การผลิตเซลล์ตั้งต้นประสาทจากเซลล์ต้นกำเนิดตัวอ่อนลิงชนิดธรรมดาและชนิดตัดต่อพันธุกรรมโปรตีนเอพีพีและเทา

นางสาวอภิษฎา คล่องแคล่ว

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
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NEURAL PROGENITOR CELL LINES DERIVATION FROM WILD-TYPE AND TRANSGENIC APP/TAU RHESUS MONKEY EMBYONIC STEM CELLS

Apitsada Khlongkhlaeo

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biotechnology

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NEURAL PROGENITOR CELL LINES DERIVATION

FROM WILD TYPE AND TRANSGENIC APP/TAU

RHESUS MONKEY EMBYONIC STEM CELLS

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Philosophy.

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อภิษฎา คล่องแคล่ว : การผลิตเซลล์ตั้งต้นประสาทจากเซลล์ต้นกำเนิดตัวอ่อนลิงชนิดธรรมดาและชนิดตัดต่อพันธุกรรมโปรตีนเอพีพีและเทา (NEURAL PROGENITOR CELL LINES DERIVATION FROM WILD-TYPE AND TRANSGENIC APP/TAU RHESUS MONKEY EMBRYONIC STEM CELLS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 119 หน้า.

วัตถุประสงค์ของงานวิจัยคือการพัฒนาวิธีการผลิตเซลล์ตั้งต้นประสาท พร้อมทั้งและศึกษาผลของวิธีการผลิตเซลล์ตั้งต้นประสาทและศึกษาคุณสมบัติเซลล์ตั้งต้นประสาทที่ได้หลังจากการเปลี่ยนแปลงจากเซลล์ต้นกำเนิดตัวอ่อนของลิงชนิดธรรมดา และชนิดตัดต่อพันธุกรรมโปรตีนเอพีพีและเอพีพี /เทา ซึ่งเซลล์ที่ตัดต่อพันธุกรรมนี้จะใช้เป็นเซลล์จำลองของโรคอัลไซเมอร์ โดยการทดลองนี้เริ่มจากการชักนำให้เซลล์ต้นกำเนิดตัวอ่อนของลิงชนิดธรรมดาให้เปลี่ยนแปลงไปเป็นเซลล์ตั้งต้นประสาทด้วยวิธีการที่แตกต่างกันสองวิธี ได้แก่ วิธีที่เริ่มต้นจากการเลี้ยงเซลล์ต้นกำเนิดตัวอ่อนแบบไม่มีเซลล์พี่เลี้ยง (feeder-free based method) และวิธีการชักนำเซลล์ตั้งต้นประสาทผ่านทางเอ็มบริออยบอดี (embryoid body; EB) (EB-based method) ซึ่งผลการศึกษาพบว่าเซลล์ตั้งต้นประสาทจากทั้งสองวิธีมีลักษณะของเซลล์ที่คล้ายกัน ทั้งทางสัณฐานวิทยาของเซลล์และการแสดงออกของยีนที่จำเพาะ นอกจากนี้ทั้งสองวิธียังสามารถผลิตเซลล์ตั้งต้นประสาทได้ปริมาณมาก (>97% ของประชากรเซลล์ทั้งหมด), ไม่มีการปนเปื้อนของเซลล์ที่ไม่ใช่เซลล์ประสาท (เซลล์จากมีโซเดิร์มและเอ็นโดเดิร์ม), และเซลล์มีความสามารถในการเปลี่ยนแปลงไปเป็นเซลล์ประสาทที่สมบูรณ์ได้ อย่างไรก็ดีพบการแสดงออกของยีนจีเอฟเอพี (Glial fibrillary acidic protein; GFAP) ในระดับที่สูงมากในเซลล์ตั้งต้นประสาทที่ได้จากวิธี feeder-free based method นอกจากนี้การศึกษาครั้งนี้ได้ใช้ลิฟ (leukemia inhibitory factor; LIF) เป็นส่วนประกอบหนึ่งของน้ำยาเลี้ยงเซลล์ตั้งต้นประสาท ซึ่งมีรายงานว่า ลิฟสามารถกระตุ้นการเปลี่ยนแปลงของเซลล์ตั้งต้นปร ะสาทหนูไปเป็นเซลล์แอสโตรไซส์ (astrocyte) ได้ การศึกษานี้ไม่พบว่าวิธีการชักนำที่ต่างกันมีผลต่อการแสดงออกของลิฟรีเซปเตอร์ (LIF receptor) แต่ร ะดับการแสดงออกของยีนจีเอฟเอพีที่แตกต่างกันนั้นสอดคล้องกับการแสดงออกยีนทีแอลเอ็ก (nuclear receptor tailless; TLX) ซึ่งเซลล์ตั้งต้นประสาทจากวิธี feeder-free based method มีระดับการแสดงออกของทีแอลเอ็กต่ำ และอาจทำให้เซลล์ตั้งต้นประสาทจากวิธีนี้มีแนวโน้มที่จะเปลี่ยนแปลงไปเป็นเซลล์แอสโตรไซส์ หรือมีผลต่อความเป็นเอกพันธุ์ (homogeneity) ของประชากรเซลล์ตั้งต้นปร ะสาท นอกจากนี้วิธีการ EB-based method ยังสามารถชักนำให้เกิดเซลล์ตั้งต้นประสาทจากเซลล์ต้นกำเนิดตัวอ่อนชนิดตัดต่อพันธุกรรมโปรตีนเอ

พีพีและเอพีพี/เทา ซึ่งเซลล์ตั้งต้นประสาทจากเซลล์ชนิดตัดต่อพันธุกรรมนี้ สามารถแสดงลักษณะของเซลล์ตั้งต้นประสาท อย่างเช่น สัณฐานวิทยาของเซลล์ การแสดงออกของยีนจำเพาะ และคุณสมบัติการเปลี่ยนแปลงไปเป็นเซลล์ประสาท ได้เช่นเดียวกับเซลล์ต้นกำเนิดตัวอ่อนของลิงชนิดธรรมดา ในการศึกษานี้ยังคงพบความสอดคล้องกันของระดับการแสดงออกของยีนจีเอฟเอพีและยีนทีแอลเอ็ก และจากการตรวจสอบการแสดงออกของยีนเอพีพี และยีนเทา พบว่ามีการเพิ่มขึ้นของยีนทั้งสองเมื่อเซลล์เปลี่ยนจากเซลล์ต้นกำเนิดตัวอ่อนไปเป็นเซลล์ตั้งต้นประสาทและเซลล์ประสาท แต่ไม่พบความแตกต่างกันของเซลล์ระหว่างเซลล์ชนิดธรรมดากับเซลล์ชนิดตัดต่อพันธุกรรม

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2558 ลายมือชื่ออาจารย์ที่ปรึกษา _____

APITSADA KHLONGKHLAEO : NEURAL PROGENITOR CELL LINES DERIVATION FROM WILD-TYPE AND TRANSGENIC APP/TAU RHESUS MONKEY EMBRYONIC STEM CELLS. THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 119 PP

NEURAL PROGENITOR CELL/ NEURAL PROGENITOR CELL DERIVATION/ EMBRYONIC STEM CELL/ NON-HUMAN PRIMATE

The objectives of this research were to develop and establishment neural progenitor cell (NPC) lines and to study the influence and the capacity of NPC derivation methods on the properties of NPCs derived from wild type, and transgenic APPs/tau rhesus monkey (*Macaca mulatta*) embryonic stem cells (rhESCs). The NPCs were obtained from the common NPC derivation protocols: feeder-free and EB-based methods. The NPCs from both protocols shared the similar morphology and gene expression profiles. They were able to provide a high yield (>97%) of NPC population, and no contamination of non-neural lineage cells (mesoderm and endoderm), as well as the potential to differentiate into mature neurons *in vitro*. Interestingly, the elevated expression of Glial fibrillary acidic protein (GFAP) was exhibited in NPCs derived from the feeder-free method. In this study, the leukemia inhibitory factor (LIF) was used as a component in the neural proliferation medium. Although, it has been reported to promote the astrocyte differentiation of NPCs in mice, this study showed that the different NPC induction protocols did not affect the expression of LIF receptor. The enhanced GFAP expression was correlated with the reduced expression of nuclear receptor tailless (TLX). This suggested their higher tendency to differentiate

toward astrocyte lineage or heterogeneity of NPCs in the feeder-free method. Moreover, the EB-based induction protocol was able to induce the NPC differentiation of the transgenic (tg) APPs, and transgenic APPs/human tau ESCs. The tg ES cell lines had the ability to differentiate into NPC cell lines possessing various similar properties to the wild type such as morphology, gene expression, and neural differentiation potential. Additionally, this study showed the correlation between GFAP and TLX expressions in NPCs. Although, the gene expression analysis of tg APP mutations in NPCs and neurons revealed the elevated expression of total SwAPP and IndAPP transcription as well as human tau compared to ESCs, the difference of tg APPs NPCs and neuron compared to wild type could not be detected in this study.

School of Biotechmology Student's Signature _____

Academic Year 2015 Advisor's Signature _____

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CONTENTS

Page

ABSTRACT (THAI) I

ABSTRACT (ENGLISH) III

ACKNOWLEDGEMENTS V

CONTENTS VII

LIST OF TABLES X

LIST OF FIGURES XI

LIST OF ABBREVIATIONS XIII

CHAPTER

I INTRODUCTION 1

1.1 Introduction 1

1.2 References 3

II REVIEW OF LITERATURES 4

2.1 Nonhuman primates for embryonic stem cell research 4

2.2 Early development of the vertebrate central nervous system 5

2.3 Neural progenitor cells 7

2.4 Neural progenitor cell inductions of embryonic stem cells 11

2.5 Neural progenitor cells for neurodegenerative disease 17

2.6 Alzheimer's disease 20

2.6.1 The amyloid precursor protein (APP) 23

2.6.2 Molecular genetics of Alzheimer's disease 28

CONTENTS (Continued)

Page

2.6.3 Amyloid plaques and neurofibrillary tangles 31
2.7 APP transgenic models of Alzheimer's disease 32
2.8 Nonhuman primate models of Alzheimer's disease study 35
2.9 Nonhuman primates embryonic stem cell of AD study 36
2.10 References 37
III SUCCESFUL OF NEURAL PROGENITOR CELL LINES DERIVATION FROM RHESUS MONKEY EMBRYONIC STEM CELLS 48
3.1 Abstract 48
3.2 Introduction 49
3.3 Materials and Methods 51
3.4 Results 55
3.5 Discussion 70
3.6 Conclusion 78
3.7 References 79
IV NEURONAL PROGENITOR CELLS INDUCED FROM TRANSGENIC APPs AND APPs/HUMAN TAU EMBRYONIC STEM CELLS 88
4.1 Abstract 88
4.2 Introduction 89
4.3 Materials and Methods 92

CONTENTS (Continued)

Page

4.4 Results 96

4.5 Discussion 107

4.6 Conclusion 109

4.7 References 110

V OVERALL CONCLUSION 114

APPENDIX..... 115

BIBLIOGRAPHY..... 119

LIST OF TABLES

Table **Page**

2.1 Human embryonic stem cell differentiated into neural progenitors using different protocols 13

3.1 Comparison of characteristic of NPC population from two derivation method 77

มหาวิทยาลัยเทคโนโลยีสุรนารี

LIST OF FIGURES

Figure **Page**

2.1 Cross section through the forming neural tube 6

2.2 Cell lineages in the developing central nervous system 7

2.3 ESC-derived neural rosettes in vitro bear striking resemblance to the neural tube 10

2.4 Step differentiation protocols to induce neural differentiation using EBs and cocultured with stromal cells 19

2.5 Triple immunohistochemical labeling using specific antibodies to Aβ42, HLA-DR and GFAP show patterns of commonly two types of amyloid plaques in AD brain 23

2.6 The structure and process of APP 26

2.7 Overview of possible pathways for biogenesis, clearance and fibrillisation of Aβ 27

3.1 Neural progenitors induction of rhESC in adherent monolayer without feeder cells 57

3.2 The morphological observation of the cells during differentiation of rhESC to NPCs in EB-based culture 58

3.3 Comparison of cell morphology of rhESC-derived NPC from feeder-free culture and suspension culture 59

3.4 Characterizations of rhESC-derived NPC 61

3.5 Gene expressions of non-neural lineage and neural lineage markers 64

LIST OF FIGURES (Continued)

Figure **Page**

3.6 Quantitative real-time PCR showed the expression of astrocytic genes 66

3.7 Differentiation of rhNPCs into neurons..... 70

4.1 ADrES and rQdES1 colonies stained the ESC pluripotency markers..... 97

4.2 Morphology of Rosettes-like structure and neural progenitors after 1 week of induction 98

4.3 Characterization of NPCs derived from tg AD and WT monkey ESCs..... 99

4.4 Transgenic AD and WT monkey NPCs were capable of differentiating into neural cell types 102

4.5 Elevated expression of tg APPs and rhesus APP during neural induction and differentiation 105

4.6 The expression of human tau increased after ADrES2-ESC differentiated into NPCs and neurons..... 107

LIST OF ABBREVIATIONS

ES cell	=	Embryonic Stem cell
NPC	=	Neural Progenitor cell
EB	=	embryoid body
rhESCs	=	Rhesus embryonic stem cells
KO-DMEM	=	knockout-Dulbecco's modified Eagle's medium
KSR	=	Knock-out Serum Replacement
bFGF	=	basic fibroblast growth factor
MEFs	=	mouse embryonic fibroblast
GFAP	=	glial fibrillary acidic protein
AFP	=	alpha-fetoprotein
TTR	=	transthyretin
HNF1β	=	hepatocyte nuclear factor;
GATA4	=	GATA binding protein 4
VEGFA	=	vascular endothelial growth factor A
RUNX1	=	runt-related transcription factor 1
LIF	=	leukemia inhibitory factor
TLX	=	nuclear receptor tailless
MAP2	=	microtubule-associated protein 2
TH	=	neuralfilament and tyrosine hydroxylase
tg	=	transgenic
wt	=	wild type

LIST OF ABBREVIATIONS (Continued)

Sw	=	Swedish
Ind	=	Indiana
APP	=	amyloid precursor protein
Aβ	=	β-amyloid peptide
ml	=	milliliter
µl	=	microliter
mM	=	millimolar

CHAPTER I

INTRODUCTION

1.1 Introduction

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass (ICM) of early stage embryos. They have infinite replication potential and ability to differentiate into all adult cell types (Evan and Kaufman, 1981). These properties render them a promising source for the *in vitro* study of developmental processes and future therapeutic approaches. Although, mouse and human ES cells are both isolated from ICM. The mouse ES cells exhibit fundamental difference, particularly in their morphology, cell surface and molecular markers, cellular biochemistry, and their ability to withstand dissociate into single cells which are important for long term maintenance (reviewed by Vazin and Freed, 2010). Moreover, mouse ES cells can easily be propagated in medium supplemented with serum and recombinant Leukemia Inhibitory Factor (LIF) which recruits the signal transducer and activator of transcription 3 (STAT3). The LIF/STAT3 pathway plays a central role in the control of self-renewal and pluripotency of mouse ES cells (reviewed by Burdon et al., 2002). Unlike mouse, several growth factors have been used to maintain self-renewal of primate ES cells such as FGF2, Nodal/Activin, TGFβ1, and Wnt. Nevertheless, supplement FGF is the most common and effective culture condition for the maintenance of primate ES cells (reviewed by Lanner and Rossant, 2010).

Nonhuman primate ES cells provide an invaluable model system that can be used to avoid the ethical issue and the possibility to develop a preclinical model close to human, for assess the benefit, safety and stem cell transplantation technology.

ES-derived neural progenitor cells (NPCs) are promising candidates for future transplantation strategies, due to their potential to differentiate into all neural lineages, including neuron, astrocyte, oligodendrocyte, and specific subtypes of neural cells, such as motoneuron, dopaminergic neuron, retinol neuron (reviewed by Erceg et al., 2009). Another application for ES cell-based neural differentiation protocol is the opportunity to study developmental events which cannot be studied in living humans, due to ethical reasons. The potential of ES cells for neural progenitor differentiation has been demonstrated. Two strategies which were developed based on the mouse model have been used to drive neural lineage differentiation of monkey and human ES cells. The first protocol is based on adherent culture in feeder-free condition which allows us to directly visualize the process of neural conversion and eliminate the contamination of feeder cells (Dhara and Stice, 2008). However, the maintenance of pluripotency in rhesus monkey ES cells without feeder cells remains a challenging issue (Erceg et al.,2008; Dhara et al., 2008). Therefore the efficiency of NPC line establishment is challenging. The other protocol involves an embryoid body (EB) and its three-dimensional nature recapitulates the environment of early embryo development. EB is generated by suspension culture ES cells. In 5-7 days they develop an outer layer of extraembryonic endoderm surrounding an epiblast-like core. The juxtaposition of these cell types is required for neural induction in the embryo (Germain et al., 2010). Therefore, the goal of this study was to develop NPC line, and the methods for NPC derivation from rhesus ESCs (Rhesus macaque; *Macaca*

mulatta) will be compared characteristic, gene expression, homogeneity, and capable of further differentiation to mature neurons

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CHAPTER II

REVIEW OF LITERATURES

2.1 Nonhuman primates for embryonic stem cell research

Although many of basic researches have been done with rodent embryonic stem cells (ES cells), these models are different from primate ES cells. They are different in cell and colony morphology, molecular biology, pluripotentcy maintenance, and growth factor requirements. For example, LIF (leukocyte inhibitory factor) is essential for maintaining undifferentiated state of mouse ES cells, but not primate ES cells (Pau and Wolf, 2004). Several molecular markers are only specific to primate ES cells (e.g., SSEA-3, SSEA-4, TRA-1-60), while others are specific to mice (e.g., SSEA-1) (Suemori and Nakatsuji, 2006). Although, many human ES cell lines are available, ethical concerns have limited their application. Thus, nonhuman primates (NHP), specifically old world monkeys, such as rhesus macaques (*Macaca mulatta*) are valuable animal models for understanding basic biology and an ideal animal models for the preclinical testing of ES cell-derived therapies because of genetic and physiological conditions similar to humans.

ES cells have two important propoties; rapid proliferation to produce large number of cells and capable differentiate methods into all cell types. Different differentiation methods have been established to differentiate to neurons, glia, cardiac muscle, hematopoietic cells, and endothelial cells for basic and applied research. The cells are valuable for research developing treatment of human disease.

2.2 Early development of the vertebrate Central nervous system

The story begins with the blastocyst stage, embryo that consists of an outer trophectoderm cell layer which develops to placenta, surrounding the inner cell mass that compose of pluripotent stem cells. The inner cell mass gives rise to the epiblast which differentiates to form the three primitive germ layers including ectoderm, mesoderm and endoderm. During gastrulation, a primitive streak forms in the epiblast at the caudal end of the bilaminar embryo. Cell migrating through the primitive streak form the mesoderm and endoderm, and remaining epiblast become ectoderm. The response of dorsal ectodermal cells to primitive induction is to thicken, and formation of a neural plate (Carlson, 2009). Neurulation consists of the epithelial neural plate begins to furrow and the two neural ridges generate on either side of neural groove move towards each other to form the neural tube (fig. 2.1). The neural plate and early neural tube consists of neural stem cells, which can be divided into two main categories: the neuronal progenitor cells and glial progenitor cells. The neuronal progenitor cells give rise to neuroblasts which is cellular precursor of neurons. The other major progenitor cell is the glial line. Glial progenitors split into several lines. One, the O-2A progenitor cell, is a precursor to two lines of glial cells that ultimately form the oligodendrocytes and type 2 astrocytes. Another glial lineage gives rise to type 1 astrocytes. The third glial lineage is radial progenitor cells which give rise to radial glial cells. These cells act as guide wires in the brain for the migration of the young neurons (fig 2.2) (Carlson, 2009). Neurogenesis can also occur in adult mammalian brain, gliogenesis continues throughout life and neurogenesis has been detected in two main areas: the subventicular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) in the dentate gyrus of hippocampus (Bazan et al., 2004).

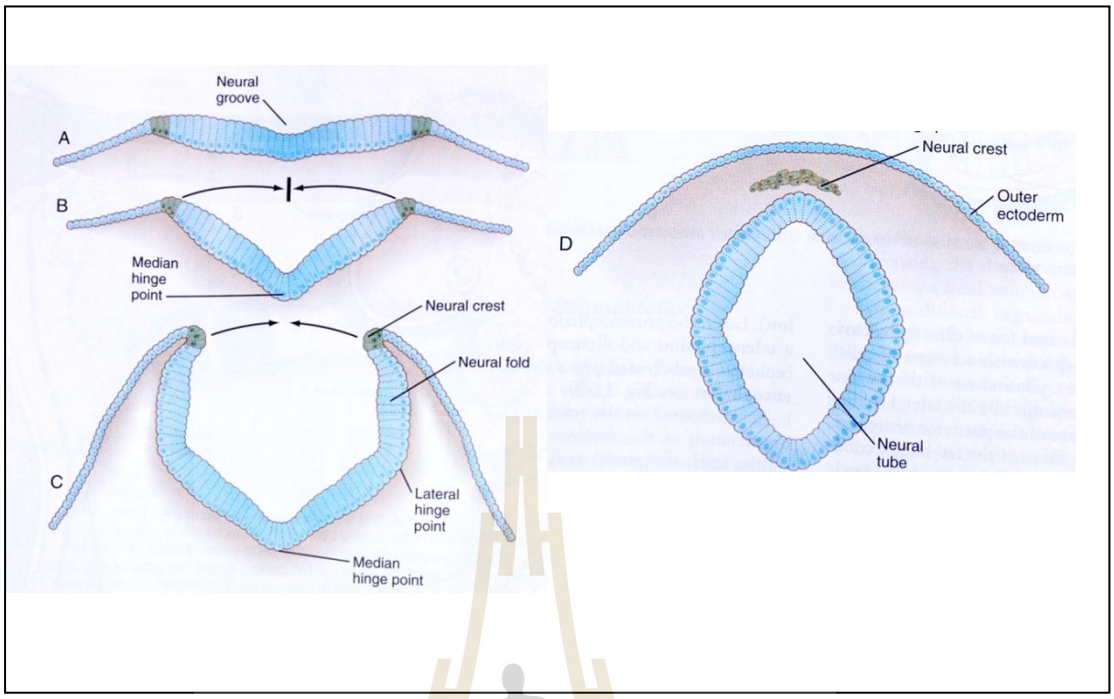

Figure 2.1 Cross section through the forming neural tube. A, Neural plate. B, Neural fold. C, Neural folds apposing. D, Neural tube complete (Neural crest before and after its exit from the neural epithelium is shown in green) (Carlson, 2009).

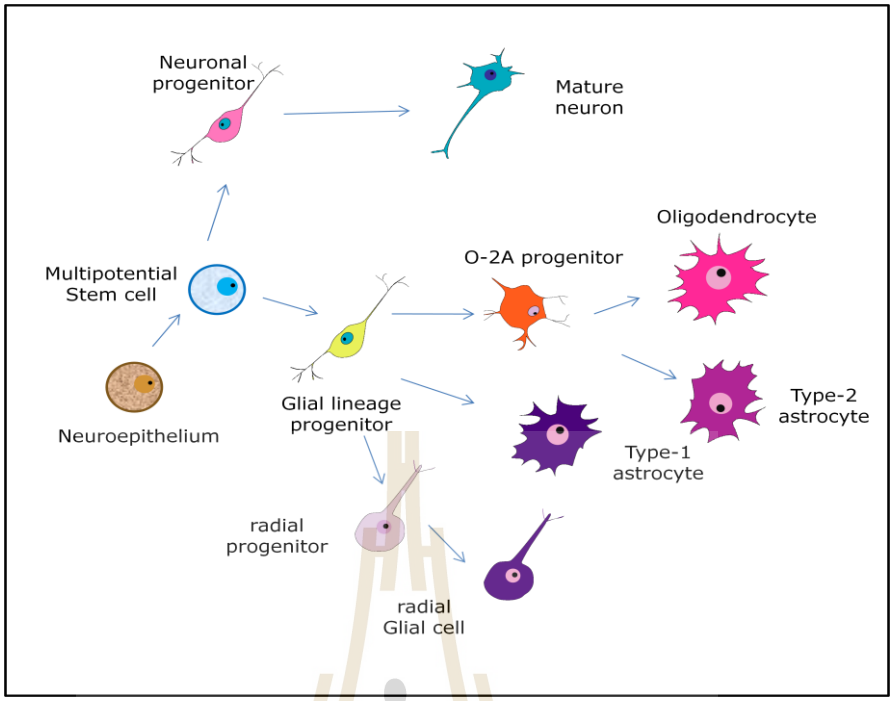

Figure 2.2 Cell lineages in the developing central nervous system. (Modified from Carlson, 2009)

2.3 Neural stem cells/neural progenitor cells (NPCs)

Neural Stem cells are undifferentiated cells that have the ability to generate multiple mature neural cell types (multipotency) and unlimited capacity for self-renewal. However, progenitors are proliferative cells with a limited capacity for self-renewal (Lillien, 1998). Several markers were often used for characterization of NPCs by based on gene expression and immunocytochemistry. Nestin is an intermediate filament protein, which expressed in neural stem cells and progenitors in the central nervous system (CNS) (Roy et al., 2000; Sun et al., 2008). However, nestin was also detected in non-neural tissues, such as the pancreas, muscle, heart, tooth and testis (reviewed by Bazan et al., 2004). Besides nestin, the RNA-binding protein Musashi1 which regulates the translocation of target mRNA during neural

development. Musashi1 appears specifically in undifferentiated neural stem/precursor cells during both embryonic and adult stages (Kaneko et al., 2000). Transcription factors are other candidate molecules that have specific expression pattern in NPCs. The HMG-box transcription factors of the Sox family play key role in the maintenance of multipotency of progenitors. Sox1 is one of the earliest transcription factors to be expressed in ectodermal cells committed to the neural fate and expressed by neural plate epithelia during neural tube ensure (Pevny et al., 1998). Sox2 is another one of the Sox family which is a universal neural progenitor and stem cell marker in the vertebrate CNS. The expression of Sox2 inhibits neuronal differentiation and maintains their progenitor identity. In contrast, the inhibition of Sox2 is associated with a loss of NPC markers and the expression of neuronal markers (Graham et al., 2003). Another member of Sox subfamily group B1, Sox3 is also expressed in NPCs (Dhara et al., 2008). Transcription factor Pax6 is a key regulator in neuronal fate determination as well as the proliferation of neural stem cells. Pax6 is essential for neural stem cell self-renewal ability and interacts with Neurog2, Ascl1, and Hes1 which are regulators of neurogenesis. Removing Pax6 reduces stem cell self-renewal by increasing expression of key cell cycle regulators, resulting in early neurogenesis (Sansom et al., 2009). Moreover, Pax6 regulates the proliferation of NPC through direct modulation of the Sox2 expression during the late development stage of cortical subventicular zone in mice (Wen et al., 2008). Another marker that appears to be characteristic of NPC is the mediate cell adhesion molecules. They have the additional advantage that, being antigens that show on the cell surface, so, they can be used for immunochemical isolation of NPCs. Polysialylated neural cell adhesion molecules (PSA-NCAM), CD9, CD15, CD81, CD95 and CD271 are cell

surface marker that was identified in human NPCs (Klassen et al., 2001; Gerrard et al., 2005; Van Strien et al., 2014).

The signature feature of neuroprogenitors in culture is the formation of neural rosette (Wilson and Stice, 2006), the radial arrangements of columnar cells. This distinctive morphology has mimic developing neural tube (fig 2.3). The rosette-like cells express many neuroepithelial cells markers of the neural tube such as nestin, NCAM, Pax6, Sox1 as well as Musashi (Li et al., 2005; Gerrard et al., 2005; Sonntage et al., 2007). However, Perrier and colleague (2004) reported that rosette structure exhibited Sox1, Pax6, nestin, and NCAM in the absence of specific dorsoventral markers suggests that these cells might resemble to neural plate rather than a neural tube stage (Perrier et al., 2004). In addition, these structures are capable of differentiating into various region-specific neuronal and glial cell types suggest that neural rosettes contain multipotential neuroprogenitors (Li et al., 2005; Gerrard et al., 2005; Sonntage et al., 2007). Neural rosettes can be manually isolated and subcultured on polyonithine/laminin treated substrates in proliferating monolayer cultures of neuroprogenitors and the purity of adherent neuroprogenitor cultures approached 90% as assayed by expression of Pax6 and nestin (Perrier et al., 2004).

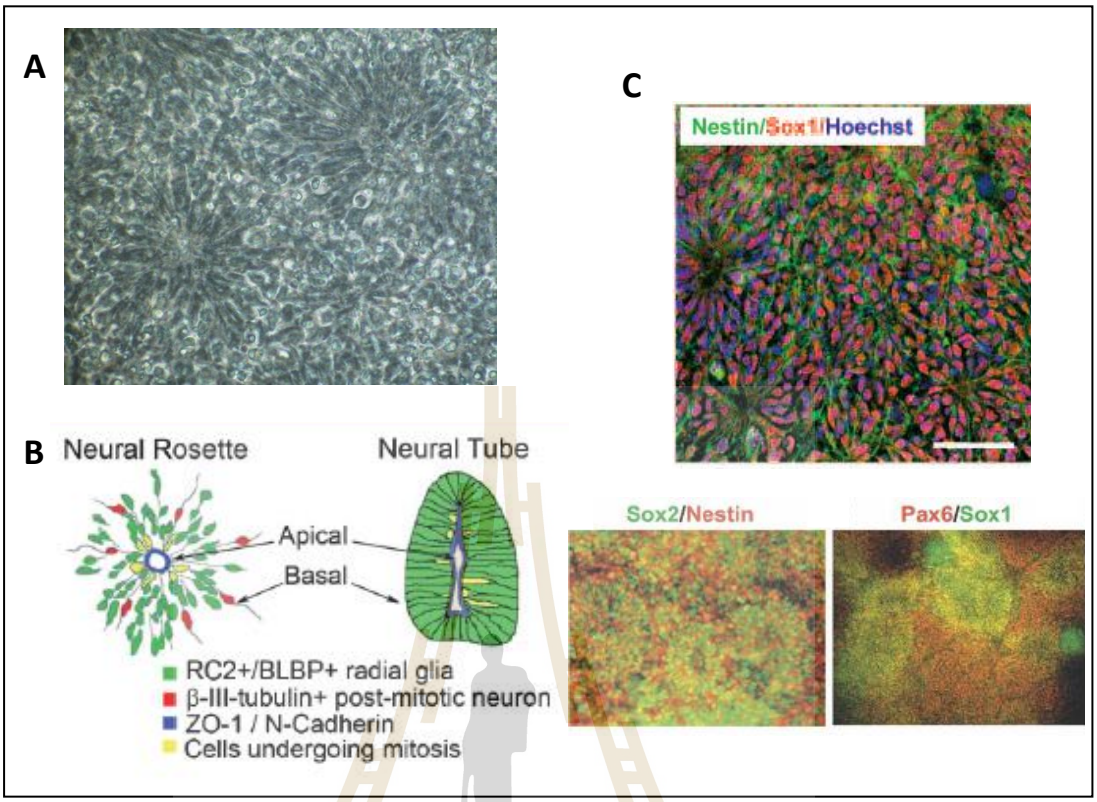

Figure 2.3 ESC-derived neural rosettes in vitro bear striking resemblance to the neural tube. Bright-field microscope showed neural rosette stricture (A). Apicobasal polarity is similar between rosettes and the neural tube, with tight junctions (indicated by ZO-1 expression, blue) at the apical surface forming a lumen. Radially arranged progenitors in the rosette, as well as the neural tube, express RC2 and BLBP (green) (B). Pools of neural stem cells remain in the rosettes (labeled by Sox1, nestin, Pax6 and NCAM). Nuclei are labeled with Hoechst in blue (C). This figure is modified from Perrier et al., 2004, Sonntag et al., 2007 and Elkabetz et al, 2008.

2.4 Neural progenitor cell inductions of nonhuman primate embryonic stem cells

Base on pluripotency of ES cell, it is not only able to reveal the mechanism of cell differentiation, but also provide specific cells for cell therapy and pharmaceutical screens. *In vitro*, neural differentiation of monkey and human ES cells can be easily induced by prolonged culture without replacing feeder cells. However, it is difficult to achieve homogeneous differentiation of ES cells into neural progenitors. Studying neural specification in vitro will help us to further improve neural cells differentiation from ES cells and investigate neural development. The neural differentiate of human ES cells can be induced and enhance under *in vitro* condition by supplementary growth factors, growth factor antagonist, or morphogen. There are three strategies to drive ES cells differentiation into NPCs. First approach is spontaneous differentiation of hES cells into highly purified neural lineages (Gerrard et al., 2005). Propagating the ES cells in feeder-free adherent culture and adding factors into the medium to induce neural differentiation (reviewed in table 2.1). The formation of ectodermal derivatives can be induced by prolonged cultured in induction medium. This monolayer culture allows visualization of the process of neural conversion and avoids the generation of EBs and co-culture with stromal cells, which reduce the risk of contamination with non-neural cells and the use of other cell types. Second approach is the formation of three-dimensional aggregates called embryoid bodies (EBs) using ES cells as the initial step of differentiation. Pluripotency of ES cells can be maintained in the present of growth factors (LIF for mouse, bFGF for monkey and human), and feeder layers. However, when cultured in the absence of growth factors in petri-dish, ES cells differentiate spontaneously, and form EB. This structure

develops an outer layer of extraembryonic endoderm surrounding an epiblast core, mimicking the two cell layers observed in the egg cylinder stage mouse embryo or the bi-laminar germ disc of the human embryo. Interactions between these cell layers facilitate neural ectoderm specification (Germain et al., 2010). The epiblast layer can generate derivatives of all three primitive germ layers (Itskovitz-Eldor et al., 2000). The last strategy is co-culture with stromal cells to promote neural differentiation (Perrier et al., 2004). Stromal calls are loose connective tissue cells found in number of organ, such as gonad and bone marrow. They provide matrix-support for other cells in the organ to function. The differentiation is promoted by stromal-derived inducing activity (SDIA). SDIA suppresses the mesodermalizing effects of BMP4 on ESCs (Kawasaki et al., 2000). This neural induction culture system was first described for mouse ESC and has been used to generate midbrain dopaminergic (DA) neurons (Kawasaki et al., 2000; Perrier et al., 2004). However, the SDIA neural-inducing effects have been adapted to a wide range of neuronal cell types (Kawasaki et al., 2002; Mizuseki et al., 2003). Numerous stromal cell lines, such as PA6, MS5, S2, were used for this purpose (Kawasaki et al., 2000; Perrier et al., 2004; Sonntag et al., 2007). Although, SDIA provides an efficient method for neural differentiation, co-culture technique can introduce unknown factors of non-human origin via culture and may obscure the mechanisms involved in neural differentiation (Dhara et al., 2008).

Table 2.1 Human embryonic stem cell differentiated into neural progenitors using different protocols.

Growth condition	Growth factor	Day of derivation (day)	Yield of NPC	Ref.
Feeder free-adherent culture	KSR, NEAA, bFGF, N2	22	>97% nestin+	Dhara et al., 2008
Feeder free-adherent culture	N2, B27, noggin, bFGF	75	>97 %nestin+ >93%musashi+ >91%PSA-NCAM+	Gerrard et al., 2005
Feeder free-adherent culture	KSR, bFGF, noggin, RA	30	>94%β-tubulin III+ >92%MAP2+ >90%synaptophysin+ >89%neurofillament+	Baharvand et al., 2007
Feeder free-adherent culture	RA, bFGF, B27, insulin, progesterone, putrescin, sodium selenite, holotransferin	28	>87% nestin+ >84% pax6+ >93%BLBP+	Erceg et al., 2008
EB-base culture	Isulin, sodium selenite, transferrin, fibonectin	19-26	>74%nestin+ >63%musashi+	Kuo et al., 2003
EB-base culture	N2, bFGF	26	>85% β-tubulin III	Cho et al., 2008
EB-base culture	KSR, NEAA, N2, bFGF	26	ND	Zhou et al., 2008

Table 2.1 (Continues).

Growth condition	Growth factor	Day of derivation (day)	Yield of NPC	Ref.
EB-base culture	N2, bFGF	17	ND	Tibbitts et al., 2006
Stromal cells co-culture	KSR, N2, SHH, FGF8, BDNF, GDNF, TGFβ3, dibutyryl cAMP, AA	28	>90%pax6+	Perrier et al., 2004
Stromal cells co-culture	N2, AA, bFGF, EGF, AA	28	>95%nestin+	Hong et al., 2007
Stromal cells co-culture	N2, bFGF, SHH, FGF8, BDNF, AA	30	>74%NCAM	Sonntag et al., 2007

Abbreviations: KSR, Knockout serum replacement; NEAA, Non-Essential Amino Acids; N2, N2 supplement; RA, retinoic acid; SHH, sonic hedgehog; BDNF, brain-derived neurotrophic factor; GDNF, glail cell line-derived neurotropic factor; TGFβ3, transforming growth factor type β3; AA, ascorbic acid; EGF, epidermal growth factor; ND, no data

The most often used factors that promote neural differentiation are N2 supplement and FGF2. The following is a brief discussion on the roles of these factors related to neural differentiation.

N2 supplement was used in neural induction culture in adherent culture on laminin or in suspension as EBs (Table 1). N2 supplement is a chemical defined reagent which comprise of human transferrin, recombinant insulin, progesterone, putrescine and sodium selenite. N2 supplement promotes neural differentiation, but not neural survival (Babu et al, 2007). Use of N2 for 14 days increase musashi1 expression (Svendsen et al, 1995). Insulin is the key component to promote differentiation of hESC. Insulin (20 µg/ml) has been reported to induce the activation of the insulin-like growth factor (IGF) and may promote neuronal precursor differentiation (Arsenijevic and Weiss, 1998).

Basic fibroblast growth factor, also known as bFGF, bFGF is the most commonly used mitogen in neural stem cell culture. It is necessary for neural stem cell proliferation (Carpenter et al., 1999; Wilson and Stice, 2006). bFGF is a member of the FGF family act through FGF receptors (FGFRs) to activate cascades of kinase signaling, including Raf, MEK (mitogen-activated and extracellular signal-regulated kinase), and MAPK (mitogen activated protein kinase) (Nutt et al., 2001). The loss of FGF or FGF receptors leads to significant reduction in stem cell proliferation (Sommer and Rao, 2002; Sun et al., 2008). However, the addition of a high dose (>5ng/ml) of bFGF in neural precursor culture, resulted in differentiation and astrocytes (Nistor et al., 2011). Interestingly, cell from non-neuronal origin can also be induced by bFGF to adopt neuronal phenotypes neuronal transdifferentiation of human amniotic epithelial cells (Niknejad et al., 2010) and bone marrow stromal cells (Yang et al., 2008), indicating its potent neuronal-inductive effects.

The other popularly used growth factors to generate neural progenitors are noggin and retinoic acid (RA). Noggin is a BMP-antagonist secreted from notochord

and floor plate that reduces the effect of bone morphogenetic protein (BMP) involved in dorso-ventral patterning (Graham et al., 1994: Shimamura and Rubenstein, 1997). Treatment with noggin reduce the expression of ESC markers, up-regulated the expression of neural progenitor marker, such as Sox1, nestin, Pax6, and the neuronal marker MAP2 (Gerrard et al., 2005), and suppress non-neuronal differentiation (Yao et al., 2006). hESC treated with noggin, that potentially binds to BMP-2,4, and7, generated NPCs and also down-regulate the expression of AFP, GATA6, suggesting preventing formation of extra-embryonic endoderm. Noggin has also been shown to up-regulate the expression of phosphorylated Smad1 (Pera et l., 2004) and down-regulate the BMP-2 target protein Id2 and Id3, suggesting that noggin increase neurogenesis using Smad1-mediated signaling and repress the promoter activation induced by neurogenic bHLH factors (Miyazono and Miyazawa, 2002). In addition, noggin not only improve the neuronal induction efficiency of hESCs, but also inhibite differentiation to glial cells (Zhou et al., 2008). Retinoic acid (RA) is also used in NPC induction medium (Baharvand et al., 2007). RA plays important roles in neural development such as axon regeneration in the adult, neural differentiation patterning of neural plate and neural tube in the early embryos (Maden, 2007). For neuronal differentiation, RA increase the proportion of neuronal cells in cultures, induce extensive outgrowth of processes, and the expression of neuron-specific molecules (Schuldiner et al., 2001). RA mediate neuronal and glial differentiation occurs by the activation of genes that involve transcription factors (BRN2, Sox1, Sox6), cell signaling molecules or associated cell structures (ceramide, PSEN1, MAP2) (Dhara and Stice, 2008). Moreover, RA and LIF synergistically activate the promoter of *gfap*, and induce astrocyte differentiation of NPCs (Asano et al., 2009).

Chemical and growth factors are a crucial factors for cell survival and cell fate. LIF is a member of interleukin-6 (IL-6) cytokine family that signaling through a receptor complex for LIF receptor and gp130. LIF activate a cascade of signaling through JAK-STAT3 (Janus kinase-Signal transducer and activator of transcription 3), MEK (MAPK/ERK kinase), and PI3K (phosphoinositide 3-kinase) pathways (Patterson, 1994). In fact, activated LIF signaling promotes proliferation, reduced caspase-mediated apoptosis and reduced reactive oxygen species (ROS) in culture of hNPCs (Majumder et al., 2012). LIF was found to inhibit differentiation, maintain multipotency of NPCs (Carpenter et al., 1999; Babu et al., 2007), increase overall expansion rate, prevent senescence and allow long term culture of neural stem cells (Wright et al., 2003). Additionally, LIF has been reported to influent the regulation of GFAP expression in rodent neural stem cells leading to an enhanced activation of astrocyte differentiation (Wright et al., 2003; Takizawa et al., 2001; Fan et al., 2005). The other commonly used supplement in the NPC culture is B27. B27 supplement provide a variety of lipid-rich compound, protein, and growth factors which enhance primary neuronal growth and long term survival while suppress glial cell growth (Brewer et al., 1993).

2.5 Neural progenitor cells for neurodegenerative disease

Cultured neural stem/progenitor cells are powerful tool for basic and applied neurobiology. A nonhuman primate neural stem/progenitor cell provides a model system to investigate neural development, biochemistry and cell biology. They also offer a renewable cell source, including neural cells and specific subtypes of neural precursors for neurodegenerative disease studies and would be suitable for

pharmaceutical and toxic screening. For examples of differentiated ES cells into neural cells and specific subtypes of neural precursors are summarized in the figure 2.4. The first report of ES cells differentiation by Kang and colleagues (2007). They produced mature oligodendrocytes from hESCs by addition of the thyroid hormone at a specific stage after the induction of oligodendrocyte precursor by EGF and PDGF. In 2005, Li and colleagues generate early and late neuroectodermal cells and in the presence of SHH that can be differentiated into spinal motorneurons. Because of the important roles of dopaminergic (DA) cells in the control of many brain functions and degeneration in Pakinson's disease, DA neurons are therefore one of the most interesting cell types for cell replacement therapy. Cho and colleagues (2007) reported a protocol to produce a large number of DA neurons.

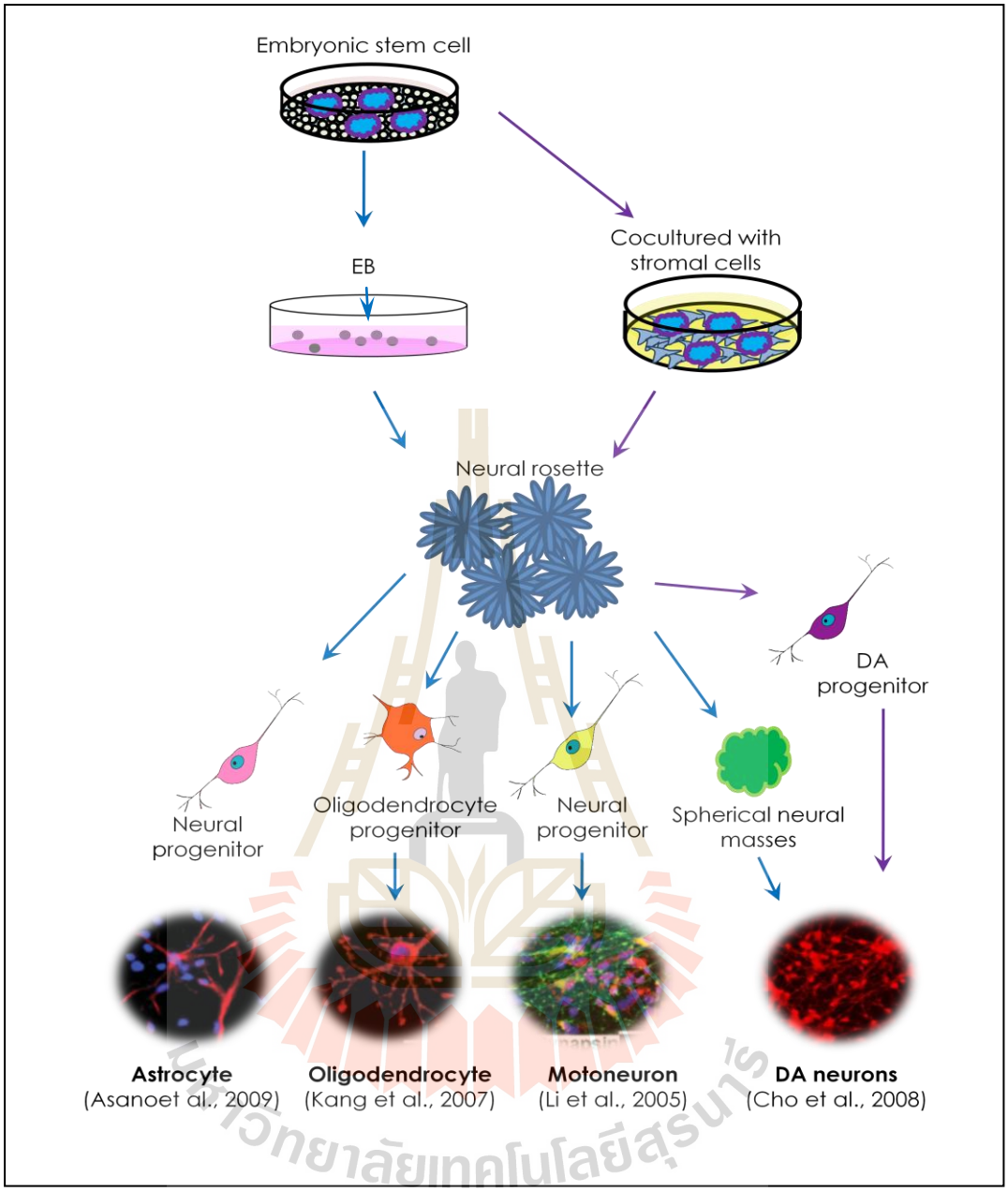

Figure 2.4 Step differentiation protocols to induce neural differentiation using EBs and cocultured with stromal cells. Neural rosette is the source of neural progenitor cells, which differentiate into neurons and glia cells.

2.6 Alzheimer's disease

In 1906, Alois Alzheimer gave a lecture at a congress in Tubingen, Germany, on the first case of the disease that later named Alzheimer's disease (AD). He described the two lesions that pathologist still use to make the histological diagnosis of AD, senile plaques and neurofibrillary tangle (Graeber et al., 1997). Alzheimer's disease is the most common age-related neurodegenerative disorder currently affecting more than 20-30 million people worldwide. 2050, European region alone will be around 11.2 million AD patients (Wancata et al., 2003). At present, there is no efficient therapy available. AD develops differently among individuals, suggesting that more than one pathologic process may lead to the same outcome. The first symptom marking the transition from normal aging to Alzheimer disease is forgetfulness. This transitional stage, known as amnestic mild cognitive impairment (MCI), is characterized by dysfunction in memory with retention of normal cognitive ability in judgment, reasoning, and perception. As amnestic MCI progresses to Alzheimer disease, memory loss becomes more severe that along with language, perceptual, and motor skills deterioration. The mood becomes unstable the individual becomes irritable and more sensitive to stress that become intermittently angry, anxious, or depressed. In advanced stages, the individual becomes unresponsive and loses mobility and control of body functions; death after a disease onset ranging 2 to 20 years (Roger, 2010).

In AD brain, major pathological hallmarks are widespread neural loss, formatting intraneuronal neurofibrillary tangle (NFT), and extracellular amyloid-β (Aβ) plaques. NFTs consist of paired helical filaments. The neurofibrillary lesions are found in the cytoplasm of cell bodies and apical dendrites as NFT. Tangles are formed

by hyperphosphorylation of a microtubule-associated protein knows as tau. Tau is a normal microtubule-binding protein physiologically located in neuronal axons in the brain. Tau promotes microtubule assembly and stable organization of the actin cytoskeleton, intracellular vesicle transport anchoring of phosphatases and kinases. Tau phosphorylation is regulated by the balance between multiple kinase and phosphates, i.e. GSK-3β, CDK5, MAPK, PP-1, PP-2A and JNK. In amyloid precursor protein (APP) transgenic mice increased JNK and MAPK activation was observed and a decrease in Aβ levels caused decreased activity of GSK-3β. Additionally, downregulation of PP2A levels, one of the major tau phosphatases, is found in the brains of AD patients (Brandt, et al., 2005; Brouwers, et al., 2008). Tau protein can be phophorylated in at least 30 sites. In AD brain, tau is hyperphosphorelated and glycosylated. Hyperphosphorylation of tau mediates a decrease in the binding of normal tau to microtubules and other microtubule-associated protein, results in isolated tau in neuron and its subsequent aggregation, with the formation of paired helical filaments (PHFs) and NFTs. The main consequence is a disorganization of the neuronal skeleton leading to neuronal cell death and eventually dementia (Gotz, et al., 2004; Flirski and Sobow, 2005; Blennow, et al., 2006; Huang and Jiang, 2009)

The other classical hallmarks of AD pathology is amyloid plaque. This structure is a heterogeneous class of protein aggregate of fibrillary Aβ forms rich in a cross-β-pleated sheet structure. The β-sheet conformation forms stable fibrillary structures that can be dispersed in aqueous solution only with nonphysiological denaturing reagents, such as formic acid. (Gunther and Strittmatter, 2010). The amyloid plaques initially formed in myelinated areas of the basal neocortex.

The layer II and III are most susceptible to plaque formation. The depositions increase and spread into the adjoining neocortical areas and the hippocampal (Braak and Braak, 1997). There are commonly two types of amyloid plaques (fig 2.5): diffuse plaques containing mainly unstructured amyloid, which is not associated with inflammatory cells and dense core plaques consists of a dense central core of radiating amyloid fibrils together with their ability to attract microglia and macrophages that initiate inflammatory response. In addition, they contain nuclear and cytoplasmic component which resistant to proteolysis by lysosomes released during necrosis of neuronal cells (D'Andrea and Nagele, 2010). Aβ is the most proteinaceous component of plaques. Aβ is a normal soluble cellular metabolite comprised of two predominant forms with different COOH-termini, Aβ40 and Aβ42. While Aβ40 is the major species produced in human, Aβ42 is an initiating molecule and a major composition of amyloid plaques (Sisodia and Price, 1995; Ling, et al., 2003; McGowan, et al., 2005; Czirr, 2008). Analysis of photo-induced, crosslinked oligomers by dynamic light scattering, Western blot, and electron microscopy (EM) revealed that Aβ40 formed smaller aggregates (monomer to tetramer), while Aβ42 preferentially formed larger, pentamer/hexamer aggregates. Analysis of secondary structure revealed higher amounts of β-sheet content in Aβ42 preparations as compared with Aβ40, which may facilitate early assembly into larger oligomers (Bitan, et al., 2003; Dasilva, et al., 2010). Mice expressing high levels of Aβ40 do not develop manifested amyloid pathology. In contrast, mice expressing lower levels of Aβ42 accumulate insoluble Aβ42 and develop compact amyloid plaques (McGowan, et al., 2005). These studies suggested that Aβ42 is a major cause of amyloid pathology. Moreover, natural and synthetic Aβ oligomers have been shown to inhibit hippocampal long-term

potentiation, disrupt memory, decrease synaptic density, and suppress synaptic plasticity to induce the dendritic spine retraction, (Rowan, et al., 2005; Shankar, et al., 2008; Selkoe, 2008; Tomiyama, et al., 2008).

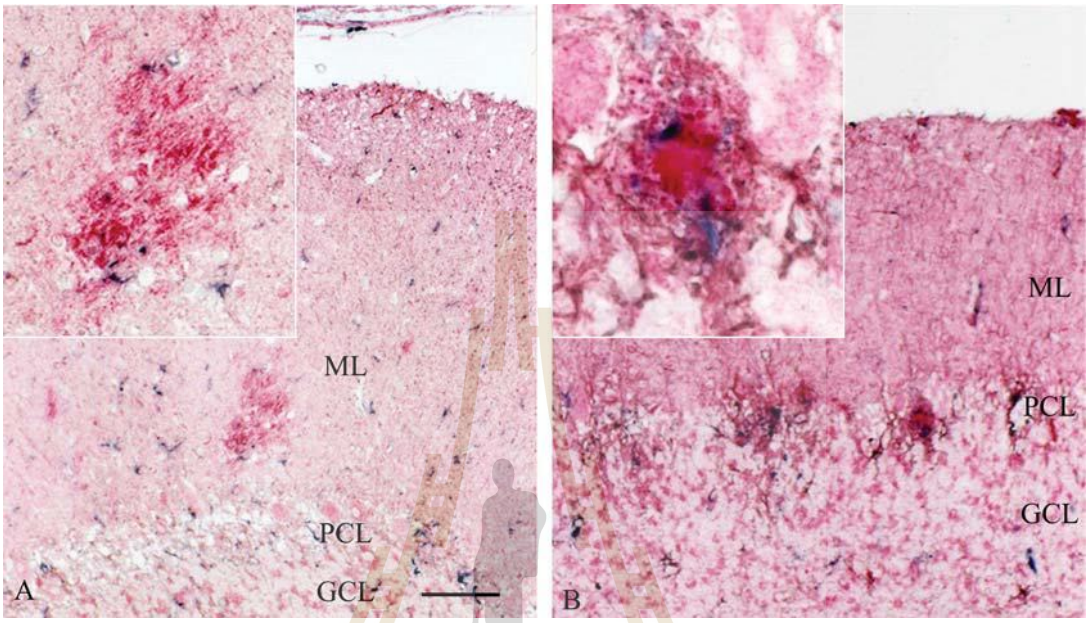

Figure 2.5 Triple immunohistochemical labeling using specific antibodies to Aβ42, HLA-DR and GFAP show patterns of commonly two types of amyloid plaques in AD brain: diffuse plaque (A) and dense core plaque (B). Microglia (blue) and astrocyte (black) are found within and surrounding the dense core plaque. Anatomical regions of the cerebellum are labeled: ML, molecular layer; PCL, Purkinje cell layer; GCL, granular cell layer. Insets are higher magnification. Bar = 25 μm; 100 μm for insets (D'Andrea and Nagele, 2010).

2.6.1 The amyloid precursor protein (APP)

APP is a member of type I integral membrane glycoproteins with a large ectodomain and a shorter cytoplasmic carboxy-terminal (C-terminal) tail and is

expressed in many tissues (Hardy, 1997; Zheng and Koo, 2006; Czirr, 2008). The mammalian APP family proteins are abundantly expressed in the brain. The human *APP* gene is located on the long arm of chromosome 21 and contains at least 18 exons. Alternative splicing of APP generates APP mRNAs translating several isoforms that range from 365 to 770 amino acid residues. The most prevalent isoforms are APP770, APP751, and APP695, the latter being the predominant isoform in neural tissue. (Huang and Jiang, 2009; Perreau et al, 2010). The function of APP is an important cell constituent that plays a role in the recognition of extracellular signal, cell adhesion and apoptosis. In neurons, APP is required for synaptogenesis, synapse remodeling and neurite outgrowth. The expression of APP is increased during neuronal maturation, differentiation and during traumatic brain injury (Hung, et al., 1992; Buoso, et al., 2010; Perreau, et al., 2010). Interestingly, APP is the precursor of Aβ which is the major component of amyloid plaques found in the brains of AD patients. Aβ is the key molecule in AD pathology. The production of Aβ occurs via cleavage of APP by different protein complexes, called secretases. There are two pathways to cleave APP. First is the non-amyloidogenic pathway. Approximately 90% of all APP molecules are cleaved by the α-secretase, resulting in a membrane-bound C-terminal fragment (CTFα/C83) and the soluble APPα (sAPPα) which is thought to have neuroprotective properties (Stein, et al., 2004; Czirrr, 2008) and promotes neuritogenesis (Porayette et al., 2009). Since this cleavage occurs at the lys16-leu17 bond within the Aβ domain, it prevents deposition of amyloidegenic peptide (Nunan and Small, 2000; Ling, et al., 2003). The C-terminal stub (CTFα) is further processed by γ-secretase which lead to the release of the APP intracellular domain (AICD) and the formation of a 3 kDa protein fragment termed p3 that is

released into the extracellular or luminal space. Different proteins have been reported to interact with AICD including those that are necessary for AICD-dependent function in signal transduction, apoptosis or the modulation of cytoskeletal dynamics (Buoso, et al., 2010). The cleavages by α-secreatase, cut APP within the Aβ sequence, and prevent the formation of Aβ. A secondary pathway involves the cleavage of APP by β-secretase. The first cleavage is mediated by the β-secretase and releases a shorter soluble fragment from membrane, sAPPβ and leaves a C-terminal membrane-associated fragment (C99 or CTFβ) (Vardy, et al., 2005: Ling, et al., 2003: Buoso, et al., 2010). CTFβ is then processed by γ-secretase, released the AICD and produce Aβ peptides. Aβ40 is the predominant cleavage product *in vitro* and *in vivo*, Aβ42 is the less abundant and less soluble (Yan et al., 2003). Besides these two peptide species, γ-cleavage also produces to a lesser extend longer and shorter Aβ forms ending after 46, 45, 43, 38, 37, 34 or 33 amino acid residues (Ling, et al., 2003; Haas, 2004; Czirr, 2008). Under normal condition, Aβ is released from neuronal membrane into the extracellular fluid. Aβ is usually removed from our brains by clearance mechanisms (fig. 2.7a). The clearance of excessive Aβ is either through uptake mechanisms involving LDL-receptor-related protein (LRP) endocytosis complex with alpha-2-macroglobulin (A2M), apolipoprotein E (APOE) or by direct proteolytic degradation by Aβ degrading enzyme, including insulin-degrading enzyme (IDE), neprilysin (NEP), endothelin converting enzyme (ACE), cathepsin D, gelatinous A, matrix metalloendopeptidase-9 (MMP-9), coagulation factor Xia and α2-macroglobulin complexes. However, when the concentration of Aβ is increased by overproduction or defective clearance, Aβ self-aggregates assemblies ranging from oligomers to protofibrils, fibrils and amyloid plaques (fig 2.7b). In addition, the decrease or

deficient of clearance of Aβ in the brain and cerebrospinal fluid (CSF) is the main cause of Aβ accumulation in the aporadic (late-onset) AD (Ling, et al., 2003; Huang and Jiang, 2009; Mucke, 2009).

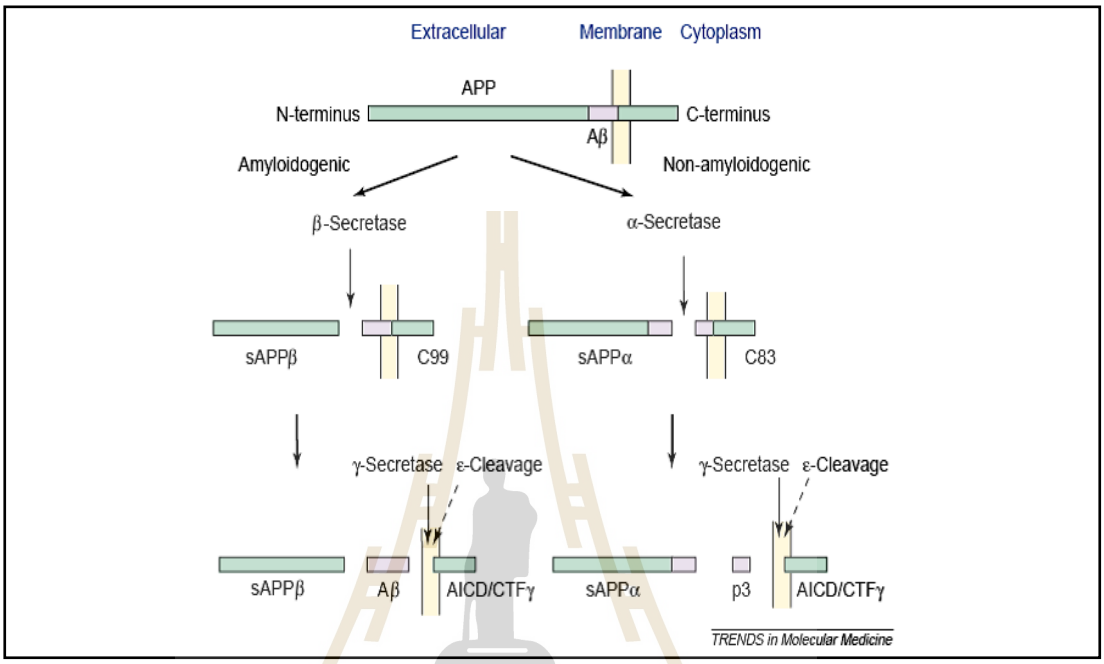

Figure 2.6 The structure and process of APP. APP is a type 1 transmembrane protein consisting of a long N-terminal extracellular segment (ectodomain), a transmembran domain and a shorter intracellular C-terminal portion (the cytoplasmic domain). Aβ (purple box) constitutes part of the transmembrane domain and an adjacent short fragment of the extracellular domain. β-secretase cleaves APP at the N-terminus of Aβ to produce sAPPβ and a 99 amino acid C-terminal fragment (C99). C99 is further cleavage by γ-secretase within the transmembrane domain to produce Aβ and the smaller AICD (CTFγ). Alternatively, APP is processed by α-secretase to produced sAPPα and a 83 amino acid C-terminal fragment (C83), which in turn is cleaved by γ-secretase to produce the P3 peptide and AICD (Vardy, et al., 2005).

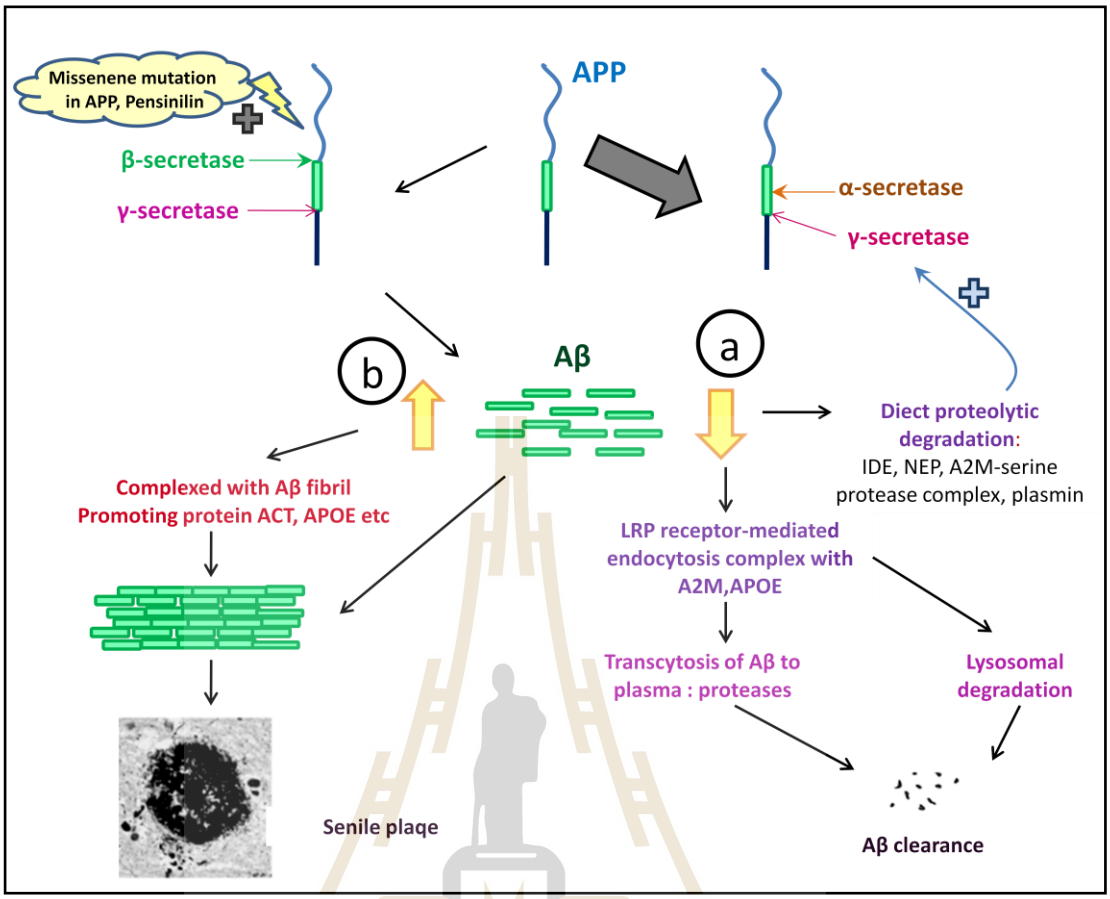

Figure 2.7 Overview of possible pathways for biogenesis, clearance and fibrillisation of Aβ. Aβ is derived from APP protein through cleavage of β- and γ-secretase. Under normal condition, Aβ is usually removed from the brain by clearance mechanisms (a). However, when its concentration is increased by overproduction or defective clearances, Aβ self-aggregates from oligomers to protofibrils, fibrils and amyloid plagues (b). This figure was modified from Ling et al. 2003. *The International Journal of Biochemistry and Cell Biology* 35; 1505–1535.

Huang and Jiang (2009) hypothesized the etiology of AD, including: 1) the gene mutation hypothesis, *Amyloid-β protein precursor* (APP), *presenilin 1/2* (*PS1/2*) or/and *apolipoprotein E* gene mutations/polymorphisms induce overexpression or deficient removal of Aβ, and the accumulated Aβ induces neuronal toxicity; 2) the oxidative stress hypothesis of Aβ-metal ion complex, Aβ and copper/zinc ions form an Aβ-metal ion complex which produces reactive oxygen species (ROS), such as O_2^-, H_2O_2 and OH. ROS affect neuronal lipid, protein and DNA oxidative degradation; 3) the coupling signal transduction hypothesis of Aβ receptors, Aβ binds to receptors on the neuronal membrane, and receptors interact with downstream coupling proteins which regulate neuronal apoptosis; and 4) the hyperphosphorylated tau protein hypothesis, The loss of neuronal Ca^{2+} homeostasis induces tau's hyperphosphorylation, the hyperphosphorylated tau loses the capability to bind microtubules and subsequently induces neuronal apoptosis.

2.6.2 Molecular genetics of Alzheimer's disease

Alzheimer's disease has been divided into two types: early-onset familial form of AD (FAD) and late-onset which 25 to 40% of patients are related to genetic factors. The mutation can be inherited in an autosomal dominant manner, which causes FAD that usually emerges at younger ages (beginning before the age of 65 years) than idiopathic AD (Blennow et al., 2006; Agca et al., 2008). The central hypothesis for the cause of AD is the amyloid cascade hypothesis which is an imbalance between the production and clearance of Aβ in the brain. Support for this hypothesis includes the finding that the mutations implicated in the familial disease. The first mutation causing the familial form of the disease was identified in *APP* gene on chromosome 21. This mutation causes an abnormal production of the amyloid

protein (Aβ) species. Moreover, patients with Down's syndrome who have an extra copy of chromosome 21 (trisomy 21), show overexpression of the normal *APP* gene. This causes AD-like pathology and symptoms during middle adult years (Beyreuther et al., 1993; Czirr, 2008). The first mutation in FAD families was found in this gene of a British family. In mutation carriers, a valine was replaced by an isoleucine at codon 717 (Val717Ile). This mutation was termed "the London mutation''. Since then, additional allelic variants have been reported. Immediately upstream of the Aβ domain, double-point mutations at codons 670 and 671 were discovered in a Swedish FAD family. The Swedish mutation consists a double base pair substitution that results in lysine and methionine being replaced by aspartic acid and leucine (Lys670Asp and Met671Leu) at the N-terminus of Aβ. This mutation leads to enhanced cleavage at the β-secretase (BACE1) cleavage and increased amyloid beta (Aβ) formation. In additional, the missense mutations at APP717 such as Indiana mutiation which consist a mutation of valine at residue 717 substituted by phenylalanine (V717F) lead to an increased the Aβ42/ Aβ40 ratio and promote accumulation of Aβ42 in the brain, which result in the enhancement of amyloid fibril formation (Tamaoka et al., 1994; Gotz et al., 2004).

Another family of genes identified in FAD is the presenilin 1 (*PSEN1*) on chromosome 14 and presenilin 2 *(PSEN2*) genes on chromosome 1 account for most cases of familial disease (Blennow et al., 2006). These proteins are catalytic subunit of γ-secretase which is the enzymatic complex that cleaves amyloid beta peptide from APP. (Moehlmann, et al., 2002; Shen and Kelleher, 2007; Czirr, 2008; Tackenberg, 2009). Presenilin1 (PS1) is a 467-amino acid integral membrane protein. Some of the mutations in the gene such as: His163Arg, Ala246Glu, Leu286Val and Cys410Tyr.

Most display complete penetrance, but a common mutation, is Glu318Gly and this predisposes individuals to familial Alzheimer disease (Shen and Kellesher, 2007). Presenilin 2 (PS2) is 448-amino acid peptide, which shows very similar in structure and function to PS1. Mutations in the PS2 gene are rare, such as Thr122Pro, Asn141Lie, Met239Val and Met239Ile. Studies in transgenic PS1 and PS2 mutations animal models have revealed a clear genotype to phenotype relationship; These missense mutations cause a selective and marked increase in Aβ42 levels *in vivo* and appear to disrupt the processing of Aβ that lead to the overproduction of Aβ (Qian et al., 1998; Kukar, et al., 2005; Shen and Kellesher, 2007; Czirr, 2008; Tackenberg, 2009). In addition, since the expression of human mutant PS1 in a PS1 null background murine is sufficient to elevate Aβ42 in mouse brains (Qian et al., 1998), FAD mutant PS1 may possess a gain-of-function activity and selectively increase the γ-secretase-mediated cleavage of APP at residue 42 of the Aβ region of APP (Xia, 2000). On the other hand, there is no evidence that any of these mutations play a major role in the more common, sporadic or non-familial of late-onset Alzheimer's which affect above the age of 65 years (Wang, et al., 2008). Although, there is no evidence that autosomal dominant inheritance of specific mutated genes causes late-onset AD, genetic risk factor does appear to play a role in developing the disease. This increased risk is related to the *APOE* gene found on chromosome 19 coding an apolipoprotein E protein. This protein is a polymorphic glycoprotein that combines with fats (lipids) in the body to form molecules called lipoproteins. Lipoproteins are responsible for packaging cholesterol and other fats and carrying them through the bloodstream. ApoE plays an essential part in the binding to receptors for the uptake of chylomicrons and lipoprotein called very low-density

lipoproteins (VLDLs) (Gustaw-Rothenberg, 2008). VLDLs remove excess cholesterol from the blood and carry it to the liver for processing. Apolipoprotein E is produced at high levels in the brain by glia cells. Three alleles of *APOE* gene: ε2, ε3, and ε4 are studied extensively. The finding that increase risk is linked to inheritance of APOE ε4 allele. The Apoε4 allele is associated with a 2-3-fold increased risk of contracting the disease when one copy is present, and when there are two copies the risk is increased by as much as 12 times (the ApoE gene is co-dominant) (Pericak-Vance, et al., 2000; Gustaw-Rothenberg, 2008). However, the Apoε4 alleles do not necessary or sufficient for AD, and the inheritance of one or two Apoε4 alleles does not predict AD to lead to certainly. Non-Mendelian disease etiology suggests that an epigenetic component could be involve the late-onset AD (Wang, et al., 2008).

2.6.3 Amyloid plaques and neurofibrillary tangles

The pathological characteristics of AD (Aβ senile plaques, tau NFT and loss of neuron) are not independent events. There are two possible signal transduction pathways for the hyperphosphorylation of tau induced by overloading Aβ. The cumulative oxidative stress induced by overloading Aβ will produce reactive oxygen species (ROS), which disrupt neuronal membrane lipids, proteins, nucleic acids and mitochondrial respiration. Mitochondrial damage is associated with, and may indeed promote, degeneration and cell death (Huang and Jiang, 2009). Moreover, Aβ mediated neuronal apoptosis through ROS-mediated activation of ASK1. Apoptosis signal-regulating kinase (ASK1) is known as mitogen-activated protein kinase kinase kinase 5 (MAP3K5), that activates the JNK (c-Jun N-terminal Kinase) signaling cascade (Kadowaki et al., 2005). The study of Atzori and colleagues (2001) showed that the important kinase enzyme of tau phophorylation, JNK are colocallized

with hyperphosphorylated tau *in vivo* and may induce hyperphosphorylation of tau *in vitro*, suggested that the overproduction of Aβ or defective of clearance mechanism promote production of ROS. This subsequently activates JNK and induces the phosphorylation of tau. While tau hyperphosphorylation may be mediated by ROS, the Aβ may affect tau phosphorylation through disrupted the activation of the phosphatidylinositol 3-kinase-activated protein (PI3K)/Akt in mouse primary hipocampal neurons (Townsend et al., 2007). Inhibition of PI3K induced a form of cell death associated with the activation of GSK-3β (Kaytor and Orr, 2002). The glycogen synthase kinase 3β (GSK-3β) is a proline-directed serine/threonine kinase, is an important kinase enzyme of the hyperphophorylation of tau. In addition, receptors on the neuronal membrane may also mediate receptors-signal transduction pathways are involved in the hyperphosphorylation of tau induced by Aβ via GSK-3β. Therefore, accumulation of Aβ may induce the increase of tau phosphorylation through activation of this enzyme (Wang et al., 2006; Hooper, Killick and Lovestone, 2008; Huang and Jiang, 2009)

2.7 APP transgenic models of Alzheimer's disease

Invertebrate is common model to study the genetics and biology of the disease quickly. The most commonly used invertebrate models in the neurogenerative studies are fruit fly (*Drosophila melanogaster*) and the nematode (*Ceanorhabditis elegan*). These models express a homolog of APP. Both of these proteins are similar to APP throughout their length, but lack an Aβ sequence and thus do not produce Aβ peptide (Rosen et al., 1989; Daigle and Li., 1993; Link 1995). The overexpression of human APP constructs induced neuronal apoptosis in *Drosophila* (Rosen et al., 1989) and the

overexpression of Drodophilla APP homolog (APPL) cause fail of wings expansion, the disruption of axon transport, and the deficiency of neuropeptide-containing vesicles in the distal processes of peptidergic neurons (Torroja et al., 1999). The triple transgenic flies that express human APP, the β-site APP-cleavage enzyme BACE, and presenilins show Aβ plaque in the retina with age-dependent neurodegenerative and shorten life span (Greeve et al., 2004). Studies of APP in *C. elegan* show that the expression of human Aβ42 in the body wall muscle induces progression paralysis and shortens life span with Aβ deposition that bind the amyloid-specific dye thioflavin S (Link 1995; Fay et al., 1998) and knockout of the amyloid precursor-related gene (apl-1) causes lethality during early larva development while overexpression causes defects in brood size (Ewarld and Li, 2010). Although, these invertebrates are a power genetic model with low cost, small size, and short life span make them well suited for drug screening and determination of the cell biology involved in AD pathology. These models have a brain anatomy very different from humans, making a direct comparison difficult.

Mouse is the most widely used system for modeling human neurodegenerative diseases. The mouse is closely related to humans through the evolutionary radiation of Epitheria about 75 million years ago, which resulted in the generation of rodents and primates. The general outline of development is similar in mice and humans, and there are syntenic relationships between mouse and human genes across much of the genome (Carver and Stubbs, 1997). Moreover, mice have a short life span, short generation time, low cost, and high environmental control that is possible. Unlike, non-human primates, dogs, and polar bears, age mice do not develop plaque-like or show any sign of their endogenouse Aβ deposition (Selkoe et al. 1987). However, the

human mutant APP transgenic mice exhibited extracellular plaques and many plaque having dense compact cores which presented from about 6-9 months of age, the number increased with age and they were found in the cerebral cortex, the hippocampus and also in the corpus collosum (Howlett and Richardson, 2009).

Several transgenic mouse models of AD have been generated to study the effect of APP mutation. The transgenic APP mice have produced that express wild-type APP, APP fragments, Aβ and FAD-associated mutations. The FAD-associated mutant APP mice followed by the development of transgenic mice, which overexpressing APP containing the Indiana mutation. Dora and colleagues (1995) reported the production of transgenic mice that expressed the human APP mutation (V717F) was driven by a platelet-derived growth factor (PDGF) -β promoter. These transgenic showed human APP overexpression at least 10-fold higher than endogenous mouse APP and began to exhibit human Aβ deposition in the hippocampus, corpus callosum and cererbral cortex at 6-9 months of age. Moreover, synaptic and dendritic density were reduced in the molecular layer of the hippocampal dentate gyrus of these transgenic. In 1996, Hsaio and colleagues developed the transgenic mouse line, Tg2576 which overexpressing the APP695 Swedish transgene under the control of the hamster prion protein promoter (*PrP*). These transgenic mice showed 5-fold increase in Aβ40 and 14-fold increase in Aβ42. The plaques which stained with Congo red dye were found in hippocampus, cortex, subiculum, and cerebellum at around 9-11months of age. The AD-like pathology can be found in other mouse models such as APP23 mouse (APP751Swe) (Struchler Pierrat et al., 1997), APP/Ld mouse (APP695Lon) (Moechars et al., 1999). A further development of mutant APP mice involved the expression of transgenes containing both Swedish

and Indiana. TgCRND8 mouse was described by Chishti and colleagues (2001). These mice contained APP695Swe/Ind under the control of a hamster prion protein promoter. The plaques present at 43 days in the subiculum and then were found in the frontal cortex at 65 days, spreading to the cortex and hippocampus by 101 days, to the thalamus (111 days) and striatum and cerebral vasculature (196 days). The thiofavin S and Congo red positive Aβ amyloid deposit were found at 3 months, indicating the presence of a β-sheet conformation and plaque density continued to increase with age. Moreover, this transgenic model causes the premature death dependent on background stain, indicating the importance of genetic background on the effects of APP overexpression. Although, transgenic mice seem to be a great model which can represent AD pathology, the fundamental characteristic, including lifespan, and an age-dependent repression of broad-spectrum neuronal genes (Loerch et al., 2008), suggesting that the assumption of biochemical equivalence between human and mouse aging is inaccurate.

2.8 Nonhuman primate models of Alzheimer's disease study

Nonhuman primates are useful for study of age-related changes in the brain and AD research since they share relatively close common ancestry with humans. Therefore, they have comparable brain structures and cognitive capabilities. Age-related amyloid deposition in the brain has been studied. APP imunoreactivity is presented in both young and aged monkeys, but only old monkey shows senile plaques which resemble plaques that occur in elder human. Aβ deposition can be detected in aged monkey brain resembling the pathology observed in early stages of AD patient (Martin et al., 1991). However, Neuritic plaques were observed in the

hippocampus and neocortex in the aged rhesus monkeys, but were distinct from those in human in that the dystrophic neurites in the monkey plaques lacked paired helical filaments and tau immunoreactivity. The chimpanzee brain, on the other hand, showed only diffuse plaque-like structures in the hippocampus and neocortex. Neither chimpanzee nor rhesus monkey brains evidenced neurofibrillary tangles. (Gearing et al., 1994)

The evidence to date indicates that nonhuman primates may represent the closet natural animal model for human AD and they can be useful for testing diagnostic and therapeutic agents targeting aggregated form of Aβ. However, the high cost, relatively long life span, and limited access to these experimental animals restrict their wider use as AD model.

2.9 Nonhuman primates embryonic stem cell of AD study

Embryonic stem (ES) cells are self-renewing and represent their ability to differentiate into all derivative three germ layers: ectoderm, mesoderm and endoderm. They could be an unlimited source of cells for replacement therapy for ageing or diseased cells and tissue, or representative of diseases model. ES cells were first established in 1981, from the inner cell mass (ICM) of mouse embryo by two groups, Gail R. Martin and Martin Evans and Matthew Kaufman. There are many types of animal that embryonic stem cells were derived from such as cow, rabbit and nonhuman primates (NHPs). Human embryonic stem cells (hES cell), first isolated in 1998 (Thomson et al., 1998), have the potential to generate cells with the ability to differentiate into all tissues of the adult and appear to be immortal in culture. They also serve as a model cell for studying developmental processes of human embryos

and fetuses at both cellular and organism levels. However, ethical concerns involve the ability of these cells to contribute to chimeric offspring and germ line (pluripotency). Human ES cell lines were claim to exhibit different properties from murine ES cells such as morphology of undifferentiated colonies and the expression pattern of stem cell markers. Nonhuman primate ES cells have potential to cross the divide between mouse and human and answer pluripotency question that cannot be asked using human ES cells. These cells were established which including rhesus, marmoset, and cynomolgus monkey as well as human (Kuo et al., 2003). They share characteristics, cell culture system and ability of differentiation into cells of multiple lineages *in vitro* such as neuron and glial cells.

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CHAPTER III

SUCCESFUL OF NEURAL PROGENITOR CELL LINES DERIVATION FROM RHESUS MONKEY EMBRYONIC STEM CELLS

3.1 Abstract

The effects of culture media and growth factors are important on neural progenitor cells (NPCs) derivation. The common NPC derivation protocols are adherent monolayer culture (feeder-free method) and the suspension culture of embryoid body followed by adherent culture (EB method). The risk of contamination of other lineage cells can still be a concern. A monolayer culture with feeder-free conditions has been reported to generate NPC. Here, we investigated the influence of NPC derivation methods on the properties of NPCs derived from rhesus monkey (*Macaca mulatta*) embryonic stem cells (rhESCs). In this study, rhESCs were induced to NPC differentiation by two different conditions; first, differentiation through embryoid body (EB) structure and rosette selection and second, ESCs were cultured in a monolayer culture system. The cells from two experimental groups were exposed to the same induction factors. Cell morphology, neural and non-neural lineage markers were evaluated. We also measured the expression of leukemia inhibitory factor (LIF) receptors and nuclear receptor tailless (TLX) that may cause the raised GFAP expression. Moreover, the potential to differentiate into mature neurons was observed.

Both methods were successful in deriving NPCs with relatively homogenous population based on the expression pattern of Nestin (>97%) and Pax6 (>99%) by flow cytometry analysis. The qRT-PCR showed no significant difference of non-neural lineage and common NPC specific genes. However, the expression of GFAP in feeder-free derivation group was higher than the EB based group (>400 fold). The different NPC induction processes do not affect the expression of LIFR and the LIFR is not involved in the extremely high expression of GFAP of monkey NPCs in the feeder-free based group but the enhanced GFAP expression was consistent with the lower expression suggest their higher tendency in differentiating toward astrocyte lineage or heterogeneity of NPCs. The NPC from two different methods have the potential to differentiate into mature neurons *in vitro*.

3.2 Introduction

The prevalence of neurodegenerative disease is increasing. Cell therapies and stem cell applications are among the most promising options in biomedicine. Embryonic stem cells (ESCs) are self-renewing pluripotent cells which can differentiate into cells and tissues which could be derived from all 3 germ layers (Heins et al., 2004; Francis Pau and Wolf, 2004). This potentiality with high proliferation rate holds promise to serve as a potential source of cells for investigation of neurodevelopment disease and cell therapies to treat many neurodegeneration diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis. (Erceg et al., 2009; Dantuma et al., 2010).

Although, mouse is the most commonly used animal models, however, some fundamental characteristics are different from human. Nonhuman primate (NHP) is

one of the best models for understanding human physiology and diseases. NHP ES cells and human ES cells show most similarities and provided relevant information than mouse ES cells. NHP ES cells were established from various sources, including rhesus, marmoset, and cynomolgus monkey as well as human (Kuo et al., 2003). They share characteristics, including morphology, cell surface marker expression, cell culture system and ability of differentiation into cells of multiple lineages *in vitro* (Chen et al., 2007). Thereby, NHP ES cells can play important intermediate transitioned from rodent systems to human clinical applications, especially in the case of transplantation.

Neural stem cells can be isolated and expanded from adult brain at the subventricular zone (SVZ) and the subgranular zone (SGV) of the dentate gyrus. Unfortunately, neural stem cells are difficult to obtain in large amounts from human brain tissues since limitations are not only the number of neural stem cells available, but also the ethical concerns on using human brain tissues as the cell source. In addition, the expansion of primary neural stem cells is limited in culture and with limited plasticity after repeated passaging *in vitro* (Carpenter, et al., 1999; Roy, et al., 2000). Several groups reported many protocols to induce neural progenitor cells (NPCs) differentiation of ESC. NPCs derived from NHP ES cells have multipotency. Cells were found to differentiate into the major cell types in the central nervous system: neuron and, glial cells (Kuo et al., 2003; Calhoun et al., 2003; Tibbitts et al., 2006). Highly enriched neuronal precursor population can be utilized to produce specific cell types, such as dopaminergic neurons, motor neurons. There are two widely used methods; one of them is spontaneous differentiation, ESCs are cultured in suspension and allowed to form an aggregated mass known as an embryoid body (EB)

(Li et al., 2005;Cho et al., 2008; Zhou et al., 2008; Kim et al., 2012). EBs consist of differentiating cells representing the three embryonic germ layers (Itskovitz-Eldor et al., 2000) and create a microenvironment that promotes cell-cell interaction that plays a crucial role in the development and function of multicellular organisms. Exogenous cytokines and growth factors are used to stimulate EBs to differentiate towards a specific lineage such as retinoic acid (Carpenter et al., 2001; Schuldiner et al., 2001) and bone morphogenetic proteins (BMPs) (Kramer et al., 2000; Chadwick et al., 2003). Although the EB method is usually used for ESCs differentiation to neural lineage, the contamination of non-neural cells is common, and because the EB has a multilayer structure, the cells would be exposed to different concentrations of growth factors (Sachlos and Auguste, 2008). On the other hand, the feeder-free method results in no feeder cell preparation, and the cells could be exposed homogeneously to growth factors. However, the maintenance of rhESC without feeder cells remains a challenging issue (Erceg et al.,2008; Dhara et al., 2008). These studies investigate the effect of NPC derivation methods on the resulted NPCs with the same media components for the induction of rhesus ESCs (Rhesus macaque; *Macaca mulatta*) in order to compare characteristics, gene expression, homogeneity, and capability of further differentiation to mature neurons.

3.3 Materials and methods

3.3.1 ESCs culture

NPCs were generated from rhesus Rhesus embryonic stem cells (rhESCs) (rZH-2, estrablished at National Primate Research Center, Emory University, Atlanta, GA). They were maintained in a culture medium composed of

knockout-Dulbecco's modified Eagle's medium (KO-DMEM) supplemented with 20% Knock-out Serum Replacement (KSR; Invitrogen), 1 mM glutamine, 1% non-essential amino acids and supplemented with 4 ng/ml of human basic fibroblast growth factor (bFGF; Chemicon). Half of the ES medium was replaced every other day. ESC colonies were passaged by mechanically dissociation into small clumps and plated on a freshly prepared mitomycin C inactivated mouse embryonic fibroblast (MEFs).

3.3.2 Adhesive culture with feeder-free and NPCs derivation

rhESCs were mechanically passaged and grown without feeder on 35 mm tissue culture plate coated with 20 µg/ml Poly-l-ornithine/1 µg/ml Laminin (P/L)with MEF conditioned medium. MEF conditioned medium was ES medium used for the culture MEF cells for 24 hrs. After four to five manual passages in the feeder-free culture, ESCs were trypsinized by 0.25% trypsin-EDTA and then plated on P/L-coated dishes. Cells were then cultured in rhNPC induction medium with DMEM/F12 supplemented with N2 supplement (Invitrogen), 4 ng/ml bFGF, and 2 mM L-glutamine for two weeks. Fresh medium was replaced every day. At day 14, cells were dissociated by accutase (Life Technology) for 5 minutes at 37 °C followed by culturing in neural proliferation medium that compose of Neurobasal A medium (Invitrogen Inc) supplemented with 20 ng/ml bFGF, 10 ng/ml LIF (Sigma) and B27 supplement (Invitrogen Inc). Fresh medium was replaced every other day, and NPCs were passaged at 80-90% confluency.

3.3.3 EB formation and NPCs derivation

rhESC colonies were mechanically dissected into small clumps and removed from MEF feeder. The rhESCs clumps were then cultured in suspension in

35mm petri dish for six to seven days to form EB in ES culture medium without bFGF. Fresh medium was replaced every other day. EBs were then transferred into 35-mm culture dishes coated with P/L (10-20 EBs per dish) and cultured in a pre-induction medium composed of DMEM/F12 media supplemented with 0.5x N2 supplement and 2mM L-glutamin for three days. At day four of the differentiation, the cells were induced and differentiated into rhNPC by using the same induction medium as described for adherent feeder-free culture. After six to seven days, rosette-like structures were manually picked up, transferred onto a P/L-coated dish and cut into small pieces for culture. For expansion, the rosette-like structures were incubated in accutase and then cultured in a neural proliferation medium.

3.3.4 Differentiation of rhesus neural progenitor cells to mature neurons

To determine the neuronal differentiation potential of rhNPCs derived by the two different methods, NPCs (~$3x10^4$ cells) were plated onto P/L coated 35-mm dishes for neural differentiation. NPCs were first cultured in neural differentiation medium compose of DMEM/F12 supplemented with N2 supplement (1:100; Invitrogen) and 1X B27 supplement (1:50; Invitrogen) for four days. On day five, 200 ng/ml SHH (R&D Systems) and 100 ng/ml FGF8 (R&D Systems) were added. At day eight, 160 µM ascorbic acid was supplemented into the medium until day 14. Fresh medium was replaced every other day.

3.3.5 Immunofluorescent staining

The cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 15 min. The fixed cells were washed for five minutes, three times in PBS and incubated with blocking buffer consisting of 0.2% triton-x 100 (for intracytoplasmic markers) (Sigma), 3 mM sodium azide (sigma), 0.1% saponin

(Sigma), 2% BSA (Sigma), and 5% house serum (Hyclone) in PBS (-) for 30 min. In order to detect NPC, the cells were incubated in primary antibody with PBS for eight hrs. The NPC markers were Nestin (Chemicon), Musashi, and SOX-2. After being washed six times for five minutes each with PBS, the cells were incubated with fluorescent-tagged secondary antibodies in PBS for two hrs. They were later washed five times for five minutes each and the cells were co-stained with DAPI. After staining, the specimens were covered with PBS before examination with a fluorescence microscope according to standard protocols.

3.3.6 Real-time Polymerase Chain Reaction

Total RNA was extracted from early passages (P2 and P3) of rhNPCs cell lined using TRIzol reagent (Ambion) and subsequently treated with Turbo DNase (Ambion). RNA concentration was measured with the Nanodrop. Three micrograms of RNA were reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the protocol of the manufacturer. The expression level of 14 genes containing 6 NPC specific genes (*Sox2*, *Pax6*, *Nestin*, *Musashi*, *TLX*, and *NPDC1*), Endoderm specific genes (*AFP*, *TTR*, and *HNF1B*), Mesoderm specific genes (*GATA4*, *VEGFA*, and *RUNX1*), astrocyte markers; *GFAP* and *S100β* were evaluated by quantitative RT-PCR using Taqman Gene Expression Master Mix (Applied Biosystems). The primers are commercially available rhesus Taqman assay from ABI. The relative levels of gene expression of the target RNA were normalized against *GAPDH* expression. The plotted data represents the mean values of at least three independent cell lines ± SEM and the statistical significance was evaluated using one-way ANOVA with GLM procedure by SAS.

3.3.7 Flow cytometry

The NPCs were dissociated into single cells using 0.25% trypsin and washed with PBS without calcium ion (Ca^{2+}) and magnesium ion (Mg^{2+}). The cells were harvested ($1x10^6$ cells per sample) in a cold FACS wash buffer and then the cells were permeabilized using BD-Perm-2 for 10 minutes in the dark. The samples were stained with anti-Pax6 or anti-nestin antibodies at a dilution of 1:100 for 30 min at 4 ºC in the dark followed by submerging in the appropriate Alexafluor^{488}-conjugated secondary antibody for 30 min. The control group was prepared in parallel by staining cells with secondary antibodies. After being washed in FASC wash buffer, the cells were fixed by 1% PFA. Flow cytometric analysis was performed on BD FACS Calibur (BD Bioscience) and the data was analyzed using FlowJo analysis software.

3.4 Results

3.4.1 Differentiation of rhesus ESCs to neural progenitor

In order to compare the efficiency in deriving NPCs by feeder-free and EB derivation methods, we first look at the emergence of neuroepithelial structure or neural rosette. For feeder-free adherent culture, the first sign of neural differentiation emerged at day 8 of feeder-free culture in the presence of N2 supplement and bFGF. Neural rosette has radial arrangement of elongated columnar cells and is the signature morphology of neuroprogenitors in culture (Fig 3.1d). At day 14 in culture, these cells had developed an NPC-like bipolar morphology (Fig 3.1e), and then were cultured for one week in a neural proliferation medium. Although, the cells expanded rapidly in this condition, plating density appeared to be an important factor for the proliferation of rhESC-NPC on Poly-L-orithin/laminin condition.

For EB-based method, ES cell clumps were cultured in suspension in low-adherent petri dish to allow the formation of cell aggregates called EB (Fig 3.2b). The EBs were transferred onto P/L-coated culture dishes and cultured in induction medium. The development of compact columnar cells emerged from the EBs and formed neural rosette-like and neural tube-like structures five to seven days after induction (Fig 3.2f-g). One of the major challenges in deriving NPCs was to eliminate non-NPCs. The rosette-like structures were mechanically isolated from the whole clump of cells, especially from the flattened cells (Fig 3.2d). Rosette-like structures were cut into smaller pieces for expansion in neural proliferation medium. After induction, these cells were passage emzymatically by using Accutase every five to seven days. Moreover, the NPCs were cryopreserved after 3 passages and subsequently thawed for testing the recovery of cells. More than 30 NPC lines were derived by the two approaches. The morphology of NPCs derived by the two methods was similar (Fig 3.3). This result demonstrated that the two NPC derivation methods (feeder-free and EB methods) can derive NPC-like cells with no obvious different in morphology.

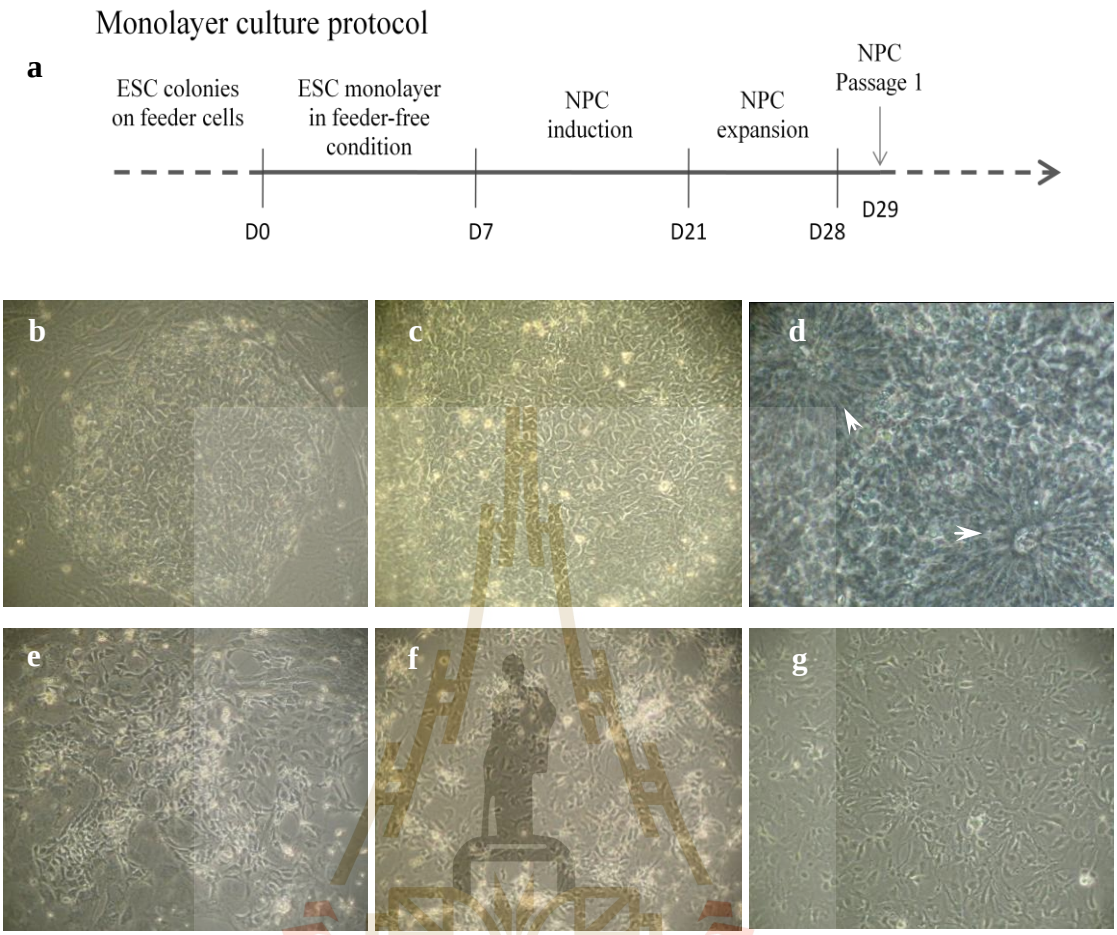


Figure 3.1 Neural progenitors induction of rhESC in adherent monolayer without feeder cells. Schematic diagram for monolayer culture induction methods (a). rhESC colony (b) were cultured on MEF feeder layer before transfer to feeder free condition (c) after four passages without feeder, the pluripotent cells were cultured in neural induction medium. Neural rosette-like structure (indicated with white arrows) formed at day 8 of differentiation (Original magnification, x400) (d) Only NPC survived in induction medium until 14 days (e) For expansion, the cells were cultured in neural proliferation medium one week before passage (f) rhESC-derived NPC at P1 (g). Original magnification, x100 under phase contrast microscope.

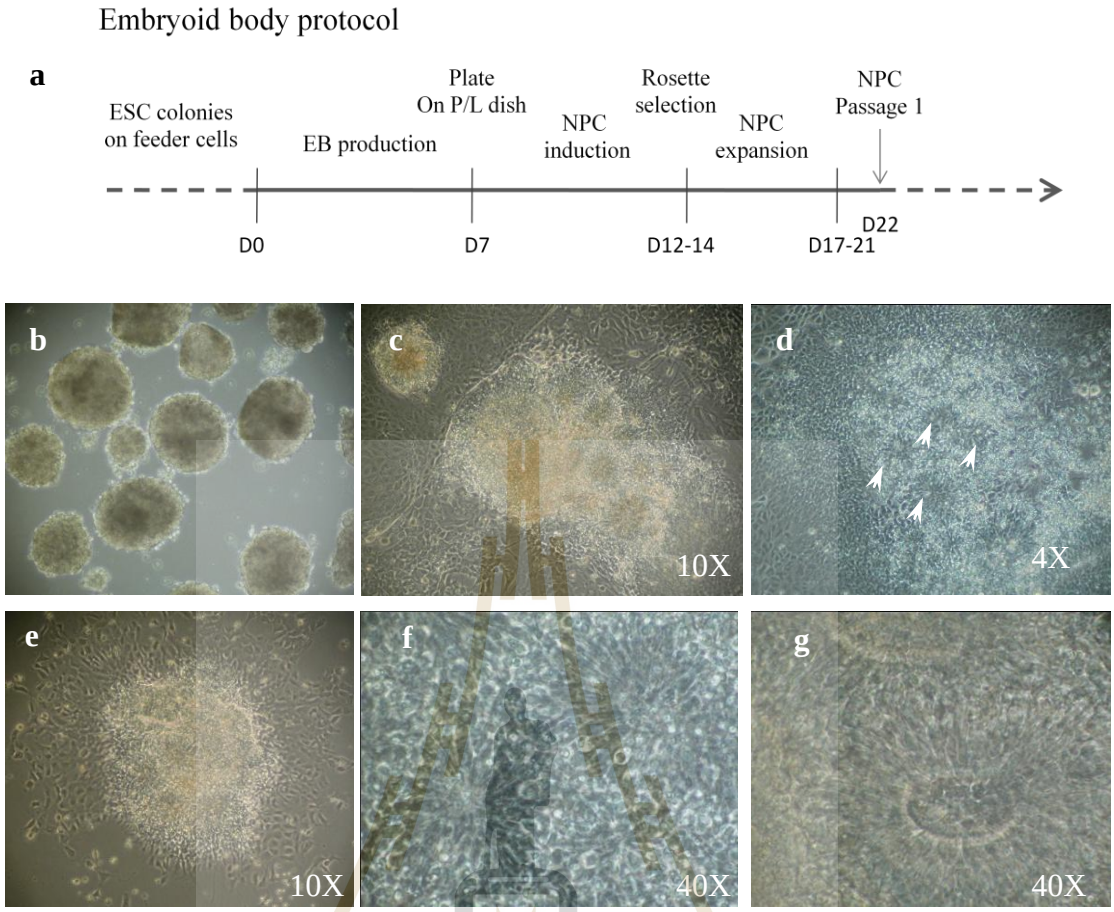


Figure 3.2 The morphological observation of the cells during differentiation of rhESC to NPCs in EB-based culture. Schematic diagram for embryoid body induction protocol (a). rhESCs were transferred and cultured as floating cell aggregates in non-adherent culture dish. Spherical cystic EBs formed five to seven days later (Original magnification, x100)(b). After the pre-induction, adherent clump cells were cultured in induction medium and showed small neural rosette-like structures (Original magnification, x100) (c). The rosette-like and neural tube-like structures (inside the selected area) were isolated from unwanted cells (Original magnification, x100) (d) After selection, clump of cells were cut and transferred to new culture dish. They still showed rosette structures (Original magnification, x100)

(e). Neural rosette-like structure (Original magnification, x400) (f) and neural tube-like structure (Original magnification, x400) (g) emerged at day seven differentiation. Under a phase contrast microscope.

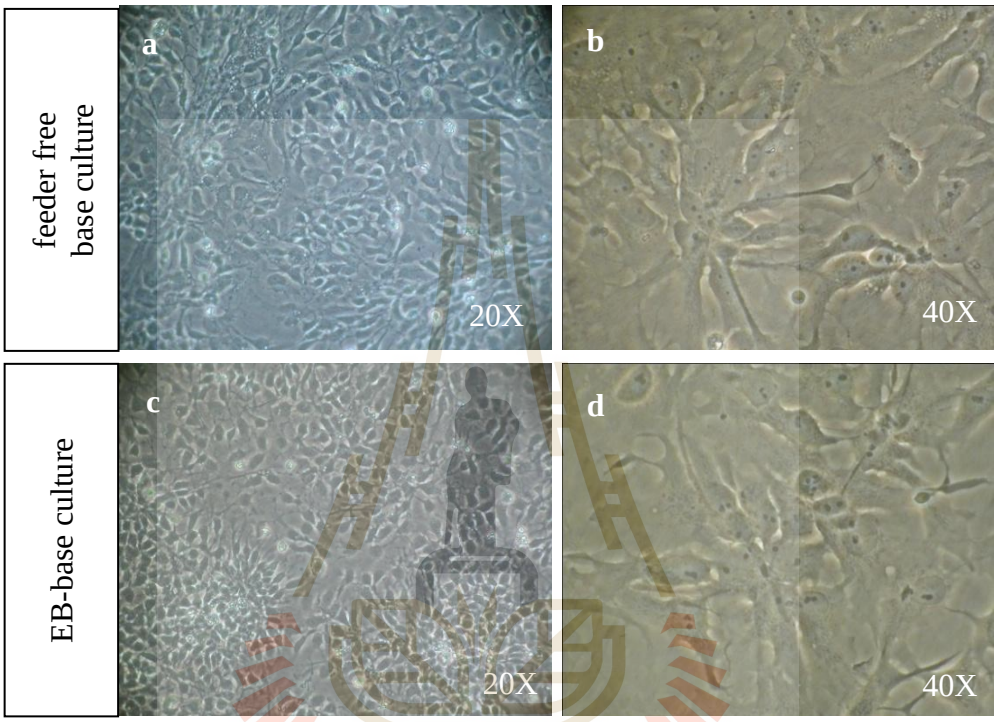


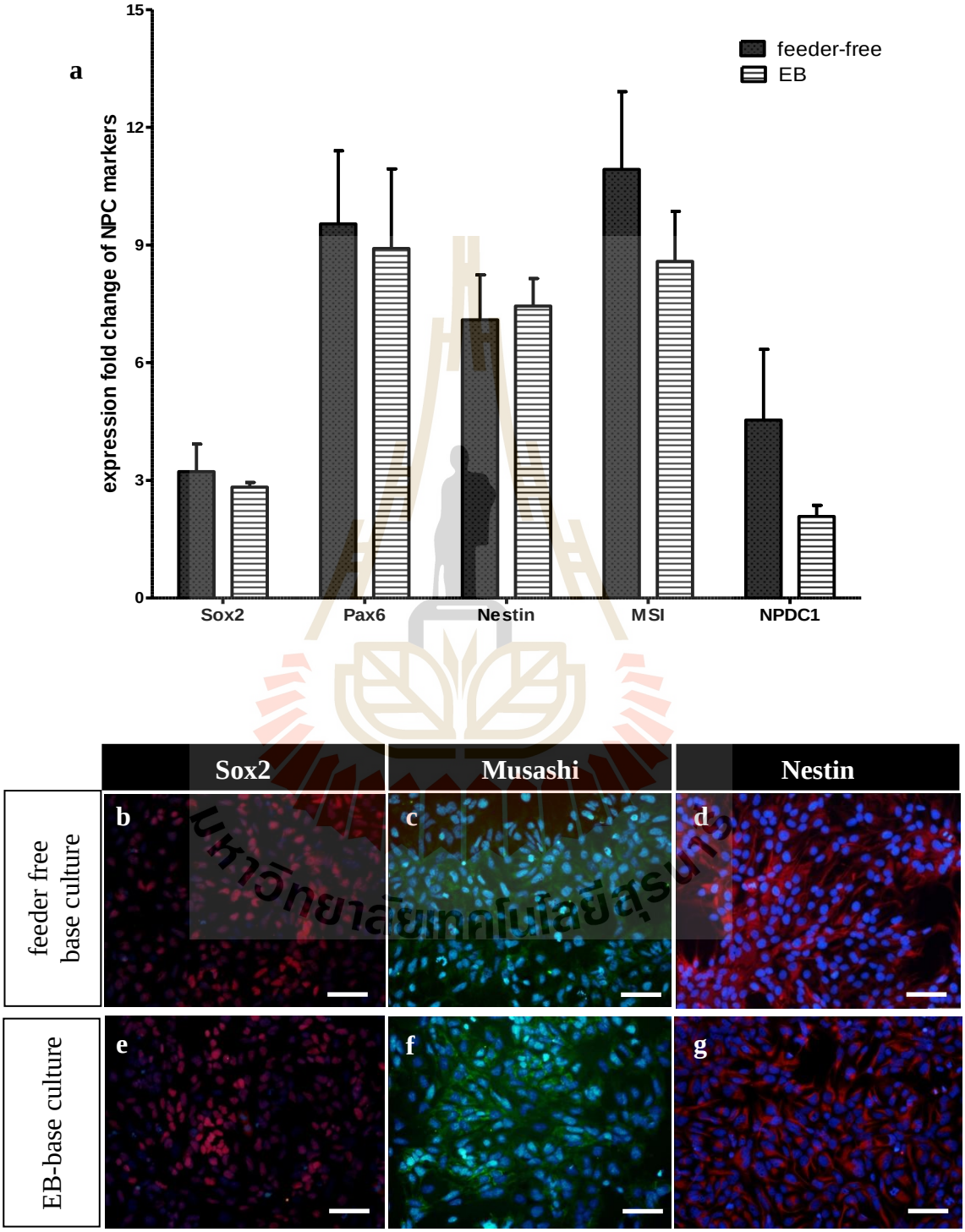
Figure 3.3 Comparison of cell morphology of rhESC-derived NPC from feeder-free culture (a,b) and suspension culture (c,d)

3.4.2 Expression of NPCs specific markers of rhESC-derived NPC

The rhNPCs derived from ESC by the two different derivation methods were evaluated to confirm the expressions of NPC-related genes. An up-regulation of the NPC markers was revealed by real-time PCR after derivation from ESC. Similar levels of NPC specific genes including Sox2, Pax6, Nestin and Musashi1 were expressed in rhNPCs from different derivation methods. An up-regulation was shown

in both methods with *Sox2* 3.22±0.70 and 2.83±0.12 fold and *Nestin* 7.09±1.15 and 7.44±0.70 fold compared with the ES cells (feeder free and EB-based groups respectively). Both the feeder free-based group and the EB-based group showed significantly elevated levels of factor *Pax6* at 9.54±1.86 fold and 8.91±2.02 respectively. *Musashi1*, which is the other NPC-related marker, was also not significantly elevated by 10.93±1.98 fold (feeder free-based group) and 8.58±1.28 fold (EB-based group). The up-regulation of *NPDC1* was higher in the feeder free-based group (4.53±1.56 fold) than the other group (2.08±0.29 fold), which was shown by real time PCR. However, they are not significant different. NPDC1 (Neural Proliferation Differentiation and Control-1) is specifically expressed in the nervous system and neural cells in culture when they stop dividing and start to differentiate (Evrard, at al., 2004) (Fig.3.4a). Immunofluorescent staining confirmed the NPC markers. Cells from both derivation methods were positive for specific NPC markers in the results (Fig.3.4b-g). Based on these results, it is suggested that two different derivation methods generated the cells that express common NPC specific genes in similar levels.

Flow cytometry analysis of single cell suspension rhESC-derived NPC revealed 97.0% and 99.4% of the cells were nestin positive (Fig. 3.4i-j), and 99.1% and 99.5% of the cells were Pax6 (Fig. 4g-h) from feeder free and EB-based conditions respectively, indicating that both cultures have a high yield of NPCs.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Musashi
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feeder free base culture
EB-base culture
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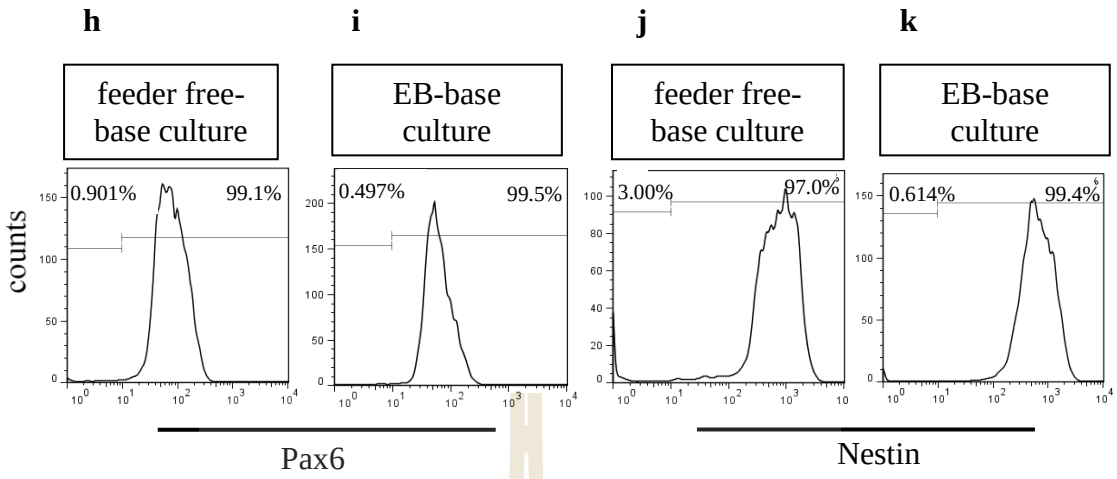


Figure 3.4 Characterizations of rhESC-derived NPC. Neural progenitor cell markers were measured by real-time PCR (a). Presented bar are the mean of fold change relative to rhesus ESC genes expression ± SEM (samples run in duplicate, n=3, *P* <0.05). NPC from feeder-free (b-d) and EB (e-g) culture expressed NPC specific markers; Sox2 (b,e), Musashi (c,f), and Nestin (d,g). Scale bars 50 µm. Flow cytometry analysis of rhESC-derived NPC from feeder-free (h,j) and EB (i,k) culture. The histogram shown 99.1% cells from feeder-free and 99.5% cells from EB-based condition were Pax-positive (h-i) and 97.0% and 99.4% from feeder-fee and EB condition respectively were Nestin-positive (j-k). The x-axis represents the intensity of marker expression and y-axis shows the number of cells at a specific intensity.

3.4.3 Expressions of non-neural lineages and astrocyte markers of rhESC-derived NPC

The prevention of other lineage cell generation during derivation process is a challenge that needs to be overcome if a homogeneous population is to be generated. To examine the contamination of non-neural lineages, the expression of mesoderm and endoderm specific markers were determined. Gene expression analysis showed that non-neural lineage genes including GATA4, VEGFA, (mesoderm lineage), AFP, TTR, and HNF1B (endoderm lineage) were down-regulation compared with expression levels of undifferentiated ESC. The only RUNX1 from feeder free-based group showed up-regulation, however, the result was not significantly different from the ESC gene expression level (Fig 3.5). These results indicated no non-neural lineage cells in the NPC population. Furthermore, the level of glial fibrillary acidic protein (GFAP) mRNA expression in NPCs from feeder-free conditions was much higher than that of the cells from the suspension condition in early passage (Fig 3.6a). Immunochemistry confirmed that some of NPCs from feeder-free based group express GFAP. The result showed that some of the cells expressed GFAP (Fig 3.6b). In the adult forebrain, GFAP-expressing cells intend immature astrocytes in unidentified development stage (Bonaguidi et al. 2005). In contrast to the case of GFAP, the alternative astrocytic marker, S100β was not statistically up-regulated compared with ESC and there were no significant differences between the two groups (Fig 3.6a). This was probably because S100β expression determines the late development stage of neural development after which GFAP-expressing cells lose their NPC potential and differentiate into mature astrocyte (Raponi et al., 2007). These findings indicate that feeder-free conditions generate early differentiation of astrocyte.

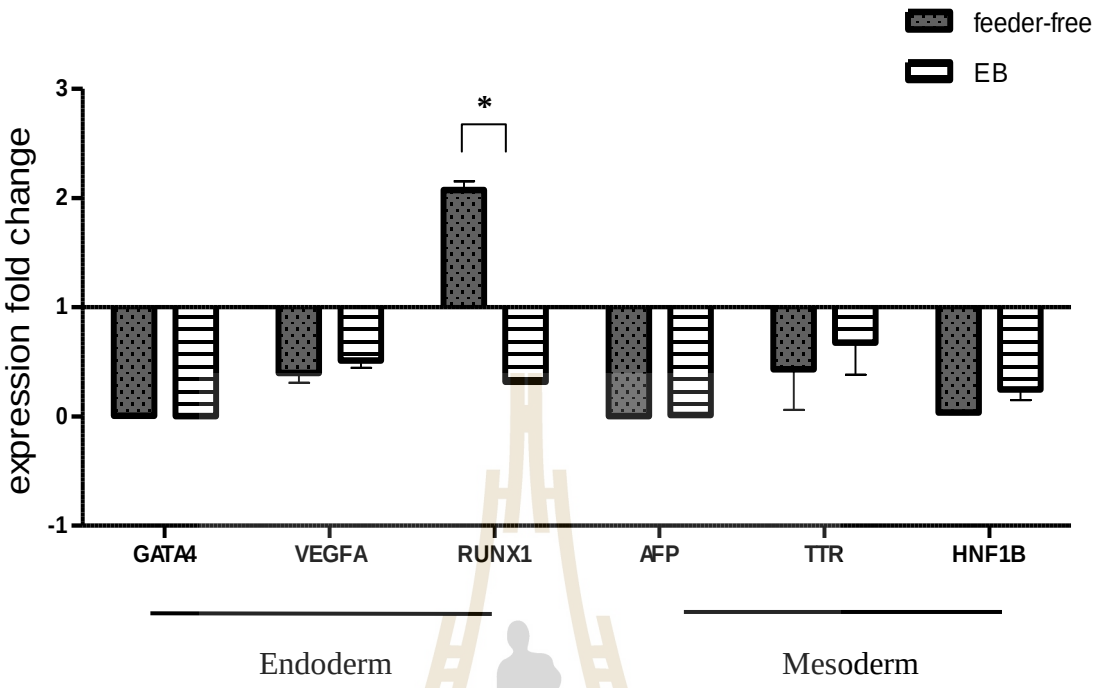


Figure 3.5 Gene expressions of non-neural lineage and neural lineage markers. Quantitative real-time PCR indicated that three independent rhesus neural progenitor cell lines from monolayer and cell suspension cultures showed similar gene expression pattern of non-neural lineage; mesoderm and endoderm. Presented bar are the mean of fold change relative to rhesus ESC genes expression ± SEM (n=3, P <0.05).

3.4.4 The expression of GFAP does not related to LIF receptor expression in rhNPC

In fact, growth factors and cytokines are crucial in maintaining cell division and the rate of growth and inhibition of differentiation in NPC cultures. One of the most important components in our neural proliferation medium is the leukemia inhibitory factor (LIF). LIF is known for its ability to maintain mouse ES cells isolated

from the inner cell mass of embryo in an undifferentiation state. Moreover, this cytokine can prevent the differentiation of NPCs and maintain multipotency for a considerably longer period and the component of B27 supplement provided factors in the long term culture (Carpenter et al., 1999, Wright et al., 2003; Babu et al., 2007). In murine, the activation of LIF receptors has been reported to promote astrocyte differentiation of NPCs by activating the transcription factor signal transducer and activator of transcription 3 (STAT3) (Pitman et al., 2004, Fan et al., 2005). We hypothesize that feeder free-based protocol may induce LIFR transcription in NPC cause the raised GFAP expression. The expressions of LIFR of the NPCs were evaluated. The results from the real-time PCR showed a reduction of LIFR after NPC derivation compared with undifferentiated ESC (Fig 3.6e), indicating that LIFR on rhesus NPCs possibly does not have as much effect to the expression of GFAP as murine. Moreover, the levels of LIFR were not different between the two groups (Fig 3.6e), indicating that the different NPC induction processes do not affect the expression of LIFR and the LIFR is not involved in the difference of GFAP expression between NPCs from the feeder free-based group and EB-based group.

3.4.5 The expression of GFAP related to TLX expression in rhNPC

Several studies reported that the nuclear receptor tailless (TLX) plays an important role in vertebrate brain functions. It belongs to the nuclear receptor; NE2E orphan NR family. TLX and is expressed in the neuroepithelium of developing central nervous system. TLX has been reported to act as repressor of cell cycle inhibitors to maintain the undifferentiated state, self-renewal, and proliferation of rodent adult-derived progenitor cells. In addition, TLX can repress the expression of GFAP, astrocyte differentiation and activate neuronal lineage commitment (Shi et al.,

2003; Elmi et al., 2010; Li et al., 2012). We further examined whether the expression of TLX related to the raised GFAP expression of NPCs. As shown in Figure 3.6d, the expression of TLX in the feeder free-based group was substantially lower than that of the EB-based group, which was consistent with the higher GFAP expression of the feeder free-based group. This implies that the different derivation methods lead to different intracellular conditions which decrease the expression of TLX and affect the GFAP expression.

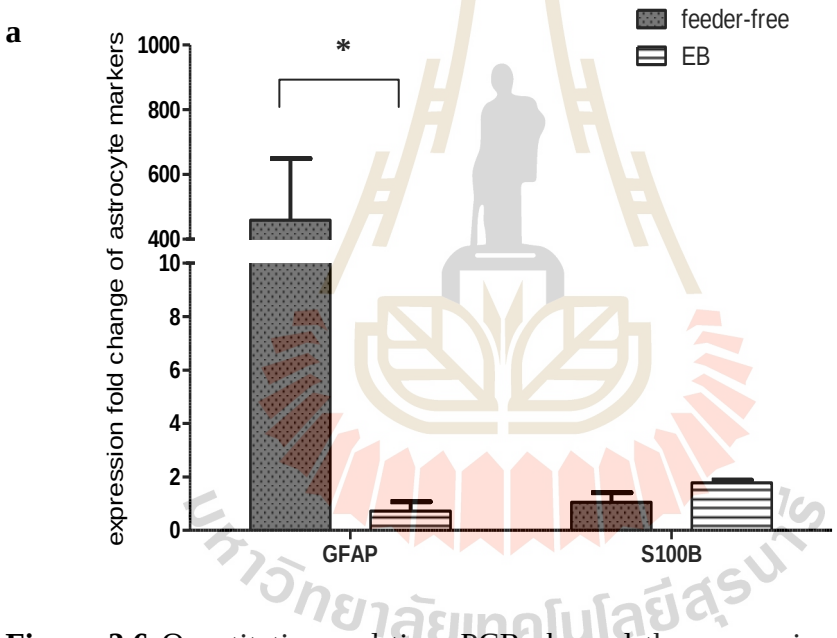


Figure 3.6 Quantitative real-time PCR showed the expression of astrocytic genes; GFAP and S100β at the early passages (a) and the genes that might involve the GFAP expression of NPCs including TLX (d) and LIF receptor (e) were evaluated. Presented bar are the mean of fold change relative to rhesus ESC genes expression ± SEM (n=3, P <0.05). The expression of GFAP of feeder-free group (b) and EB-based group (c) were confirmed by immunocytochemistry.

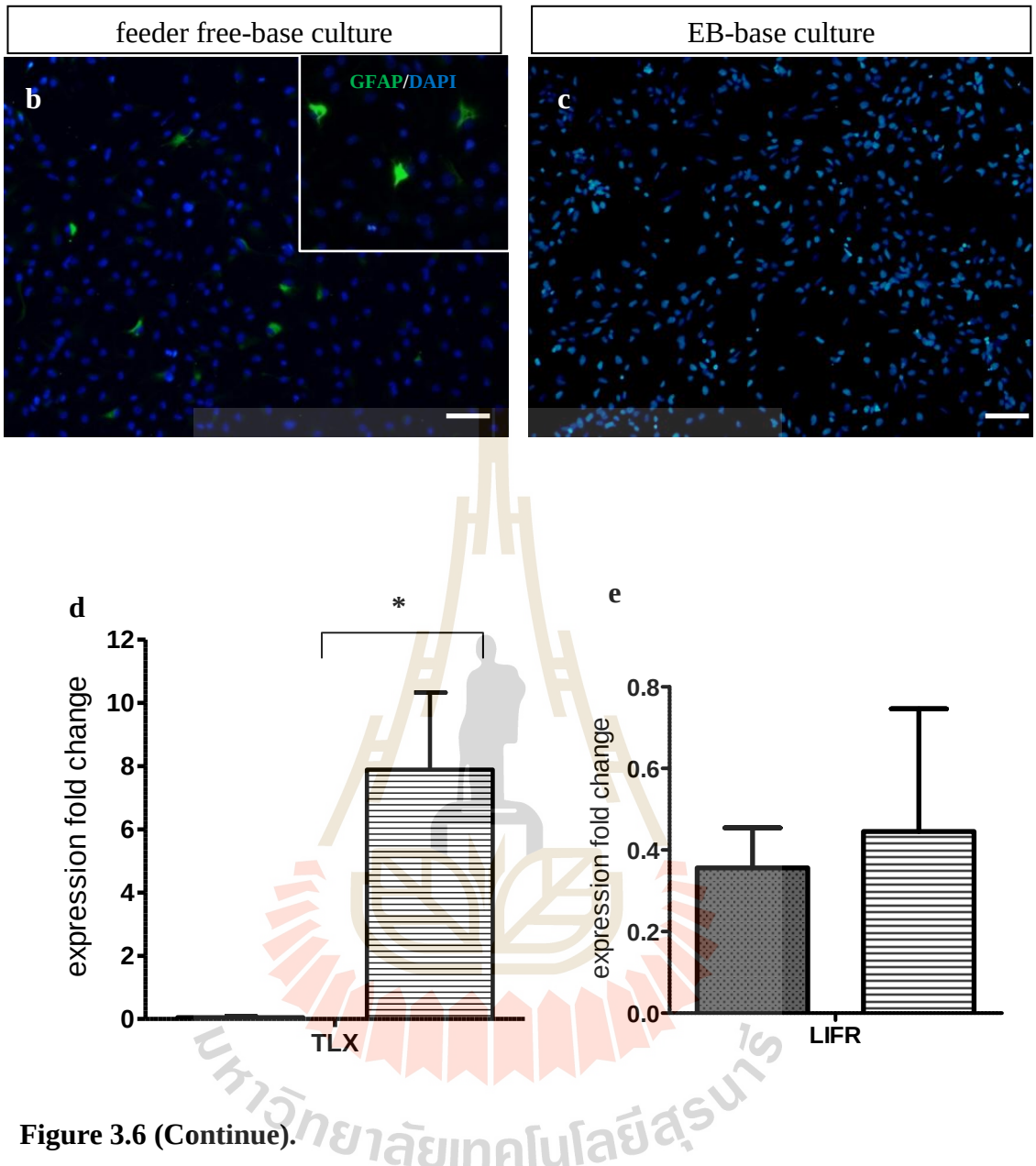
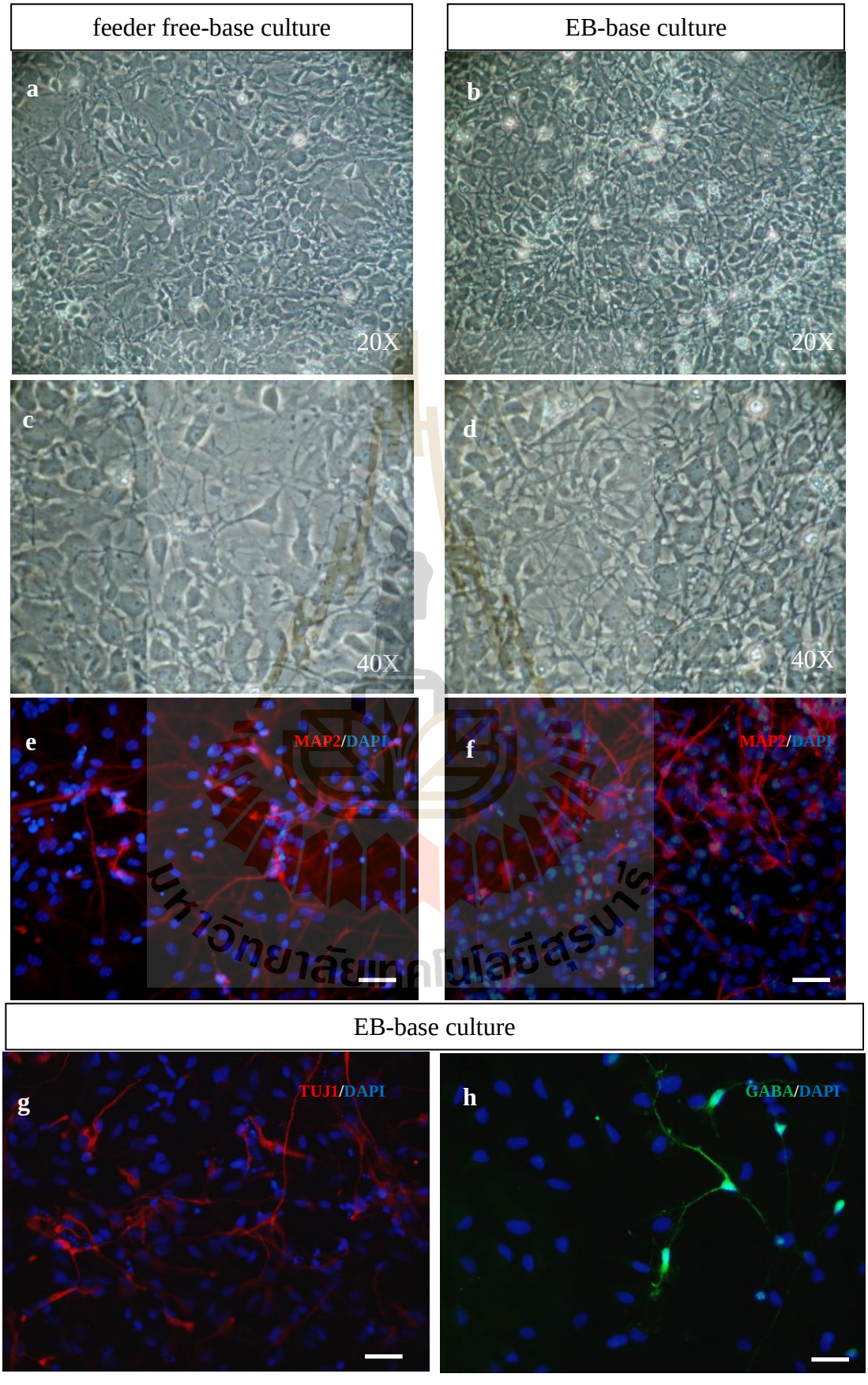


Figure 3.6 (Continue).

3.4.6 Differentiation of rhESC-derived NPC into neuron

In the next step, we examined whether NPCs have the ability to differentiate mature neurons. NPCs were plated ~3x10^4 cells into P/L coated 35-mm dishes for differentiation cultures. Neuronal differentiation was triggered by FGF2 and LIF withdrawal from the proliferation medium and added with N2, SHH, FGF8, and Ascorbic acid. By the end of second week of neuronal differentiation, differentiated

cells from EB-based group developed highly branched morphology and elongated spindle processes (Fig 3.7b,d). Immunocytochemistry staining confirmed neuronal maturation; presence of the neuron-specific microtubule associated protein MAP2 positive cells (Fig 3.7f), and Tuj1 positive (Fig 3.7g). These cells possessed the potential to become neuronal specific-subtype such as GABA (Fig 3.7h) and tyrosine hydroxylase (TH) (Fig 3.7i) positive cells. Unfortunately, the cells from the feeder free-based group showed fewer branches and spindle processes (Fig 3.7a,c) and displayed only MAP2 positive (Fig 3.7e). Moreover, the cells from two methods revealed up-regulation of neuron-specific microtubule associated protein MAP2 expressions when compared with NPCs (Fig 3.7j). These results suggested that the progenitor cells from two different methods have the potential to differentiate into mature neurons. Moreover, NPC from the EB-based group can differentiate into specific-subtype neuron.


feeder free-base culture
EB-base culture
a
20X
b
20X
c
40X
d
40X
e
MAP2/DAPI
f
MAP2/DAPI
EB-base culture
g
TUJ1/DAPI
h
GABA/DAPI

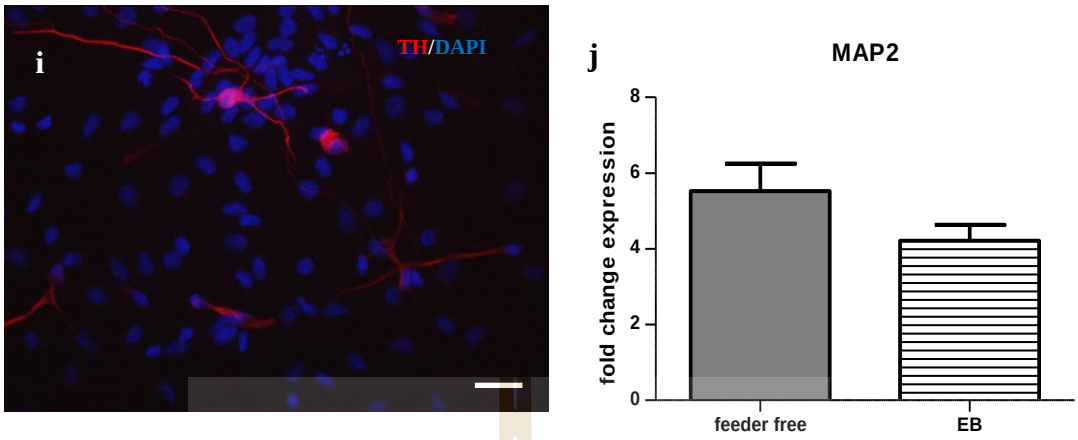


Figure 3.7 Differentiation of rhNPCs into neurons. rhNPCs from EB-based group showed highly branched morphology and elongated spindle processes, consistent with neuronal morphology (b,d). Nevertheless, cells from feeder free-based group showed less branched and spindle processes (a,c). Immunolabeling revealed MAP2 (f), Tuj1 (g), GABA (h), and TH (i) positive cells in EB-based group and presented only MAP2 positive in feeder free-based group (i). Scale bars 50 µm. qRT-PCR revealed the expression of MAP2 of neurons from two groups (e). Presented bars are the mean of fold change relative to NPC gene expression ±SEM (n=3, $P<0.05$).

3.5 Discussion

Several methods have been proven to be able to induce NPCs. Most of the methods focus on growth factors that were added in the induction culture. However, the processes by which the cells are obtained are also important. This study demonstrated how the NPCs could be different when derived from ESCs treated with the same induction medium but with different methods. ESC differentiation towards

NPCs, which involved the formation of EB, is a conventional protocol. In another protocol, the monolayer of ECS without serum and feeder cell condition has been provided to diminish zoonotic transmission.

In this study, we minimized the use of growth factors to induce differentiation. Only bFGF and 1x N2 supplement were added for NPC induction. N2 is a commercially serum-free supplement often used with primary nerve and neural stem cell culturing. Moreover, N2 can promote neuronal differentiation of hippocampal precursor cell and induce ESCs to differentiate into neural progenitors (Li et al., 2005; Babu et al., 2007; Dhara et al., 2008; Sun et al.,2008). bFGF is the most commonly used mitogen in neural stem cell culture. It is necessary for cell expansion and it also enhances proliferation of neural stem cells. The loss of FGF or FGF receptors leads to significant reduction in stem cell proliferation (Carpenter et al., 1999; Sommer and Rao, 2002; Sun et al., 2008). However, the expansion of neural stem cells, which were isolated from adult brain in the presence of bFGF requires a high cell density to support cell proliferation (Taupin et al., 2000). bFGF is widely used in combination with EGF in neural stem cell cultures. EGF is important in maintaining stemness properties of precursor cells isolated from the adult murine dentate gyru (Taupin et al., 2000). The presence of EGF and bFGF promote the proliferation of human neural stem cells (Sun et al., 2008). Unfortunately, several studies reported that EGF receptors play a oncogenic role and uncontrolled activation of these receptors or their ligands has been correlated with various types of human cancer such as brain cancer (Libermann et al., 1984; Moscatello et al., 1995; Zhang et al., 2004). Other exogenous growth factors may associate with tumor formation including Platelet-derived growth factor (PDGF), Vascular endothelial growth factor

(VEGF), and Sonic hedgehog (SHH) (Plate et al., 1993; Takano et al., 1996; Dahmane et al., 2001).

The first observable morphological results were the slightly elongation of cells along with the aggregation of the cell, into rosette structures. These rosette-like cells are the source of the neuroectoderm. They express many of the markers of neuroepithelium cells, which have been identified as characteristic of neural progenitor cells. The rosette-like cells often appear in the range of 2-4 rosettes on adherent EB after 5 days in the induction medium. However, these rosette-like cells could not be easily maintained over many passages. The conclusion could be drawn from the observation that they normally disappeared after 2-3 passages. In this study, the time-course of NPC induction from feeder-free conditions took at least 3-4 weeks for 1 cell line while the EB protocol took only 2 weeks. However, retaining the rosette-like structure was more challenging in the case of the feeder free-based culture. We found that the cell growth, cell survival, and rosette formation of NPCs in culture is highly dependent on cell density. Therefore, the maintenance of pluripotency of the starting ESC and keeping them healthy in the absence of serum without a feeder layer is the most important step. This finding is consistent with previously published reports that the efficiency of neural induction, proliferation and neuronal differentiation are dependent on cell density (Tropepe et al., 1999; Lanka and Ramasamy, 2007; Babu et al., 2007; Main et al., 2013). Because cell density of neural progenitor cells affect the production of reactive oxygen species (ROS) (Limoli et al., 2004). ROS level in the low-density culture affected the mitochondria membrane potential, mt DNA damage and the degradation of metabolic enzyme Aconitase. This enzyme prevents oxidant-induced mitochondrial dysfunction and apoptosis (Penduri et al., 2009). Furthermore,

mtDNA damage in neural stem cells can be a cause of a shifted differentiation lineage, with more potential to differentiate into astrocyte (Wang et al., 2011). Our results clearly indicated that rhESC-derived NPC from both different methods showed NPC-related genes expression. Quantitative PCR analyses revealed significant up-regulation of NPC marker genes compared with the original cells; 1) a transcription factor Sox2, which is expressed at the earliest development in the nervous system and acts as a neural stem cell marker which is a criteria of self-renewal and multipotential differentiation (Zappone et al., 2000; Ellis et al., 2004), 2) the RNA binding protein Musashi1 which regulates the translocation of target mRNA during neural development and is expressed in CNS progenitor cells (Kaneko et al., 1999), 3) Pax6, the transcription factor which is expressed in early neuroectoderm relates to the long-term generation of neurons and neurogenesis of striatal neural stem cells (Kallur et al., 2008; Zhang et al., 2010) and 4) the intermediated filament protein, Nestin is widely used as a marker of multipotent neural stem cells. Nevertheless, there was no significant difference in the presence of NPC markers, when compared between the feeder free-based and the EB-based groups, indicating the relevance to neural development of the cells from both derivation methods.

Although, the EB structure reflects the early development stage that contains three germ layer cells, the selection of the rosette-like cells can yield high purity cells. Our results showed that 99.5% of cells were Pax6-positive and 99.4% were Nestin-positive. The purity of the cells population from the feeder free-based group was also remarkably high Pax-positive at 99.1% and 97.0% were Nestin-positive. Not only that, the purity of the population was also confirmed by the analysis of the expression of several non-neural genes. qPCR data showed down-regulation of endoderm genes

which increased during the processes of EB development (alpha-Fetoprotein; AFP, transthyretin; TTR, and hepatocyte nuclear factor; HNF1β) (Abe et al., 1996). We also investigated the expression of mesoderm-associated genes including GATA binding protein 4 (GATA4), vascular endothelial growth factor A; VEGFA. These markers were significantly down regulated compared with undifferentiated ESC in the EB-based and feeder free-based groups. GATA4 is expressed in lateral mesoderm and is require for tissue specification and differentiation (Rojas et al., 2005). VEGF is essential for angiogenesis and a cardiovascular system (Haigh, 2008). Nevertheless, and runt-related transcription factor 1; RUNX1 from the feeder free-based group showed up-regulation, however, the result was not significantly different from the ES cells gene expression level. This study would seem to support that these two differentiation methods are specific for neural induction.

The culture and maintenance system are important to preserve the multipotency of cells. Long-term culture might be another factor that is involved with the potential of the differentiation. From the observations, some populations of long term, cultured (>15 passage, depending on each cell line and freeze-thaw cycle) NPCs showed a process or processes extending from the cell body, which probably is cell spontaneous differentiation. Adding some chemical and growth factors is also a crucial factor. LIF is a member of interleukin-6 (IL-6) cytokine family. These factors signal through a receptor complex for LIF receptor and gp130. In fact, LIF was found to inhibit the differentiation of neural progenitor cells and maintained multipotency for a longer period and culture (Carpenter et al., 1999; Babu et al., 2007). Additionally, LIF has been reported to have an influence on the regulation of the GFAP gene and protein expression in rodent neural stem cells through the Janus kinase/signal

transducers and activators of transcription (JAK/STAT) pathway leading to an enhanced activation of astrocyte differentiation (Wright et al., 2003; Takizawa et al., 2001; Fan et al., 2005). Our results exhibited dramatically increased GFAP genes after induction by the feeder free-based culture system but no up-regulation of alternative astrocyte markers; S100β. Studies in mice deficient in LIF shows that the number of GFAP-positive cells in the hippocampus in the mutant is much lower than in the wild type. On the contrary, S100β staining shows an equivalent number of positive cells, in comparison with wild type (Bugga et al., 1998). This result is consistent with Pitman and colleges study (2004). They reported that the activation of LIFR enhanced the expression of GFAP but did not significantly generate S100β-positive cells, indicating that the effect of LIFR activation was the induction of a GFAP expression which influences the maturation of astrocytes, rather than the generation of astrocyte. We investigated whether the LIF activation involved with the GFAP expression in the feeder free-based group. qPCR data revealed no significant difference of the LIFR expression, in comparison with the EB-based group which showed a low expression of GFAP. This indicates that LIF does not affect the GFAP expression in monkey NPC. In mice, the LIF/STAT3 pathway plays a central role in the control of self-renewal and pluripotency of the mouse's ESCs (Daheron et al., 2004). Unlike mouse ESCs, the LIF/STAT3 pathway in monkey ES cells can be activated by LIF but is does not affect the inhibition of their differentiation and maintenance of self-renewal, which is correlated with the study in human ES cells (Pera et al., 2000; Sumi et al., 2004). LIF receptor and the signaling subunit gp130 are expressed in hES cells and LIF can activate the LIF/STAT3 pathway signaling. However, this signaling is unable maintain the pluripotent state and self-renewal (Daheron et al., 2004). Together this

data indicated that monkey cells provide more relevant information than mouse cells and represents a very useful tool for studying the neurogenesis and development. Interestingly, the expression of the orphan nuclear receptor TLX, also known as NR2E1, which highly expressed in an undifferentiated adult neural stem cell and essential for self-renewal, proliferation, GFAP expression and astrocyte differentiation of neural stem cells (Shi et al., 2004, Yokoyama et al., 2008, Sun et al., 2010, Li et al., 2012). Our results showed the correlation of the TLX expression and the GFAP expression. The feeder-free based group which has a high GFAP expression showed a much lower TLX expression relative to the EB-based group. This result suggested that the low expression of TLX was because of the high expression of GFAP found in the feeder free-based NPCs might lead to the differentiation into astrocyte. Moreover, different environments, provided by different induction methods can affect the expression of intracellular biological chemistry in the cells, even though the same chemicals and growth factors inducted them.

Under neuronal differentiation conditions, NPCs from two different induction methods could differentiate into neuron. They expressed neuronal marker MAP2, however, only differentiated cells from the EB-based group showed the expression of the alternative neuronal marker TUJ1, tyrosine hydroxylase (TH), an enzyme expressed in dopaminergic neurons and GABA positive cells. This result suggested that the different environments, provided by different induction methods could affect the neuronal differentiation potential of NPCs. The comparison of the two derivation methods can conclude in the table below.

Table 3.1 Comparison of characteristic of NPC population from two derivation methods.

Title	**Feeder free based method**	**Embryoid body based method**
Time (ESC to NPC cell line)	29 days	15-17 days
Rosette-like structure	Yes	Yes
Cell morphology	Normal NPC morphology no observable difference	
NPC-related genes	Yes	Yes
Yield of NPCs	High	High
Contamination of non-neural lineage genes	No	No
differentiate into neurons	Yes	Yes
specific-subtype neurons	N/A	TH+, GABA+
GFAP expression	High	Low
LIFR expression	No significant difference	
TLX expression	Low	High

3.6 Conclusion

Currently, there are several existing methods to induce NPC from monkey ESC and they have been published for different purposes. Different based mediums, growth factors, growth factor antagonists, morphogens and supplements have been experimented to find the best solution to induce differentiation of ESCs into NPCs. There were attempts to supply nutrients and induce neural differentiating pathways to mimic the *in vivo* environment. However, the bodies of knowledge that still need to be clarified are the differentiation methods, which involve the quality of the cells. In this study, we compared the efficiency of two types of culture methods that aim to induce neural progenitor cell induction. Here we report that the population of NPCs from two simple culture systems could be generated in high purity. The qPCR results showed no up-regulation of non-neural lineage genes after induction. However, the different culture systems subsequently provided different environments caused the expression of several genes such as GFAP, TLX to change. These may have affect on self-renewal, proliferation, and differentiation potential. All of this, the use of derivation methods is dependent on the experimental purposes, the skill of researcher, and the laboratory facilities.

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CHAPTER V

OVERALL CONCLUSION

Due to similar characteristics of monkey and human cells in terms of morphology, cell biology, biochemistry, and culture system, several methods have been reported to produce the NPCs from monkey ESCs through different applications. In this study, the development and the comparison of the efficiency in deriving rhNPCs from rhESCs by two different methods was therefore examined. The research results suggested no difference between homogeneity and the expression of NPC markers in NPCs derived from the two methods. One interesting finding was that the expressions of GFAP and TLX were respectively up- and down-regulated in feeder-free derived NPCs. This phenomenon suggested these cells could possibly have higher tendency or commitment to the astrocytic lineage compared to those derived from EBs. Nonetheless, NPCs induced by the two methods were capable of differentiating into neurons and could be maintained in culture. Moreover, this study showed that the EB-based NPC derivation method was able to be applied to induce the NPC differentiation of tg AD ESCs and these progenitor cells might be helpful to cellular studies of AD pathology, mechanism, drug screening and impacts on disease cure.

CHAPTER IV

NEURONAL PROGENITOR CELLS INDUCED FROM TRANSGENIC APPs AND APPs/HUMAN TAU EMBRYONIC STEM CELLS

4.1 Abstract

Alzheimer's disease (AD) is the most common type of dementia. It is histopathologically characterized by β-amyloid-containing plaques, tau-containing neurofibrillary tangles, reduced synaptic density and neuronal loss in particular brain areas. For the familial forms of AD, the pathogenic mutations have been identified in the gene encoding region which is the precursor of the Aβ peptide, Amyloid precursor protein (APP). Moreover, the mutations can be indicated by tau which also leads to neurodegeneration and dementia. Neural progenitor cells (NPCs) are self-reproducible used as a powerful tool for investigating the neural development, neurodegenerative disease studies and suitable for pharmaceutical and toxic screening. Previous studies showed the efficient protocols to induce NPC differentiation of rhesus embryonic stem (ES) cells. The transgenic (tg) APPs ES cells with Swedish (Sw) and Indiana (Ind) APP mutation, and the transgenic APPs and human tau ES cells were induced to differentiate into NPCs by using the EB-based protocol. The results showed that the tg ES cell lines had the ability to turn into NPC cell lines and the tg APPs cloud not affect the differentiation potential into NPCs of ES cells. Cell morphology,

gene expression analysis, and cytochemistry showed no difference between tg and wild-type NPCs. However, the expression of TLX in NPCs derived from rQdES1 ES cells was low with the consistency of highly expressed GFAP and S100Bwhich are general astrocytes markers. This implied that the low expression of TLX might affect the astrocyte differentiation of NPCs in rQdES1 cells. Although, the tg APPs progenitors from two tg ES cells cell lines had the potential to differentiate into mature neuron, the differentiation ability into specific-subtype neuron was diversified. The difference between transgenic cell lines was likely due to the individual potential of each cell line. The expression analysis of tg APP mutations in the cells revealed the increase in total transcript of SwAPP and IndAPP expression as well as human tau protein in NPCs and neurons compared to ES cells which correlated with the trend of common rhesus APP expression. There was no significant difference in tg APPs between NPCs and neuron.

4.2 Introduction

Alzheimer's disease (AD) is the most common form of dementia worldwide, which is characterized by a progressive and global deterioration of mental function, most notably in cognitive performance (Blennow et al., 2006; Abdul-Hay et al., 2009). The pathological hallmarks of AD are neurofibrillary tangles and amyloid plaques (Blennow et al., 2006; Imbimbo et al., 2007; Abdul-Hay et al., 2009; Lammich et al., 2010). Amyloid plaques consist of a proteinaceous core composed of 5- to 10-nm fibrils of β-amyloid peptide (Aβ) surrounded by dystrophic neurites, astrocytic processes, and microglial cells. Amyloid-β consists of 40 (Aβ40) to 42 (Aβ42) amino acids generated by the cleavage of amyloid precursor protein (APP) (Imbimbo et al.,

2007). The mutation of APP gene is one of a cause of an abnormal production of the Aβ species. This causes AD-like pathology and symptoms during middle adult years or early-onset familial form of AD (FAD) (Beyreuther et al., 1993; Czirr, 2008). One of the mutation leads to enhanced cleavage at the β-secretase cleavage site, promoting the amyloidogenic cleavage of APP, and increased amyloid beta (Aβ) formation lead to an increased proportion of Aβ42 being produced and secreted. This Aβ domain mutation is called Swedish mutation consist a double base pair substitution that results in lysine and methionine being replaced by aspartic acid and leucine at codons 670 and 671 (Met670Asp and Met671Leu) (Gotz et al., 2004). Mutations at the γ'-site affects the γ-secretase activity result the APP C-terminal fragments are cleaved. These C-terminal mutations relative increase Aβ42 and the Aβ42/Aβ40 ratio. The examples of C-terminal APP mutations are Australian (L723P), Belgian (K724N), and Indiana (V717F) (Brouwer et al., 2008). The transgenic mouse carrying both the Swedish double mutation and the Indiana mutation show the high production of Aβ with age-dependence increasing and Enzyme-linked immunosorbent assay (ELISA) analysis reveals highly increase Aβ42/Aβ40 ratio in neocortical and hippocampula tissue. Moreover, this transgenic model develops oligomeric Aβ or senile plaque deposited in the dentate gyrus and the neocortex, and represents an early impairment in acquisition and learning reversal (Chishi et al., 2001; Lopez-Toledano and Shelanski, 2007).

Embryonic stem (ES) cells are self-renewing and represent their ability to differentiate into all derivative three germ layers: ectoderm, mesoderm and endoderm. These characteristics could be useful in drug screening, replacement therapy for ageing or diseased cells and tissue, or representative of diseases model. Although, mouse is the most commonly used animal models, some fundamental characteristics

are different from human. Monkey is one of the best models for understanding human physiology and diseases. With longer lifespans than rodent, primates develop amyloid plaque and other pathological features that are absent in wild-type or nontransgenic rodent (Wu et al., 2008). Primate ES cells were established as well as human. They share characteristics, cell culture system and ability of differentiation into cells of multiple lineages *in vitro* such as neurons (Kuo et al., 2003).

The central nervous system disease which involves neural loss, including Alzheimer's disease can be investigated by the clinical symptoms. However, cellular and molecular studies during early neurogenesis are necessary. Although, neural progenitor cells can be isolated and expand from adult brain in the subventricular zone (SVZ) and the subgranular zone (SGV) of the dentate gyrus, these cells are difficult to obtain large-scale from primary culture. These isolated cells contain a mixture of progenitor cell types with various differentiation potential, the limited expansion in culture and failing plasticity after passaging (Carpenter, et al., 1999; Roy, et al., 2000). Several studies report neural progenitor cell induction from monkey ES cell (Kuo et al., 2003; Calhoun et al., 2003; Tibbitts et al., 2006). The inductions of neuronal progenitor cells from transgenic APP monkey ES cells have not been reported. In this study, we showed effective protocol to induce neuronal progenitor cells (NPCs) differentiation from two transgenic Rhesus macaque (*Macaca mulatta*) ES cell lines. First, ES cells carried the Swedish mutation (Sw) hAPP and Indiana mutation (Ind) hAPP, and second, transgenic ES cells contained the transgenic Sw/Ind hAPP and human tau protein. These progenitor cells might be helpful for the cellular studies of AD pathology, mechanism, drug screening and impacted to cure of disease.

4.3 Materials and methods

4.3.1 Transgenic monkey ES cells culture

Pluripotent Transgenic AD monkey ES cells were obtained from Dr. Anthony Chan. They were established at the department of human genetics, Emory University School of Medicine. Briefly, mature metaphase II rhesus monkey oocytes, from adult healthy females with regular menstrual cycle were infused by lentivirus into the perivitelline space (PVS). A construction of lentiviral-based vector was pFUW which increase transcription level, RNA stability and minimize position effect. In addition, the ubiquitin (U) promoter is high expression level without tissue specificity (Chan and Yang, 2009). The human APP695 containing Swedish-Indiana mutations and tau were inserted into a lentiviral vector. The PVS injection was followed by ICSI and transgenic embryos were selected to establish an ES cell line. A polymerase chain reaction (PCR) was used to confirm the inserted genes. Common monkey ES cells; Nanog, Oct4, Sox2, SSEA4 and TRA-1-60 were used to determine the stem cell properties.

The transgenic (tg) and wildtype (wt) ES cells were maintained in culture medium composed of knockout-Dulbecco's modified Eagle's medium (KO-DMEM) supplemented with 20% Knock-out Serum Replacement (KSR; Invitrogen), 1 mM glutamine, 1% non-essential amino acids and supplemented with 4 ng/ml of human basic fibroblast growth factor (bFGF; Chemicon) Half of the ES medium will be changed every other day. ES cells colonies were passaged by mechanically dissociated into small pieces and then replace to freshly mouse embryonic fibroblasts (MEFs) layer, inactivated by mitomycin C. For expansion, undifferentiated colonies were

manually cut into multiple small pieces with a glass pipette under a phase-contrast microscope, and transferred onto a fresh MEF feeder layer.

4.3.2 Differentiation of tg monkey ES cells into NPCs

Transgenic and wt ES cell colonies were removed from MEFs feeders and dissociated into small clumps by mechanical dissection. The ES cells clumps were allowed to form floating cell aggregates (embryoid body) by culturing in suspension in low attachment dishes for 6-7 days with changed of ES culture medium without bFGF every other day. They were then transferred to 35-mm culture dishes coated with P/L (10-20 EBs per dish) and cultured in pre-induction medium consisting of DMEM/F12, 0.5x N2 supplement, and 2mM L-glutamine for 3 days. At day 4 of differentiation, the cells were induced differentiation into NPCs by the induction medium, including DMEM/F12, 1X N2 supplement, 4 ng/ml bFGF, and 2 mM L-glutamine for 2 weeks. Medium was changed every day during the induction time. After 6-7 days, rosettes-like structure appeared and manually picked with a glass pipette under a phase-contrast microscope. The rosettes-like structures were transferred to another P/L-coated dish and were cut into small pieces to multiply the number of rosette-like structures. For expansion, the rosette-like structures were incubated in Actase and then cultured in neural proliferation medium; neurobasal medium with 20 ng/ml bFGF, 10 ng/ml LIF, 1X bB27 supplement and 2 mM L-Glutamine. NPCs can be frozen in 10% DMSO and thawed when required.

4.3.3 Differentiation of NPCs into mature neuron

To determine the neuronal differentiation potential of rhNPCs from tg and wt ES cells, NPCs (~$3x10^4$ cells) were plated on P/L coated dishes. Medium was changed to differentiation medium containing DMEM/F12, N2 supplement (1:100;

Invitrogen), B27 supplement (1:50; Invitrogen). Four days later, 200 ng/ml SHH and 100 ng/ml FGF8, and after another 4 days, medium added 160 µM ascorbic acid until day 14. Medium was changed every other day and growth factors were always freshly added before using.

4.3.4 Immunofluorescent staining

The cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 15 min. The fixed cells will be washed for 5 min, 3 times in PBS and incubated with a blocking buffer consisting of 0.2% triton-x 100 (for transcription factor markers), 3 mM sodium azide, 0.1% saponin, 2% BSA, and 5% house serum in PBS (-) for 30 min. In a case of detecting NPC, the cells were incubated with primary antibody with PBS for 8 h. The NPC markers are Nestin, Musashi, PAX-6, and SOX-2. After washing 6 times 5 min each with PBS, the cells were incubated with fluorescence -tagged secondary antibody in PBS for 2 h. They were later washed 5 times for 5 min each and the cells were co-stained with DAPI. After staining, the specimens were covered with PBS before examination with a fluorescence microscope according to standard protocols. For counting of neurons, βIII tubulin (TUJ1) positive cells of each sample will be examined and images will be taken at different regions of the culture. A total of three 4-well dishes of differentiated cells will be used in this study. All images will be taken at the same magnification, and the total number of cells in each image will be counted and categorized as those with nuclear staining with neural marker. The plotted data represent the mean values of at least 3 independent cell lines ± standard error and the statistical significance was evaluated using one-way ANOVA with GLM procedure by SAS.

4.3.5 Real-time Polymerase Chain Reaction

Total RNA was extracted from early passages (P2 and P3) of rhNPCs cell lined using TRIzol reagent and subsequently treated with Turbo DNase. RNA concentration was measured with the Nanodrop. Three micrograms of RNA were reverse transcribed using High Capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol. The expression level of 5 NPC specific genes (Sox2, Pax6, Nestin, Musashi, TLX) were evaluated by quantitative RT-PCR using Taqman Gene Expression Master Mix. The primers were detailed in Table 1 with commercially available rhesus Taqman assay from ABI. Quantitation of tg hAPP mRNA expression including Swedish mutation (Sw; forward sequence – 5'AATATC AAGACGGAGGAGATCTCTGAA'3; reverse sequence – 5'CTTCATATCCTGA GTCATGTCGGAAT'3; probe sequence – 5'CTGCATCCAGATTCAC'3), and Indiana mutation (Ind; forward sequence – 5'GGTGGGCGGTGTTGTCA'3; reverse sequence – 5'TTTCTTCTTCAGCATCACCAAGGT'3), were performed using custom-designed gene-specific Tagman assays. The relative levels of gene expression of target RNA were normalized against GAPDH expression. Non-transgenic cells were used for negative control. The plotted data represent the mean values of at least 3 independent cell lines $\pm$ standard error and the statistical significance was evaluated using one-way ANOVA with GLM procedure by SAS.

4.4 Results

4.4.1 Derivation and characterization of NPCs derived from tg ES cells

Tg APPs monkey ES cells derived from transgenic embryo (ADrES-2) which overexpressing human APP containing Swedish-Indiana mutations and human tau (Sw/IndAPPs-hTau), and AD monkey embryo (rQdES-1) which carried only human APP Swedish-Indiana mutations (Sw/IndAPPs). Wild type (wt) cell lines (rZH-2 and rZH-11) were generated from nontransgenic control monkey. Stem cell lines expressed pluripotent stem cell markers, including nanog, OCT4, Sox2, SSEA4, and Tra-1-60 (fig 4.1). To derived NPC, The protocol for this study involved EB formation and neural-rosette selection with the same induction medium as the previous study. NPCs derived from tg ES cells showed the rosette-like radial arrangement of elongated columnar cells and displayed NPC morphology (fig 4.2 c,e). No observable difference of cell morphology between tg and wild-type NPCs (fig 4.2 d,f). NPCs were expanded and cryopreserved with high viability and maintain neural differentiation competency. qRT-PCR analysis revealed elevated expression of NPC markers: *Sox2*, *Pax-6*, *Musashi-1* (*MSI*), *Nestin*, and *TLX* (fig 4.3b). The expression of NPC markers was confirmed by immunocytochemistry. Transgenic NPCs stained for Sox-2, Musashi, and Nestin (fig 4.3 a). Together, the results revealed that the cells derived from tg APPs ES cell lines have the ability to differentiate into NPC cell lines and the tg APPs could not affect differentiation potential into NPCs of ES cells. Interestingly, expression of TLX in NPCs derived from rQdES1 ES cells was low which was consistent with the high GFAP and S100B expression. GFAP and S100B are general astrocytes markers. This implies that the low expression of TLX may affect astrocyte differentiation of NPCs in rQdES1 cells.

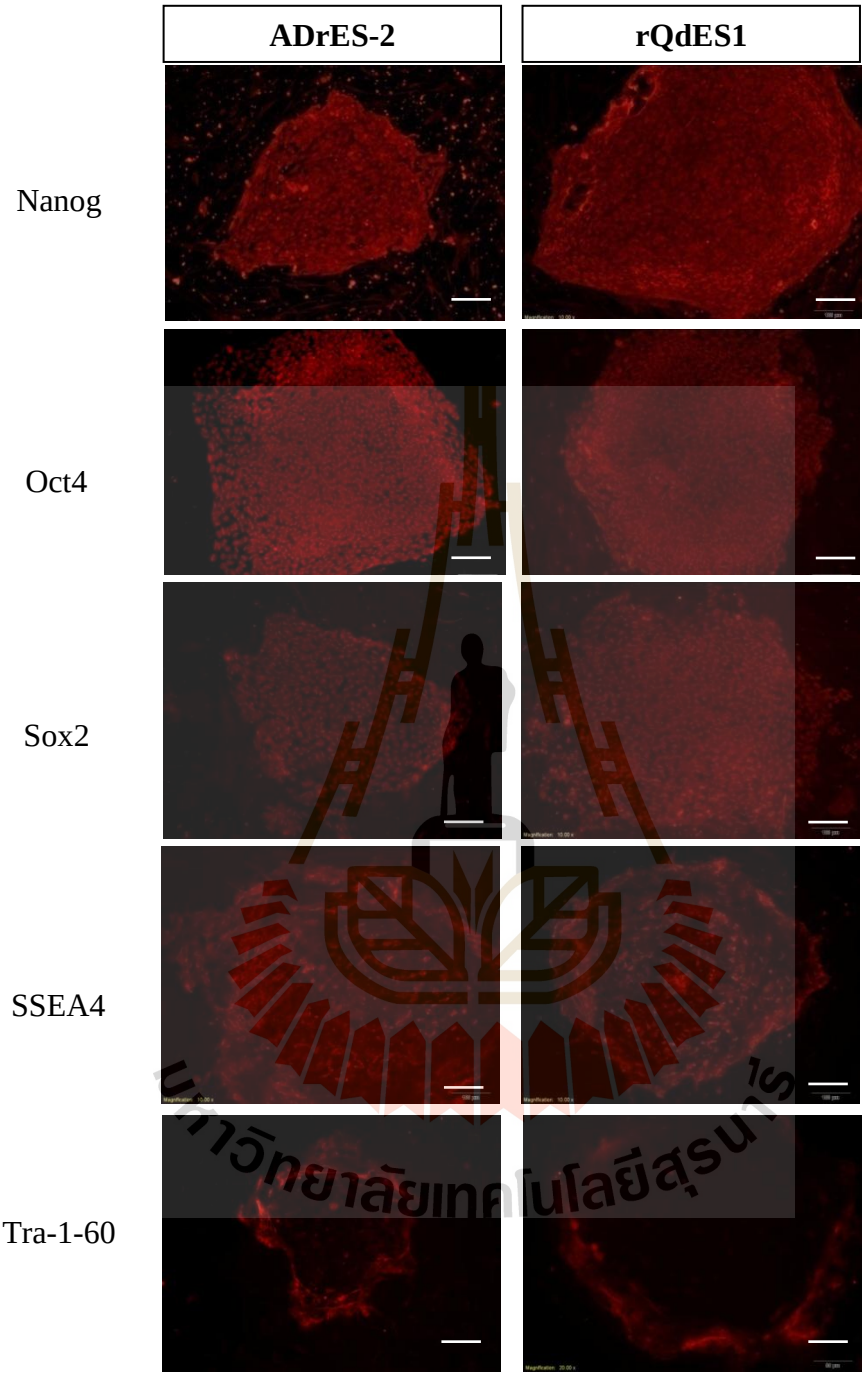

Figure 4.1 ADrES and rQdES1 colonies stained the ES cell pluripotency markers; Nanog, Oct4, Sox2, SSEA4, and Tra-1-60. Bar = 100 µm.

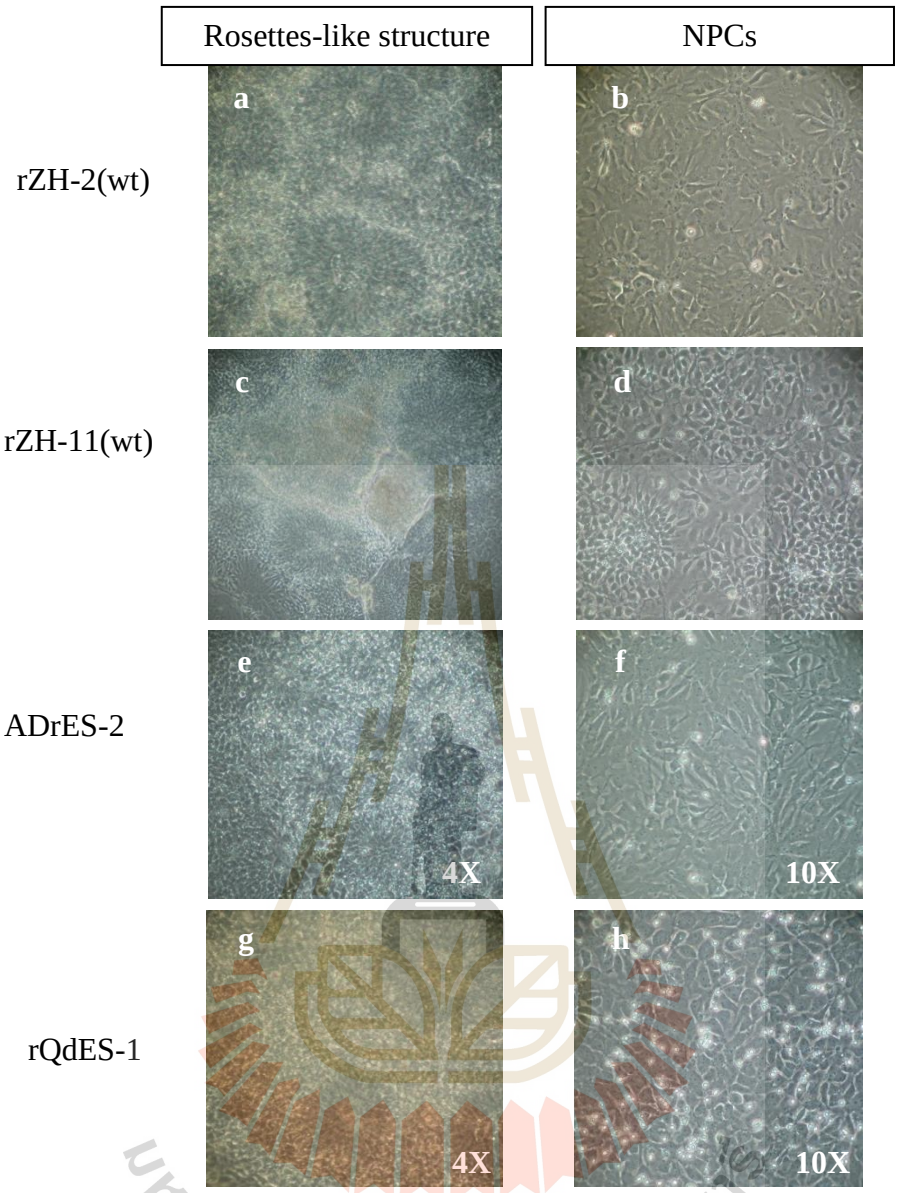

Figure 4.2 Morphology of Rosettes-like structure and neural progenitors after 1 week of induction. Transgenic APPs rhES cell were induced into rosettes-like structure (ADrES-2 (e) and rQdES-1 (g), Original magnification, x40) and NPC cells lines (ADrES-2 (f) and rQdES-1 (h)) (Original magnification, x100). They exhibited the same observable morphology as rosettes-like structure (a,c) and NPC (b,d) which induced from wt rhES (rZH-2 and rZH-11, respectively) cell lines.

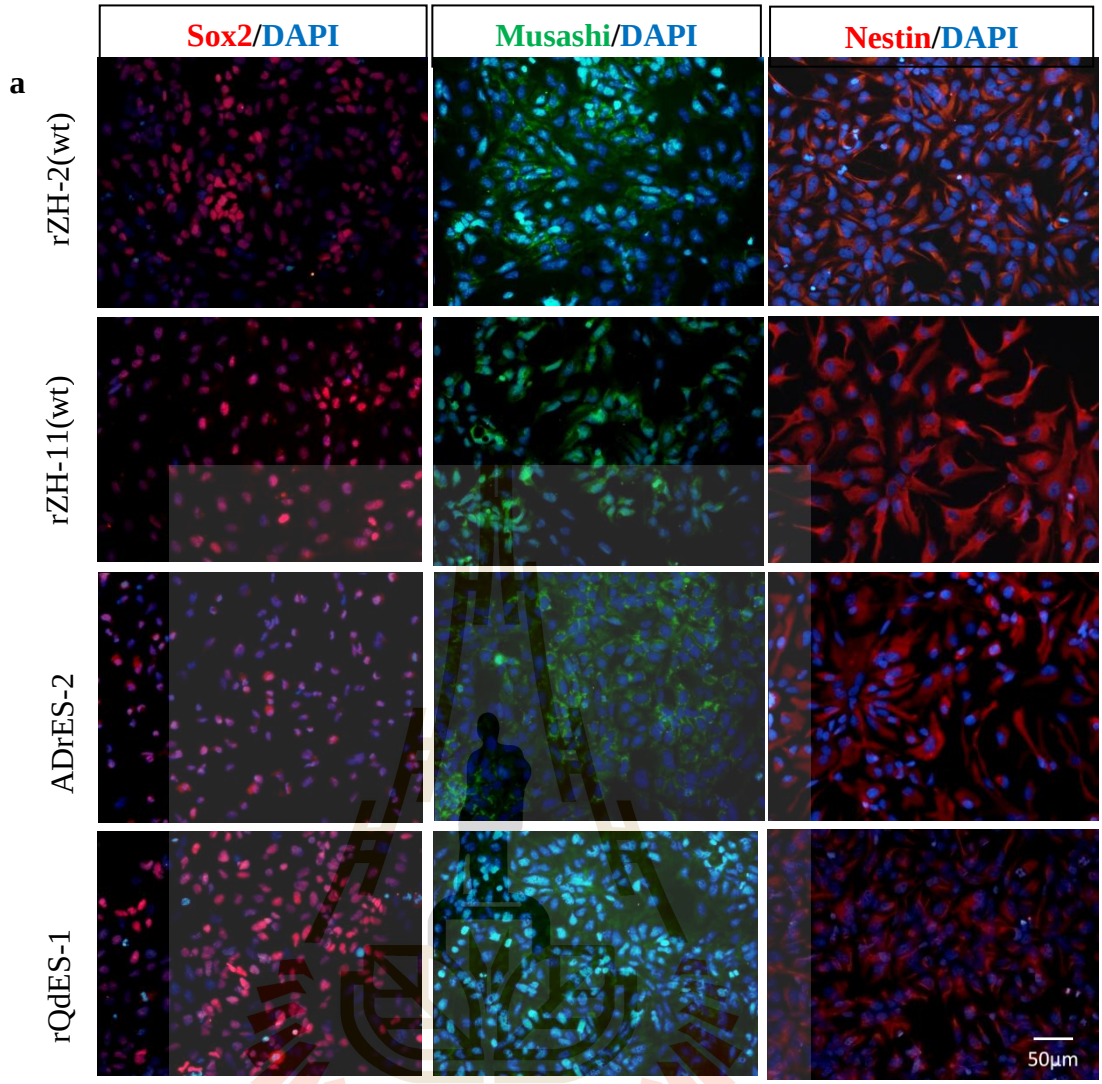

Figure 4.3 Characterization of NPCs derived from tg AD and wt monkey ES cells. NPCs are positive for neural precursor markers, including SOX2, Musashi 1 (MSI1), and Nestin, confirmed by immunocytochemistry (a) and quantitative RT-PCR (qRT-PCR). RT-qPCR shows elevated expression of NPC markers: SOX2, Nestin, MSI1, andPAX6 (b). The expression of astrocyte markers: GFAP and S100β also were evaluated (c). Expression values were normalized to GAPDH. Presented bar is the mean of fold change relative to ES cell gene expression ± SEM (samples run in duplicate, n=4, P <0.05). Scale bars 50 µm.

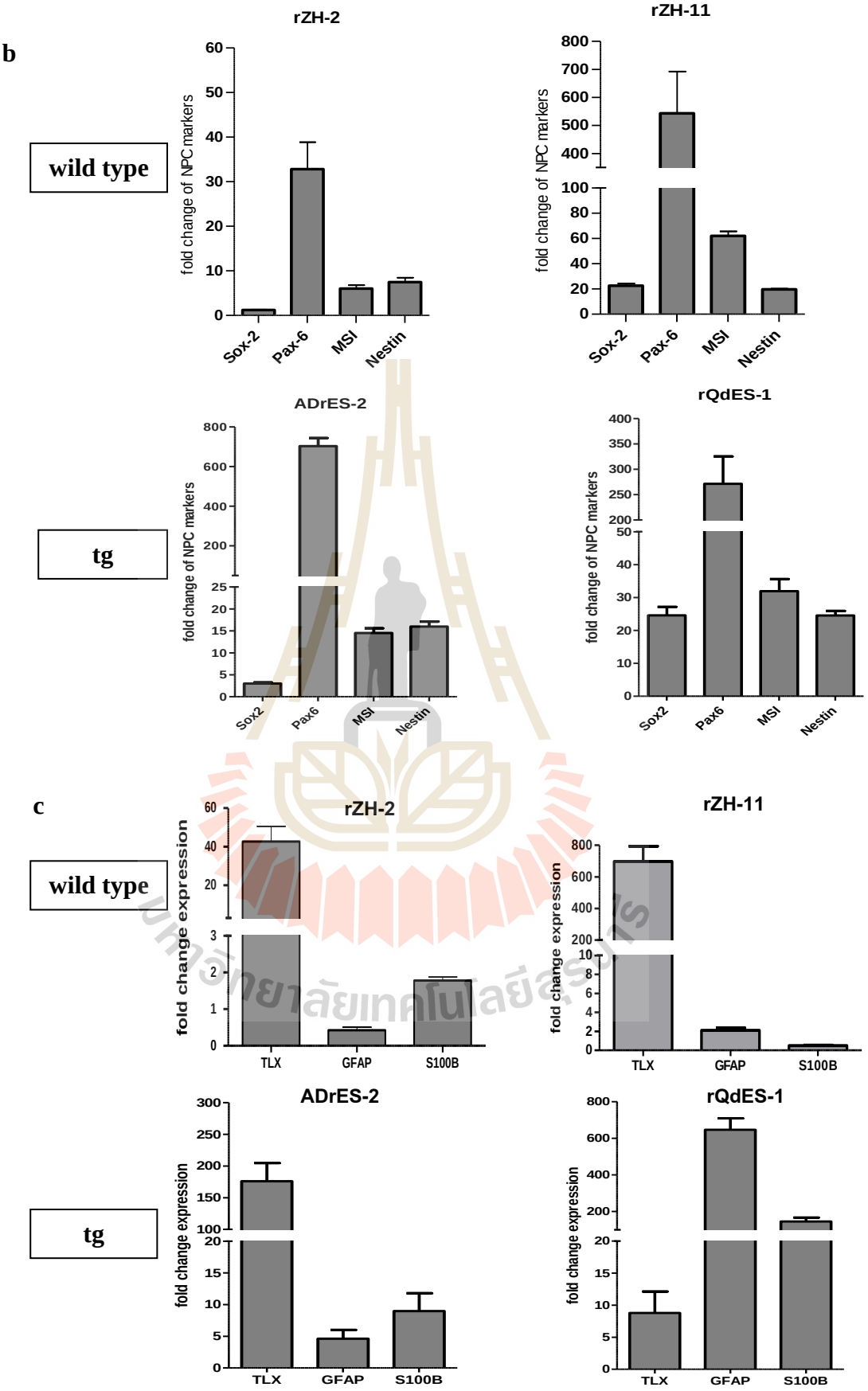

Figure 4.3 (Continue).

4.4.2 Transgenic APPs monkey NPCs generate neuronal markers positive cells following *In Vitro* neural differentiation

To determine that tg APPs-NPCs were capable of differentiating to neuron. NPCs were plated ~$3x10^4$ cells into P/L coated 35-mm dishes for differentiation cultures. Neuronal differentiation was triggered by FGF2 and LIF withdrawal from the proliferation medium and added with N2, SHH, FGF8, and Ascorbic acid. By the end of the second week of neuronal differentiation, differentiated cells from two tg APPs-NPC groups exhibit highly branched morphology and elongated spindle processes. The difference of cell morphology of wt and tg cells were unobservable by light microscope (fig4.4 a). Neurons derived from tgAPP NPCs showed microtubule-associated protein 2 (MAP2) and Tuj1 (fig4.4 a) which are neural-specific structural protein. The quantity of neuron was determined by immunostaining. The result showed no significant difference of Tuj1 positive cells of wt and tg cells (fig4.4 b). Moreover, Neurons derived from ADrES2-NPCs expressed neurotransmitter γ-aminobutyric (GABA) which is a marker expressed in GABAnergic neurons, but not in cells from rQdES1 (fig4.4 c). However, neurons derived from rQdES1 NPCs expressed additional markers: glial fibrillary acidic protein (GFAP), neuralfilament and tyrosine hydroxylase (TH) (fig4.4 c). TH is an enzyme which is used as a marker for dopaminergic neurons. These results suggested that the tg APPs progenitor cells from two tg ES cells cell lines have potential to differentiate into mature neuron. However, the potential of differentiation into specific-subtype neuron was different. It might be individual character of each cell line.

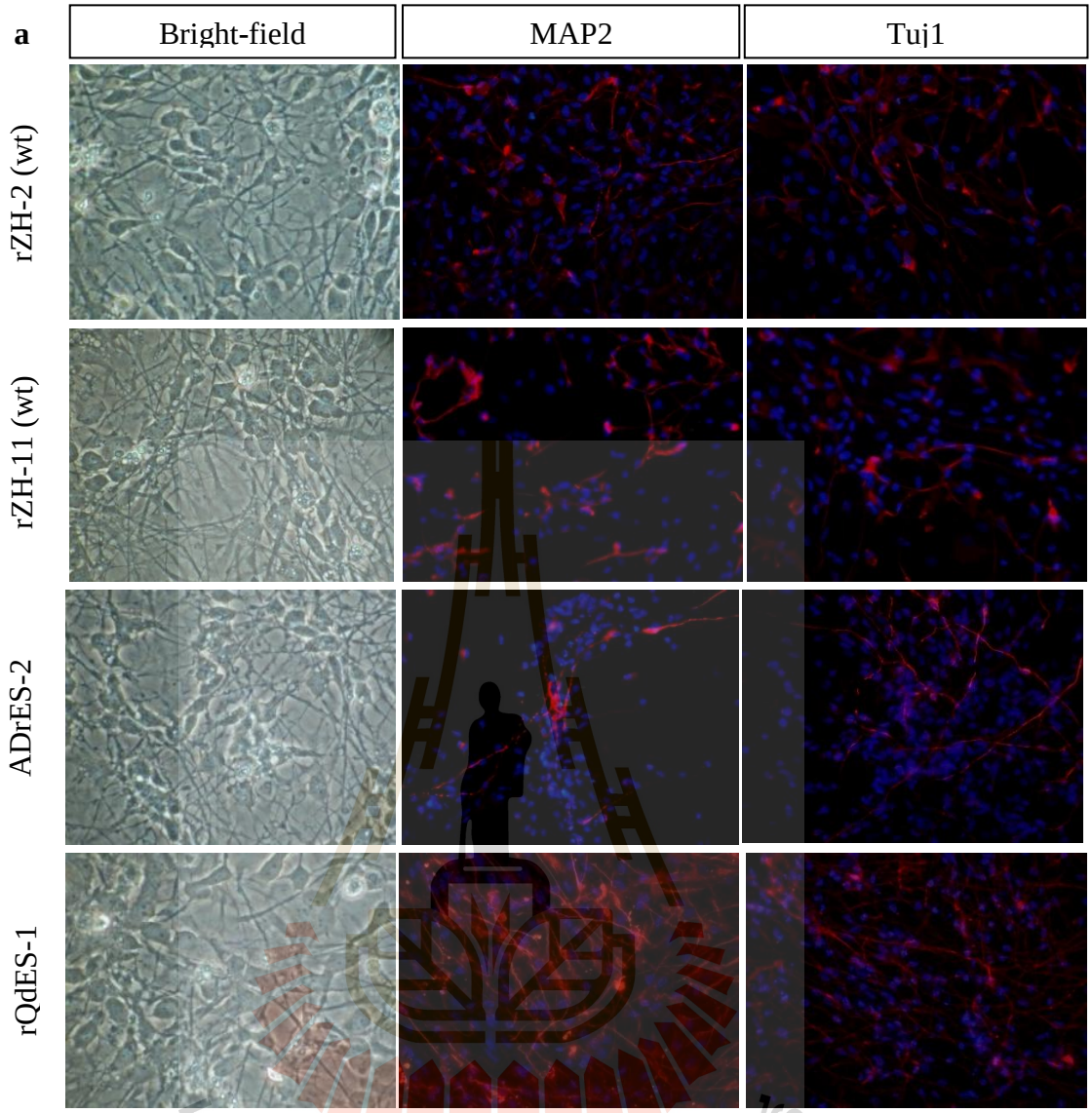

Figure 4.4 Transgenic AD and wt monkey NPCs were capable of differentiating into neural cell types. The bright-field image showed cell morphology after neural differentiation (first column). Immunocytochemistry revealed expression of neural cell markers: MAP2 (second column), Tuj1 (third column) (a). Quantity of neurons was investigated. Error bars indicate the SEM. Data represent three biological replicates (b). The differentiated NPCs stained for neuronal-specific type markers such as GABA and TH (c).

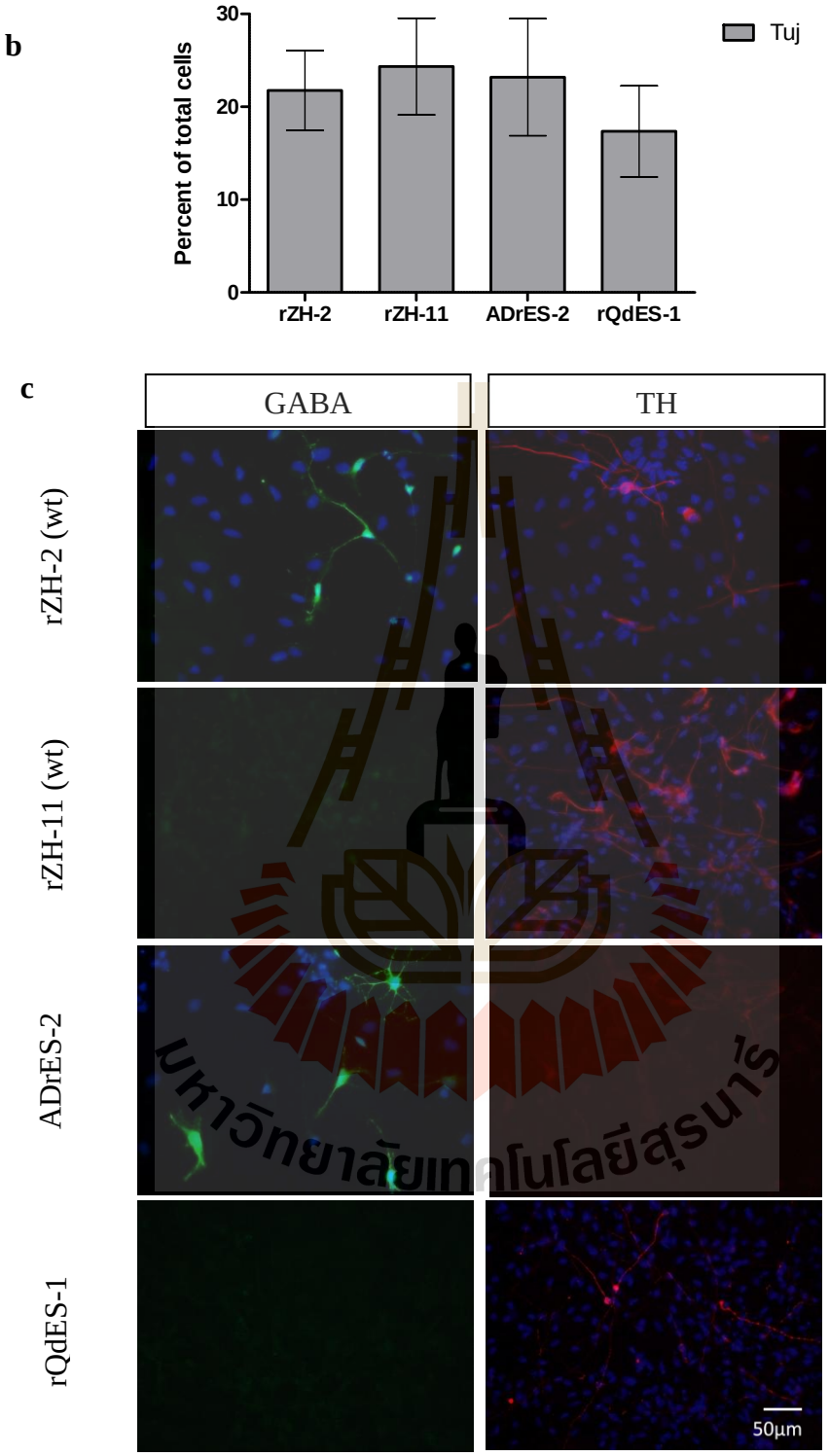

Figure 4.4 (Continue).

4.4.3 Expression of tg APPs genes and human Tau in ES cells, NPCs, and neuron

In AD brain, the major pathological hallmark is extracellular amyloid plaques which consist of Aβ peptide. The Swedish mutation enhanced cleavage at the β-secretase (BACE1) cleavage and increased amyloid beta (Aβ) formation (Gotz et al., 2004). While Indiana mutation lead to an increased the Aβ42/ Aβ40 ratio and promote the accumulation of Aβ42 in the brain, which result in the enhancement of amyloid fibril formation (Tamaoka et al., 1994). To investigate the expression of Swedish and Indiana APP mutations in the tg cells. We analyzed mRNA levels of tg APPs using qRT-PCR. The result revealed elevated expression of total SwAPP and IndAPP transcript in NPCs and neurons compared to ES cells which correlated with the trend of common rhesus APP expression. There was no significant difference of tg APPs between NPCs and neuron (fig 4.5 a,b). When the expression of tg APPs in rQdES-1 were compared with ADrES-2, the tg APPs expression of rQdES-1 cell lines were higher than ADrES-2 in ESC, NPC and neuron (fig 4.5 c). Moreover, we can detect GFAP positive cells from rQdES-1 after neuronal differentiation (fig 4.5 d). This result may correlate with the high expression of astrocyte markers (GFAP and S100β) in NPC from rQdES-1 cell lines.

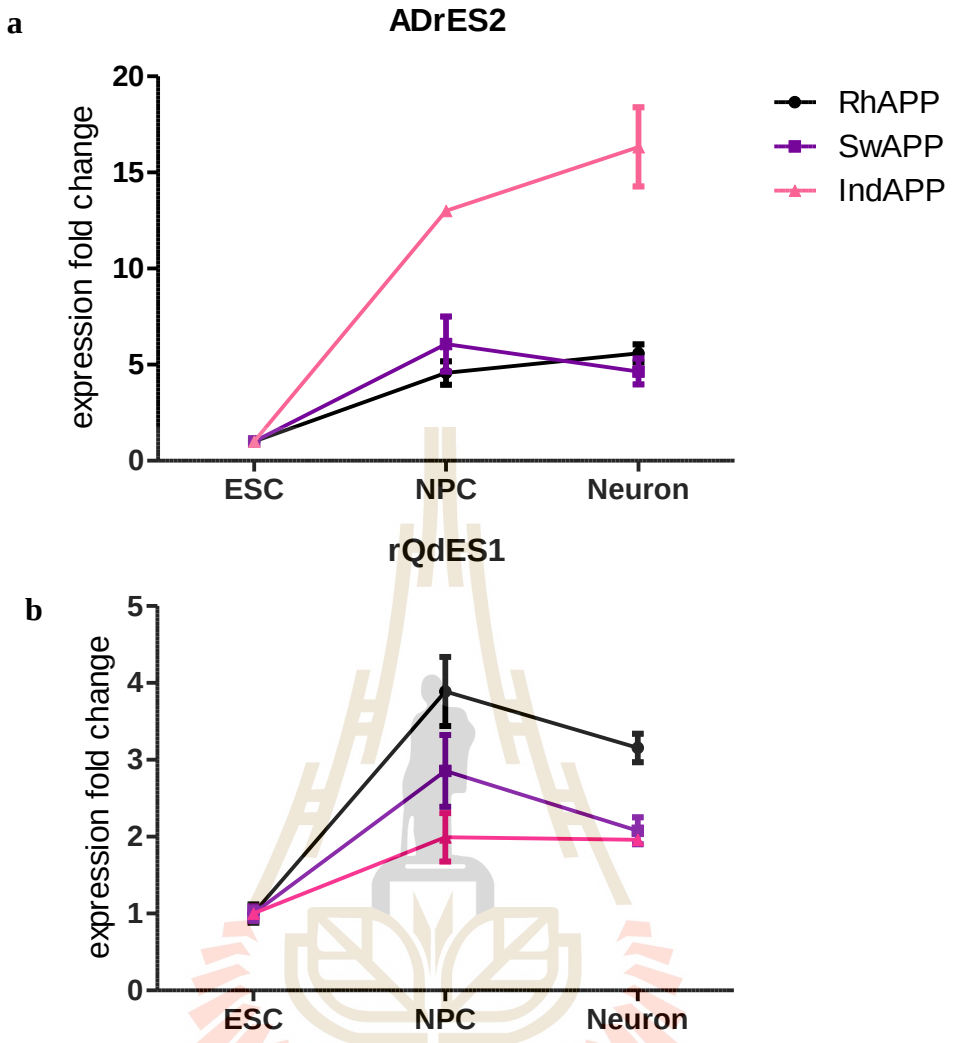

Figure 4.5 Elevated expression of tg APPs and rhesus APP during neural induction and differentiation. qRT-PCR analysis showed expression levels of tg and normal APP from tg cells lines compared to ES cells (a,b). Comparison of transgenic genes expression between rQdES-1 and ADrES-2 (c). Moreover, cells from rQdES-1 stained for GFAP after neuronal differentiation (d). Nuclear staining is shown using Hoechst (blue). Scale bars 50 µm.The results were from three biological replicates. qRT-PCR samples run in duplicate. Data and error bars are represented as mean ± SEM.

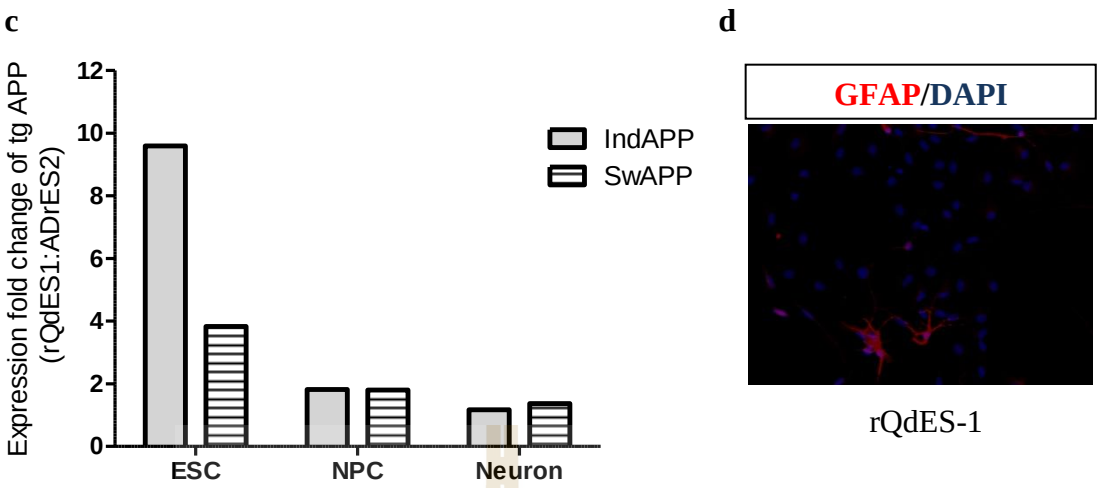

Figure 4.5 (Continue).

The other AD pathology Neurofibrillary tangles (NFT) which are formed by hyperphosphorylation of tau. Tau is a microtubule-associated protein located in neuronal axons that binds to the microtubule surface and promotes microtubule assembly (Brandt, et al., 2005). The expression of human tau was observed by qRT-PCR in ADrES2 cells. Results showed tau expression increase after NPCs and Neurons differentiation of ES cells. No significant difference of tau expression in NPC and neuron (fig 4.6).

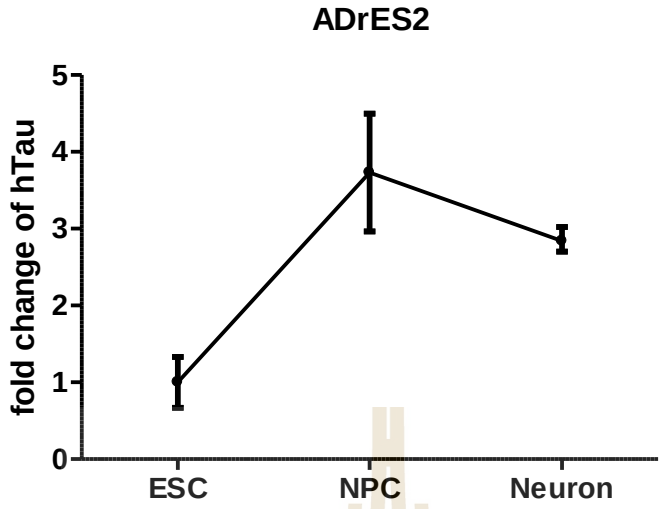

Figure 4.6 The expression of human tau increased after ADrES2-ES cell differentiated into NPCs and neurons. The results were from three biological replicates. qRT-PCR samples run in duplicate. Data and error bars are represented as mean ± SEM.

4.5 Discussion

From previous studies, the efficient protocols for NPC induction were developed. The EB base protocol can generate NPCs from wt monkey embryonic stem cell lines as well as tg APPs ES cell lines. We established two stable and expandable NPC lines. They exhibited rosette-like structure and neural progenitor-like morphology. qRT-PCR revealed up-regulation of NPC specific genes during NPC differentiation and the expression of NPC specific genes were confirmed by immunocytochemistry. Transgenic APPs-NPCs were capable of differentiating into neural cell types. Moreover, the expressions of GFAP and S100B were high correlated with low expression of TLX in NPCs derived from rQdES-1 ES cells. GFAP and

S100B are general astrocytes markers. These results correlated with the chapter III study. The low expression of TLX may affect GFAP expression and induce astrocyte differentiation. After neuronal differentiation, rQdES-1 was the only one which GFAP positive cells could be detected by immunocytochemistry. Moreover, several studies reported that APP induces glial differentiation of NPCs. A high concentration of secreted APP increased the population of GFAP-positive cells of differentiated human neural stem cells in a dose-dependent manner and extensive present of robust GFAP immunostaining in the hilus and SGZ in AD patient brain compared to controls (Kwak et al., 2006; Boekhoorn et al., 2006). However, we could not observe the expression of GFAP in the other tg cell line; ADrES2 cells. So, the expression of tg genes in ADrES-2 was compared with rQdES-1. The result revealed that the expression of Sw and Ind APP in rQdES-1 was higher than ADrES-2 in every stage (ESC, NPC and neuron).

The cytotoxicity of the Aβ peptides in the human and mouse brain has been intensely studied and the APP transgenic models have been generated. Due to one of the major pathological features of AD are senile plaques which consist of Aβ protein. Although, The studies of the double transgenic SwAPP and IndAPP mouse models showed the production of Aβ is enhanced with aged-dependence, increase Aβ42/Aβ40 ratio, and developed oligomeric plaques in the brain (Chishi et al., 2001; Lopez-Toledano and Shelanski, 2007). The expression of SwAPP in PC12 cells stimulated the basal secretion which contributes to the altered neurotransmitter pathology of AD (Lee et al., 2008). These studies showed tg APPs were increased during NPCs differentiation of ES cells but the difference of cell morphology could not be detected in this study as well as the expression of NPC markers compared with NPC derived

from wt ES cells. NPCs derived from tg APPs cells can differentiate into mature neurons and specific-subtype neurons. The difference between transgenic genotype may explain the disparity of the type of neuron.

Overexpression of tau affects shape, growth and alters the distribution of mitochondria of N2a cells (Ebneth et al., 1998), and involves extensive lobulation along the nuclear envelope in SH-SY5Y cells (Monroy-Ramirez et al., 2013). The expression of human tau was observed by qRT-PCR. Results showed tau expression increase after NPC and Neuron differentiation of ES cells. No significant difference of tau expression in NPC and neuron. However, the cell morphology change could not be detected.

4.6 Conclusion

Cultured neural progenitor cells are attracting increasing interest from neuroscientist. *In vitro*, expansion of human NPCs provides a model system to investigate neural development, biochemistry and cell biology. They also offer a renewable source of cells, including neural cells and specific subtypes of neural precursors for neurodegenerative disease studies and would be suitable for pharmaceutical and toxic screening. Monkey could provide more pertinent information than mouse and have potential to cross the divide between mouse and human and answer some question that cannot be asked using human cells because of limitation of ethic and safety concern. Monkey and human cells share characteristics, cell biology, biochemistry, and culture system. This study showed the protocol that can induce NPCs differentiation of tg AD ES cells and these tg neural progenitor cell lines could be a useful model for AD study.

4.7 References

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APPENDIX

Monkey ES cell base solution

1) Knockout DMEM (4°C)	77.50 ml
2) Knockout Serum (KSR) (20%)	20 ml
3) NEAA (100x)	1 ml
4) L-Glutamine	1 ml

PBS (-)

1) NaCl	10.0000 g
2) KCl	0.2500 g
3) Na_2HPO_4	1.4400 g
4) KH_2PO_4	0.2500 g
5) Ultra pure water	1.0000 L

0.25% Trypsin/EDTA

1) Trypsin	0.2500 g
2) EDTA	0.0400 g
3) PBS (-)	100 ml

0.1% Laminin

1) laminin	0.1000 g
2) PBS (-)	100 ml

2% Poly-l-ornithine

1) poly-l-ornithine	0.2000 g
2) PBS (-)	10 ml

Hoechst 33342 (stock)

1) Hoechst 33342	0.0020 g
2) DMSO	1.0000 ml

Blocking solution

Final conc. 100 ml

1) Triton X-100 0.2%	200 µl
2) Sodium azide 3 mM	19.50 mg
3) Saponin (mass/V) 0.1%	100 mg
4) BSA (mass/V) 2%	2 g
5) Horse/Donkey serum (V/V) 5%	5 ml
6) PBS (-)	95 ml

Neural differentiation medium

1) DMEM/F12	9.800 ml
2) B27 supplement	200 µl
3) N2 supplement	100 µl
4) SHH (100 µg/ml)	20 µl
5) FGF8 (100 µg/ml)	10 µl
6) ascorbic acid (160 mM)	10 µl

Monkey NPC induction medium

1) DMEM/F12	9.800 ml
2) N2 supplement	100 µl
3) bFGF (20 µg/ml)	2 µl
4) L-glutamine (200 mM)	100 µl

Monkey NPC proliferation medium

1) Neurobasal A medium	9.800 ml
2) B27 supplement	200 µl
3) bFGF (20 µg/ml)	10 µl
4) LIF (100 µg/ml)	1 µl

PCR primers used in transgenic gene evaluation for real-time PCR

Genes	Context sequence (5' to3')
Swedish mutation	forward – AATATC AAGACGGAGGAGATCTCTGAA reverse – CTTCATATCCTGAGTCATGTCGGAAT
Indiana mutation	forward – GGTGGGCGGTGTTGTCA reverse – TTTCTTCTTCAGCATCACCAAGGT

PCR primers used in neural progenitor derivation studies for real-time PCR

Genes	Context sequence (5’ to3’)
GAPDH	TCCAGGAGCGAGATCCCTCCAAAAT
Sox2	GGCCCCGGCGGAAAACCAAGACGCT
Pax6	ATGCAGAACAGTCACAGCGGAGTGA
Musashi	TTTGAGCAGTTTGGGAAGGTGGACG
Nestin	CCACGTACAGGACCCTCCTGGAGGC
TLX	ACAAAGACGCCGTGCAGCACGAGCG
NPDC1	CGGATCTCGCCTGGGGACCAGCGGC
GFAP	CCGTTCCAAGTTTGCAGACCTGACA
AFP	AGTCTGCTTTGCTGAAGAGGGACAA
GATA4	CAGCTCCGTGTCCCAGACGTTCTCG
TTR	TTGCCTCTGGGAAAACCAGTGAGTC
RUNX1	ACAGTGCTTCATGAGAGAATGCATA
VEGFA	TCATCACGAAGTGGTGAAGTTCATG
HNF1B	AAGCTGTCAGGAGTGCGCTACAGCC
LIFR	AAAGAGTGTCTGTGAGGGAAGCAGT

BIOGRAPHY

Apitsada Khlongkhlaeo was born in Kalasin, Thailand on March 21st, 1985. She finished high school from Princess Chulabhorn's college Mudaharn. In 2007, she received Bachelor's Degree (B.Sc.) in Biology from Khonkaen University, Khonkaen, Thailand. Her Bachelor's Degree study was supported by Human Resource Development in Science Project (Science Achievement Scholarship of Thailand, SAST). She then continued her Ph.D. in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. Her study was supported by The Strategic Scholarships for Frontier Research Network for the Ph.D. program. Her Ph.D. thesis title was neural progenitor cell lines derivation from wild-type and transgenic app/tau rhesus monkey embyonic stem cells. Parts of this study have been presented as poster in Commission on Higher Education Congress V: University Staff Development Consortium CHE-USDC Congress V on November 14-16, 2013 at Chonbury, Thailand.