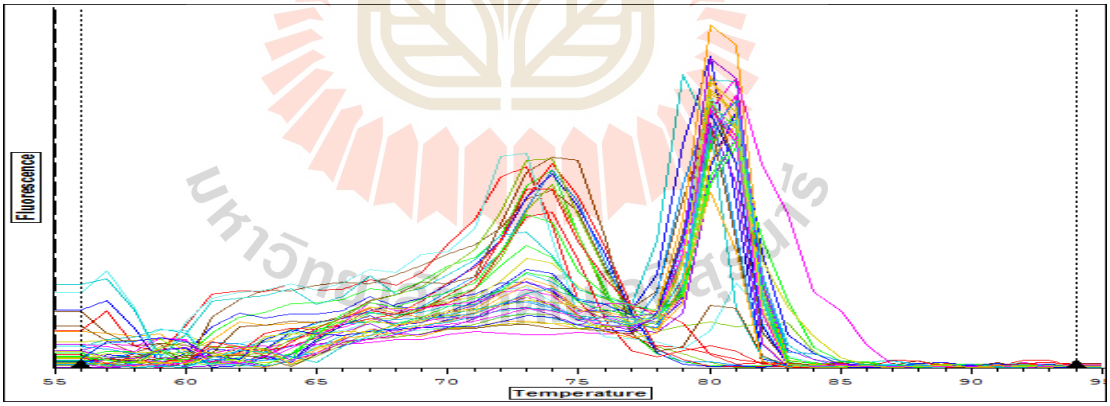
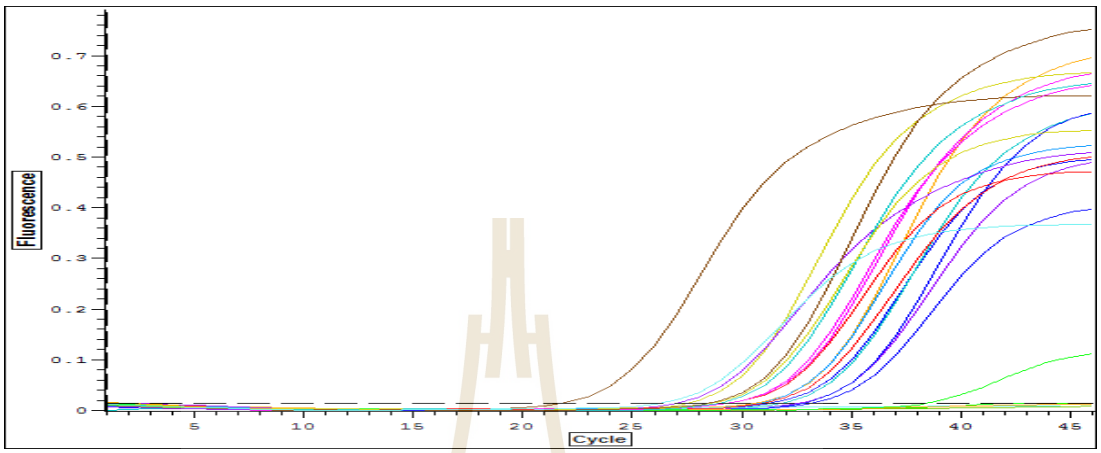


Figure 8. A representative validation curve of real time PCR for *NF-L* gene.

(A) The amplification curve for the *NF-L* gene.



(B) The dissociation curve of PCR products of *NF-L* gene.

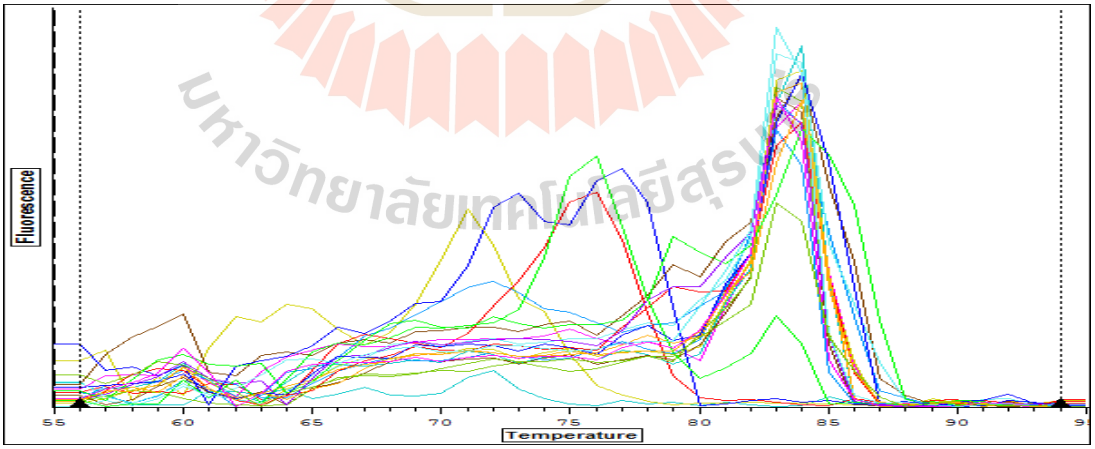
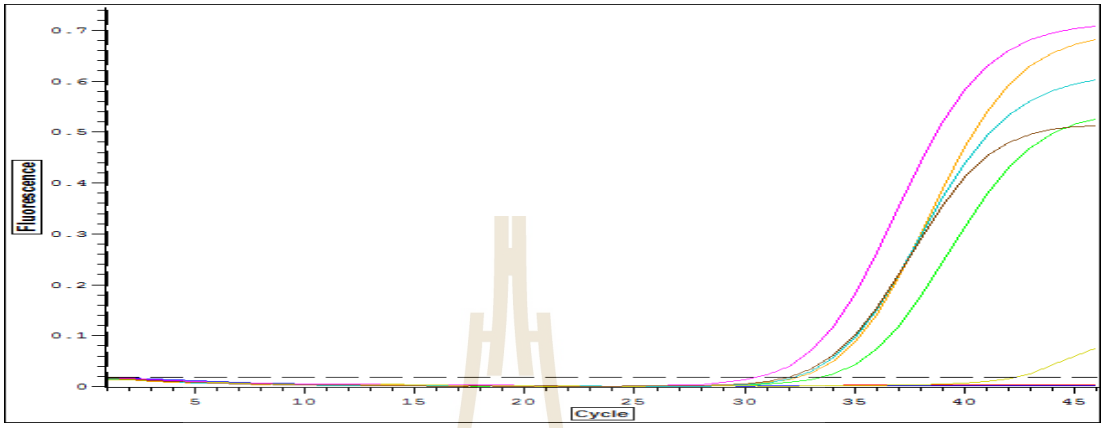


Figure 9. A representative validation curve of real time PCR for *S100B* gene.

(A) The amplification curve for the *S100B* gene.



(B) The dissociation curve of PCR products of *S100B* gene.

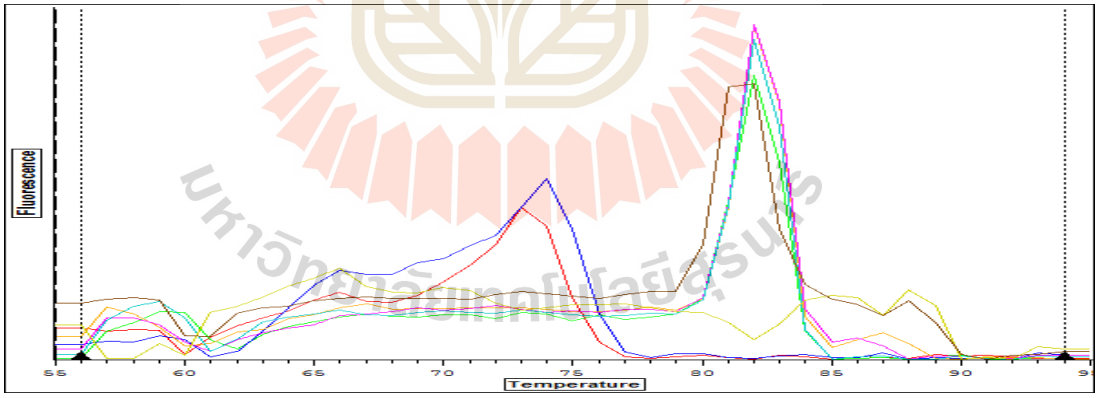
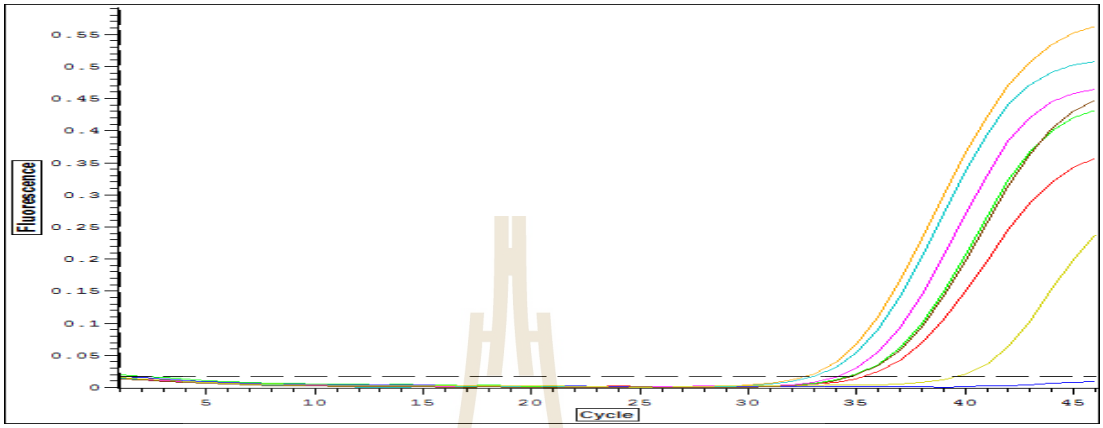


Figure 10. A representative validation curve of real time PCR for *GFAP* gene.

(A) The amplification curve for the *GFAP* gene.



(B) The dissociation curve of PCR products of *GFAP* gene.

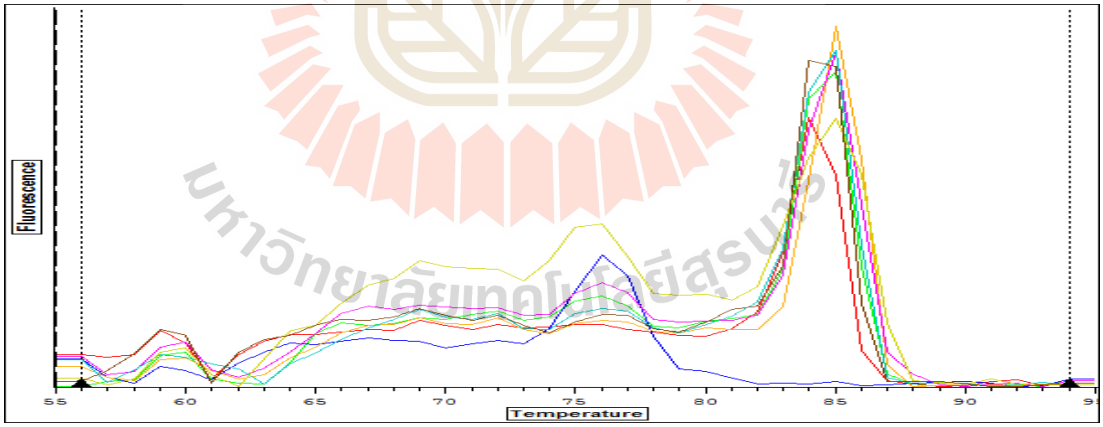
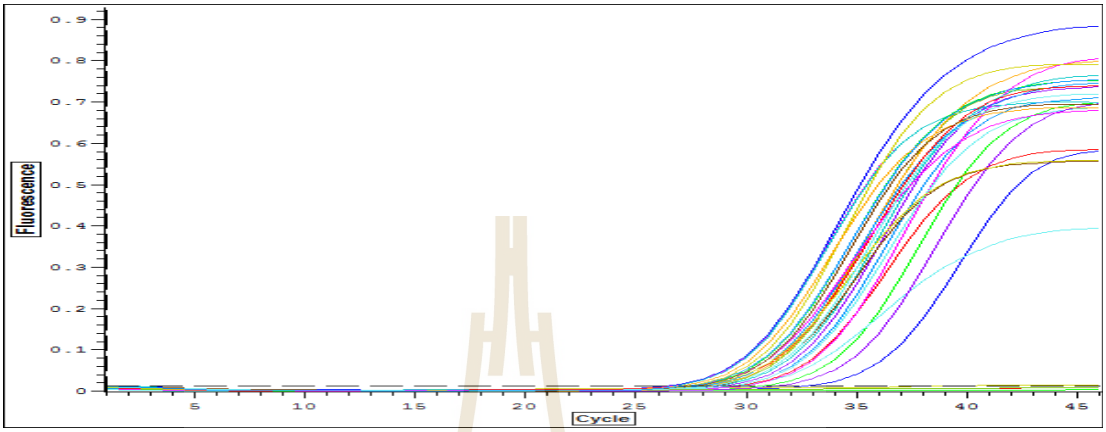
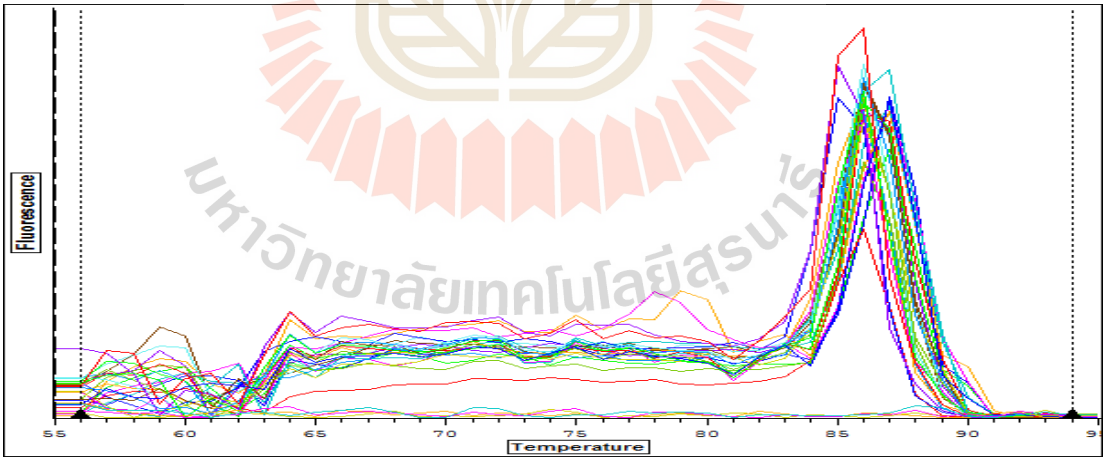


Figure 11. A representative validation curve of real time PCR for *CNPase* gene.

(A) The amplification curve for the *CNPase* gene.



(B) The dissociation curve of PCR products of *CNPase* gene.



BIOGRAPHY

Athip Licmahroen was born in Bangkok, Thailand on March 26th, 1985. He finished his high school from Benchamarachuthit School in Chantaburi. In 2007, he received a Bachelor Degree (BS) in Sports Science from Chulalongkorn University, Bangkok, Thailand. After that, he received a Master Degree (MS) in Health and Physical Active with concentration on clinical exercise physiology from School of Education, University of Pittsburgh, United States of America in 2009. Then, he became interested in stem cell research and its application on exercise physiology. To strengthen his research skills, he decided to obtain another Master Degree (MS) in Biotechnology at Suranaree University of Technology, Nakhon Ratchasima, Thailand. His MS study was supported by research assistant grant from Assoc. Prof. Dr. Rangsun Parnpai. His MS thesis was the effect of 8 bromo-cyclic GMP on neural transdifferentiation of humand adipose stem cells. Parts of this work have been presented as poster presentation at the 2nd Internal Institute of Agricultural Technology Colloquium on August 5th – 6th, 2014 at Suranaree University of Technology, Nakhon Ratchasima, Thailand and the 11th Annual Conference of Asian Reproductive Biotechnology Society on November 2nd – 8th, 2014 at The Sukosal Bangkok Hotel, Thailand.