ประสิทธิภาพการพัฒนาเปลี่ยนแปลงของเซลล์ต้นกำเนิดหนูเม้าส์ให้เป็นกลุ่มเซลล์ประสาท

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THE DIFFERENTIATION POTENTIAL OF MOUSE STEM CELLS INTO NEURONAL LINEAGE

Miss Nuttha Klincumhom

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Theriogenology
Department of Obstetrics Gynaecology and Reproduction
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การทดลองที่ 1 เพื่อศึกษาและเปรียบเทียบการพัฒนาเปลี่ยนแปลงของเซลล์ต้นกำเนิดจากตัวอ่อน และเซลล์อินดิวซ์พลูลิโพเท็นสเต็มเซลล์หนูเม้าส์ให้เป็นกลุ่มเซลล์ประสาทด้วยวิธีการเลี้ยงแบบเอ็มบริออยบอดี (EB) จากการศึกษาพบว่าทั้งเซลล์ต้นกำเนิดจากตัวอ่อนและเซลล์อินดิวซ์พลูลิโพเท็นสเต็มเซลล์แสดงคุณสมบัติของเซลล์ประสาท เมื่อทำการตรวจสอบเซลล์ที่ถูกเปลี่ยนแปลงด้วย Pax6 Nestin และ Tuj-1 อย่างไรก็ตามพบว่า เซลล์อินดิวซ์พลูลิโพเท็นสเต็มเซลล์เปลี่ยนแปลงไปเป็นเซลล์ที่ให้ผลบอกต่อ nestinได้น้อยกว่า กลุ่มเซลล์ต้นกำเนิดจากตัวอ่อนอย่างมีนัยสำคัญ (6.12±1.61 และ 74.36±1.65, P<0.05) จากการทดลองสามารถสรุปได้ว่า ประสิทธิภาพในการเปลี่ยนแปลงไปเป็นเซลล์ประสาทของเซลล์อินดิวซ์พลูลิโพเท็นสเต็มเซลล์จำเป็นต้องพัฒนาปรับปรุง อย่างไรก็ตาม อินดิวซ์พลูลิโพเท็นสเต็มเซลล์จากหนูเม้าส์แสดงประสิทธิภาพในการเปลี่ยนแปลงไปเป็นเซลล์กลุ่มประสาทได้

การทดลองที่ 2 เพื่อศึกษาความสามารถของเซลล์ต้นกำเนิดจากตัวอ่อนที่ตัดยีน Rybp ออก (D11 Rybp-deficient ES cells) ในการเปลี่ยนแปลงไปเป็นเซลล์กลุ่มประสาทในห้องทดลองด้วยวิธีการเลี้ยงแบบเอ็มบริออยบอดี โดยทำการเปรียบเทียบกับเซลล์ต้นกำเนิดจากตัวอ่อนปกติ (R1 wild type ES cells) หลังจากเลี้ยงเอ็มบริออยบอดีเป็นเวลาแปดวัน พบการเรียงตัวแบบ rosette-liked structure ซึ่งประกอบด้วยเซลล์ที่ให้ผลบวกต่อ Pax6 และ Musashi-1 จากเซลล์ภายในเอ็มบริออยบอดีที่ได้จากเซลล์ต้นกำเนิดจากตัวอ่อน R1 และ D11 การแสดงออกของยีนต่อเซลล์ต้นตอประสาท (Nestin, Musashi-1) และเซลล์ประสาท (TUJ-1, NeuroD1) ของเซลล์ต้นกำเนิดจากตัวอ่อน D11 นั้นต่ำกว่าในเซลล์กลุ่มควบคุม นอกจากนั้นร้อยละของเซลล์ที่ให้ผลบวกต่อ nestin ที่ได้จากเซลล์ต้นกำเนิดจากตัวอ่อน D11พบว่า ต่ำกว่าเซลล์ต้นกำเนิดจากตัวอ่อน R1 อย่างมีนัยสำคัญ (42.62±8.84 และ 80.42±4.27, P<0.05) จากการศึกษาสรุปได้ว่าเซลล์ D11 Rybp-deficient ES สามารถถูกเหนี่ยวนำให้เปลี่ยนแปลงไปเป็นเซลล์กลุ่มประสาทได้ในห้องทดลอง อย่างไรก็ตามประสิทธิภาพยังคงด้อยกว่าในกลุ่มเซลล์ต้นกำเนิดจากตัวอ่อนปกติ

การทดลองที่ 3 เพื่อศึกษาผลของ TGF-β1 inhibitor โดยใช้ A83-01ต่อการพัฒนาและเปลี่ยนแปลงไปเป็นเซลล์กลุ่มประสาทของเซลล์ต้นกำเนิดจากตัวอ่อนในช่วงที่เลี้ยงแบบ EB ใน suspension ผลการศึกษาพบว่า ขนาดของ EB ที่เลี้ยง TGF-β1 inhibitor (592.6±27 μm)เล็กกว่า EB ในกลุ่มควบคุม (631.7±14.1 μm, P< 0.01) อย่างมีนัยสำคัญ เซลล์ที่เกาะกลุ่มกันใน EB พบว่าให้ผลบวกต่อเซลล์ต้นตอประสาท Pax6 และเซลล์ประสาท NeuN กระจายอย่างทั่วถึงในเอ็มบริออยบอดีในทั้งสองกลุ่ม จากการวิเคราะห์การแสดงออกของยีนด้วย qRT-PCR พบว่าการยับยั้ง TGF-β1 กลุ่มที่ใช้ A83-01 ทำให้เกิดการเปลี่ยนแปลงไปเป็นเซลล์กลุ่มประสาทของเซลล์ต้นกำเนิดจากตัวอ่อนได้เร็วกว่ากลุ่มควบคุม ซึ่งตรวจได้จากระดับการแสดงออกของยีนพลูลิโพเท็น Oct4 ที่ลดลงอย่างต่อเนื่อง และยังพบว่า ลดระดับของยีนที่จำเพาะต่อเซลล์ glia อีกด้วย จากการทดลองนี้สรุปได้ว่าการยับยั้ง TGF-β1 มีประสิทธิภาพในการกระตุ้นเซลล์ต้นกำเนิดจากตัวอ่อนไปเป็นเซลล์ประสาทได้

การทดลองที่ 4 เพื่อศึกษาประสิทธิภาพของการปลี่ยนแปลงไปเป็นเซลล์ประสาทนำสั่งของเซลล์ต้นกำเนิดจากตัวอ่อน โดยใช้ TGF-β1 inhibitor (A83-01) ระหว่างการเลี้ยงแบบ EB ใน suspension คุณลักษณะของเซลล์ประสาทของเอ็มบริออยบอดีอายุแปดวันทำการตรวจได้ด้วยวิธีอิมมูนโนฮิสโตเคมี พบว่าเซลล์ที่เปลี่ยนแปลงจากงทั้งสองกลุ่มให้ผลบวกต่อเซลล์ต้นตอประสาท Pax6 และเซลล์ประสาทระยะเริ่มต้น Tuj-1 จากการวิเคราะห์ด้วย qRT-PCR พบว่าเซลล์ต้นตอประสาทนำสั่งซึ่งจำเพาะต่อ Olig2 สามารถผลิตได้จากเอ็มบริออยบอดีที่ใส่ A83-01 ซึ่งพบว่า มีระดับยีนที่สูงกว่ากลุ่มควบคุม (4.20±0.20 เทียบกับ 0.73±0.09, P < 0.01) อย่างไรก็ตามระดับยีนเซลล์ประสาทนำสั่งส่วนท้ายที่จำเพาะต่อ Hoc8 ของกลุ่มควบคุม มีระดับที่ต่ำกว่ากลุ่มที่เอ็มบริออยบอดีเลี้ยงด้วย A83-01 (14.73±2.61 เทียบกับ 2.37±0.42, P < 0.01) จากการศึกษาสรุปได้ว่า TGF-β1 มีผลต่อการเจริญและการเปลี่ยนแปลงของเซลล์ต้นตอประสาทนำสั่ง

ภาควิชา สูติศาสตร์-เธนุเวชวิทยาและวิทยาการสืบพันธุ์ ลายมือชื่อนิสิต.....

สาขาวิชา วิทยาการสืบพันธุ์สัตว์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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NUTTHA KLINCUMHOM: THE DIFFERENTIATION POTENTIAL OF MOUSE STEM CELLS INTO NEURONAL LINEAGE. ADVISOR: PROF. MONGKOL TECHAKUMPHU, D.V.M., DOCTORAT 3e CYCLE, CO-ADVISOR: ASST. PROF. THEERAWAT THARASANIT, D.V.M., Ph.D., PROF. ANDRAS DINNYES, D.V.M., Ph.D., 115 pp.

EXP. 1 aimed to investigate and compare the differentiation ability of mouse embryonic stem (ES) and induced pluripotent stem (iPS) cells into neuronal lineage using embryoid body (EB) formation. We determined for the first time the differentiation potential of mouse Sleeping beauty transposon (SB)-mediated iPS cells to form neuronal progenitor cells (NPCs) and neurons. Both mouse ES and SB-iPS cells exhibited the neuronal characteristics detected by Pax6, Nestin and Tuj-1. However, SB-iPS cells produced less nestin-positive cells than ES cells (6.12±1.61 vs 74.36±1.65, respectively). In conclusion, ES and SB-iPS cells show a difference in their capacity to differentiate towards the neuronal lineage. Even though the neuronal differentiation rates of iPS cells needs to be improved, our results are encouraging and show that SB-iPS cells are capable of forming neurons.

EXP. 2 aimed to investigate the ability of D11 Rybp-deficient ES cells to generate the neuronal population *in vitro* via EB formation comparing with R1 wild type ES cell line. After 8 days of EB suspension culture, the rosette-liked structures contained Pax6 and Musashi-1 positive cells has been found in both R1 and D11-derived EBs. Several neuronal gene expression both NPCs (Nestin and Musashi-1) and neurons specific markers (Tuj-1 and NeuroD1) of D11 derived cells were lower than those in controls. The percentage of nestin-positive cells derived from D11 ES cells were found to be significantly lower than those derived from R1 ES cells (42.62±8.84 vs 80.42±4.27, respectively This results suggested that Rybp-deficient ES cells can be induced to generate NPCs and neurons *in vitro*. However, the efficiency was poorer when compared to the wild-type.

EXP. 3 aimed to investigate the effect of TGF-β1 inhibitor using A83-01 on the development and neuronal differentiation potential of mouse ES cells during EB suspension period. Our results showing that the diameters of EBs treated with A83-01 (592.6±27 μm) were significantly smaller than the control group (631.7±14.1 μm, $P< 0.01$). Eight-day-old EBs derived from the two groups similarly contained a complex network of mixed population of early and mature neurons as indicated by Pax-6 and NeuN staining, respectively. Comparing to the control, TGF-β1 inhibitor potentially suppressed transcription factor Oct4 while rapidly up-regulation of neuronal associated genes (Sox1 and MAP2). Furthermore, this inhibitor also down-regulated astrocyte related gene (GFAP) compared favorably to non-treated control. It is concluded that selective TGF-β1/ALK inhibitor efficiently stimulates the cell fate alteration from ES state toward neuronal lineages.

EXP. 4 aimed to examine the efficacy of motor neuron differentiation of ES cells treated with TGF-β1 inhibitor (A83-01) during EB suspension culture. The neuronal characteristic of 8 day-old EBs was examined by immunohistochemical analysis on cross-sectioned EBs. Our result demonstrated that the aggregated ES cells differentiated into NPCs as they expressed Pax-6 and Tuj-1. Quantiative RT-PCR analysis revealed that treatment the EB with selective TGF-β1 inhibitor up-regulated the motor neuron progenitor Olig2 at higher levels than that obtained from the control (4.20±0.20 vs. 0.73±0.09, $P<0.01$). In contrast, mRNA expression levels of motor neuron Hoxc8 of a control group were significantly higher than the TGF-β1 inhibitor treated group (14.73±2.6 vs. 2.37±0.42, $P<0.01$). the differentiated cells expressed a neuronal marker (Tuj-1), motor neuron progenitor marker (Olig2), developing motor neuron progenitor (Isl-1) and functional motor neuron marker (ChAT). We concluded that TGF-β1 signaling appears to affect generation and differentiation fate of motor neuron progenitors.

Department: Obstetrics Gynaecology and Reproduction
Field of Study: Theriogenology
Academic Year: 2012

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LIST OF ABBREVIATIONS

ALP	alkaline phosphatase
ALK	anaplastic lymphoma receptor tyrosine kinase
ALS	amyotrophic lateral sclerosis
bHLH	basic helix-loop-helix
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BMPs	bone morphogenetic proteins
BSA	bovine serum albumin
CNS	central nervous system
D	day
DA	dopaminergic
DAPI	4′- 6′-diamidino-2-phenylindole
DED	death effector domain
DEDD	DED-containing DNA-binding protein
DEDAF	death effector domain associated factor
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxynucleic acid
DPBS	Dulbecco's Phosphate-Buffered Saline
E	the day of embryonic development
EB	embryoid body
EC	embryonal carcinoma
EGFP	enhanced fluorescent green protein
ERKs	extracellular signal-regulated kinases
ES	embryonic stem
FACS	fluorescence-activated cell sorting
FADD	Fas associated protein with death domain
FBS	fetal bovine serum
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde phosphate dehydrogenase

GDFs	growth and differentiation factors
GDNF	glial cell-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescence protein
HD	Huntington disease
ICM	inner cell mass
IGF-1	insulin like growth factor 1
iPS	induced pluripotent stem
IU	international unit
KCl	potassium chloride
Klf4	krueppel-like factor 4
LIF	leukemia inhibitory factor
MAP2	microtubule-associated protein 2
MEF	mouse embryonic fibroblast
mg	milligram
min	minute
ml	milliliter
mM	millimole
MND	motor neuron diseases
mRNA	messenger ribonucleic acid
NEAA	non essential amino acid
NEP	neuroepithelial
NeuN	neuronal-specific nuclear protein
NPCs	neuronal progenitor cells
Oct4	octamer-binding transcription factor 4
Pax6	paired box protein 6
PBS	phosphate buffered saline
PcG	polycomb group protein
PD	Parkinson disease
PFA	paraformaldehyde
PNS	peripheral nervous system
poly 2-HEMA	poly 2-hydroxyethyl methacrylate

qRT-PCR	quantitative real time polymerase chain reaction
RA	retinoic acid
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
Rybp	Ring1 and YY1 binding protein
SB	sleeping beauty
SD	standard deviation
SDIA	stromal cell-derived inducing activity
sec	second
SEM	standard error of mean
SHH	sonic hedgehog
SMA	spinal muscular atrophy
Sox	sex determining region Y-box
SSEA	stage specific embryonic antigen
TGF-β	transforming growth factor β
Tuj-1	neuron-specific class III beta-tubulin
v	volume
w	weight
μM	micromole
μg	microgram
μm	micrometer
β-ME	β-Mercaptoethanol

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Most neurological disorders are considered as incurable disease due to the limited ability of nervous system during adulthood to regenerate upon their injury. Many neurological problems are caused by degeneration and damage/loss of specific populations of neuronal and glial cells. However, there are few effective drug treatments, probably due to the lack of appropriated disease-study models *in vitro* and poor understanding of disease mechanisms in cellular and molecular levels. The pluripotent stem cells have become an interested issue because of its theoretical ability to provide an unlimited source of required cells.

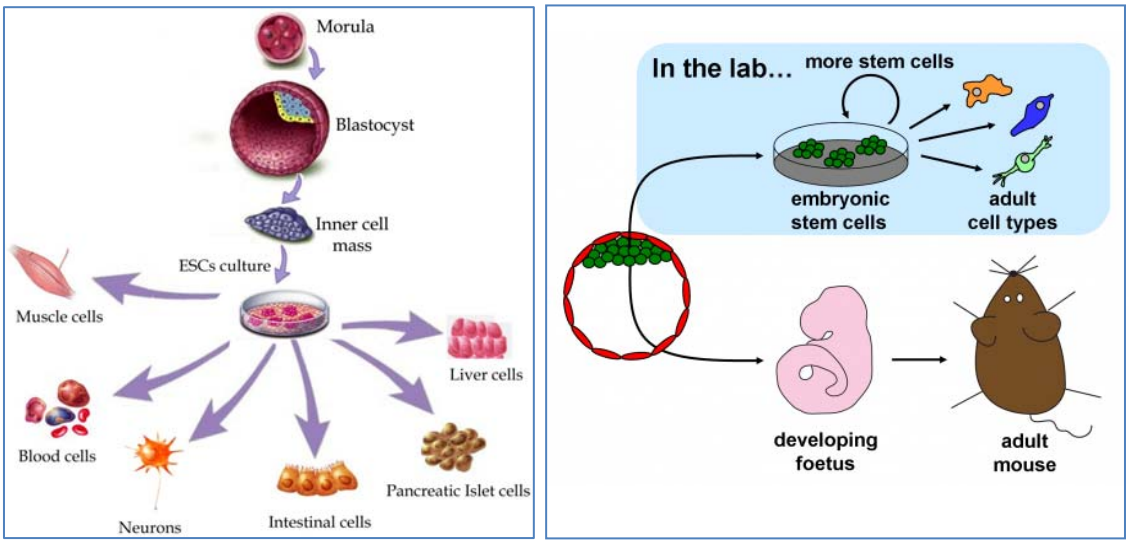

Figure 1. Differentiation potential pluripotent stem cells. Mouse embryonic stem cells derived from an inner cell mass of blastocyst stage (A). ES cells serve as study model of embryogenesis (B). (http://www.intechopen.com/books/stem-cells-in-clinic-and-resear ch/stem-cell-therapy-for-neuromuscular-diseases; http://www.eurostemcell. org/factsh eet/embryonic-stem-cells-where-do-they-come-and-what-can-they-do)

Embryonic stem cells are pluripotent cells derived from the inner cell mass of the blastocyst stage embryo that can differentiate *in vivo* and *in vitro* into all cell types of the adult animals (Evans and Kaufman, 1981). Mouse embryonic stem (ES) cells holds much promise to use as a model for the investigation of mammalian neurogenesis, the generation of neurons for pharmacological testing and development of potential cell therapy applications to overcome incurable neurological diseases such as stroke, spinal cord injuries, Alzheimer's and Parkinson's disease (Langston, 2005; Taupin, 2006). ES cells have been successfully generated into neurons and glia by several strategies (Bain et al., 1995; Okabe et al., 1996). They serve as valuable models to study *in vivo* neurogenesis, drug screening and cell transplantation (Dinsmore et al., 1996).

Nevertheless, undifferentiated ES cells should not be used in regenerative therapies because these cells may cause tumor formation called teratoma after transplantation. Thus, several approaches have been developed to achieve *in vitro* neuronal differentiation starting from embryonic stem (ES) cells, aimed at generating neuronal stem/ precursor cells as defined as multipotent that have ability to self-renew and differentiate toward all neural cell types in the central nervous system (CNS) (Fisher, 1997; Gage, 2000). In order to induce neuronal differentiation, ES cells are classically allowed to aggregate into three-dimensional structure in suspension culture so-called embryoid body (EB). The committed cells within the EB are then induced to differentiate into neuronal lineages by retinoic acid treatment (Bibel et al., 2004). However, efficiency of this technique is marginal, in terms of the cell purity and the number of neuronal cells obtained. Improving strategy for neuronal differentiation of ES and iPS cells is therefore required for studying the cellular and molecular mechanisms of early embryo development and neurogenesis and also for generation of neural cells for cell transplantation therapies or regenerative medicine.

1.2 Literature Review

1.2.1 Mouse embryonic stem (ES) cells

Mouse embryonic stem (ES) cells were first isolated from the inner cell mass of mouse blastocysts in pre-implantation stage and continuously grown on

mitomycin-treated mouse embryonic fibroblasts (Evans and Kaufman, 1981; Martin, 1981). Indeed, several attempts have been made to establish the embryonic stem cell line in many species such as chicken, pig, cow, monkey and human (Thomson et al., 1995; Pain et al., 1996; Notarianni et al., 1997; Cibelli et al., 1998; Thomson et al., 1998). However, only three species of mammals, including mouse, monkey, and human, have yielded long-term cultures of self-renewing ES cells. They have been defined as pluripotent cells as they have capabilities to proliferate into an undifferentiated state, to self-renew, and give rise to any cell types in the body through three primary germ layers including ectoderm, mesoderm and endoderm that can be observed *in vitro* by embryoid bodies (EBs) (Rungarunlert et al., 2009).

The pluripotent ability of ES cell can also be proven by the production of chimeric mice following injection of the ES cells into mouse blastocysts or the formation of teratomas after injection of the ES cells under the skin or the kidney capsule of immune-deficient mice. In cell culture system, the property of ES cells is maintained by the underlying layer of undividing feeder cells derived from mouse embryonic fibroblast (MEF). These cells essentially provide the ES cells with all required signals for pluripotency. Among pathways that maintain the pluripotency of mouse ES cells, leukemia inhibitory factor (LIF), a glycoprotein cytokine is a well-known factor which involved with STAT3 to regulate transcriptional event in ES cells (Boeuf et al., 1997). The evidence that LIF can maintain the ES cell property in feeder-free culture system strongly indicates that this cytokine plays an important role in maintaining the ES in undifferentiated stage. Transcription factor Oct4, a member of the POU transcription factors, has proven to be required for maintenance of pluripotent fate of the inner cell mass (ICM) in blastocyst (Nichols et al., 1998; Niwa, 2001). Nanog is also the transcription factor expressed in ES cells and is defined as one of the key factors that maintain undifferentiated state of ES cells. The expression levels of Oct4 and Nanog gradually decrease following ES cells differentiation (Chambers et al., 2003; Mitsui et al., 2003). Sox2, a member of the *Sox* (SRY-related HMG box) gene family, has a unique function in maintaining the undifferentiated state of the epiblast cells in relation with the activation of Oct4 transcription (Avilion et al., 2003). In addition, mouse ES cells have been shown to express a stage-specific embryonic antigen-1 (SSEA-1) as it has been presented on the surface of pre-

implantation embryos and teratocarcinoma stem cells (Solter and Knowles, 1978). SSEA-1 is a carbohydrate-associated molecule which has biological properties involving in the controlled interactions of cell surface during development. In contrast, SSEA-3 and SSEA-4 but not SSEA-1 is expressed in human ES cells, suggesting the differences of specific pluripotency pathways between the species (Reubinoff et al., 2000). For *in vitro* differentiation assay, ES cells are cultured in suspension in the absence of extrinsic factors, spherical multi-cellular structure called EBs are formed, and the individual cells comprising the structure begin to differentiate along various lineages in a disorganized pattern (Keller, 1995). In order to induce differentiation of ES cells into a specific lineage, the molecular mechanism that controls ES differentiation has become an intense topic of interest because these particular cell lineages have potential application for regenerative medicine and also for in-depth understanding the disease mechanism.

1.2.2 Neuronal differentiation of ES cells

In embryogenesis, the proper orchestrated sequential organization of lineage specification is essentially needed for cells development starting from an inner cells mass to postnatal maturation (Slack, 2008). Upon neuronal development, neuroepithelial cells form as single layer of neural plate and neural tube that present in the spinal cord of rat embryos (E10.5). The neuroepithelial cells are self-renew and multipotent cells which can differentiate into numerous cell types in the central nervous system (CNS) including neurons, oligodendrocytes and astrocytes (Kalyani et al., 1998) and peripheral nervous system (PNS) derivatives (Mujtaba et al., 1998). During generation of neuroepithelial cells into several neuronal cell types in early neurogenesis, radial glial cells have been exhibited in ventricular zone which is mostly comprised of progenitor cell bodies. Thereafter, differentiation of NEP cells and redial glial cells operates through the generation of the two types of restricted precursors at E13.5 including neuronal-restricted precursor or neuronal progenitor cells (NPCs), that can generate multiple types of neurons (Mayer-Proschel et al., 1997) and glial-restricted progenitor cells or glia that can generate into oligodendrocytes and astrocytes (Rao and Mayer-Proschel, 1997; Rao et al., 1998).

The developmental transition of pluripotent cells in the early stage of embryo into neuronal lineage has been emulated by *in vitro* differentiation of ES cells which is capable of generating neurons, astrocytes and oligodendrocytes (Fraichard et al., 1995). A variety of neuronal production strategies have been established aimed at rendering the high yield of homogenous neuronal population of desired neuronal cell types. Since then, many studies allowed ES cells to form EBs by culturing in suspension without factors that maintain the undifferentiated or pluripotent phase of stem cells such as LIF and feeder layer cells (O'Shea, 1999; Desbaillets et al., 2000) or with medium supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (Reynolds and Weiss, 1996; Lee et al., 2000). Additionally, EBs can be produced at various differentiation stages by arranging their culture conditions such as cell density (Koike et al., 2007), concentration of retinoic acid (RA) (Okada et al., 2004). In the past decade, the stromal cell lines derived from skull bone marrow such as PA6 cell line has been shown to promote neuronal differentiation of ES cells (Kawasaki et al., 2000). The active components responsible for the dopaminergic differentiation with a high proportion of neurons expressing tyrosine hydroxylase are referred to as stromal cell-derived inducing activity (SDIA) (Barberi et al., 2003; Perrier et al., 2004). PA6 cell line can also retain the neuronal-inducing activity even after separating ES cells from co-cultured PA6 by filter membrane or being fixed with paraformaldehyde (Ohse et al., 2009). Other investigators have shown that the generation of mature neurons from ES cells cultured on semi-permeable membrane as hollow fibers could also be established by using condition medium derived from the PA6 cell lines (Yamazoe and Iwata, 2006). Moreover, the condition medium derived from many cell types also provided the neuronal-inducing factors for neuronal induction of ES cells such as astrocytes (Nakayama et al., 2003) and dorsal root ganglia (Kitazawa and Shimizu, 2005). Although differentiated neurons derived from co-culture with PA6 cells had ability to survive *in vivo* after transplanted into the ischemic brain (Takagi et al., 2005; Hayashi et al., 2006), the providing growth factors and mechanisms underlying neuronal lineage commitment induced by these techniques remain to be studied.

ES cells can be differentiated into neurons by culturing as a monolayer on substrate-coated plate (McKay, 1997). Several proteins in extracellular matrix which

used as substrate-coated plate in monolayer culture also have an impact on neuronal differentiation of the ES cells. Previous study revealed that ES cells grown on fibronectin and laminin and poly-L-ornithine increased the proportion of glial cells and neurons, respectively (Goetz et al., 2006). The differentiation of ES cells as monolayer can be optimized by cell density. For instance, ES cells cultivated at a low density gave rise to spherical colonies that expressed the neuronal marker Nestin (Tropepe et al., 2001). Additionally, highly gene-expressed neuronal cells and the number of cells with a mature neuronal morphology increased substantially in monolayer culture condition at optimal cell density (Lorincz, 2005).These reasons have shown the great prospect of neuronal differentiation of mouse ES cells which may serve as useful platforms to study gene function and biological events in neuronal development and also to optimize the strategies of ES cell differentiation into neuronal lineages for future stem cell transplantation.

1.2.3 Generation of induced pluripotent stem (iPS) cell lines

Since the first ES cell lines derivation from early mouse embryos in 1981, the number of researched topics associated with these pluripotent cells has dramatically raised up according to their biological and a variety of prospect clinical applications (Evans and Kaufman, 1981; Martin, 1981). Theoretically, pluripotent ES cells have been isolated from an inner cell mass of blastocyst stage embryos. They can be clonally proliferated in culture indefinitely, while their capability to differentiate both *in vivo* and *in vitro* into several cell types of the body throughout primary three germ layers still be persisted. Undoubtedly, by their pluripotent features, a variety of neuronal cells types in CNS including neurons and glia can be *in vitro* generated as in embryo. Then, these cells have expected as biological platform for studying the underlying mechanism in neurological diseases.

In 2006, mouse induced pluripotent stem cells have been firstly established from skin fibroblasts by retroviral-mediated transduction of four reprogramming factors (Klf4, Sox2, Oct4 and c-Myc) which exceedingly revolted the field of stem cells-based therapeutic application. Later on, the human iPS cells have been successfully established from skin fibroblasts using the retroviral transduction of the same set of reprogramming factors (Masaki et al., 2007; Takahashi et al., 2007b).

Another set of reprogramming factors has also demonstrated ability to generated human iPS cells by using Nanog and Lin28 instead of c-Myc and Klf4 (Yu et al., 2007). However, the risks of insertional mutagenesis due to the integration of exogenous genetic elements into chromosomes still impede the future aspect of resultant iPS cells in medical applications.

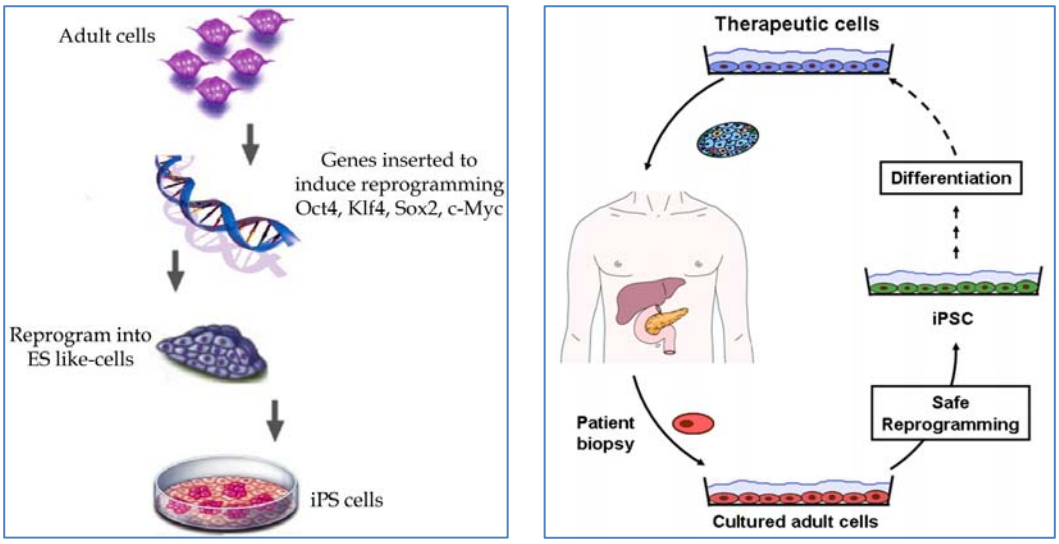

Figure 2. Generation of iPS cell lines. Induction of somatic cells by reprogramming factors (A). The schematic of cell-based therapy using iPS cell lines (B). (http://www.intechopen.com/books/stem-cells-in-clinic-and-research/stem-cell-therapy-for-neuromuscular-diseases; https://research.cchmc.org/stemcell/iPSC)

To overcome the cautious concerns associated with residual exogenous reprogramming genes and chromosomal integrations, several studies have attempted to develop the new strategies of iPS cells generation including use of minimal numbers of reprogramming genes into host genome by depletion of exogenous cMyc (Nakagawa et al., 2008) or use of only two transcription factors Oct4 and Sox2 (Huangfu et al., 2008). Moreover, the efficiency of retroviral mediated iPS cells generation has been improved by using the combination of small molecule including TGF-β inhibitor and MEK (Lin et al., 2009). Relative recently, there was a study reported that replacement of Oct4 with the nuclear receptor Nr5a2 increased the reprogramming efficiency of iPS cells generation from mouse somatic cells (Heng et al., 2010).

Non-integrating vectors such as using episomal plasmid has been demonstrated to generate iPS cells by driving the reprogramming factors into cell genome without integration of viral elements (Okita et al., 2008). The direct delivery of recombinant cell-penetrating proteins has presented capability to establish both mouse and human iPS cells (Zhou et al., 2009). The administration of synthetic modified mRNA has been demonstrated the ability to efficiently converse the human somatic cells into iPS cells (Warren et al., 2010). Meanwhile, piggyBac transposon, the sequence of transposable DNA, has applied as non-viral method to deliver the reprogramming factors for somatic cell mediation (Kaji et al., 2009; Woltjen et al., 2009). Sleeping beauty is the another potential transposon element which has been demonstrated to prosperous benefit for gene therapeutic application in hematopoietic disorders such as B-cell lymphoma (Izsvak et al., 2009). Lately, our laboratory has been successfully established the transposon mediated iPS cell line using Sleeping beauty (SB) transposon which offers the interested promise of alternative cell source in stem cell field and future clinical application (Muenthaisong et al., 2012)

1.2.4 Neuronal differentiation of iPS cells

It is now vastly enlightened that iPS cells are particular to ES cells characteristics including morphology, proliferation, gene expression, teratoma formation, chimera production and ability to differentiate into all cell types of the body (Maherali et al., 2007). Since the iPS cells have been established via retroviral transduction, they have also exhibit ability to spontaneously differentiate into early post-mitotic neurons in mouse and then by neuronal induction method in human (Okita and Yamanaka, 2011).

A variety of neuronal cell types could be induced from pluripotent stem cells by several strategies. Using EB formation, iPS cells can be induced to differentiate into neuronal lineages in growth factors and concentration dependent manners such as NPCs, dopaminergic and motor neurons (Karumbayaram et al., 2009; Mak et al., 2012). By these floated-cells aggregation treated with retinoic acid (RA), our SB-transposon mediated iPS cells can be reprogrammed from pluripotent stage into neuronal progenitor cells and early post-mitotic neurons (Klincumhom et al., 2012). Co-culturing with PA6 stromal cells also produced the dopaminergic neurons from

undifferentiated iPS cells (Zhou and Freed, 2009). In a monolayer culture system, the directed differentiation of iPS cells into neuronal cells can be achieved by a defined medium with sequential supplements depending on desired types of neurons. It has been reported that the synergistic action of two inhibitors of SMAD signaling including Noggin and SB431542, promoted the neuronal differentiation of iPS cells under adherent culture conditions (Chambers et al., 2009). Instead of RA-treated EB in suspension, RA has been used to directly generated high-yield (>95%) of NPCs and neurons from human ES colonies (Parsons et al., 2011). Advancement in the differentiation strategies of iPS cells toward the neuronal lineages will increase the number and specificity of neuronal subtypes that can be generated *in vitro*.

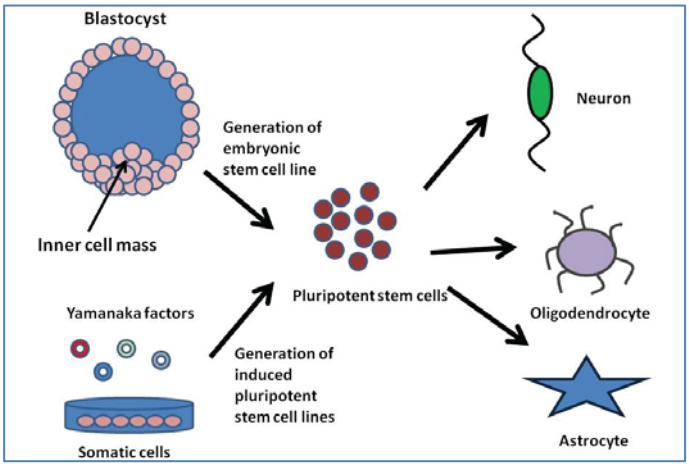

Figure 3. The potential of neuronal differentiation of iPS cells. iPS cells are resemble to ES cells properties which can induced to differentiated into the three main cell types found in the central nervous system including neurons, oligodendrocyte and astrocyte (Willerth, 2011)

To date, many patient-specific iPS cells of neurological diseases have been established and successfully differentiated into particular neuronal cell types such as motor neurons from amylotrophic lateral sclerosis (ALS)-derived iPS cells (Dimos et al., 2008), dopaminergic neurons from Parkinson disease (PD)-derived iPS cells (Soldner et al., 2009; Cooper et al., 2010) and neuronal progenitor cells and neurons from Huntington's disease (HD)-derived iPS cells (Castiglioni et al., 2012).

Meanwhile, the transplantation of patient specific iPS cells has been also studied on the survival and functions of differentiated cells in host lesions (Hargus et al., 2010). Recently, the path of differentiation process of somatic cells has directly induced into the desired specific cells types/tissues neglecting the embryonic-liked or pluripotent stage called induced transdifferentiation. Recently, by combination of three transcription factors (Ascl1, Brn2 and Myt1l), functional neurons can be directly generated from mouse fibroblasts (Vierbuchen et al., 2010) and also in human (Ambasudhan et al., 2011).

Thereafter, several researches developed this novel technique continuously to improve the efficiency and control of neuronal subtype specification, for example, dopaminergic neurons (Pfisterer et al., 2011). It has paved an alternative trail which prospected as rapid and efficient methods for generating several functional specific neuronal cell types in nervous system. In addition, this technique has therefore overcome the risk of tumoriogenesis from pluripotent cells at transplantation site, although the methods efficiency still needs to be considerably addressed. The ongoing progresses in stem cell fields encourage to further improve differentiation protocols and cell purification methods, as well as novel strategies to generate patient-specific iPS cells.

1.2.5 Role of retinoic acid in embryo development and neuronal differentiation

Retinoic acid (RA) is well known as the biologically active form of retinol (vitamin A) and has been shown to be an essential key during embryogenesis and central nervous system (CNS) development (Ross et al., 2000; Maden, 2001). RA first appears in the mouse embryo at the mid-primitive streak to late allantoic bud stages (day 7.5) (Ulven et al., 2000). RA and its molecular machinery (RARs, RXR, binding proteins) are present as essential key in the developing CNS. Insufficient or lack of RA and its molecular machinery can disrupt nervous system development. In mouse day 10.5 and day 13 embryo, retinoids differently distribute in the CNS. The forebrain, midbrain and hindbrain express extremely low levels of all-trans-RA, while the neural tube that will develop into the spinal cord expresses the highest level (Horton and Maden, 1995). Furthermore, most studies have concluded that the neurons show the characteristics of CNS neurons rather than peripheral nervous

system (PNS) neurons (Pleasure et al., 1992; Strubing et al., 1995; Dunlop et al., 1998). Former study indicated that the gastrulation stage is the critical period of mammalian embryo development for neurogenesis, when the developing CNS is radically posteriorized by exposure to the active retinoid all-trans-retinoic acid (RA) (Durston et al., 1989).

In vitro, RA has utilized to mouse ES cells induced concentration- and time dependently differentiation of cardiac, neuronal, adipogenic, myogenic, and vascular smooth muscle cells (Rohwedel et al., 1999). RA induced neuronal differentiation in variety of cell types for example neuronal crest cells (Henion and Weston, 1994), PC12 rat pheochromocytoma (Hens et al., 1998), human neuroblastoma (Cervello et al., 1997; Hill and Robertson, 1997) and human embryonal carcinoma (EC) cells (Przyborski et al., 2000). RA has been recognized to be one of important extrinsic factors that can induce neuronal differentiation of mouse ES cells *in vitro*. A significant induction of neuronal differentiation was achieved by the application of high concentrations of RA in early embryogenesis, when neuroepithelial cells are generated in EB formation derived from ES cells (Bibel et al., 2004). RA has been demonstrated to promote several specific transcription factors in nervous system in a concentration-dependent manner such as Pax-6, sonic hedgehog (SHH), Islet, HB9, Lim3, Phox2b, Lim l/2, and En- 1 (Renoncourt et al., 1998; Okada et al., 2004). Relative recently, it has been reported that addition of retinoic acid (RA) to rapidly dividing mouse ES cells led to the generation of a uniform population of neuronal progenitors that displayed the characteristics of radial glial cells and neurons (Bibel et al., 2007). The ES-derived neuronal culture provides valuable gene and protein expression profiling and the study of cell differentiation between wild-type and mutant cells (Quinn et al., 2010).

1.2.6 Function of Rybp in embryo development and neurogenesis

The polycomb group (PcG) proteins are the family of proteins which is first discovered in Drosophila (*Drosophila melanogaster*) and involved in epigenetic modifications of chromatin as repressive transcription factors. The PcG proteins have defined as transcriptional repressors cooperated in contrary effect with Trichorax (trxG) proteins activator group. For this reason, these groups of proteins are important

in many aspects of cell proliferation and differentiation during development as gene expression regulator (Ringrose and Paro, 2004). In addition, they have also involved in many biological rule of the cells including self-renew of stem cells (Valk-Lingbeek et al., 2004).Rybp (Ring1 and YY1 binding protein), a member of the Rybp/Yaf2 zinc finger-containing protein family, has been defined as crucial gene for embryo implantation and development. This gene has characterized as interacting partner of polycomb group protein member (PcG). Rybp acts as transcriptional repressor via binding with two types of Ring1 proteins (Ring1A and Ring1B) and M33 at the same C-terminal domain and also interacts directly with YY1, a transcription factor partially related to the product of the Drosophila pleiohomeotic gene, which has been known as the essential components of mammal PcG. Owning to its ability to bind and interact with several transcriptional factors, PcG products, and mediator of apoptosis, Rybp has indicated as multifunctional regulator during organogenesis such as adaptor protein, transcriptional repressor, cell-cycle regulator, tumor suppressor, apoptosis process (Martinez and Cavalli, 2006; Stanton et al., 2007; Gonzalez et al., 2008; Chen et al., 2009).

Rybp, cooperating with YAF2, have been implicated to be essential regulator of apoptosis process in embryo development (Stanton et al., 2006). Rybp has been called as Death effector domain associated factor (DEDAF) according to heterotypical binding with several Death effector domain (DED)-containing proteins such as pro-Caspases 8, pro-Caspase10, Fas associated protein with death domain (FADD) and DED-containing DNA-binding protein (DEDD) (Zheng et al., 2001). Rybp is required for a protein implicated in neuronal apoptosis named Hippi to function as an adapter protein for Caspase 8-mediated apoptosis, while YAF2 has been shown the opposite function to inhibit apoptosis. At E9.0, *in situ* hybridization analysis of Rybp mRNA is mostly showed to express in cells of the developing CNS with highest levels in the spinal cord and forebrain of mouse embryo (Schoorlemmer et al., 1997; Garcia et al., 1999). Previous study demonstrated that the spatially and quantitatively appropriate Rybp gene expression is required for normal morphogenesis of the CNS. The inadequate of Rybp level can cause CNS malformations including forebrain overgrowth and defective neuronal tube closure, while, the embryonic death (E 5.5-6.0) has reported in Rybp-deficient embryos (Pirity et al., 2005). In addition, the role

of Rybp has also addressed in mouse retina and lens development. From the study using *in vivo* mouse models, Rybp has been observed in lens, corneal epithelium, corneal stroma, optic nerve and perioptic mesenchyme. Therefore, several ocular defects has demonstrated from transgenic mice both ablation and overexpression of Rybp resulting in retinal coloboma, an incomplete closure of the optic fissure, corneal neovascularization and lens opacification (Pirity et al., 2007). However, Rybp deficient ES cells are not lethal as in transgenic null embryos. Differentiation of ES cells into neuronal lineages thus represents a new opportunity to explore the molecular function of Rybp which is still unclear in neurogenesis.

1.2.7 Transforming growth factor (TGF)-β1 in ES cells differentiation

The TGF-β1 is polypeptide member that belong to TGF-β superfamily which is comprised of Bone morphogenetic proteins (BMPs), Growth and differentiation factors (GDFs), Activin, Nodal and TGF-β family. These factors have been known as pleiotropic factor as their effects depend on their concentration as well as the combination with other growth factors, and the signaling transduction is modulated through SMAD phosphorylation (ten Dijke and Hill, 2004). The expression and function of transcription factors in TGF-β superfamily have been conserved in many species and necessitate in both the adult organism and the embryo development including cell growth, cell differentiation, cell apoptosis and cellular homeostasis (Massague and Chen, 2000). Over expression of TGF-β superfamily members, Nodal, in mouse ES cells induces up-regulation of both mesodermal and endodermal cell markers while the neuroepithelial markers were down regulated. Recent study found that hippocampal neural progenitor cell proliferation and production of neurons and astrocytes has inhibited by TGF-β1 (Buckwalter et al., 2006). The modulation of NPC proliferation demonstrated to be involved with cell-cycle arrest action of TGF-β1 which is compatible with the limit proliferation potential of adult neural stem cells (Wachs et al., 2006).

The activin/nodal signaling pathway has been implicated in the inhibition of neuronal differentiation and in the maintenance of pluripotency in ES cells (Vallier et al., 2005). The loss of either Nodal or BMPs, allowed neural tissue formation in default pathway (Tropepe et al., 2001; Levine and Brivanlou, 2007). Recent studies

suggested that the inhibition of both activin/nodal and BMPs of the TGF-β signaling pathways are significantly enriched a functional neural in adherent cultures (Chambers et al., 2009). Additionally, inhibition of TGF-β1 activin receptor-like kinases using the SB431542 down-regulated the pluripotency of ES cells and converted the cell fate from undifferentiated stage into neuronal identity (James et al., 2005; Patani et al., 2009). In the previous study reported that A83-01, a selective inhibitor of TGF-β type I receptor ALK5 kinase, type I activin/nodal receptor ALK4 and type I nodal receptor ALK7, potentially inhibited the epithelial-to-mesenchymal transition induced by TGF-β than SB431542 at the same concentration (Tojo et al., 2005). Thus TGF-β inhibitors and it related molecules may be much utility for induction of proliferation and differentiation of many cell types including stem cells.

1.2.8 Differentiation of ES and iPS cells into spinal motor neurons

Mouse ES cells have been manifested their ability to differentiate into several neuronal cell types of nervous system. Multipotency of ES-derived neuronal progenitor cells (NPCs), region-specific neurons such as dopaminergic, GABAnergic and motor neurons have been generated sequentially upon growth factors-dependent manner (Barberi et al., 2003). The functional mature motor neurons derived from ES cells have been used as model to study the state of motor neuron diseases (MND), including amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) (Lunn et al., 2011).

The development of spinal motor neurons during embryogenesis has been organized sequentially. After ectodermal cells were converted into rostral neural fate, neural inductive signal like RA has led the caudalization of neural cells, then the motor neurons can be generated via ventralization of the spinal motor progenitors which is directly dependent upon the SHH activity (Jessell, 2000; Wilson and Edlund, 2001). Therefore, the efficient differentiation of mouse ES cells into specific neural subtype of CNS like spinal motor neurons probably involved with the many signaling factors aimed to recapitulate the steps of motor neurons generation in embryo. The successful establishment of motor neurons can be achieved though orchestration of multiple signals such as retinoic acid (RA) and sonic hedgehog (SHH) which show significant activities to differentiate cells into caudo-ventral pattern in nervous system

and induction of floor plate in developing neural tube and spinal cord patterning (Marti and Bovolenta, 2002; Maden, 2006).

Exposure to these morphogens promoted the differentiation fate of both ES and iPS cells into spinal motor neurons which involved several developmental steps related concentration-dependent manner. With several conditions including embryoid body formation, stromal cells co-culturing, ES and iPS cells were induced for differentiation in order to enrich the functional motor neurons in culture (Li et al., 2005; Karumbayaram et al., 2009). In addition, several transcription factors including Pax6, Nkx6.1, bHLH protein and Olig2 have been also suggested to play potential role in specific position and stage of differentiation and development of motor neurons (Pituello et al., 1995; Lee et al., 2005; Karumbayaram et al., 2009).

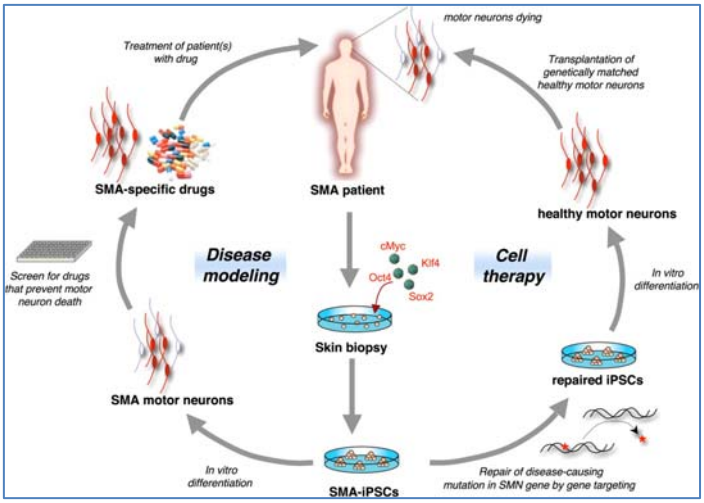

Figure 4. The prospect applications of iPS cells technology for cell therapy and disease modeling of SMA (Stadtfeld and Hochedlinger, 2010)

1.3 Objectives of the thesis

1. To generate the neuronal progenitor cells and neurons derived from ES and iPS cells using EB formation
2. To investigate the efficiency of neuronal differentiation between Rybp-deficient ES cell line *in vitro*

3. To determine the effect of TGF-β1 inhibitor on cells differentiation of mouse ES cells-derived EBs during suspension culture
4. To determine the effect of TGF-β1 inhibitor on the development potential of mouse ES cells-derived motor neurons

1.4 Hypothesis

1. Mouse ES and iPS cells can be differentiated into NPCs and neurons *in vitro*
2. Rybp-deficient ES cells can generate neuronal cells *in vitro* with less efficiency compared to the wild type ES cells
3. TGF-β1 inhibitor enhances the development of ES cells to differentiate toward neuronal lineage during EBs development
4. TGF-β1 inhibitor effect on the development of ES cells to differentiate toward motor neurons

1.5 Keywords: neuronal differentiation, embryonic stem cells, induced pluripotent stem cells, embryoid body, Rybp, TGF- β1 inhibitor, motor neurons, mouse

1.6 Research merits:

1. The development of cultured conditions for efficient neuronal differentiation of mouse ES and iPS cells
2. The knowledge of Rybp function in neurogenesis and embryo development using *in vitro* neuronal differentiation of mouse ES cells as investigative model
3. The knowledge in the effect of TGF-β1 inhibitor on EB formation and morphology, proteins and gene expression of differentiated neurons
4. The knowledge about the effect of TGF-β1 inhibitor on motor neurons differentiation

CHAPTER II

GENERATION OF NEURONAL PROGENITOR CELLS (NPCs) AND NEURONS FROM MOUSE SLEEPING BEAUTY TRANSPOSON-GENERATED INDUCED PLURIPOTENT STEM (iPS) CELLS

2.1 Abstract

Mouse embryonic stem (ES) and induced pluripotent stem (iPS) cells can be used as models of neuronal differentiation for the investigation of mammalian neurogenesis, pharmacological testing and development of cell based therapies. Recently, mouse iPS cell lines have been generated by Sleeping Beauty (SB) transposon-mediated transgenesis (SB-iPS). In this study, we determined for the first time the differentiation potential of mouse SB-iPS cells to form neuronal progenitor cells (NPCs) and neurons. Undifferentiated SB-iPS and ES cells were aggregated into embryoid bodies (EBs) and cultured in neuronal differentiation medium supplemented with 5 μM all-trans retinoic acid. Thereafter, EBs were dissociated and plated to observe further neuronal differentiation. Samples were fixed on day 10 and 14 for immunocytochemistry staining using the NPCs marker (Pax6 and Nestin) and neuron marker (βIII-tubulin/Tuj1). Nestin-labeled cells were analyzed further by flow cytometry. Our results demonstrated that SB-iPS cells can generate NPCs and differentiate further into neurons in culture, although SB-iPS cells produced less nestin-positive cells than ES cells (6.12±1.61 vs 74.36±1.65, respectively). In conclusion, the efficiency of generating SB-iPS cells-derived NPCs needs to be improved, however, given the considerable potential of SB-iPS cells for drug testing and as therapeutic models in neurological disorders, continuing investigation of their neuronal differentiation ability is required.

2.2 Introduction

Pluripotent stem cell research holds great promise for revolutionizing the future of medicine, and especially, for the regeneration of damaged and diseased organs. Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of pre-implantation blastocyst-stage embryos, which can differentiate *in vivo*

and *in vitro* into all cell types of an adult animal (Evans and Kaufman, 1981). Mouse ES cells are used as model systems for neurological diseases and investigation of mammalian neurogenesis. In particular, they can be used in the generation of neurons for pharmacological testing and development of models for cell therapy applications, which may help to overcome incurable neurological diseases, such as stroke, spinal cord injuries, Alzheimer's and Parkinson's disease (Langston, 2005; Taupin, 2006).

Recently, a novel alternative method has been developed to establish induced pluripotent stem (iPS) cells. Mouse and human iPS cells have been directly reprogrammed from adult cells (e.g. fibroblasts) by the introduction of pluripotency transcription initially by Oct4, Sox2, c-Myc, and Klf4, known as Yamanaka factors (Takahashi and Yamanaka, 2006) or complementing/replacing them partially with other factors such as Nanog and Lin28 (Liao et al., 2009). These iPS cells resemble ES cells with respect to morphology, proliferation, gene expression, teratoma formation and in their ability to differentiate into all three germ layers, and in the case of mouse iPS cells, also form mouse chimeras (Maherali et al., 2007; Takahashi et al., 2007a). The iPS cells might also be useful for patient- and disease-specific cell transplantation, through their differentiation potential into several different cell lineages, including cardiac cells (Zwi et al., 2009), hepatic cells (Iwamuro et al., 2010), hematopoietic cells (Tolar et al., 2011) and neurons (Hu et al., 2010).

To date, iPS cells have been established by several methods, such as viral transduction (Yamanaka, 2007), recombinant cell-penetrating proteins (Zhou et al., 2009), administration of synthetic modified mRNA (Warren et al., 2010) and recently, by transposon-transposase mediated transgenics using the PiggyBac transposon (Kaji et al., 2009; Woltjen et al., 2009). Transposons are sequences of DNA, which have the capability to change their positions within the genome by use of a transposition mechanism. Besides the PiggyBac, another transposon, the Sleeping Beauty (SB) was engineered by the molecular reconstruction of the inactive Tc1/mariner element found in the salmonid fish genome (Ivics et al., 1997) and has been used as a powerful tool to introduce genes into various cell types (Essner et al., 2005; Izsvak et al., 2009). Very recently SB has been reported by us to be a suitable tool for mouse iPS cell line generation (Muenthaisong et al., 2012) and offering an alternative method for the efficient generation of iPS cells.

Neuroepithelial (NEP) cells are multipotent cells in the neural tube, which have the capability to self-renew and give rise to neurons in the central nervous system (CNS) and in the peripheral nervous system (PNS), such as glial cells and ependymal cells (Pevny and Rao, 2003). Differentiation of NEP cells occurs via the generation of two major types of progenitor cells including neuroblasts or neuronal progenitor cells (NPC) that can generate into multiple kinds of neurons (Mayer-Proschel et al., 1997; Kalyani et al., 1998). The developmental conversion of the undifferentiated ICM in the early embryo into committed neurons has been partially emulated by *in vitro* differentiation of ES cells (Okabe et al., 1996). It has been reported that ES cells are able to form neurons, astrocytes, and oligodendrocytes (Abranches et al., 2009). Early development and neuronal differentiation of mouse ES cells has been extensively studied *in vitro* and for the initial steps of neuronal induction, most strategies include use of aggregates of a few hundred stem cells, so called embryoid bodies (EB). Although EBs consist of several cell types of many lineages, it has been shown that supplementation with RA can induce the formation of a relatively uniform glutamatergic neuronal population (Bibel et al., 2007).

Retinoic acid (RA) is a biologically active form of retinol (vitamin A) and has been demonstrated to have a significant role during embryogenesis and CNS development (Ross et al., 2000; Maden, 2001). RA first appears in the mouse embryo at the mid-primitive streak to the late allantoic bud stage (E7.5) (Ulven et al., 2000). *In vitro*, RA applied to mouse ES cells induced concentration- and time dependent differentiation towards neuronal, cardiac, myogenic, adipogenic, and vascular smooth muscle cell types (Rohwedel et al., 1999). Furthermore, RA has been considered to be an important inductive signal for neuronal differentiation of mouse ES cells *in vitro* (Lu et al., 2009). Induction of neuronal differentiation has been achieved by the application of RA (10^{-6} to 10^{-7} M) at early stages of development (Fraichard et al., 1995; Strubing et al., 1995). Recently, a study has reported that the addition of RA, when used at the concentration of 5 μM added to 4-days old EBs in suspension for a further 4 days, induced mouse ES cells to form high yields of NPCs which exhibit the characteristics of Pax6-positive radial glial cells (Bibel et al., 2004).

In this study, our goal was to demonstrate the differentiation potential of iPS cells to form NPCs using a mouse model. We compared the differentiation capacity of

mouse ES and SB-iPS cells into neuronal progenitor cells and neurons via EB formation.

2.3 Materials and methods

Materials and Cell culture condition

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) and culture media were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA), unless specified otherwise. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Medium was changed daily for mouse ES and iPS cell cultures and on every second days during differentiation.

2.3.1 Mouse pluripotent cell cultures

Mouse (129SV/Ola) HM1 ES cells (Selfridge et al., 1992) (kindly provided by Dr. Jim McWhir, Roslin Institute, Roslin, UK) and the mouse B5 iPS cell line derived by SB-transposition from the C57BL/6 mouse background (Muenthaisong et al., 2012) were cultured on mitomycin C inactivated mouse embryonic fibroblasts (MEFs) as described (Magin et al., 1992). The pluripotent cells were maintained in ES medium: Dulbecco's modified Eagle's medium (DMEM) containing 15% (v/v) fetal bovine serum (FBS, Sera Laboratories International, West Sussex, RH17 5PB, UK), 0.1 mM non-essential amino acids (NEAA), 0.1 mM β-mercaptoethanol (β-ME), 50 U penicillin/ml, 50 µg streptomycin/ml and 1,000 U/ml mouse leukemia inhibitory factor (LIF, ESGRO, Chemicon International, Temecula, CA). The cells were passaged prior to reaching 70% confluency (approximately every 1-2 days). ES cells at passage 25, and SB-iPS cells at passage 22, were cultured on gelatin-coated dishes in the presence of LIF (2,000 U/ml) in ES medium for at least one passage prior to differentiation, in order to deplete potentially present MEF cells from the system.

2.3.2 *In vitro* neuronal differentiation

Mouse pluripotent cells were induced to differentiate into the neuronal lineage as previously described, with some modifications as shown in Figure 5 (Bibel et al., 2007). Mouse ES and iPS cell colonies were harvested into single cells using 0.05% (w/v) Trypsin, then seeded at a density of 3×10^5 cells/ml in differentiation medium

(ES medium without LIF) onto bacteriological dishes pre-coated with Poly 2-hydroxyethyl methacrylate (Poly-HEMA) to prevent cell attachment. Pluripotent cells were allowed to aggregate in suspension and form EBs for 4 days. Five µM all-trans RA were then added to the medium and EBs were cultured for a further 4 days. Thereafter, 8-day old EBs were dissociated and plated onto Poly-L-ornithine and laminin (Roche, CA) coated dish at a density of 2×10^5 cells/cm^2 in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F-12) containing 3 mg/ml D-(+)-Glucose, 3 mg/ml AlbuMaxI, 50 U/ml penicillin, 50 µg/ml streptomycin, 1% (v/v) N2 supplement and 10 ng/ml recombinant human basic Fibroblast Growth Factor (bFGF). Two days later the medium was changed to DMEM/F12:Neurobasal medium (1:1), 1 mM glutamax, 3 mg/ml AlbuMaxI, 50 U/ml penicillin, 50 µg/ml streptomycin, 0.5% (v/v) N2 Supplement and 1% (v/v) B27 supplement. The medium was renewed every second day until day 14. The cells were harvested for further analyses on day 10 and 14 after the start of the EB formation. The experiments have been replicated three times.

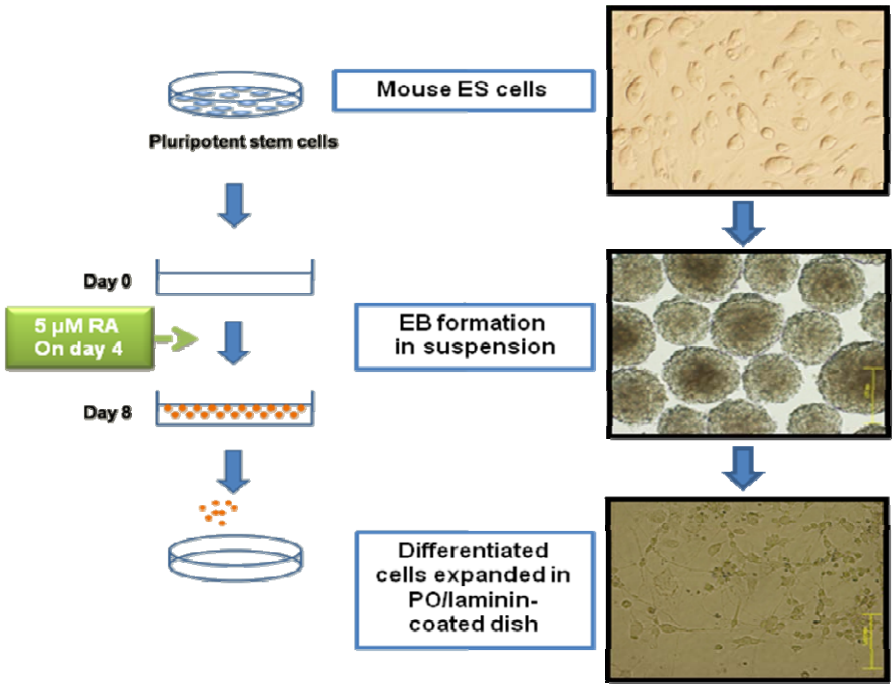

Figure 5. The schematic of neuronal differentiation of mouse pluripotent cells using EB formation method

2.3.3 Immunocytochemical analysis

ES and SB- iPS cells were prepared for characterization by 2-dys culture (until reaching 70% confluency) on gelatin-coated coverslips. Differentiating cells following dissociation of EBs were plated onto poly-L-ornithine/laminin-coated coverslips for 2 and 6 days (referred as day 10 and 14, respectively). Cells were washed with phosphate-buffered saline (PBS) and fixed with 4% (v/v) paraformaldehyde (PFA) for 15 min at room temperature (RT). Fixed cells were washed and stored in PBS at 4°C until analysis. Permeabilization was performed using 0.2% (v/v) Triton X-100 (for intracellular staining) for 30 min at RT. Cells were then blocked with 3% (w/v) Bovine serum albumin containing 0.5% (v/v) Tween20 in PBS for 30 min. The cells were then sequentially incubated with the following primary antibodies diluted in blocking solution overnight at 4°C: pluripotent marker Oct4 (sc9081, dilution 1:100, Santa Cruz Biotechnology, Santa Cruz, CA; mouse), Sox2 (SC-20088, dilution 1:100, Santa Cruz Biotechnology, Santa Cruz, CA; rabbit), Nanog (AF-2729, dilution 1:20, R&D Systems, Minneapolis, MN; mouse), neuroepithelial marker Pax6 (Pax6, dilution 1:200, DSHB; mouse), neuronal progenitor marker Nestin (Rat-401, dilution 1:200, DSHB; mouse) and neuron marker βIII-tubulin (Tuj1, dilution 1:2,000; Covance, PRB-435P; rabbit). The cultures were washed 3 times with PBS and then incubated with fluorescent-labeled secondary antibodies (Alexa fluor® 488, Alexa fluor®594 and Alexa fluor® 647-labeled goat IgG; dilution 1:2,000; Gibco) for 1 hour at RT. After three washes with PBS, the cells were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) in Vectashield mounting medium (Vector laboratory, Temecula, CA). The images were taken on a Zeiss AxioImager fluorescent microscope using Digital Image Processing Software (AxioVision 4.8.1, Carl Zeiss MicroImaging GmbH, Germany).

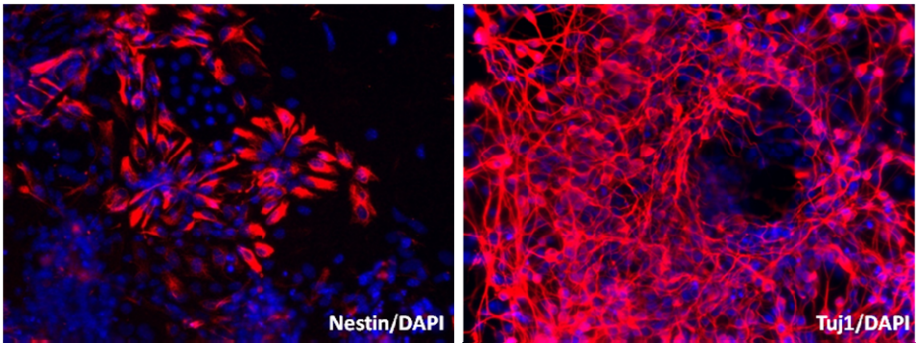

Figure 6. Immunocytochemical analysis for neuronal differentiation. HM1-derived differentiated cells were expressed NPCs marker; Nestin and neuron marker; β-III tubulin or Tuj1 after EB dissociation and plating on PO/laminin (20X, magnification)

2.3.4 Flow cytometric analysis

On day 10 of the neuronal differentiation process, cells were trypsinized into single cells, washed with PBS and centrifuged at 1000 rpm for 5 min. Cells were fixed in 4% PFA for 15 min at RT. Following washing with PBS, cells were stained with the primary antibody Nestin (Rat-401, dilution: 1:50, DSHB; mouse) in 0.1% (v/v) Triton X-100 in PBS for 1 hour at RT. Cells were washed once with 0.05% Tween-20 in PBS, then incubated with Alexa fluor® 647-labeled secondary antibody (goat IgG; dilution 1:500; Gibco) for 1 hour at RT. The cells were washed and re-suspended in PBS. Flow cytometry was performed using a Becton-Dickson (Palo Alto, Temecula, CA) FACS Calibur flow cytometer.

2.4 Statistical analysis

Data concerning flow cytometric analysis are expressed as mean ± SEM and include at least three independent experiments. Statistical analyses for comparison between nestin-positive cells derived from ES and iPS cells were conducted using the Student's *t* test. A $P < 0.05$ was considered statistically significant.

2.5 Experimental designs

Mouse ES and iPS cells were examined for pluripotent characterization by immunocytochemical analysis prior neuronal differentiation. Pluripotent cells were induced to differentiate into the neuronal lineage using EB formation method. Eight-

day-old EBs were observed for morphology analysis. The differentiated cells-derived from ES and SB-iPS cells were collected on day 10 and day 14 of differentiation (referred as 2 days and 6 days after EB dissociation, respectively).

The differentiated cells were investigated for NPCs and neuronal characteristic by immunocytochemical analysis. On day 10, flow cytometric analysis was performed in order to compare the neuronal differentiation ability between ES and SB-iPS cell lines (Figure 7).

2.6 Results

2.6.1 Immunocytochemical analysis of pluripotent cells

The SB-iPS cell lines exhibited characteristics typical for pluripotent stem cells including ES cell-like morphology, strong alkaline phosphatase (ALP) positivity, and pluripotency marker gene expression patterns (verified by quantitative real-time PCR) as it was recently described (Muenthaisong et al., 2012). In this study, iPS cells were examined for immunofluorescent staining patterns as shown in Figure 8. The results showed that SB-iPS cell lines expressing the pluripotency markers Oct4, Sox2 and Nanog when cultured in an undifferentiated state.

2.6.2 Characterization of neuronal phenotype

To investigate the ability of neuronal differentiation of mouse ES and SB-iPS cells, these pluripotent cells were induced to differentiate into NPCs and neurons through EB formation and supplementation of RA (Rungarunlert et al., 2011). The morphology of 8-day old EBs derived from ES and SB-iPS cells are shown in Figure 5A and 5B. We found that the 8-day old EBs derived from ES and SB-iPS cells show the spherical structures with various sizes. SB-iPS cells-derived EB were form smaller aggregate when compared with those from ES cell line. Two days after plating the cells onto culture dishes (day10 of differentiation), the cells exhibited a neuron-like appearance with neurite processes organized in a network (Figure 9C and 9D).

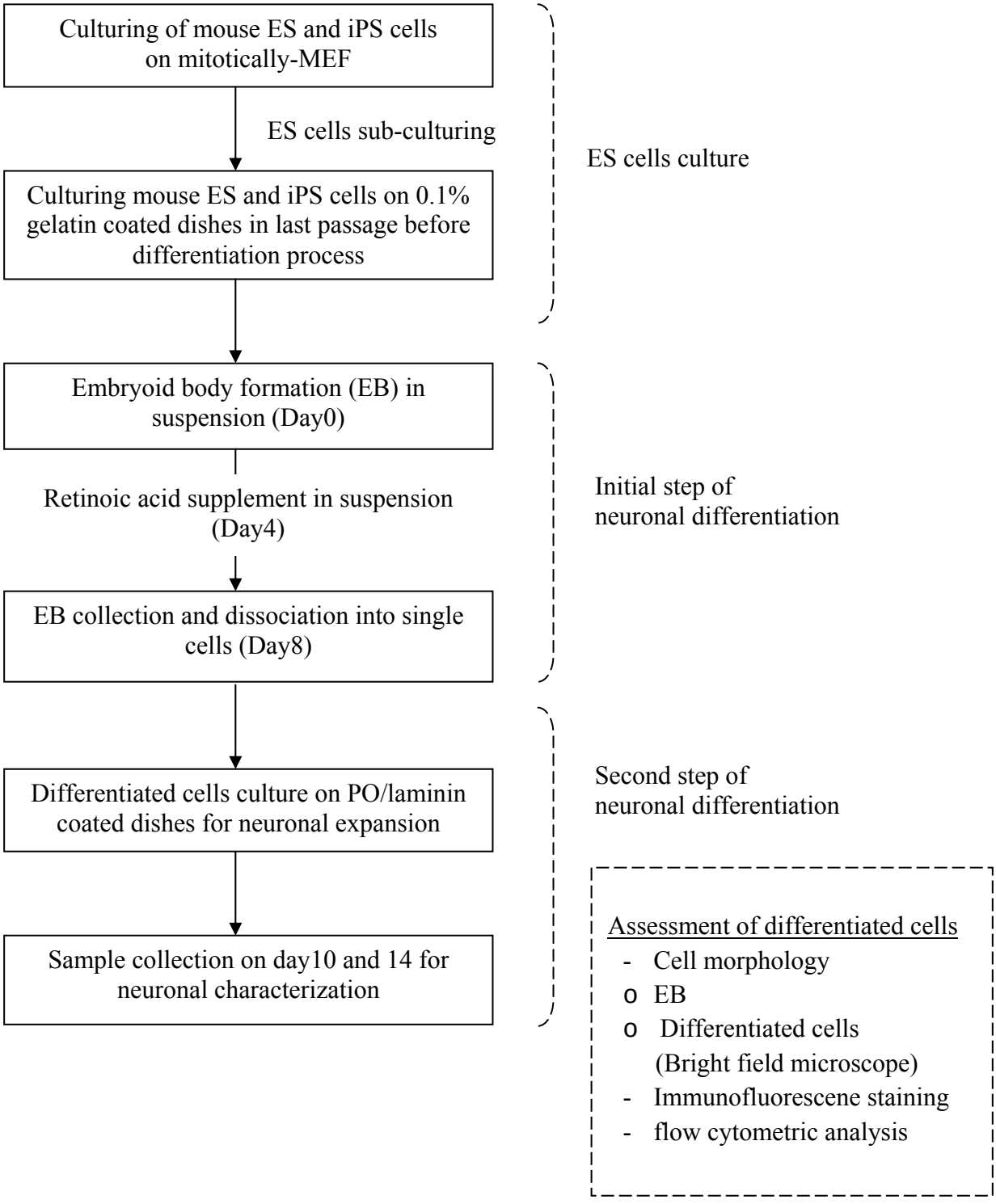

Figure 7. Diagram of experimental design for neuronal differentiation of HM1 ES and SB-mediated iPS cell lines

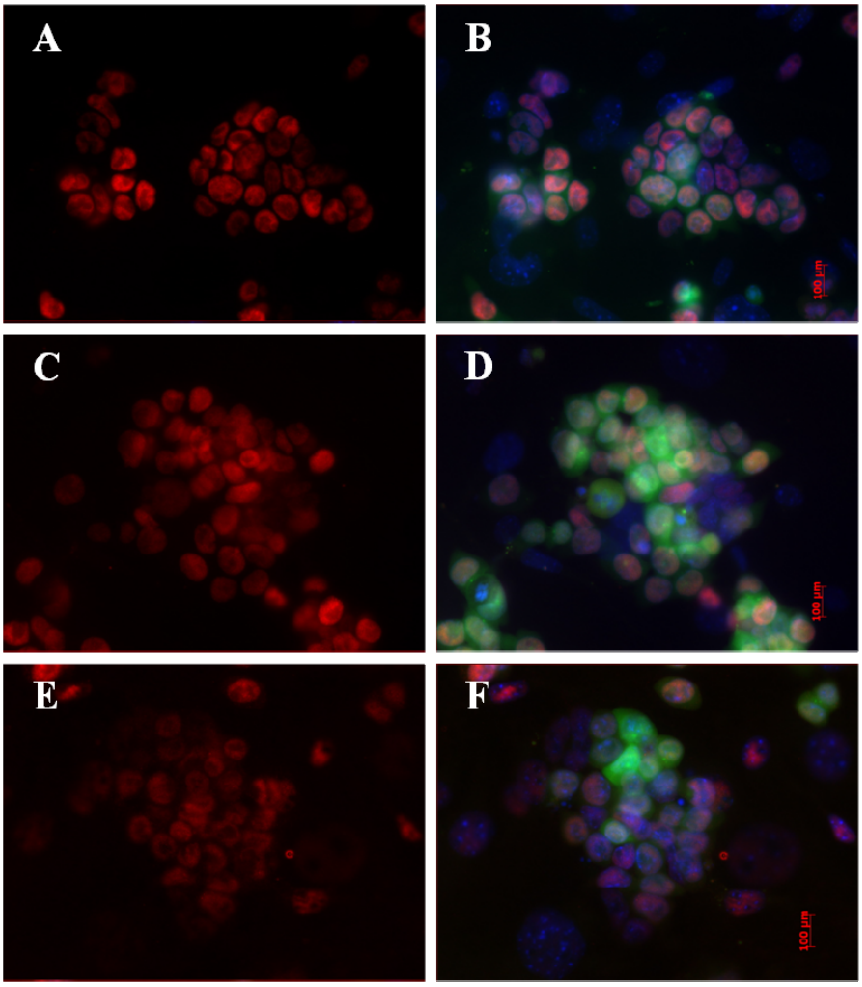

Figure 8. Immunocytochemical analysis of iPS cell line. Mouse SB-mediated GFP-expressing iPS cells (green) were maintained in an undifferentiated stage before neuronal differentiation. The cells were stained with the pluripotent marker Oct4 (A-B), Sox2 (C-D), Nanog (E-F) (red) and the nuclear marker DAPI (blue). Scale bars: 100 μm.

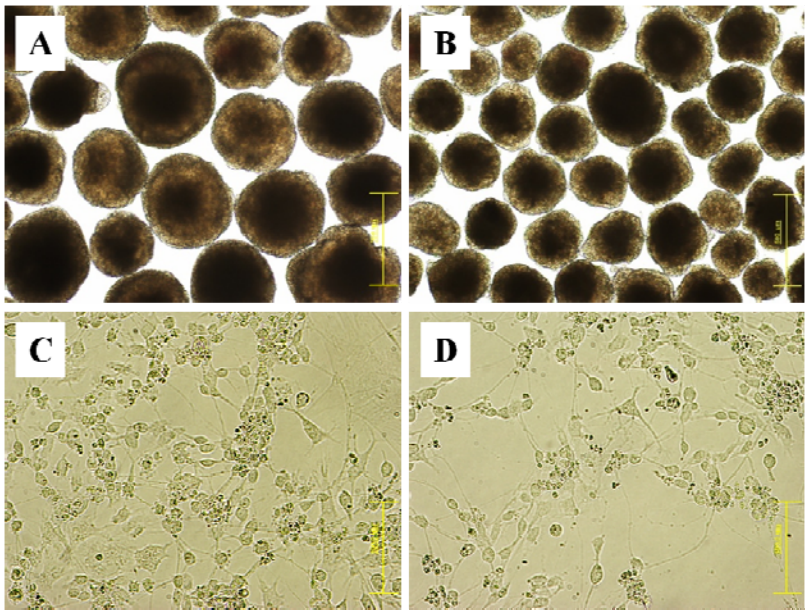

Figure 9. The morphology of mouse ES and iPS cells upon neuronal differentiation. Phase-contrast images demonstrate 8 day-old EBs derived from mouse HM1 ES (A) and SB-mediated iPS cells (B). The cells were induced to differentiate into the neuronal lineage using RA. Neuron-like phenotype such as neurite processes can be observed 2 days after EB dissociation in both ES (C) and iPS (D) cells. Scale bars: 500 µm (A and B) and 100 µm (C and D).

2.6.3 Immunocytochemical analysis of differentiating cells

The result of the immunostaining showed that undifferentiated ES and SB-iPS cells expressed the pluripotency markers Oct4, Sox2 and Nanog (Figure 8). After performing the neuronal differentiation procedure (see Materials and Methods) ES and SB-iPS cells subsequently expressed neuronal markers, as well. Within two weeks in culture ES and SB-iPS cells were able to differentiate into cells expressing Pax6, a neuroepithelial marker (Suter et al., 2009) (Figure 10A and 10B) and nestin, a specific antibody against the intermediate filament protein of NPCs (Lin et al., 1995) (Figure 10C and 10D, respectively). In particular, SB-iPS cells started to generate few amount of neurons indicated by early post-mitotic neuronal marker Tuj-1 (Lee and Pixley, 1994) as shown in Figure 10F, then the neuronal population were gradually increased by day 14 (Figure 10H). Additionally, with approximately 2-3 times lower in number when compared to ES cells (Figure 10E and 10G). Our results

demonstrated that mouse ES and SB-iPS cells have the ability to generate NPCs and differentiate further into neurons through EB formation in culture.

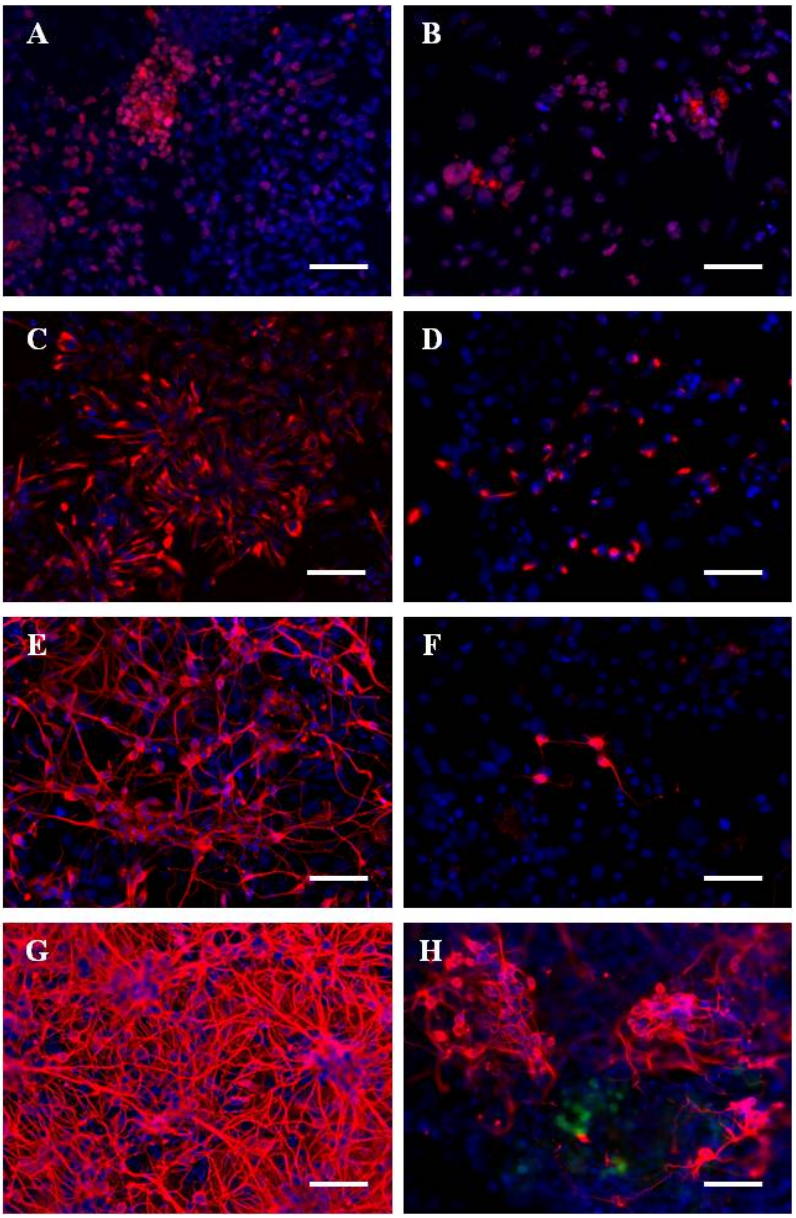

Figure 10. The developmental potential of mouse ES and iPS cells to differentiate from pluripotent stage into neuronal lineage. Immunocytochemical analysis showed the differentiated ES- and iPS-derived neuroepithelial cells (Pax-6) (A and B) and NPCs (nestin) on day 10 (C and D). After bFGF removal, immature neurons (Tuj1-positive cells) are generated and observed at day 10 and 14 from ES (E and G) and iPS cells (F and H), respectively. Scale bars: 200 μm

2.6.4 Comparison of neuronal efficiency between ES and iPS cells

A quantitative analysis was performed by flow cytometry on both ES and SB-iPS-derived NPC populations. Two days after EB dissociation and plating, 76.68% of the ES cell-derived NPCs were labeled by the nestin-specific antibody, while the amount of nestin-positive SB-iPS cells-derived NPCs was only 6.2% (Figure 11). However, we showed that SB-iPS cells have the potential to differentiate further into neurons, though, SB-iPS cells exhibited the unfavorable potential to generate NPCs population.

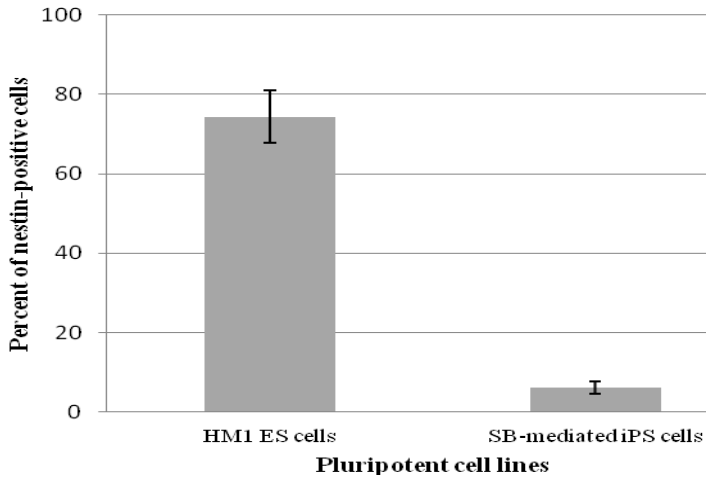

Figure 11. Quantitative analysis of nestin expression in ES- and iPS-differentiated cells by flow cytometry. Representative histogram of flow activated cell sorting (FACS) analysis showing the percent of the NPC marker (nestin) expression in SB-mediated iPS cells compared with mouse ES cells following 10 days of neuronal differentiation.

2.7 Discussion

We have investigated neuronal differentiation of mouse ES and SB-iPS cells using EB formation to initially induce the cells in the presence of RA followed by plating and culture in defined media. Our method allowed SB-iPS cells to aggregate and differentiate in suspension culture and form EBs. Embryoid bodies recapitulate many aspects of cell differentiation during early mammalian embryogenesis, and the

cells can be terminally differentiated into cell types belonging to the three germ layers (Keller, 1995). The lack of structural organization and positional information within EBs during pluripotent cell differentiation results in heterogeneity both within and between EBs. However, high yield of neuronal population can be generated from ES cell-derived EBs by using RA to commit cell fate into neuronal lineage (Kim et al., 2009). This is the first report describing the capability of mouse SB-iPS cells, reprogrammed by the Sleeping Beauty transposon, to differentiate into NPCs and neurons. This is also the first *in vitro* study where the efficiency of neuronal induction of SB-iPS cells was compared with the efficiency of that in ES cell lines. The neuronal phenotypes were observed through phase-contrast microscope (Figure 9C and 9D) and by immunofluorescence staining (Figure 10).

SB-iPS cells were investigated their ability to differentiate into neuroepithelial fate using Pax6 which was an essential transcription factor for neurogenesis in CNS including controlling neural stem cells proliferation and their multipotency (Sansom et al., 2009). Nestin is a type IV intermediate neurofilament, which expressed specifically in neuroepithelial stem cells or NPCs. In the developing embryos, nestin is expressed in both the ventricular and subventricular zones of CNS and is also expressed in radial glial cells (Hockfield and McKay, 1985; Lendahl et al., 1990). We found that differentiated cells derived from SB-iPS cells had a significantly lower level of Pax6- and nestin-positive cells than those originating from ES cells. However, SB-iPS cells did show a potential to differentiate further into neurons expressing Tuj-1, which is a marker of early post-mitotic neural cell types. There are many specific-neuronal markers, apart from Pax6 and nestin, which have been used for determination of NPC fate including SOX1 (Aubert et al., 2003), SOX2 (Ellis et al., 2004), Musashi-1 (MacNicol et al., 2008) and Cx43 (Duval et al., 2002). In order to determine a more specific cell fate of our NPCs, further investigations are required. In the future, by applying these markers (Sox1, Cx43 or Musashi-1) the cell types will be further classified and perhaps differences between ES and SB-iPS-derived NPCs can be revealed.

It has been reported that mouse iPS cells possess morphological, molecular and developmental features closely resembling those of ES cells (Takahashi and Yamanaka, 2006). A recent study showed that different pluripotent cell lines or even

subclones of the same cell line can display different potentials to form EBs or to generate NPCs. The reasons behind these differences are not yet clear. We can only speculate whether it is related to different epigenetic modifications or perhaps cell cycle-related gene expression differences (Martinez et al., 2012). One of probable reasons is the remaining of reprogramming cassette in our SB-iPS cells which the transposase system is now addressing and developed further aim to render the practical and more promising iPS cell lines for efficient and safety application issues. However, these SB-iPS cells have been differentiated into neuronal lineage even transposon construct containing. We hypothesized that the silencing of transgenes in SB-iPS cells perhaps occurred during neuronal differentiation process. Previous report revealed that the repression of the exogeneous pluripotent factors necessitate for allowing efficient cell differentiation toward lineages (Chamberlain et al., 2008). Moreover, incomplete promoter DNA methylation has been reported, which results in the retention of transcriptional memory and may predispose somatic cell-derived iPS cells to differentiate more readily into the particular lineage of their starting cell types (Bar-Nur et al., 2011; Ohi et al., 2011). Also, differences in the cell response toward neuronal-inducers have been detected during iPS cell differentiation (Hu et al., 2009). This might explain the different response of the iPS cells to neuronal differentiation stimuli, which reportedly have more variability than ES cell lines. Consequently, co-culturing of pluripotent cells with stromal cells/conditioned medium (Kawasaki et al., 2000; Yamazoe and Iwata, 2006) or as a monolayer culture in defined medium (Ying and Smith, 2003) may have different effects on ES and iPS cells.

In the future, human iPS cells may become a valuable source of neural cells for the regeneration and repair of tissue for traumatic injuries of the spinal cord and for potential treatment of neurodegenerative disorders including Parkinson's disease, Huntington's disease (Salewski et al., 2010; Schwarz and Schwarz, 2010). However, there are many basic biological and technical issues such as epigenetic modification, efficient system of transposon and transposase construct, genotoxic risk and optimal differentiation strategies which still need to be resolved (VandenDriessche et al., 2009). In conclusion, our study demonstrated that by using the same differentiation procedure, ES and SB-iPS cells show a difference in their capacity to differentiate towards the neuronal lineage. Even though the neuronal differentiation rates of iPS

cells needs to be improved, our results are encouraging and show that SB-iPS cells are capable of forming neurons. Thus, the sleeping beauty transposon-mediated reprogramming approach may be a suitable tool for obtaining these much sought after iPS cell lines.

CHAPTER III

THE DIFFERENTIATION POTENTIAL OF RYBP-DEFICIENT ES CELLS DURING EB FORMATION AND NEURONAL DEVELOPMENT

3.1 Abstract

The role of Rybp (RING1 and YY1 binding protein), the members of the mammalian polycomb-group protein, is critical at multiple stages of mouse embryogenesis and neural development. *In vivo* study showed that reduced Rybp gene expression of heterozygous embryos interfered completed closing of neural tube and early embryonic death in Rybp-deficient embryos. We aimed at investigating the efficiency of *in vitro* neuronal differentiation of Rybp-deficient D11 (*rybp* $^{-/-}$) and the wild type R1 embryonic stem (ES) cells (*rybp*$^{+/+}$) as a control cell line. Mouse ES cells were induced to differentiate into neuronal lineage via embryoid body (EB) formation and all-trans retinoic acid (RA) supplementation. For the EB morphology analysis, EBs were observed with a phase-contrast microscope. The immunocytochemistry and immunohistochemistry for proliferative cells (KI-67), apoptotic cells (cleaved caspase-3), neuroectoderm (Pax6), neuronal progenitor cells (NPCs) (Musashi-1), mature neuron (NeuN) and motor neuron (Isl-1) were performed to characterize the differentiated cells. The quantitative analyses for gene and protein expressions were also performed by qRT-PCR and flow cytometric analysis. On day 8, the rosette-like structures contained NPCs were found in both R1 and D11-derived EBs. Several neuronal gene expressions both NPCs and neurons specific markers of D11 derived cells were lower than those in controls. On day10, the percentage of nestin-positive cells derived from D11 ES cells were found to be significantly lower than those derived from R1 ES cells. This results suggested that Rybp-deficient ES cells can be induced to generate NPCs and neurons *in vitro*. However, the efficiency was poorer when compared to the wild-type. We concluded that Rybp is necessary for ES cell differentiation into neuronal lineages. Using Rybp-deficient ES cell line may provide great a opportunity to investigate developmental role of Rybp in neurogenesis *in vitro*.

3.2 Introduction

Mouse ES cells hold much promise to use as model for the investigation of mammalian neurogenesis. The development of novel strategies to give rise the functional neuron cells is challenging to translate the potential of stem cells to serve as useful platforms for study the functions of genes and biological events in neuronal development. Previous study demonstrated that the spatially and quantitatively appropriate Rybp gene expression is required for normal morphogenesis in central nervous system (CNS). Rybp levels can cause CNS malformations including forebrain overgrowth and defective neuronal tube closure, while the embryonic death has been reported in Rybp-deficient chimeras (Pirity et al., 2005). In addition, the role of Rybp has also addressed in mouse retina and lens development. From the study using *in vivo* mouse models, Rybp has been observed in lens, corneal epithelium, corneal stroma, optic nerve and perioptic mesenchyme. Therefore, several ocular defects have demonstrated from transgenic mice both ablation and overexpression of Rybp resulting in retinal coloboma, an incomplete closure of the optic fissure, corneal neovascularization and len opacification (Pirity et al., 2007). Rybp deficient ES cells are not lethal as in transgenic null embryos. Differentiation of ES cells into neuronal lineages thus represents a new opportunity to explore the molecular function of Rybp-related neurogenesis.

Here, we investigated the ability of Rybp-deficient (D11) ES cells to generate the neuronal population *in vitro* through EB formation method comparing with wild type (R1) ES cell line.

3.3 Materials and methods

Materials and cell culture condition

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) and culture media were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA), unless specified otherwise. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Medium was changed daily for mouse ES cell culture and on every second days during differentiation.

3.3.1 Mouse pluripotent cell cultures

Mouse Rybp-deficient ES cells (*rybp* $^{-/-}$) were kindly provided by Melinda K. Pirity. Wild type R1 ES cells (*rybp*$^{+/+}$) and Rybp-deficient D11 ES cells (*rybp* $^{-/-}$) (referred as D11 ES cells) were cultured on mitomycin C inactivated mouse embryonic fibroblast (MEF) cells. Pluripotent cells were maintained in ES medium: Dulbecco's modified Eagle's medium (DMEM) containing 15% (v/v) fetal bovine serum (FBS, Sera Laboratories International, West Sussex, RH17 5PB, UK), 0.1 mM non-essential amino acids (NEAA), 0.1 mM β-mercaptoethanol (β-ME), 50 U penicillin/ml, 50 µg streptomycin/ml and 2,000 U/ml mouse leukemia inhibitory factor (LIF, ESGRO, Chemicon International, Temecula, CA). Pluripotent cells were cultured on gelatin-coated dish for at least one passage before the differentiation process to deplete MEF cells.

3.3.2 *In vitro* neuronal differentiation

Mouse pluripotent cells were induced to differentiate into neuronal lineage as previously described (Klincumhom et al., 2012). Mouse ES cell colonies were harvested into single cells using 0.05% (w/v) Trypsin, then seeded at a density of 3×10^5 cells/ml in differentiation medium (ES medium without LIF) onto bacteriological dishes pre-coated with Poly (2-hydroxyethyl methacrylate) (Poly-HEMA) to prevent cell attachment. Pluripotent cells were allowed to aggregate to form EBs for 8 days, then last 4 days during this period, 5 µM all-trans RA was added to the medium. Thereafter, 8-day-old EBs were dissociated and plated onto Poly-L-ornithine and laminin (Roche, CA) coated dish at a density of 2×10^5 cells/cm^2 in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F-12) containing 3 mg/ml D-(+)-Glucose, 3 mg/ml AlbuMaxI, 50 U/ml penicillin, 50 µg/ml streptomycin, 1% (v/v) N2 supplement and 10 ng/ml recombinant human FGF basic (bFGF). On day 10 of culture, the medium was changed to DMEM/F12:Neurobasal medium (1:1)**,** 1 mM glutamax**,** 3 mg/ml AlbuMaxI, 50 U/ml penicillin, 50 µg/ml streptomycin, 0.5% (v/v) N2 Supplement and 1% (v/v) B27 supplement. For EB morphology analysis, ES cells were seeded at density 1,000 cells in 200 µl of differentiation medium in each well of a 96-well plate (round bottom) for EB formation. The medium was renewed every second days.

3.3.3 EB morphology analysis

EBs were transferred into Dulbecco's Phosphate-Buffered Saline (DPBS) and visualized in bright-field during the suspension period with a Nikon TE 2000 inverted microscope (Nikon Inc., Melville, NY,) with a SpotFLEX camera (Diagnostic Instruments, Inc., Sterling Heights).

3.3.4 Histological analysis

At day 8 before EB dissociation, EBs were collected and fixed in 4% (w/v) PFA for 15 min. EBs were washed three times in PBS and embedded in paraffin, and examined for general histological analysis. Serial sections were generated and of 4-μm thickness sections were stained with proliferation marker (Ki-67) and apoptosis marker (cleaved caspase-3). For neuronal characterization, the sections were stained for neuroepithelial markers Pax6 (Pax6, dilution 1:200, DSHB; mouse), neuronal progenitor marker Musashi-1 (AB5977, dilution 1:200, Millipore; rabbit), neuronal marker NeuN (MAB 377, dilution 1: 100, Millipore; mouse) and motor neuron marker Isl-1 (40.2D6, dilution 1:50; DSHB; mouse). The images were visualized in a Zeiss AxioImager fluorescent microscope using Digital Image Processing Software (AxioVision 4.8.1, Carl Zeiss MicroImaging GmbH, Germany).

3.3.5 Immunocytochemical analysis

After EB dissociation, the differentiating cells (day10 and day14) were cultured on gelatin- and poly-L-ornithine/laminin-coated coverslips. Cells were washed with phosphate-buffered saline (PBS) and fixed with 4% (w/v) paraformaldehyde (PFA) for 15 min at room temperature (RT). Fixed cells were washed and stored in PBS at 4°C until analysis. After permeabilization with 0.2% (v/v) Triton X-100 (for intracellular staining) for 30 min at RT, cells were blocked with 3% (w/v) Bovine serum albumin with 0.5% (v/v) Tween20 in PBS for 30 min. Then cells were sequentially incubated with the following primary antibodies in blocking solution overnight at 4°C: Nestin (Rat-401, dilution: 1:200, DSHB; mouse), neuronal progenitor marker Musashi-1 (AB5977, dilution 1:200, Millipore; rabbit), β-III Tubulin (Tuj1, dilution 1:2,000; Covance, PRB-435P; rabbit) and Isl-1 (40.2D6, dilution 1:50; DSHB; mouse). The cultures were washed 3 times with PBS and then

incubated with fluorescent-labeled secondary antibodies (Alexa fluor®594-labeled goat IgG; dilution 1:2,000; Gibco) for 1 hour at RT. After three washes with PBS, the cells were counter stained with 4',6'-diamidino-2-phenylindole (DAPI) in Vectashield mounting medium (Vector laboratory, Temecula, CA). The images were visualized on a fluorescent microscope.

3.3.6 Quantitative RT-PCR assay

Total RNA will be extracted from EBs and differentiated cells on days 2, 8 10 and 14 using the RNeasy Mini Plus Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Nucleic acid concentration was measured by NanoDrop. For each sample, 500 ng RNA was reversely transcribed using the using the First-Strand cDNA Synthesis Kit (SuperScript III Kit, Invitrogen, Carlsbad, CA, USA), as per the manufacturer's instructions. After the reverse transcription, the cDNA samples were diluted to 2.5 ng/µl concentrations and dispensed to once-use aliquots. For quantitative RT-PCR (qRT-PCR) reaction SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) were carried out in Qiagen Rotor-GeneQ machine. The reaction conditions were template denaturation and polymerase activation at 95°C for 3 min followed by 40 cycles of 95°C denaturation for 5 sec, 60°C annealing for 20 sec and 72°C extension for 30 sec with a single fluorescent measurement at each cycle. When the reaction was completed, melting curves were plotted to confirm the specificity of the product. The reference gene used for analysis of the relative expression was GAPDH and the following primers (Table 1) were designed for the amplification of specific products.

3.3.7 Flow cytometric analysis

On day 8 and 10 of neuronal differentiation process, the EBs and differentiated cells were trypsinized to single cells, then washed with PBS and centrifuged at 1000 rpm for 5 min. Cells were fixed in 4% (w/v) PFA for 15 min at RT. After washing with PBS, cells were stained with primary antibodies for nestin, neuronal progenitor marker (Rat-401, dilution: 1:50, DSHB; mouse) in 0.1% (v/v) Triton X-100 in PBS for 1 hour at RT. Cells were washed once with 0.05% Tween-20 in PBS, then incubated with Alexa fluor® 647-labeled secondary antibody (goat IgG;

dilution 1:500; Gibco) for 1 hour at RT. The cells were washed and re-suspended in PBS. Flow cytometry was performed using a Becton-Dickson (Palo Alto, Temecula, CA) FACS Calibur flow cytometer.

3.4 Statistic analysis

The statistical differences were assessed by student's t-test using SPSS software (version17.0 SPSS Inc., Chicago, IL, USA). Data was expressed as a mean ± standard error of the mean (SEM), of at least three independent replications. The results were considered significantly different when P-value was less than 0.05 (*).

3.5 Experimental design

This study aimed to investigate the ability of D11 Rybp-deficient ES cells to generate the neuronal population *in vitro* through embryoid bodies (EBs) formation method comparing with R1 wild type ES cell line. Rybp-deficient D11 and wild-type R1 ES cells were induced to differentiate into neuronal lineage using EB formation method. Eight-day-old EBs were collected for histological analysis. The EBs and differentiated cells were observed for morphological analysis. Protein expression of differentiated cells assessed by immunocytochemistry and flow cytometric analysis was performed for neuronal characterization. Total RNA was extracted from EBs and differentiated cells on day 2, 8, 10 and 14 for quantitative RT-PCR.

Table 1. Primers used for determination of mRNA expression levels by quantitative RT-PCR (Experiment 2)

Gene name	Forward primer	Reverse primer	Product size (bp)
Nestin	AGTGCCCAGTTCTACTGGTGTCC	CCTCTAAAATAGAGTGGTGAGGGTTGA	125
Musashi-1	TCGCTCTCAAACGTGACAAATC	CACGGTGGAAGATGTGAAACA	120
Tuj-1	TGAGGCCTCCTCTCACAAGT	GGCCTGAATAGGTGTCCAAA	104
NeuroD1	ACGAGGAACACGAGGCAGAC	TTTTTGGGACCCCGTCTCTT	171
GAPDH	CATGTTCCAGTATGACTCCACTCACG	CCAGTAGACTCCACGACATACTCAGCA	164

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NeuroD1, Neurogenic differentiation factor 1

3.6 Results

3.6.1 Morphological and histological analysis of D11$^{(-/-)}$ derived EBs

After 2 days of EB formation, single ES cells have already aggregated and form into spherical structure floating in the differentiation medium (Figure 12C and 12D). The uniform size and shape could be observed from EBs cultured in 96-well plate seeded with equal numbers of ES cells. However, the size of ES cell-derived EBs between the two cell lines were obviously different on day 8 as the R1 ES cell-derived EBs were about 2 times larger than those observed from D11 ES cell line (Figure 12E and 12F).

In histological analysis (Figure 13), by day 8 of differentiation, both R1 and D11 derived EBs formed the cavity located mostly at the center of the EB structure. We found that this cavity was contained with the apoptotic cells detected by cleaved caspade-3 marker. While the KI-67 positive cells represented cell proliferative activity were found mostly at periphery part surrounding the apoptotic area within the EBs. At this proliferative zone, Pax6 and Musashi-1 positive cells which are neuroepithelial and NPC-specific markers, respectively, were presented and formed as rosette-like structures in both R1 and D11-derived EBs (black arrows). Outside the rosette-like structures, both R1 and D11 derived EBs were found the cells expressing the mature neuronal marker NeuN. The Isl-1 positive cells indicating the motor neuron characteristic was also presented in R1 derived EBs. However, this was hardly found in D1-derived EBs.

3.6.2 The immonocytochemistry studies on neuronal differentiation ability of D11$^{(-/-)}$ ES cell line

After 8 days of EB culture, EBs were dissociated and re-plated for further differentiation on monolayer culture into neuronal lineages. The immunocytochemical analysis showed that R1 and D11 ES cells were efficiently differentiated into neuronal lineages which were subsequently detected by early (Nestin and Musashi-1) and late (Tuj-1 and Isl-1) neuronal markers. During day 10 to day 14 of differentiation, all neuronal markers were presented in both cell lines. However, the number of neuronal markers-positive cells from D11 ES cell line was approximately lower than the wild-

type. More distinct neuronal characterization between the two cell lines could be observed on day 14. Nestin and Musashi-1 positive NPCs were formed the rosette-like pattern in R1, but not in D11 derived cells (Figure 14A and 14B). The neuronal bodies and elongated branches were illustrated by Tuj-1 marker. The thicken branches formed neuronal network was obviously showed in R1 derived cells (Figure 14C).

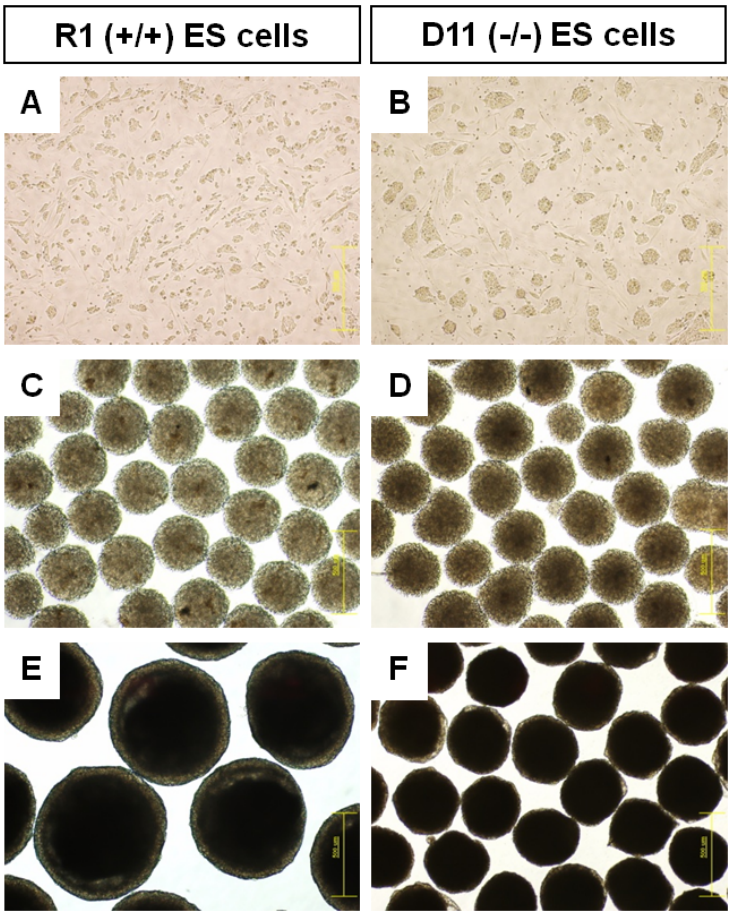

Figure 12. Embryoid body formation of $R1^{(+/+)}$ and $D11^{(-/-)}$ ES cell lines**.** Mouse R1 and D11 ES cells were maintained on gelatin at least one passage prior differentiation (A-B). The morphology analysis showed the uniform spherical ES cells-derived EBs cultured in 96-well plate (C-D). Small EBs can be clearly observed 2 days after differentiation. EBs were subsequently cultured in differentiation medium supplemented with RA until day 8. The uniform spherical EBs were observed in ES cells-derived EBs cultured in a 96-well plate (E-F). Scale bars = 500 μm

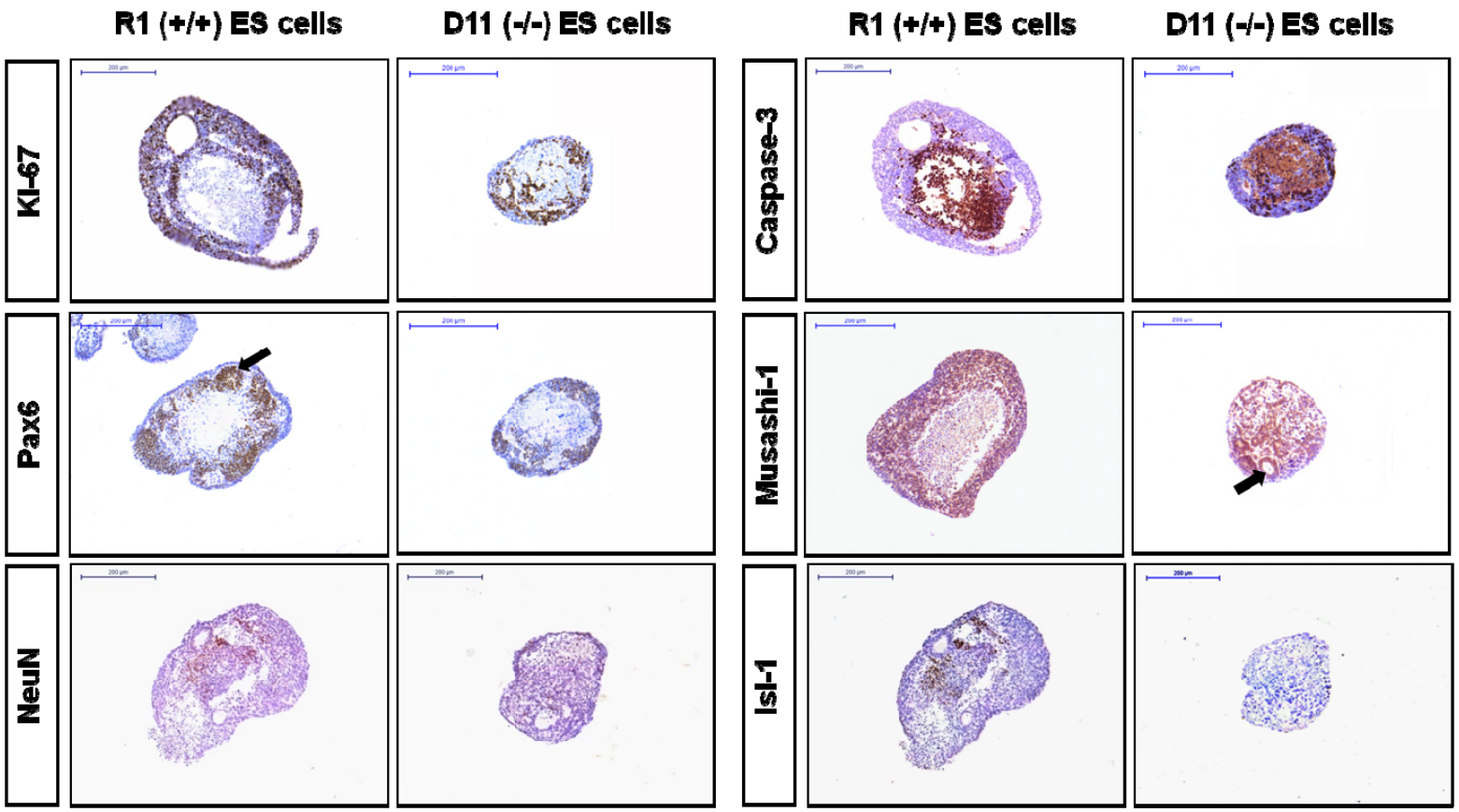

Figure 13. Neuronal differentiation of R1 $^{(+/+)}$ and D11$^{(-/-)}$ ES cells during EB formation. The cross-sections of eight-day-old EBs were investigated for proliferation marker KI-67, Apoptotic marker cleaved caspase-3. The neuronal characterization was performed using neuroepithelial marker Pax6, NPCs marker Musashi-1, neuronal marker NeuN and motor neuron marker Isl-1. Scale bars = 200 µm.

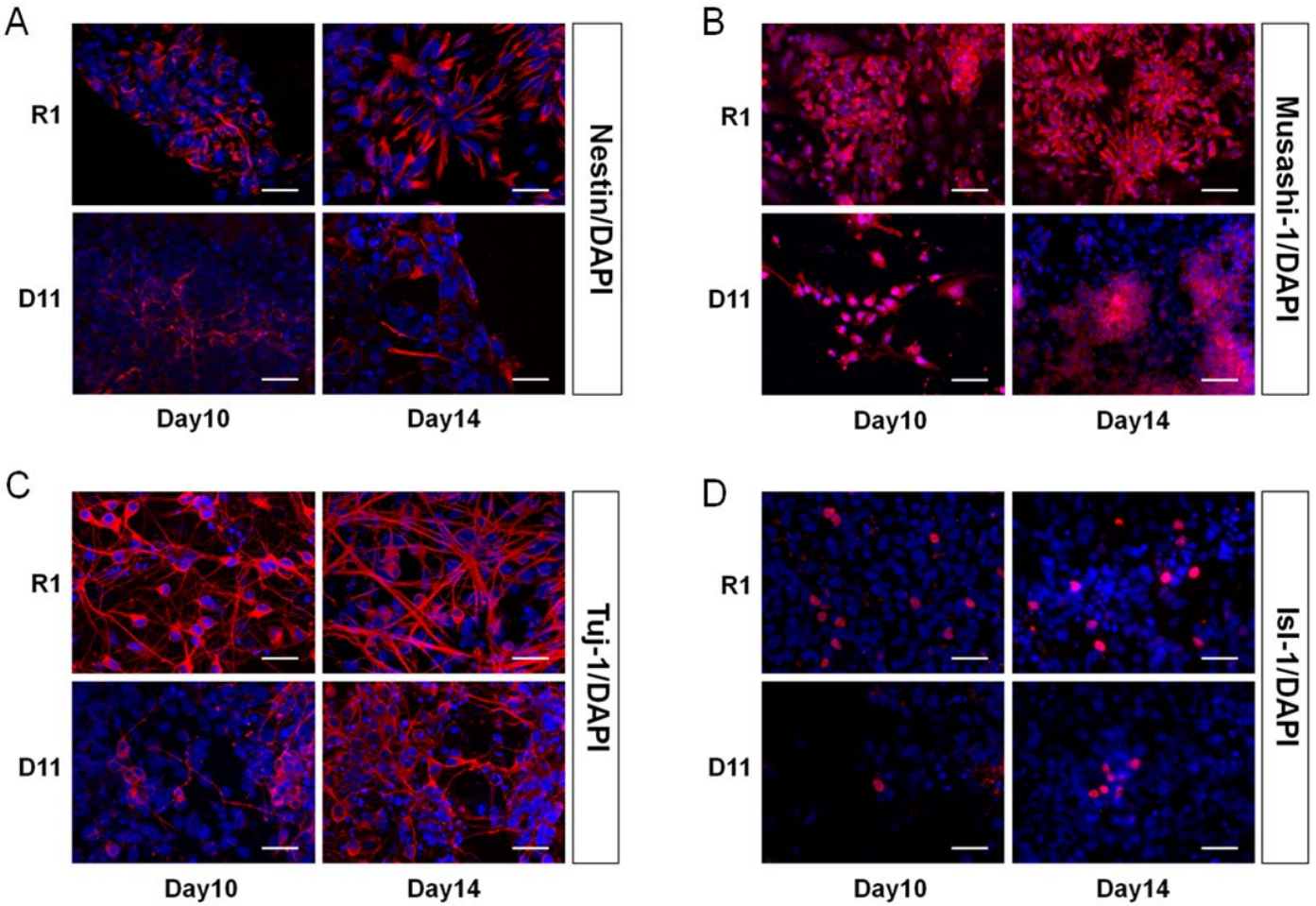

Figure 14. Neuronal characterization of R1 $^{(+/+)}$ and D11$^{(-/-)}$ derived cells. After EB dissociation and re-plating, the differentiated cells derived from R1 $^{(+/+)}$ and D11$^{(-/-)}$ ES cells were investigated for neuronal characteristic using Nestin (A), Muasashi-1 (B), Tuj-1 (C), and Isl-1 (D) on day 10 and day 14 of differentiation (red). DAPI was used for nuclei staining (blue). Scale bars = 100 μm.

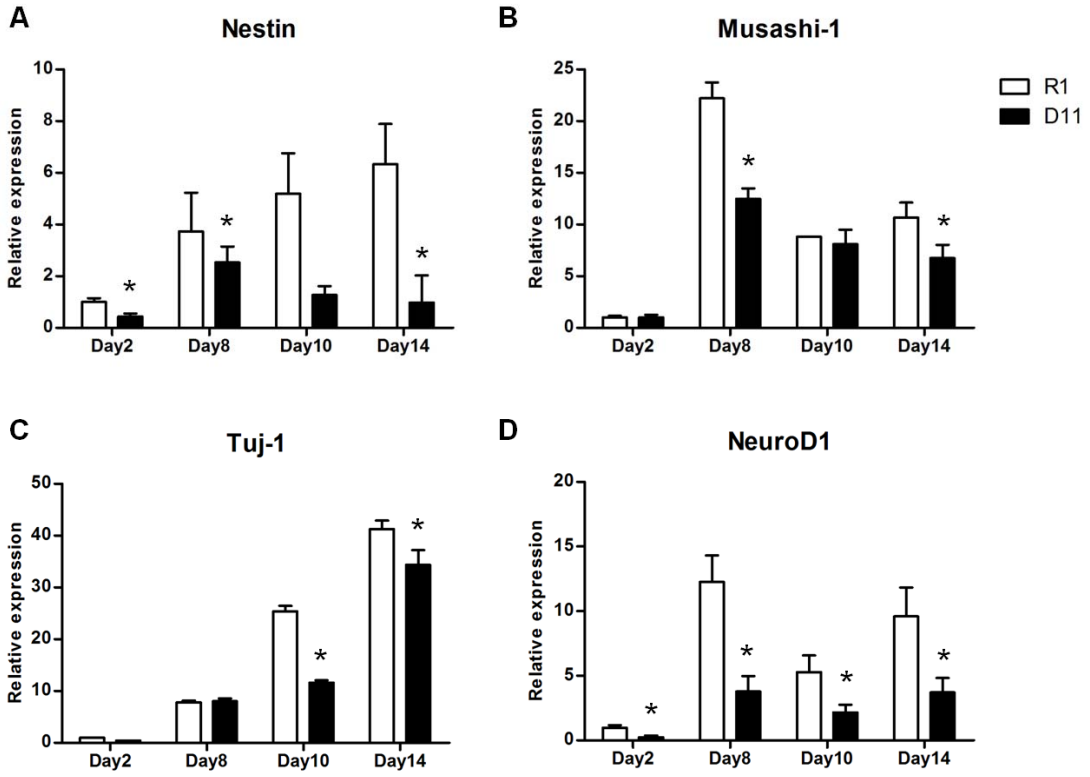

Figure 15. Developmental potential of R1 $^{(+/+)}$ and D11$^{(-/-)}$ derived cells during neuronal differentiation. The differentiated cells were investigated for gene expression levels of Nestin (A), Musashi-1 (B), NeuroD1 (C), Tuj-1 (D) and MAP2 (E) during day 2 until day 14 of differentiation using quantitative RT-PCR analysis. Gene expression was normalized to the house keeping gene, GAPDH. Results are means ± S.D. and represent the relative expression compared with mRNA levels of EB day2 derived from wild-type (R1) cell line (arbitrarily set at 1). Relative mRNA levels of differentiated cells derived from R1 (open bars) and D11 ES cells (black bars) were compared with statistical analysis indicate values that differed significantly, $P < 0.05$ (*).

3.6.3 Gene expression analysis during neuronal differentiation of D11$^{(-/-)}$ ES cells

NPCs and neurons-specific gene expression were detected from Rybp-deficient ES cells line, beginning from EB culture period. The comparison of mRNA level of neuronal characteristics of differentiated cells derived from R1 and D11 ES cells were investigated by quantitative RT-PCR analysis. In both D11 and R1 differentiated cells, the expression of NPCs genes including Nestin and Musashi-1 were up-regulated from day 2 until day 8 of EB suspension culture. Thereafter EB dissociation and re-plated on poly-L-ornithine and laminin on day 8, those gene expressions were gradually down-regulated. Interestingly, only the expression level of Nestin of R1 derived cells was still gradually increased until day 14 of differentiation (Figure 15A and 15B). R1 and D11 derived EBs were observed for mature neuronal-specific genes expression including Tuj-1 and NeuroD1. The early neuronal marker Tuj-1 was gradually up-regulated since day 2 until day 14. D11 derived cells showed similar gene pattern, but with the remarked lower level compared to those of R1 wild-type after EB disaggregation (Figure 15C). We found that expression of NeuroD1 mRNA level from both R1 and D11 derived cells were rapidly increased during EB suspension culture, beginning on day 2. The expression levels of NeuroD1 in D11 derived EBs and differentiated cells were significantly lower than wild-type group throughout differentiation culture (Figure 15D). Our study demonstrated that several neuronal gene expression both NPCs and neurons of D11 derived cells were lower than control cell line. The results indicated that loss of Rybp from ES cells can reduce their neuronal differentiation capacity *in vitro*.

3.6.4 Protein expression during neuronal differentiation of D11$^{(-/-)}$ ES cells

The related results were demonstrated by expression of NPC (Nestin) and neuronal (NeuN) markers using flow cytometric analysis. On day 8 of differentiation, the percentages of nestin positive cells were not significantly different between D11 and R1 groups (5.87±1.47 vs 16.73±7.49, respectively). Two days later (day 10), the percentage of nestin-positive cells derived from both groups marked increased. The D11-derived cells were found to be significantly lower nestin expression than those derived from R1 ES cells (80.42±4.27 vs 42.62±8.84, respectively) (Figure 16A). The similar pattern was observed in NeuN expression of both cell lines. However, there

were no significantly difference was observed between D11 and R1 ES cell lines during day 8 (0.81±0.18 vs 3.26±2.23, respectively) to day10 of differentiation (1.99±0.37 vs 4.33±0.98, respectively) (Figure 16B).

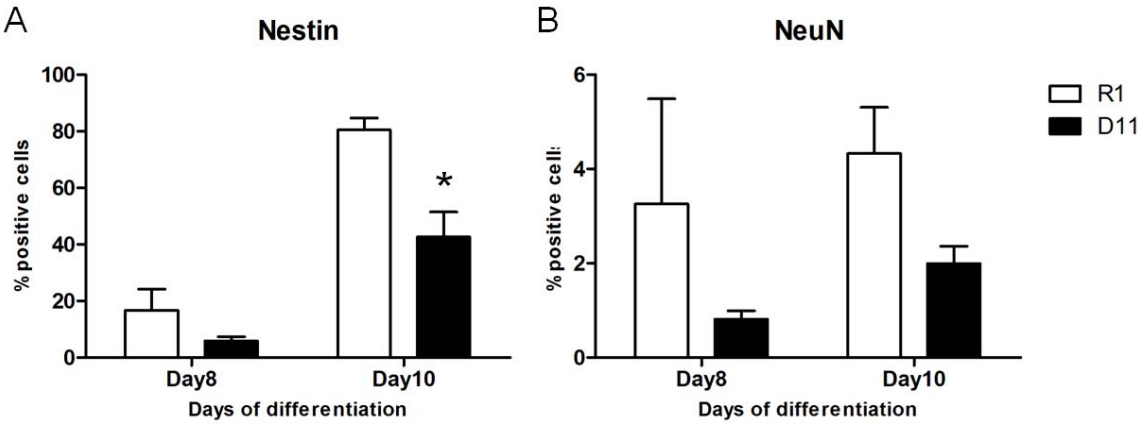

Figure 16. Quantitative analysis of neuronal gene expression R1 $^{(+/+)}$ and D11$^{(-/-)}$ derived cells by flow cytometry. Representative histogram of flow activated cell sorting (FACS) analysis showing the percentage of the NPCs marker Nestin and neuronal marker NeuN expression in D11$^{(-/-)}$ derived cells compared with wild-type R1 ES cells during day 8 to day 10 of neuronal differentiation, $P < 0.05$ (*).

3.7 Discussion

Normal embryogenesis is regulated by the orchestration of several transcription factors in concentration, stage and position dependent manner (Gage, 2000; Jessell, 2000; Altmann and Brivanlou, 2001). Here, we first investigated the role of Rybp, polycomb group (PcG) protein during neuronal differentiation *in vitro*. With many aspects, the *in vitro* neuronal development of Rybp-deficient ES cells represented negative impact resembled to that previous described *in vivo* (Pirity et al., 2005). The *in vitro* neuronal differentiation ability of Rybp-deficient ES cells through EB culture was demonstrated in this study.

In presence of RA, EB formation method initially induced the differentiation of ES cells into early neuronal fate, followed by their further differentiation into functional neurons at transplantation site (Kumar et al., 2007; Ideguchi et al., 2010).

Here, EB formation culture was used as initial step to induce undifferentiated ES cells into neuronal lineages. Both cell lines exhibited their potential to form EBs. However, we found that loss of Rybp in ES cells showed to impair EB development. Several studies revealed that ES cells differentiation using EB formation method can imitate the development of embryos (Desbaillets et al., 2000; Vallier et al., 2005; Sheridan et al., 2012). Recently study indicated that the different sizes of EBs can represent the difference of differentiation ability between ES cell lines or even among sub-clones (Martinez et al., 2012). From our results, D11-derived EBs mostly contained with apoptotic cells that is probably caused by the fact that Rybp is essential gene regulator required in many aspects of cell survival and differentiation during development (Ringrose and Paro, 2004). This result is in an agreement to the mortality of Rybp-deficient mice at early post-implantation stage (Pirity et al., 2005).

EBs were further investigated for neuronal characteristics by immunohistochemistry. The Pax-6 positive neuroepithelial cells and Musashi-1 positive NPCs were formed as rosette-like structures within both R1 and D11 derived EBs. Recent study demonstrated the same ring structures presented in 10-day-old EBs treated RA. These structures consisted of several cell types, presenting in the neural tube of early embryo (Khoddami and Becker, 1997; Chiba et al., 2005). This neural tube-like structures in culture condition can facilitate the morphological and molecular events of neural tube formation during early embryogenesis in both human and mouse (Zhang et al., 2001; Rathjen et al., 2002).

By quantitative RT-PCR and flow cytometric analysis, our study supported that Rybp is required for ES cells differentiation toward early (Nestin and Musashi-1) and late (Tuj-1, NeuroD1 and NeuN) neurons (Figure 15 and Figure 16). Recent study indicated that Rybp is necessitated for normal gene regulation to exert the proper differentiation of ES cells (Hisada et al., 2012). Similar findings reported that the neuronal differentiation ability of mutant ES cell lines has been clearly impaired when transcription factors-related neurogenesis were depleted from their genome (Muller et al., 2003; Suter et al., 2009; Quinn et al., 2010).

We notably found that loss of Rybp disrupted the generation of motor neurons in which a few Isl-1 positive cells were founded in D11-derived cells compared to the wild-type R1, observing on day 10 and day 14 (Fig. 3D). However, this result was not

surprising given that Rybp mRNA is mostly expressed in cells of the developing CNS with highest levels in the spinal cord and forebrain of mouse embryo (Schoorlemmer et al., 1997; Garcia et al., 1999). Our study thus supports the requirement of Rybp in generation of motor neurons generation during neurogenesis. However, the further quantitative analysis is required.

In this study, the neuronal development of a mutant ES cell line respectively affects the neuronal differentiation *in vitro*. Using Rybp-deficient ES cell line illustrate that Rybp gene play an important role in neurogenesis. Further studies for determining the underlying mechanism of Rybp-related neurogenesis remain elusive.

CHAPTER IV

SELECTIVE TGF-β1/ALK INHIBITOR ENHANCES NEURONAL DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS

4.1 Abstract

Study of mouse embryonic stem (ES) cells as a model provides our understanding in complex molecular and cellular events during early development and for regenerative medicine. The TGF-β1 is a polypeptide member of TGF-β superfamily that plays a central role in determining the neuronal fate of ES cells. This study examined the effect of a selective inhibitor of TGF-β1/ALK (A83-01), on neuronal differentiation.

Mouse ES cells were allowed to aggregate into embryoid bodies (EBs) and cultured in neuronal differentiation medium supplemented with or without TGF-β1 inhibitor. The effects of TGF-β1 inhibitor on neuronal differentiation of mouse ES cells-derived EBs were consecutively examined by means of morphology analysis, immunohistochemistry and quantitative RT-PCR. Two days after differentiation, EB morphology was similar between the two experimental groups. However, the diameters of EBs treated with A83-01 (592.6±27 μm) were significantly smaller than the control group (631.7±14.1 μm, $P< 0.01$). Eight-day-old EBs derived from the two groups similarly contained a complex network of mixed population of early and mature neurons as indicated by Pax-6 and NeuN staining, respectively. Comparing to the control, TGF-β1 inhibitor potentially suppressed transcription factor Oct4 while rapidly up-regulation of neuronal associated genes (Sox1 and MAP2). Furthermore, this inhibitor also down-regulated astrocyte related gene (GFAP) compared favorably to non-treated control.

It is concluded that selective TGF-β1/ALK inhibitor efficiently stimulates the cell fate alteration from ES state toward neuronal lineages. This alternative strategy would be useful for generating uniformity of neuronal population for neuronal cell-based therapy.

4.2 Introduction

Most of neurological diseases are considered as incurable diseases due to the limited capability of central nervous system (CNS) of mammal, during adulthood, to regenerate upon the injury (Davies et al., 1997). Novel strategies have been developed in attempt to repair or to replace neuronal cells, including cell transplantation, treatment with antibody against myelin-associated neurite growth inhibitors and therapeutic targeting neuronal-specific receptors (Merkler et al., 2001; Papadopoulos et al., 2002; Lee and Pfaff, 2003).

Pluripotent stem cells have become an alternative source of neuronal cells because they have theoretically capability to provide an unlimited source of required cells that can later on give rise to all three embryonic germ cell layers (Keller, 1995). Although mouse ES cells holds much promise to use as a model for investigation of mammalian neurogenesis, the generation of neurons for therapeutic proposes remains inefficient for specific neuronal diseases such as stroke, spinal cord injuries, Parkinson's and Alzheimer's diseases (Langston, 2005; Taupin, 2006). Many studies demonstrated the robust generation of neuronal cells from ES cells via spontaneous differentiation using embryoid body (EB) formation technique. The EB is the aggregation of ES cells that can spontaneously differentiate into derivatives of three-embryonic germ layers including ectoderm, mesoderm and endoderm (Okabe et al., 1996; Kurosawa, 2007). The committed cells within the EB are then induced to differentiate into neuroepithelial cells and neurons by biologically active form of retinol (retinoic acid, RA) treatment because this is an essential factor driving the CNS development during embryogenesis (Ross et al., 2000; Maden, 2001; Bibel et al., 2004). However, efficiency of neuronal differentiation within the EBs is marginal for clinical use due principally to the heterogeneous population of undifferentiated/ differentiated ES cells and its safety (Ying and Smith, 2003). Improving strategy of neuronal differentiation of ES cells is therefore prerequisite for studying the cellular and molecular mechanisms of early embryo development and neurogenesis and also providing the high-yield generation of homogenous neuronal cell subtypes for cell transplantation and regenerative medicine.

Transforming growth factor β 1 (TGF-β1) is a polypeptide member of TGF-β superfamily that is comprised of Bone morphogenetic proteins (BMPs), Growth and

differentiation factors (GDFs), Activin, Nodal and TGF-β family. These factors have been demonstrated as a pleiotropic factor as the effects depend on their concentrations as well as the combination with other growth factors. The signaling transduction of these factors is modulated through SMAD phosphorylation (ten Dijke and Hill, 2004). The expression and functions of transcription factors in TGF-β superfamily are conserved among the species studied and actively involved in cellular homeostasis and differentiation (Dunker and Krieglstein, 2000; Massague and Chen, 2000). Over expression of Nodal in mouse ES cells induced up-regulation of both mesodermal and endodermal genes but the neuroepithelial markers were down regulated (Pfendler et al., 2005). Furthermore, proliferation of mouse hippocampal neural progenitor cells and neuronal cell production were inhibited by TGF-β1 treatment (Buckwalter et al., 2006).

Since TGF-β1 stimulation appears to negatively affect neuronal differentiation, we therefore hypothesized that suppression of TGF-β1 pathway using a TGF-β1/ALKs inhibitor during EB formation would improve the efficacy of neuronal differentiation. This study examined the effects of selective TGF-β1/ALK inhibitors on neuronal differentiation of mouse embryonic stem cells.

4.3 Materials and methods

Materials and Cell culture condition

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) and culture media were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA), unless specified otherwise. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Medium was changed daily for mouse ES cell culture and on every second days during differentiation.

4.3.1 Mouse pluripotent cell culture

Mouse (129S2/SvPas) D3 ES cells (ATCC, Manassas, VA, USA) were cultured on mitomycin C inactivated mouse embryonic fibroblasts (MEFs) as previously described (Magin et al., 1992). The pluripotent ES cells were maintained in ES medium: Dulbecco's modified Eagle's medium (DMEM) containing 15% (v/v)

fetal bovine serum (FBS, ES grade Invitrogen), 0.1 mM non-essential amino acids (NEAA), 0.1 mM β-mercaptoethanol (β-ME), 0.1 mM Antibiotic-Antimycotic (Anti-Anti) and 1,000 U/ml mouse leukemia inhibitory factor (LIF, ESGRO, Chemicon International, Temecula, CA, USA). The cells were subcultured prior to reaching 70% confluency (approximately every 1-2 days). Mouse ES cells were cultured on gelatin-coated dishes in the presence of LIF (2,000 U/ml) in ES medium for at least one passage prior to differentiation, in order to deplete potentially present MEF cells from the system.

4.3.2 EB formation towards neuronal lineage

Mouse D3 ES cells were induced to differentiate into neuronal lineage as recently described (Klincumhom et al., 2012). Mouse ES cell colonies were dissociated into single cells using 0.05% (w/v) Trypsin-EDTA, then seeded at a density of 3×10^5 cells/ml in differentiation medium (ES medium without LIF) onto bacteriological dishes pre-coated with Poly 2-hydroxyethyl methacrylate (Poly-HEMA) to prevent cell attachment. Pluripotent cells were allowed to aggregate in suspension and form EBs for 4 days. All-trans RA (5 μM) was then added to the medium, and the EBs were cultured for a further 4 days. The medium was renewed every second days until day 8. The EBs were either cultured with or without TGF-β1 inhibitor (A83-01, Biovision, Milpitas, USA). The EBs were harvested for further analysis on days 2, 4, 6 and 8 of culture as shown in Figure 17. The experiments were replicated three times.

4.3.3 EB morphology analysis

Two-day-old EBs were collected into a new Petri-dish containing phosphate buffered saline (PBS) supplemented with 5% (v/v) FBS. The EB diameter was calculated after measuring the large and small diagonals of 20 EBs derived from both control and A83-01 treated groups. The measurement was performed under a phase contrast microscope (CKX41, Olympus, Shinjuku, Japan).

4.3.4 Analysis of alkaline phosphatase (ALP) activity

ES cells prepared for characterization were cultured for 2-3 days (until reaching approximately 70% confluency) on MEF-coated coverslips. Cells were washed with PBS and then fixed in 4% (v/v) paraformaldehyde (PFA) for 15 min at room temperature (RT). Fixed cells were washed and stored in PBS at 4°C until analysis. Alkaline phosphatase staining was performed using an ALP histochemistry kit (Sigma, St. Louis, USA) according to the manufacturer's protocol. The ALP-positive ES colonies typified by bright pink/red color under an inverted light microscope.

4.3.5 Immunocytochemical analysis

Cultured cells were fixed with 4% PFA for 15 min at room temperature (RT). Permeabilization was performed using 0.2% (v/v) Triton X-100 (for intracellular staining) for 30 min at RT. Cells were then blocked with 3% (w/v) bovine serum albumin containing 0.5% (v/v) Tween20 in PBS for 30 min. The cells were sequentially incubated at 4°C with the following primary antibodies diluted in blocking solution overnight : pluripotent marker Oct4 (SC-9081, dilution 1:100, Santa Cruz Biotechnology, Santa Cruz, CA; mouse) and Sox2 (SC-20088, dilution 1:100, Santa Cruz Biotechnology, Santa Cruz, CA; rabbit). The cultures were washed 3 times with PBS and then incubated with fluorescent-labeled secondary antibodies (Alexa fluor® 488 and Alexa fluor® 647-labeled goat IgG; dilution 1:2,000; Gibco) for 1 hour at RT. After three washes with PBS, the cells were counterstained with 4', 6'-diamidino-2-phenylindole (DAPI) for 20 min. Then, the fluorescently labeled cells were mounted with Vectashield mounting medium (Vector laboratory, Temecula, CA). The examination was performed using a fluorescent microscope (BX51 Olympus, Tokyo, Japan) equipped with a DP72 camera and DP2-BSW software (Olympus, Tokyo, Japan).

4.3.6 Histological analysis

Eight-day-old EBs were fixed with 4% PFA for 15 min at RT. Thereafter, fixed EBs were incubated at 4°C in 20% (w/v) sucrose solution overnight. The EBs were cryosectioned at 7-µm thickness. Prior to staining, the sections were rinsed in

PBS and incubated for 30 min in blocking solution containing 10% (v/v) FBS and 0.1% (v/v) Triton X-100 in PBS. The sections were then incubated with primary antibodies in blocking solution for 12 hours at 4°C. Antibodies used for EB immunolabeling included: neuroepithelial cell (NE) marker Pax6 (Pax6, dilution 1:200, DSHB; mouse) and mature neuronal marker NeuN (MAB 377, dilution 1: 100, Millipore; mouse). For negative control, each primary antibody was substituted with PBS to test for non-specific labeling of particular secondary antibody. Primary antibodies were detected by anti- mouse TritC secondary antibody (T2402, dilution 1:100, Sigma-Aldrich) for 1 hour at 37°C. Slides were counterstained with DAPI. The sections were analyzed with a fluorescent microscope.

4.3.7 Quantitative RT-PCR assays

Total RNA were extracted from EBs day 2, 4, 6 and 8 by using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. For each sample, 500 ng RNA were reversely transcribed using the First-Strand cDNA SynthesisKit (SuperScript III Kit, Invitrogen, Carlsbad, CA, USA), as per the manufacturer's instructions. After the reverse transcription, the cDNA samples were diluted to 2.5 ng/µl and dispensed to once-use aliquots. Quantitative real-time RT-PCR was performed on the ABI PRISM 7300 Real-time cycler (Applied Biosystems, Foster City, CA, USA) with Power SYBR Green PCR Master Mix (Applied Biosystems, WA, UK) and specific primer pairs were used as described in Table 2. For amplification, the cycling parameters consisted of 1 cycle at 50 °C for 2min and 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 sec and 60 °C for 1 min. When the reaction was completed, melting curves were plotted to confirm the specificity of the product. Relative expression was calibrated to the undifferentiated mES cells (day 0) equaling 1. Expression levels of individual transcripts were normalized to GAPDH expression.

Table 2. Primers used for determination of mRNA expression levels by quantitative RT-PCR (Experiment 3)

Gene name	Forward primer	Reverse primer	Product size (bp)
Oct4	GTCTGGAGACCATGTTTCTGAAGT	AGAACCATACTCGAACCACATCCT	105
Sox1	GGCCGAGTGGAAGGTCAT	ACTTGTAATCCGGGTGTTCCT	101
Nestin	AGTGCCCAGTTCTACTGGTGTCC	CCTCTAAAATAGAGTGGTGAGGGTTGA	125
MAP2	AAAGGCCCGCGTAGATCAC	GGGATTCGAGCAGGTTGATG	122
GFAP	CGTTAAGCTAGCCCTGGACATC	GGATCTGGAGGTTGGAGAAAGTC	107
GAPDH	CTGCACCACCAACTGCTTAGC	CAGTCTTCTGGGTGGCAGTGA	110

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Oct4, octamer-binding transcription factor 4; Sox1, Sex determining region Y-box 2; MAP2, Microtubule-associated protein 2; GFAP, Glial fibrillary acidic protein

4.4 Statistical analysis

The statistics generated in this study were assessed by student's t-test using SPSS software (version17.0 SPSS Inc., Chicago, IL, USA). Data was expressed as a mean ± standard error of the mean (SEM), of at least three independent replications. The results were considered significant difference when P-values were less than 0.05 (*) and 0.01 (**).

4.5 Experimental design

This study aimed to examine the effect of TGF-β1 inhibitor using A83-01 on the development and neuronal differentiation potential of embryoid bodies of mouse ES cells during suspension period. Mouse D3 ES cells were induced to differentiate into the neuronal lineage. For treatment group, 1 μM TGF-β1 receptor inhibitor A83-01 was supplemented in medium throughout 8 days of EBs suspension period. The EBs were harvested for quantitative RT-PCR analysis on day 2, 4, 6 and 8 after the beginning of the EB formation. Eight-day-old EBs were investigated for morphological and histological analysis. Differentiated cells were stained by neuronal markers for neuronal characterization. The experiments have been replicated three times.

4.6 Results

4.6.1 Characterization of pluripotent ES cells

A mouse ES cell line used in this study was maintained in ground state of pluripotency. They strongly expressed alkaline phosphate (ALP, bright pink-red colony; Figure 18A) and also undifferentiated ES cells marker (Oct4 and Sox-2, Figure 18B).

4.6.2 Morphology analysis of TGF-β1 inhibitor (A83-01)-treated EBs

Following EB formation using a hanging drop method, a total of 20 EBs in each group were collected and examined on day 2 of differentiation. The EB morphology was spherical shape with uniform size which was similar between the two experimental groups (Figure 19A). However, the diameters of EBs treated with

A83-01 (592.6±27 μm) were significantly smaller than those observed in a control group (631.7±14.1 μm, P< 0.01; Figure 19B).

4.6.3 Characterization of TGF-β1 inhibitor (A83-01)-treated EBs

To characterize the protein expression within EBs by immunohistochemistry, we examined the expression of neuronal markers in the cross-section of A83-01-treated EBs at day 8 of suspension period. To characterize the neuronal differentiation, we further examined for early (Pax-6, neuroepithelial cell marker) and late (NeuN, mature neuron) neuronal differentiation within the EBs by immunohistochemistry. The EBs derived from the two groups similarly contained a complex network of mixed population of early and mature neurons as the aggregated cells were positive to both Pax-6 and NeuN (Figure 20).

4.6.4 Effect of TGF-β1 inhibitor (A83-01) on gene expression during EB development toward neuronal lineage

Quantitative RT-PCR analysis showed that the mRNA expression encoding the undifferentiated ES cell markers (Oct4) in A83-01-treated EBs was more down-regulated over 8 days of culture than the control group (Figure 21A). This sequential down-regulation of the pluripotent gene occurred simultaneously with the dramatically up-regulation of Sox-1 mRNA (neuroectoderm marker). Selective TGF-β1 inhibitor significantly increased the expression of this gene in day-2 EBs compared to controls (P<0.01). However, Sox-1 mRNA of A83-01-treated EBs was later on expressed at a lower level compared to non-treated EBs (P<0.05, Figure 21B).

Similar pattern of gene expression between Sox-1 and MAP2 neuronal markers was observed during the first 4 days of EB culture but the levels of mRNA expression were similar between A83-01 treated and control groups when examined on day 6 and 8 of culture (Figure 21C). Interestingly, expression of an astrocyte marker, glial fibrillary acidic protein (GFAP), in the EBs between the two groups exhibited a contrary dynamic of mRNA expression as shown in Figure 21D. Treatment the EBs with selective TGF-β1 inhibitor significantly down-regulated the GFAP expression compared to non-treated control throughout EB suspension period (P<0.01).

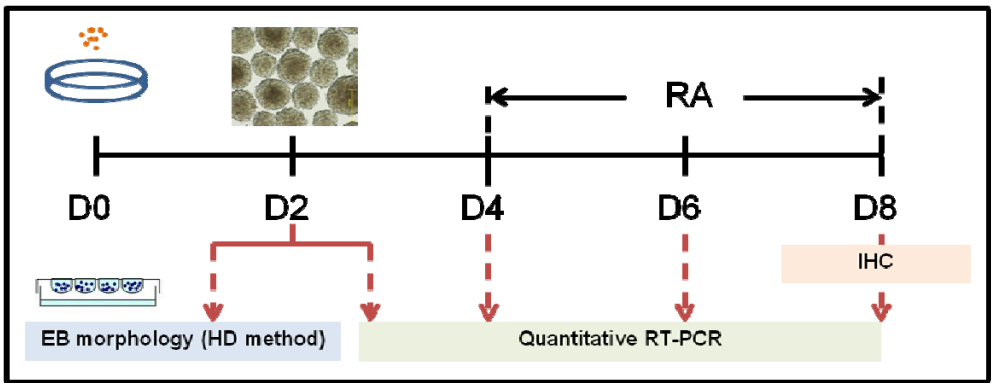

Figure 17. Schematic of neuronal differentiation using EB formation method.

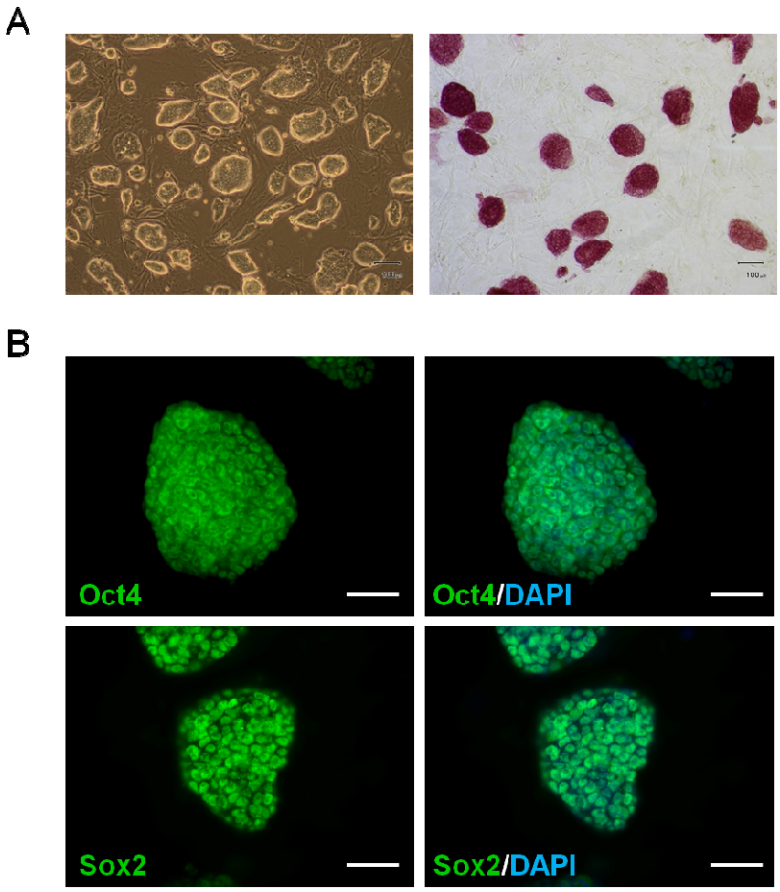

Figure 18. Characterization of mouse ES cells prior EB suspension culture. (A) Mouse ES cells were maintained undifferentiated state on mitotically inactivated MEFs and alkaline phosphatase activity identified by histochemistry (Scale bar = 100 μm). (B) Immunostaining revealing the presence of Oct4 and Sox2 in ES colonies (green). Cell nuclei were detected using DAPI staining (Blue) (Scale bar = 50 μm).

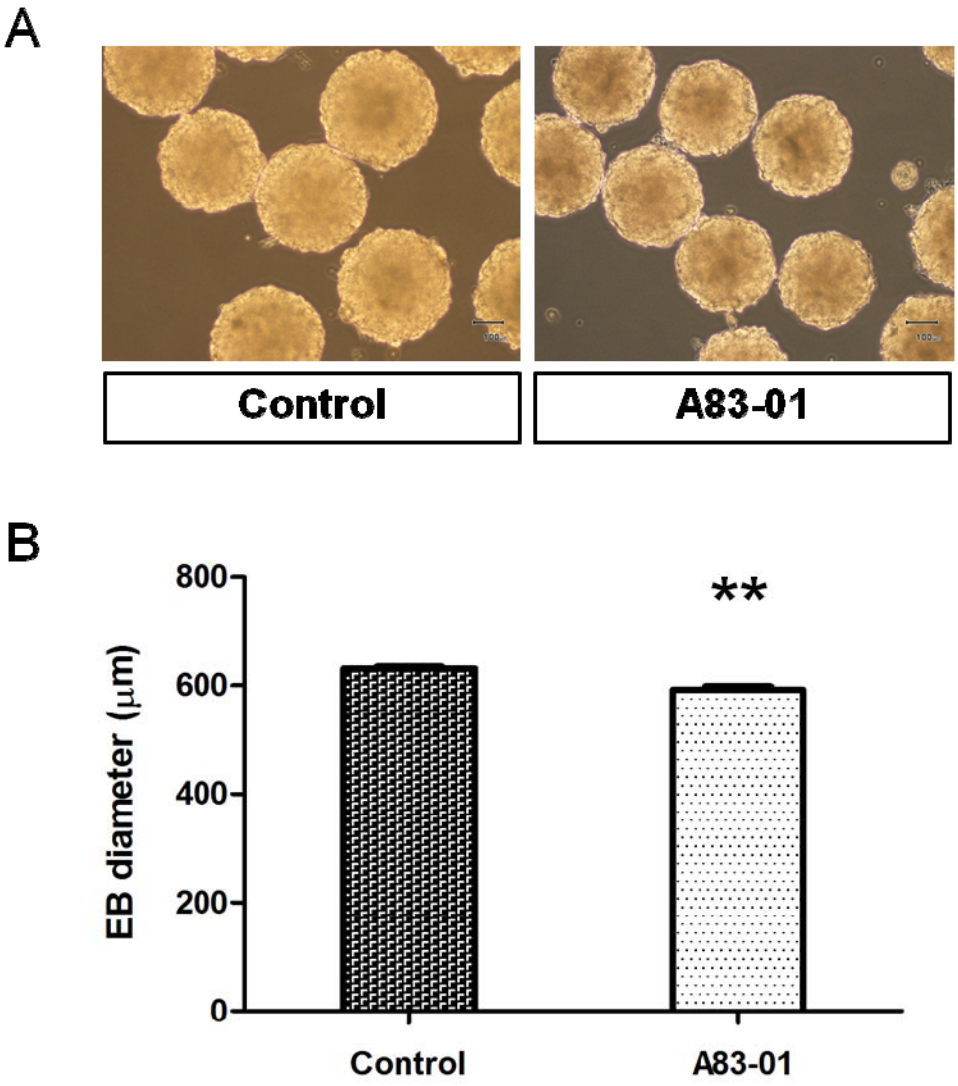

Figure 19. Morphological analysis of ES cells-derived EB formation. (A) To evaluate the size of EBs, mES cells were allowed to aggregate into 3D-structure by a hanging drop method for 2 days. (B) The diameters of two-day old EBs from control and A83-01 treated groups were measured. Data represent as mean±S.E.M. (N = 20 EBs from each group). The results were considered significant when $P<0.01$ (*).

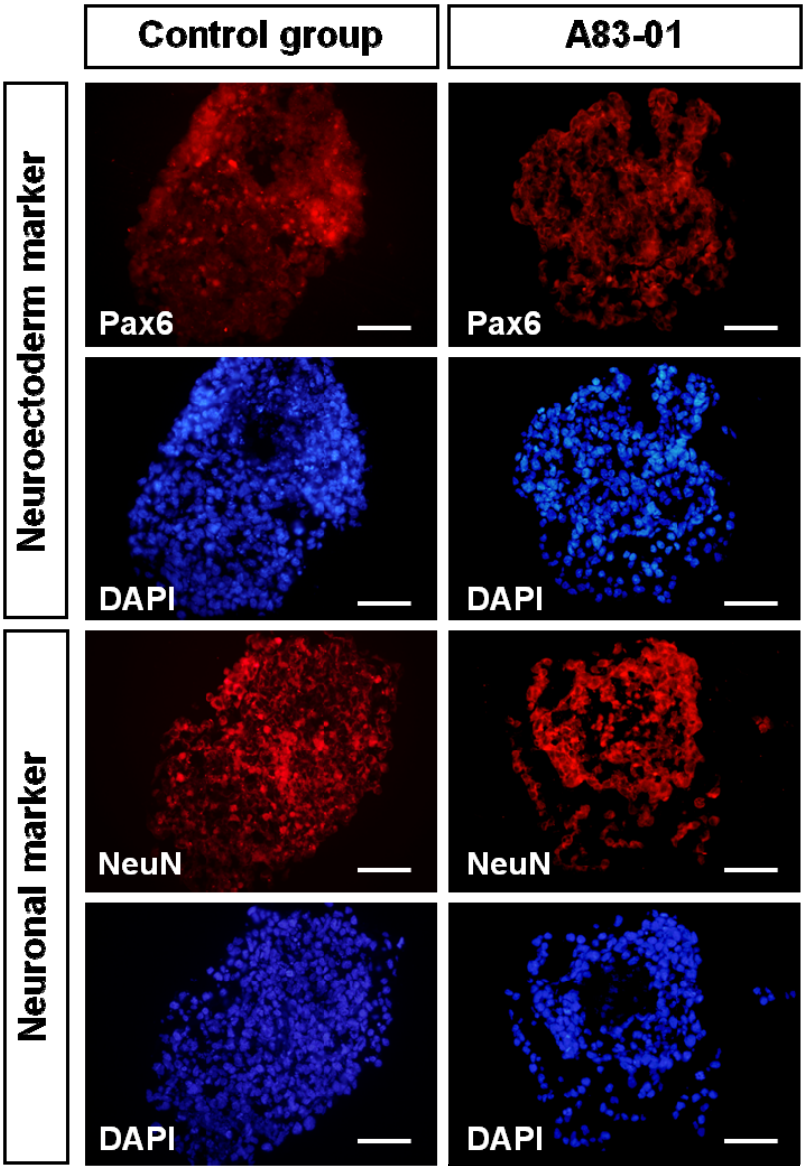

Figure 20. Characterization of neuronal phenotypes in RA-treated EBs. Cryosection (7-µm thickness) of EBs after 8 days of suspension period (see Materials and methods) counter stained with DAPI and neuronal markers. The characteristic of early neuronal stage, neuroectoderm or radial glial cells, indicates by Pax6 expression. The neuronal marker, NeuN, was used to detect neuronal maturity during formation of RA-treated EBs. Note that the expression of these markers in both groups is distributed throughout the EB. Scale bar: 100 µm.

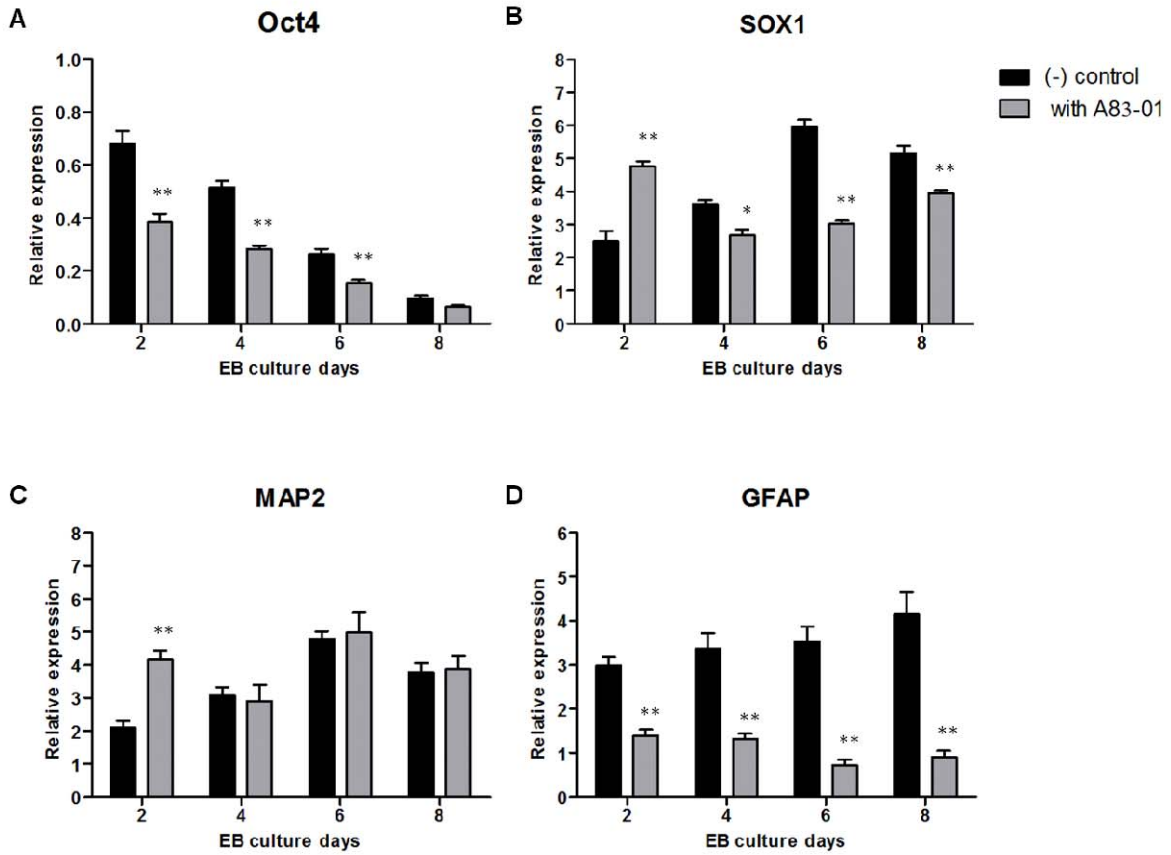

Figure 21. Gene expressions during neuronal differentiation Mouse ES cells were cultured in non-adhesive dishes for 8 days in differentiated medium supplemented with 1 μM A83-01 (grey bars). RNA was isolated from undifferentiated ES cell colonies (0 days) and from cell spheres at day 2, 4, 6 and 8, then analyzed by quantitative real-time RT-PCR. Expression of Oct4 (A), Sox-1 (B), MAP-2 (C), and GFAP (D) was normalized to the house keeping gene, GAPDH. Data shown as mean ±S.E.M. of three independent experiments. $P < 0.05$ (*) and $P < 0.01$ (**) indicate the values that differ significantly.

4.7 Discussion

In this study, we determined the effect of selective TGF-β1/ALK inhibitor (A83-01) on ES cells-derived EB development toward neuronal lineage. Several studies revealed that the TGF-β1signaling pathway is important for *in vitro* and *in vivo* neurogenesis (Gomes et al., 2005). Over-expression of TGF-β1 in transgenic mice inhibited *in vivo* neurogenesis in terms of the production of hinder neurons and immature neurons in hippocampus (Buckwalter et al., 2006). On the other hand, the TGF-β1 inhibitor such as SB431542 converted the pluripotent ES cell fate into neurons (Patani et al., 2009). In the current study, we used inhibitor (A83-01) that suppresses activin/nodal signaling pathways including TGF-β type I receptor ALK5 kinase, type I activin/nodal receptor ALK4 and type I nodal receptor ALK7. As it indicated to exert more potent in the inhibition of ALK5 than another TGF-β1 inhibitor, SB431542 (Yoshinaga et al., 2003).

By following supplementation of A83-01 in EB culture medium, the day 2-EBs derived from each group by hanging drop method were formed homogenous spheres with uniform in size as previous reports (Dang et al., 2002; Rungarunlert et al., 2011). However, at the same number of cell seeding, the A83-01-treated EBs were significantly smaller in size than the controls (Figure 19). We speculate that A83-01 may impede the anti-apoptotic effect of TGF-β1 through MAPK pathway, resulting in the reduction of cell viability and proliferation during EB formation (Chin et al., 1999). However, since we observed the marked down-regulation of pluripotent gene expression (Oct4) along with the up-regulation of neuronal markers (Sox1 and MAP2) of day 2 EB treated with TGF-β1 inhibitor (Figure 21A-21C), it is possible that the inhibition of this pathway could rapidly commit the ES cells toward neuronal lineage faster than the control. This may also coincide with the finding that the EB-mediated differentiation efficiency was dependent on the EB size. The larger EB sizes tended to differentiate toward mesoderm and endoderm, while the smaller EBs hinted their differentiation fate toward ectoderm lineage (Park et al., 2007; Peerani et al., 2007).

Although cell fate within the EBs can be successfully directed into neuronal lineage in presence of RA (Glaser and Brustle, 2005; Bibel et al., 2007), the EBs were inevitably contaminated with residual pluripotent cells (Bjorklund et al.,

2002; Barberi et al., 2003). In order to reduce the contamination of the ES cells within the EB, the ground state of ES cells is need to be suppressed. In this study, the selective TGF-β1 inhibitor, A83-01, rapidly reduced Oct4 expression similar to that observed in mouse embryos treated with TGF-β1 inhibitor, SB431542 (James et al., 2005). This inhibitor also markedly increased the rate of neuronal differentiation detected by the expression levels of early neuronal markers including of Nestin, Olig2, and Sox1 mRNA (Rohwedel et al., 1999; Okada et al., 2004). Our results showed the consecutive up-regulation of the Sox1 and MAP2 mRNA levels after supplementation of RA on day 4 which were resembles the progression of early neuronal differentiation in embryos (Loebel et al., 2003).

In adult brain, TGF-β1 and other cytokines, interleukin-1β (IL-1β) activate and involve in the modulation of GFAP expression as a neural response for cell regeneration and inflammation during brain injury and neurodegenerative diseases (Steward et al., 1990; Logan et al., 1992; Reilly et al., 1998). Two-fold increase in GFAP expression has been reported in astrocyte culture treated with TGF-β1 (Laping et al., 1994). Our results also indicated that suppression of TGF-β1 pathway significantly down-regulated the GFAP gene. Given that homogenous neuronal cells derived from the ES cells is prerequisite for clinical application. Inhibition of TGF-β1/ALK pathways during neuronal differentiation using A83-01 may become an alternative strategy for reducing the risk of pluripotent cell contamination and a more uniformity neuronal cell production.

CHAPTER V

MODULATING NEUROGENESIS IN EMBRYOID BODY USING A SELECTIVE TGF-β1/ALK INHIBITOR AFFECTS ON GENE EXPRESSION OF EMBRYONIC STEM CELL-DERIVED MOTOR NEURONS

5.1 Abstract

Mouse embryonic stem (ES) cells have been served as potential model for investigation of underlying mechanisms both cellular and molecular levels of neurological disorders. The improvement of motor neuron differentiation would enhance our understanding aimed at overcoming several incurable motor neuron diseases. In this study, we examined the effects of a selective inhibitor of TGF-β type I receptor on the efficacy of neuronal differentiation of mouse embryonic stem cells toward motor neurons. Pluripotent ES cells were induced to form as EB in suspension medium supplemented with retinoic acid using -4/+4 protocol. Thereafter, 8-day old EBs were further induced to differentiate into motor neurons on monolayer culture. Our result demonstrated that the aggregated ES cells differentiated into neuronal progenitor cells and neurons as they expressed Pax-6 and Tuj-1. Quantitative RT-PCR analysis revealed that treating the EB with the selective TGF-β1 inhibitor up-regulated the gene expression of motor neuron progenitor (Olig2) at higher levels than that obtained from the control (4.20±0.20 vs. 0.73±0.09, $P<0.01$). In contrast, mRNA expression levels of Hoxc8-specific motor neuron of a control group were significantly higher than the TGF-β1 inhibitor treated group (14.73±2.6 vs. 2.37±0.42, $P<0.01$). Immunocytochemical analysis revealed that the differentiated cells expressed a neuronal marker (Tuj-1), motor neuron progenitor marker (Olig2) and developing motor neuron progenitor (Isl-1), all of which are essential for generation of spinal motor neurons during neural tube formation. Furthermore, a small proportion of differentiated cells were also positive for choline acetyltransferase (ChAT), a marker for functional motor neurons.

We conclude that TGF-β signaling appears to affect generation and differentiation fate of motor neuron progenitors. Further investigations are required to

provide more understanding in the role of TGF-β signaling pathway on cell fate identity during spinal cord development.

5.2 Introduction

During development of mammalian embryos, ectodermal cells are converted into rostral neural fate, and the neural inductive signals such as retinoic acid (RA) lead the caudalization of neural cells. The motor neurons are then generated via ventralization of the spinal motor progenitors which is directly dependent on the sonic hedgehog (SHH) activity (Jessell, 2000; Wilson and Edlund, 2001). Unlike the embryo development, most neurons of the mammalian central nervous system (CNS) of adulthood cannot be regenerated or repaired upon injury (Gage, 2002). Therefore, several motor neuron diseases (MNDs) including amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) are classified as incurable neurological disorders due to the permanent loss of the motor neurons in the spinal cord/brain. To overcome this shortcoming, advanced technology for cell replacement via *in vitro* production of neuronal cells coupled with cell/tissue engineering has lightened a novel technique for treating these neurogenic disorders (Gowing and Svendsen, 2011). Pluripotent stem cells have capability to self-renew and to differentiate into all types of the three germ layers including neuroepithelial lineage (Keller, 1995). Thus, embryonic stem (ES) cells-derived functional mature motor neurons have increasingly become a potential model for studying the disease mechanism and ultimately for developing of novel cell-based therapy for motor neuron diseases (Lunn et al., 2011).

Mouse ES cells have been manifested their ability to differentiate into several neuronal cell types of nervous system but specific signals for successive initiation of spinal motor neuron development is required. The successful establishment of motor neurons can be achieved through orchestrated stimuli of multiple signals such as RA and SHH that have been demonstrated to potentially differentiate cells into caudo-ventral pattern in nervous system and also have ability to advance the development of floor plate of neural tube and spinal cord (Marti and Bovolenta, 2002; Maden, 2006). Exposure to these morphogens prompted the differentiation fate of the ES into spinal motor neurons in a concentration-dependent manner. Furthermore, the presence of several transcription factors including Pax6, Nkx6.1, bHLH protein and Olig2 in

neuronal population hinted the potential role in specific position and stage of motor neuron differentiation and development (Pituello et al., 1995; Muller et al., 2003; Lee et al., 2005).

To differentiate the ES cells toward neuronal lineage, several strategies have been examined but the most convenience- and cost-effective technique is the neuronal differentiation via allowing aggregated ES cells to differentiate into 3D structure resemble to embryo development, so-called embryoid body (EB) formation (Li et al., 2005; Karumbayaram et al., 2009). Although this technique allows spontaneously differentiation of the ES cells, specific cell types, for example, Pax6-positive neuronal cells, could directly be driven when cultured the EBs with specific growth factors (Bibel et al., 2007). Among growth factors affecting the neuronal differentiation, signaling via transforming growth factor family plays a crucial role in determining the cell fate in neurogenesis. Over expression of TGF-β superfamily member, Nodal, in mouse ES cells up-regulated mesodermal and endodermal cell gene expressions but the neuroepithelial markers were down regulated (Pfendler et al., 2005). The inhibition of the TGF-β members via activin/nodal and BMP pathways increased a functional neuron in culture (Chambers et al., 2009). In the current study, we examined the effects of a selective inhibitor of TGF-β type I receptor on the efficacy of neuronal differentiation of mouse embryonic stem cells toward motor neuron.

5.3 Materials and methods

Materials and Cell culture condition

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) and culture media were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA), unless specified otherwise. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Medium was replaced daily for mouse ES cell culture and on every 2-3 days during differentiation.

5.3.1 Mouse pluripotent cell culture

Mouse (129S2/SvPas) D3 ES cells (ATCC, Manassas, VA, USA) were cultured on mitomycin C inactivated mouse embryonic fibroblasts (MEFs) as previously described (Magin et al., 1992). The pluripotent cells were maintained in

ES medium: Dulbecco's modified Eagle's medium (DMEM) containing 15% (v/v) fetal bovine serum, 0.1 mM non-essential amino acids (NEAA), 0.1 mM β-mercaptoethanol (β-ME), 1% (v/v) Antibiotic-Antimycotic (Anti-Anti) and 1,000 U/ml mouse leukemia inhibitory factor (LIF, ESGRO, Chemicon International, Temecula, USA). The cells were subcultured prior to reaching 70% confluency (approximately every 1-2 days). Mouse ES cells were cultured on gelatin-coated dishes in the presence of LIF (2,000 U/ml) in ES medium for at least one passage prior to differentiation, in order to discard MEFs from the system.

5.3.2 Differentiation of mouse ES cells into motor neurons

Mouse D3 ES cells were initially induced to differentiate into the neuronal lineage via embryoid body formation as recently described (Klincumhom et al., 2012). Mouse ES cell colonies were disaggregated into single cells using 0.05% (w/v) Trypsin-EDTA, then seeded at a density of 3×10^5 cells/ml in differentiation medium (ES medium without LIF) onto bacteriological dishes pre-coated with Poly 2-hydroxyethyl methacrylate (Poly-HEMA) to prevent cell attachment. Pluripotent cells were allowed to aggregate in suspension and form the EBs for 4 days. All-trans RA (5 μM) was then added to the medium and the EBs were then cultured for a further 4 days. The EBs were either cultured with or without TGF-β1 inhibitor (A83-01, Biovision, Milpitas, USA). Thereafter, 8-day old EBs were further induced to differentiate into motor neurons as previous described with some modifications (Li et al., 2005). In brief, EBs were dissociated and plated onto Poly-L-ornithine and laminin (Roche, CA, USA) coated dish at a density of 2×10^5 cells/cm^2 in Neurobasal medium containing N2 supplement**,** 1 μM cAMP, 100 ng/ml sonic hedgehog (SHH) and 0.1 μM retinoic acid (RA) for 7 days**.** Then, Brain-derived neurotrophic factors (BDNF, 10 ng/ml), Gial cell line-derived neurotrophic factor (GDNF, 10 ng/ml) and Insulin-like growth factor (IGF-1, 10 ng/ml) were added into the medium. The concentration of SHH was reduced to 50 ng/ml onwards. The medium was renewed every second days. The experiments were replicated three times.

5.3.3 Analysis of alkaline phosphatase (ALP) activity

ES cells were prepared for characterization following 2-3 days of culture or until reaching approximately 70% confluency on MEF-coated coverslips. Cells were washed with phosphate-buffered saline (PBS) and fixed with 4% (w/v) paraformaldehyde (PFA) for 15 min at room temperature (RT). Fixed cells were washed and stored in PBS at 4°C until analysis. Alkaline phosphatase staining technique was performed using an ALP histochemistry kit (Sigma, St. Louis, USA) according to the manufacturer's protocol. The ALP-positive ES colonies demonstrates in bright pink/red color under an inverted light microscope (Olympus CKX41, Shinjuku, Japan).

5.3.4 Immunocytochemical analysis

Undifferentiated mouse ES cells and differentiated cells on day 18 were fixed with 4% (w/v) PFA for 15 min at room temperature. Permeabilization was performed using 0.2% (v/v) Triton X-100 (for intracellular staining) for 30 min at RT. Cells were then blocked with 3% (w/v) bovine serum albumin containing 0.5% (v/v) Tween20 in PBS for 30 min. The cells were then sequentially incubated with the following primary antibodies diluted in a blocking solution overnight at 4°C: Oct4 (SC-9081, dilution 1:100, Santa Cruz Biotechnology, Santa Cruz, CA; mouse) and Sox2 (SC-20088, dilution 1:100, Santa Cruz Biotechnology, Santa Cruz, CA; rabbit) for pluripotency analysis. The neuronal markers including: β-III Tubulin (Tuj1, PRB-435P, dilution 1:2,000; Covance; rabbit), motor neuron progenitors: Olig2 (MABN50, dilution 1:200; Chemicon; mouse) and motor neuron markers: Isl-1 (40.2D6, dilution 1:50; DSHB; mouse) and choline acetyltransferase (ChAT, AB143, dilution 1:100; Chemicon; rabbit) were used. The cultures were washed 3 times with PBS and then incubated with fluorescent-labeled secondary antibodies (Alexa fluor® 488, Alexa fluor®594 and Alexa fluor® 647-labeled goat IgG; dilution 1:2,000; Gibco) for 1 hour at RT. After washing, the cells were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) for 10-20 min. Vectashield (Vector laboratory, Temecula, CA) was used as a mounting medium to prevent the photobleaching during fluorescent

examination. The images were recorded using fluorescent microscope equipped with a DP72 camera and DP2-BSW software (Olympus, Tokyo, Japan).

5.3.5 Histological analysis

Seven micrometers thickness cross-sections of 8 day-olds EBs were rinsed in PBS and incubated for 30 mins in a blocking solution containing 10% (v/v) serum and 0.1% (v/v) Triton X-100 in PBS. Sections were then incubated with neuroepithelial marker (Pax6, dilution 1:200, DSHB; mouse) and early neuronal marker β-III Tubulin (Tuj1, dilution 1:2,000; Covance, PRB-435P; rabbit) as primary antibodies in blocking solution for 12 h at 4°C. As for negative control, each primary antibody was substituted with PBS in order to test for non-specific labeling of the secondary antibody. After washing with PBS, primary antibodies were detected by using anti-rabbit FITC secondary antibody (ab6717, dilution 1:250, Abcam) for 1 hour at 37°C. The sections were counterstained with DAPI and then were analyzed using a fluorescent microscope (BX51 Olympus, Tokyo, Japan).

5.3.6 Quantitative RT-PCR analysis

Total RNA was extracted from differentiated cells on day 18 of differentiation using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. RNA was reversely transcribed using the First-Strand cDNA Synthesis Kit (SuperScript III Kit, Invitrogen, Carlsbad, CA, USA), as per the manufacturer's instructions. After reverse transcription, the final concentration of cDNA samples was diluted to 2.5 ng/µl and dispensed to once-use aliquots. Quantitative RT-PCR was performed on the ABI PRISM 7300 Real-time cycler (Applied Biosystems, Foster City, CA, USA) with Power SYBR Green PCR Master Mix (Applied Biosystems, WA, UK). For cDNA amplification, the cycling parameters consisted of 1 cycle at 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 sec and 60 °C for 1 min. Gene expression of individual transcripts was detected as an amplification exceeded threshold. RT-PCR analysis for pluripotent gene expression of ES cells was performed on automated thermal cycler (Nyx Technik A6, Ramsey, MN, USA) using Go Tag® Green Master Mix (Promega, WI, USA). Our amplification protocol consisted of 1 cycle at 95 °C for 10 min, followed by 45 cycles

of 95 °C for 15 sec, 55 °C for 30 sec and 68 °C for 1 min. The PCR products were electrophoresed on 2% (w/v) agarose gel (Bio-Rad, CA, USA) stained with 0.4 mg/mL ethidium bromide (Promega, WI, USA) and were finally visualized under UV light (Syngene, CB, UK). The negative controls were performed using mouse embryonic fibroblast cells and sterile water (H_2O). The primer sequences are shown in table 3. The GAPDH (glyceraldehydes-3-phosphatedehydrogenase) was used as a housekeeping gene for endogenous normalization.

5.4 Statistical analysis

Data was expressed as a mean ± standard error of the mean (SEM), for at least three independent replicates. The student's t-test was used to compare means between control and treatment groups. The statistical analysis was performed using SPSS software (version 17.0 SPSS Inc., Chicago, IL, USA). The results were considered significant difference when P-values were less than 0.01 (*).

5.5 Experimental design

This study aimed at investigating the motor neuron differentiation treated with a selective inhibitor of TGF-β type I receptor, A83-01 during embryoid body culture period. Mouse D3 ES cells were initially induced to differentiate into the neuronal lineage via embryoid body formation. Eight-day-old EBs were examined for neuronal characteristics by histological analysis. The the differentiated cells were induced to differentiate into motor neurons. The immunohistochemistry, immunocytochemistry and quantitative RT-PCR were performed in order to examine the efficacy of motor neuron differentiation.

Table 3. Primers used for determination of mRNA expression levels by quantitative RT-PCR (Experiment 4)

Gene name	Forward primer	Reverse primer	Product size (bp)	References.
Oct4	GTCTGGAGACCATGTTTCTGAAGT	AGAACCATACTCGAACCACATCCT	105	(Otsu et al., 2011)
Sox2	ACCAGCTCGCAGACCTACAT	CCTCGGACTTGACCACAGAG	111	(Keramari et al., 2010)
Nanog	AGTGGAGTATCCCAGCATCCATT	CTGCCCCACATGGAAAGG	81	(Mitsui et al., 2003)
Olig2	CTGCTGGCGCGAAACTACAT	CGCTCACCAGTCGCTTCAT	66	(Samanta et al., 2007)
HoxC8	CGAAGGACAAGGCCACTTAAAT	AGGTCTGATACCGGCTGTAAGTTT	115	(Kruger and Kappen, 2010)
GAPDH	CTGCACCACCAACTGCTTAGC	CAGTCTTCTGGGTGGCAGTGA	110	(Otsu et al., 2011)

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Oct4, octamer-binding transcription factor 4
Sox2, Sex determining region Y-box 2; Olig2, Oligodendrocyte transcription factor 2; HoxC8, Homeobox C8 protein

5.6 Results

5.6.1 Pluripotent characterization of mouse ES cells

Mouse embryonic cell line used in this study demonstrated a typical ES morphology as ES colonies were round to oval shape with shiny appearance. The ES cells strongly expressed pluripotency markers including ALP activity (bright pink/red colored colonies, Figure 22A-22B), Oct4 and Sox2 (Figure 22C-22D). Moreover, RT-PCR analysis revealed that these ES cells also expressed pluripotency associated genes (Oct4, Nanog and Sox2) (Figure 22E).

5.6.2 The effect of A83-01 on differentiation capability of mouse ES cells into motor neurons

These undifferentiated ES cells were aggregated into spherical EBs with heterogeneous size in differentiation medium (Figure 23). Further examination on neuronal characteristics of cross-sectioned the 8 day-old-EBs using immunohistochemistry found that the aggregated ES cells differentiated into neuronal progenitor cells, irrespective to the treatment with TGF-β1 inhibitor, as they were positively stained with Pax-6 and Tuj-1 neuronal markers. To generate motor neurons, the EBs were disaggregated, and the monolayer cultured for additional 10 days. Our study demonstrated that this technique efficiently differentiated the ES cells into motor neurons. Treating the EB with a selective TGF-β1 inhibitor up-regulated the motor neuron progenitor (Olig2) at higher levels than that obtained from the control (4.20±0.20 vs. 0.73±0.09, Figure 25, $P<0.01$). In contrast, mRNA expression levels of motor neuron Hoxc8 of a control group were significantly higher than the TGF-β1 inhibitor treated group (14.73±2.6 vs. 2.37±0.42, Figure 25, $P<0.01$). In all cases, immunocytochemical analysis demonstrated that the differentiated cells expressed a neuronal marker (Tuj-1), motor neuron progenitor marker (Olig2) and developing motor neuron progenitor (Isl-1), all of which are essential for generation of spinal motor neurons during neural tube formation. Furthermore, a small proportion of differentiated cells were also positive for choline acetyltransferase (ChAT), a marker for functional motor neurons (Figure 24).

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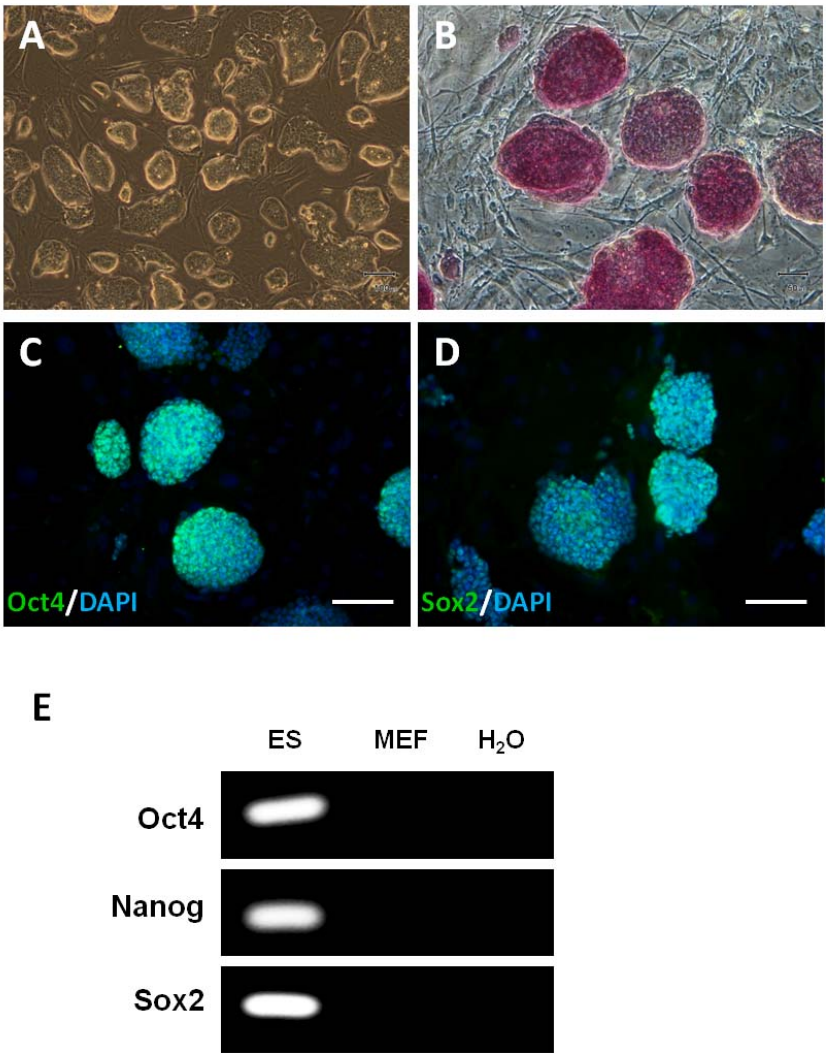

Figure 22. Characterization of pluripotent ES cells. (A) The mouse ES cells were cultured on MEF cells in ES medium supplemented with LIF. Identify by the shiny and round shape of mouse ES colonies (Scale bar = 100 μm). (B) The ALP-positive pluripotent cells were presented in bright pink/red colonies (Scale bar = 50 μm). (C-D) Immunostaining demonstrates undifferentiated ES cells detected by pluripotent markers: Oct4 and Sox2 (green color). DAPI was used for co-staining as nuclei marker (Blue color) (Scale bar = 100 μm). (E) RT-PCR analysis reveals the expressions of Oct4, Nanog and Sox2.

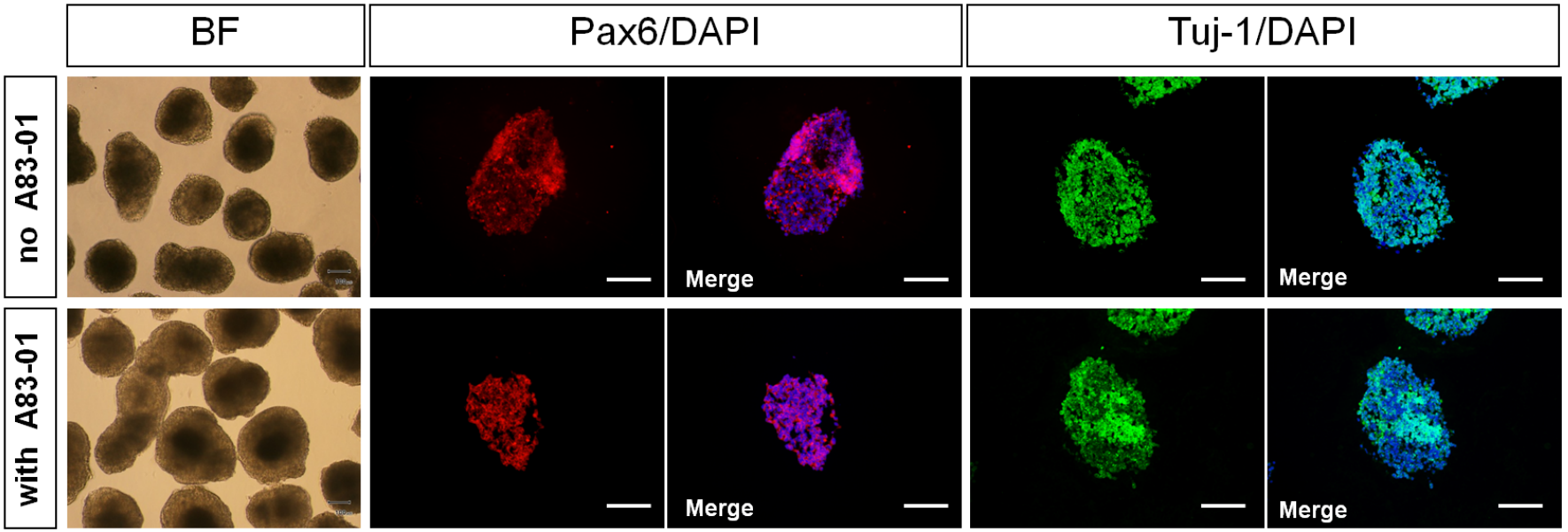

Figure 23. Morphological and histological analysis of EBs during suspension culture. The heterogeneous sizes and multiple shapes of EBs derived from mouse ES cells can be observed during 8 days of suspension period. The cross-sections of A83-01 treated EBs (lower panel) and the control group (upper panel) express neuroepithelial marker Pax6 (red) and neuronal marker Tuj-1 (green) by immunohistochemical analysis. Cell nuclei were stained using DAPI (blue). Scale bar: 100 µm.

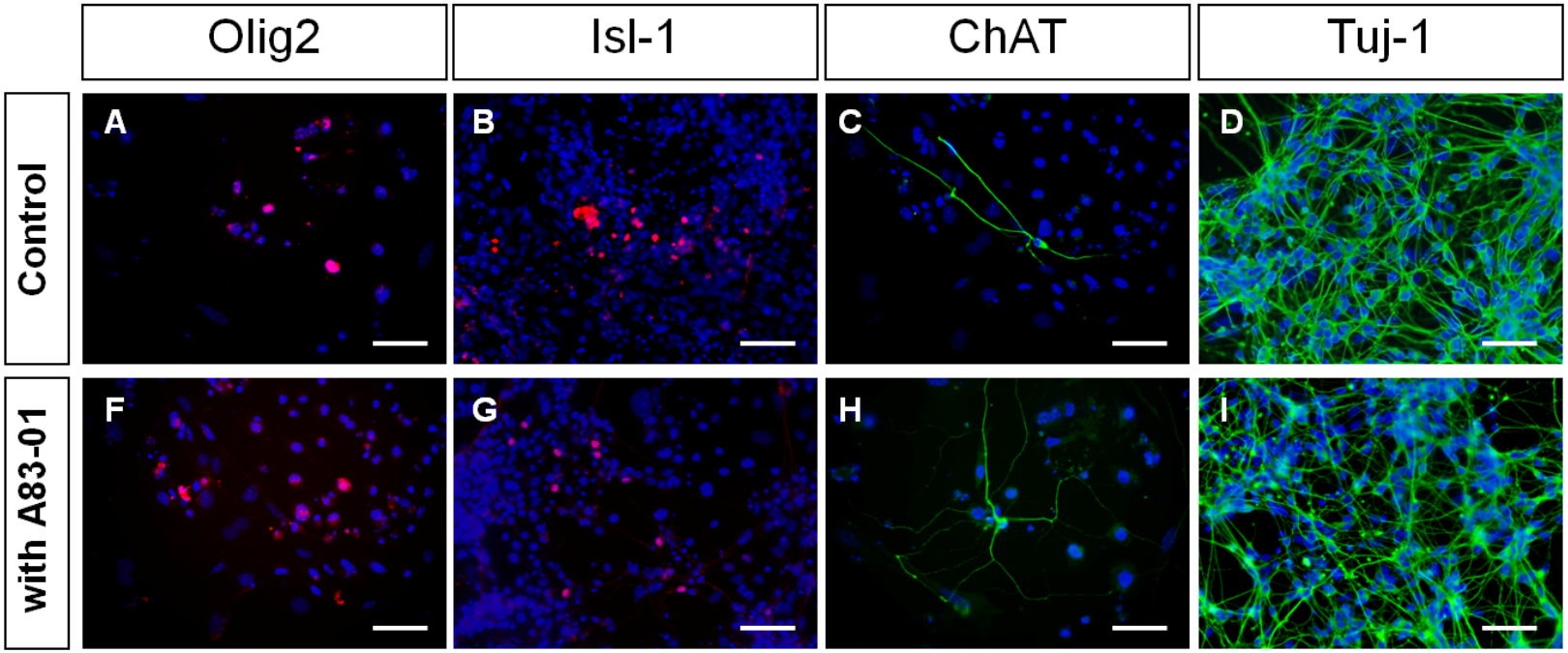

Figure 24. Motor neuron differentiation from mouse ES cells. After EB dissociation, differentiated cells were cultured on substrate-coated plate for motor neuron differentiation. By day 18 of differentiation, differentiated cells derived from A83-01 treated EBs and control were assessed for motor neuron progenitor marker (Olig2), motor neuron-specific marker (Isl-1 and ChAT), and neuronal marker (Tuj-1). The nuclei were labeled with DAPI (blue). Scale bar: 100 μm.

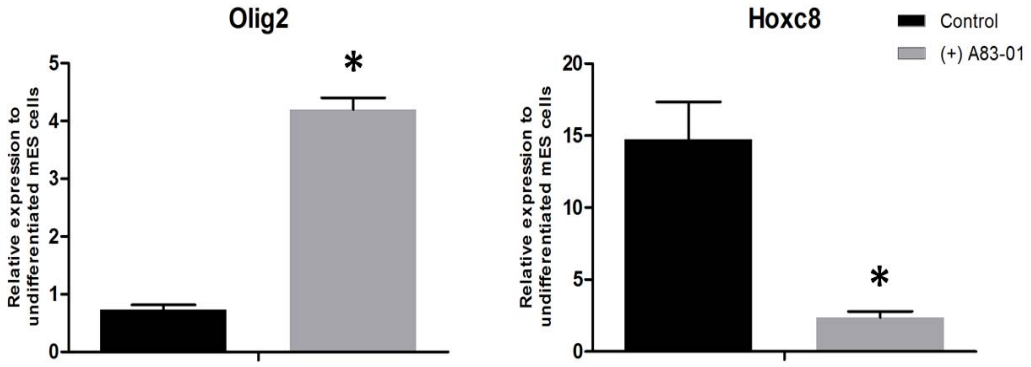

Figure 25. The effect of A83-01 on motor neuron differentiation. The differentiated cells were investigated for gene expression level of Olig2 and Hoxc8 after 18 days of differentiation using quantitative RT-PCR analysis. Gene expression was normalized to the house keeping gene, GAPDH. Results demonstrate as means ± S.E.M. of three independent experiments and represent the relative expression compared with mRNA levels of undifferentiated ES cells (arbitrarily set at 1). The statistical analyses on the relative mRNA level of differentiated cells in each gene between A83-01-treated group versus control group are showed, $P < 0.01$ (*).

5.7 Discussion

In this study, we demonstrated the effects of a selective TGF-β1 inhibitor on differentiation of undifferentiated ES cells toward motor neuron lineages through EB formation. Several techniques have been used to generate the motor neurons from embryonic and induced pluripotent stem cells such as stromal cell co-culturing and adherent culture with a defined medium (Barberi et al., 2003; Shin et al., 2005). Here, we reported that modifying neurogenic stimuli during embryoid body culture influenced on derivation of neuronal subtypes. These techniques allowed the generation of motor neuron subtypes that share some phenotypic and genotypic similarity to that of mature motor neurons (Miles et al., 2004; Karumbayaram et al., 2009; Patani et al., 2011). However, type and time of neurogenic stimuli have marked influenced on cell fate and patterning of motor neurons (Kirkeby et al., 2012).

In the current study, we differentiated the embryonic stem cells to form 3D structure of embryoid body that allows spontaneous differentiation of embryonic stem cells. This technique is quite robust and also cost- and time-effective (Keller, 1995). However, genes associated with the pluripotency of the ES cells within the EB slowly down-regulated (Bibel et al., 2004). Recently, dual inhibition of SMAD signaling (SB431542 and noggin) efficiently improved differentiation of embryonic stem cells toward neurogenic cells with a more synchronized manner (Chambers et al., 2009; Fasano et al., 2010). These TGF inhibitors completely down-regulated the pluripotency associated genes of embryonic stem cells, while neuronal gene expression was simultaneously up-regulated (Kirkeby et al., 2012). This finding is in an agreement to our previous study that the selective TGF-β1/ALK inhibitor down regulated the pluripotency-associated genes at a faster rate than that of control (Klincumhom et al., unpublished data).

As a result, although we did not observe a marked difference between EB treated with TGF-β1 inhibitor and the control, in terms of Pax-6 and Tuj-1 positive cells, these types of cells have been shown to be capable of further differentiation toward motor neurons (Briscoe et al., 2000; Li et al., 2005). Following disaggregating the day-8 EB into single cells, we further cultured of these cells in the presence of sonic hedgehog (SHH) and RA. These two factors function to ventralize and caudalize MN progenitors, respectively (Wichterle et al., 2002). However, this process is somewhat ineffective in terms of the prolonged differentiation process (Hester et al., 2011).

In the current study, although protein expressions (Pax-6, and Tuj-1) were independent to the presence of TGF-β1 inhibitor during the EB culture, the expression of Olig2 in neuronal differentiated cells from TGF-β1 inhibitor-treated EBs was significantly higher than that of control and vice versa for Hoxc8-specific caudal motor neurons. A previous report indicated that the TGF-β1 negatively affected the proliferation of the hippocampal and neuronal precursor cells by a mechanism-related to cell cycle (G0/G1 phase) arrest (Wachs et al., 2006), while inhibition of TGF-β1 restored the limited proliferation ability of retinal progenitors (Close et al., 2005). Therefore, it is possible that applying the TGF-β1 inhibitor during EB may help the produced neuronal progenitors to be maintained and proliferated in

their progenitor fate. In addition, since several transcriptional and growth factors involve the organization of spinal motor neuron identities along the axis including Isl-1, Lim3 and HB9 (Pfaff et al., 1996; Arber et al., 1999; Thor et al., 1999), modulating the TGF-β1 signaling pathway by its inhibitor therefore probably drive the neuronal progenitor to other motor neuron identities rather than Hoxc8-specific caudal motor column subtype. Further study on identifying the type of motor neurons and also its function of neuronal cells derived from EB treated with TGF-β1 inhibitor remain to be studied. Furthermore, a small number of these differentiated cells exhibited ChAT and Isl-1 were only observed. It is therefore also of importance to determine other factors that can improve the efficacy of motor neuron differentiation from embryonic stem cells.

In conclusion, our study illustrate that motor neurons can be generated from mouse ES cells after *in vitro* differentiation. Modifying TGF-β signaling positively affects neuronal differentiation toward motor neuron progenitors.

CHAPTER VI
GENERAL DISCUSSION AND CONCLUSIONS

6.1 General discussion

6.1.1 The potential of pluripotent stem cells differentiation for regenerative medicine of neurological diseases

The prospect of regenerative medicine for treating several incurable diseases such as cardiovascular, autoimmune, diabetics, Alzheimer's or Parkinson's diseases has made stem cells to become an interesting issue. Embryonic stem (ES) cells and induced pluripotent stem (iPS) cells are pluripotent cells that provide an unlimited number of cells and can differentiate into all three germ layers including ectoderm, mesoderm and endoderm. Because of these potentials, ES and iPS cells hold great prospects for repairing damaged tissue/organs or for re-constitution of healthy cell populations.

6.1.1.1 Neuronal differentiation strategies of mouse ES cells

There are various strategies leading to differentiate ES cells into neuronal lineage which focus in improving the efficiency of neuronal differentiation protocol to provide the desirable cells or specific organs for realistic medical applications. Typically, neuronal lineages derived from ES cells contain a variety of neuronal phenotypes as well as non-neuronal cells, such as glial cells. Recently, enormous interest in stem cells differentiation has arisen from both basic and medical point of views. Several recent reports have also described the generation of neuronal progenitors cells (NPCs) and neurons from mouse ES cells. NPCs have ability to further differentiate into specific neuronal subtypes such as dopaminergic and spinal motor neurons (Lee et al., 2000). Several approaches have been used to achieve *in vitro* neural differentiation starting from ES cells, aimed at generating neural stem cells (NSCs) which is defined as multipotent and have ability of self-renew and differentiation into neuron and glial phenotypes (Fisher, 1997; Gage, 2000). The most common effective approach involves neuronal differentiation is the formation of EBs

from ES cells in either presence or absence of animal serum (Bain et al., 1995; Ying and Smith, 2003). During the EB suspension culture, these aggregated cells spontaneously differentiation resemble as in embryo (Keller, 1995). The EB is the aggregation of ES cells that can spontaneously differentiate into derivatives of three-embryonic germ layers including ectoderm, mesoderm and endoderm (Okabe et al., 1996; Kurosawa, 2007). However, cell fate within the EBs can be successfully directed into neuronal lineage in presence of RA (Glaser and Brustle, 2005; Bibel et al., 2007). Meanwhile, several growth factors can be supplemented to commit the developing cells into neuronal lineages such as RA, FGF8, EGF and bFGF (Bain et al., 1995; Wang et al., 2006; Erceg et al., 2009). In presence of RA, high-yield of (>75%) NPCs population derived from ES cells has been revealed using EB formation method in our study (Klincumhom et al., 2012) (Chapter I). We thus provided the possibility of ES cell differentiation to use as control cell line, when the novel cell line liked iPS cells are needed to be study. In general perspective, neuronal differentiation of ES cells potentially provides a model system for several clinical aspects. Improving strategy of neuronal differentiation of ES cells is therefore prerequisite for studying the cellular and molecular mechanisms of early embryo development and neurogenesis and also providing the high-yield generation of homogenous neuronal cell subtypes for cell transplantation and regenerative medicine.

6.1.1.2 Neuronal differentiation and prospective cell-based therapy of iPS cells

More than a half-decade, iPS cells technology has wildly known as a great promising for regenerative medicine. This current developing technology promises to generate potential research models for understanding the cellular physiology of neurological diseases, screening of new drug as well as for clinical application as a novel source of patient specific cells-based therapy (Yamanaka, 2009). Therefore, they are prospected to be induced into neuronal lineages as a new valuable cell source for the patient-specific application in the future cells/tissue transplantation. However, the potential of iPS cells to give rise into several neuronal types as ES cells still be emphasized in several reasons including the safety of cell reprogramming methodology, unaccomplished reprogramming process, epigenetic difference which

may cause the different of proper differentiation method between iPS and ES cells or even among different iPS cells types/or sub-clones (Martinez et al., 2012). So far, the development and progress of iPS cell-based neurological therapy are flourished consecutively. The further developing researches in this field may further emphasize the cooperation of the advanced sciences, clinical applications and the ethical/safety concerns which should be move together to render this technology become a great realistic application for future regenerative medicine.

Our study demonstrates the differentiation potential of iPS cells to form NPCs using a mouse model. We compared the differentiation capacity of mouse ES and SB-iPS cells into neuronal progenitor cells and neurons via EB formation. This is the first report describing the capability of mouse SB-iPS cells, reprogrammed by the Sleeping Beauty transposon, to differentiate into NPCs and neurons. This is also the first *in vitro* study where the efficiency of neuronal induction of SB-iPS cells was compared with the efficiency of that in ES cell lines. Our study conclude that by using the same differentiation procedure, ES and SB-iPS cells show a difference in their capacity to differentiate towards the neuronal lineage. Even though the neuronal differentiation rates of iPS cells needs to be improved, our results are encouraging and show that SB-iPS cells are capable of forming neurons (Klincumhom et al., 2012). Thus, the sleeping beauty transposon-mediated reprogramming approach may be a suitable tool for obtaining these much sought after iPS cell lines. Presently, prospect utility of ES cells seems out of trend in human cell-based therapy with many reasons such as ethical and clinical applicable itself. As the pluripotent cell derivation techniques have thriving developed and more promising potential tools liked iPS cells has been emerged. However, in the period of iPS cells developing era, ES cells have yet served as valuable research model to potentially provide the fundamental knowledge of stem cell field which is still be bleary so far.

6.1.2 Neuronal differentiation of ES cells as an investigative platform of neurogenesis and gene-related neurological defects

Mouse ES cells hold much promise to use as model for the investigation of mammalian neurogenesis. The development of novel strategies to give rise the functional neuron cells is challenging to translate the potential of stem cells to serve

as useful investigative platforms for study functions of genes and biological events in neuronal development. Our study demonstrates that differentiation of ES cells into neuronal lineages can represents a new opportunity to explore the molecular function of Rybp which is still unclear in neurogenesis.

The inadequate of Rybp level can cause CNS malformations including forebrain overgrowth and defective neuronal tube closure. Besides, the embryonic death has been reported in Rybp-deficient embryos. In addition, the role of Rybp has also addressed in mouse retina and lens development. Interestingly, Rybp-deficient ES cells producing by gene modification on R1 ES cells can be generated and showed normal pluripotent ability as wild-type ES cells. Therefore, This Rybp-decifient ES cells were prospected as *in vitro* model for further studies regarding to Rybp-related embryogenesis (Pirity et al., 2005; Pirity et al., 2007).

Rybp has been shown to be necessary for normal gene regulation to exert the proper differentiation of ES cells (Hisada et al., 2012). Similar issue has been reported that the neuronal differentiation ability of mutant ES cell lines has been clearly impaired when transcription factors-related neurogenesis were depleted from their genome (Muller et al., 2003; Suter et al., 2009; Quinn et al., 2010). In Chapter III, by using the same neuronal differentiation procedure, the neuronal efficiency of Rybp-deficient ES cells was less than wild-type ES cells which showed by EB morphology, histological, immunocytochemical and gene expression analysis. We then indicated that Rybp-deficient ES cells can be induced to differentiated into neuronal lineage *in vitro*, even though, the neuronal efficiency was showed to be impaired. Recent studies demonstrated that, with the same protocol, this neuronal strategy has been achieved to efficiently commit ES cells into neuronal lineages (Bibel et al., 2007; Klincumhom et al., 2012). The sufficient of neuronal-associated growth factors, such as RA, within culture condition may partially compensated Rybp function during neuronal differentiation *in vitro*. Our study supported that mouse ES cells afford a potential study models in biological and molecular events of embryo. Importantly, using Rybp-deficient ES cell line may much facilitate a useful platform for further studies with respect to underlying mechanism of Rybp-related neurogenesis.

6.1.3 The effect of TGF-β1 inhibitor on EB formation and differentiation into neuronal lineage

Studies of early development and neuronal induction from mouse ES cells have been studied *in vitro*. Many strategies demonstrated the generation of NPCs from ES cells can be initiated by spontaneous differentiation that occurs following embryoid body (EB) formation. Although the origin and fate of neuroectoderm in this system is still unclear, major part of cells composed in the EB has been shown to be neuronal lineages after retinoic acid treatment (Plachta et al., 2004). The optimization of neuronal strategies to enrich the functional neuronal cells will translate the potential of stem cells to serve as useful investigative platforms for study functions of genes and biological events in neuronal development.

Our study in Chapter IV, we have focused on the effect of selective TGF-β1/ALK inhibitor (A83-01) on ES cells-derived EB development toward neuronal lineage. Several studies revealed the effect of TGF-β1 and its inhibitors related with neurogenesis both *in vivo* and *in vitro* (Gomes et al., 2005). Over-expression of TGF-β1 in transgenic mice inhibited *in vivo* neurogenesis in terms of the production of hinder neurons and immature neurons in hippocampus (Buckwalter et al., 2006). On the other hand, the TGF-β1 inhibitor such as SB431542 converted the pluripotent ES cell fate into neurons (Patani et al., 2009).

A83-01 is a selective inhibitor of TGF-β type I receptor ALK5 kinase, type I activin/nodal receptor ALK4 and type I nodal receptor ALK7. To assess the effect of TGF-β1 inhibitor, mouse ES cells were allowed to aggregate into EBs for neuronal induction in presence of A83-01. In this study, the selective TGF-β1 inhibitor, A83-01, rapidly reduced Oct4 expression similar to that observed in mouse embryos treated with TGF-β1 inhibitor, SB431542 (James et al., 2005). Our results showed the consecutive up-regulation of the Sox1 and MAP2 mRNA levels after supplementation of RA on day 4 which were resembles the progression of early neuronal differentiation in embryos (Loebel et al., 2003). Our results therefore indicated that A83-01 can accelerate the cell fate alteration of ES cells from undifferentiated state into neuronal lineage. We also speculated that suppression of TGF-β1 pathway significantly down-regulated the GFAP gene.

In summary, given that homogenous neuronal cells derived from the ES cells is prerequisite for clinical application, inhibition of TGF-β1/ALK pathways during neuronal differentiation using A83-01 may become an alternative strategy for reducing the risk of pluripotent cell contamination and a more uniformity neuronal cell production.

6.1.4 Differentiation of ES cells into motor neurons

The ultimate goal of stem cells is to generate functional cell types for relevant cell regenerative therapy. Mouse embryonic stem (ES) cells has been served as potential model for investigation of underlying mechanisms either in cellular and molecular levels of neurological disorders. The improvement of motor neuron differentiation is prospected to gain more understanding aimed to overcome several incurable motor neuron diseases (MND), including spinal muscular atrophy, amyotrophic lateral sclerosis (ALS), and spinal muscular atrophy (SMA) (Lunn et al., 2011). The successful establishment of motor neurons can be achieved though orchestration of multiple signals such as RA and SHH which show significant activities to differentiate cells into caudo-ventral pattern in nervous system and induction of floor plate in developing neural tube and spinal cord patterning (Marti and Bovolenta, 2002; Maden, 2006).

The effect of A83-01, TGF-β1 inhibitor, on motor neuron differentiation using embryoid body (EB) formation as initial stage of differentiation was investigated in our study (see Chapter V). Pluripotent ES cells were induced to form as EB in suspension medium supplemented with retinoic acid using -4/+4 protocol. EBs were treated with A83-01 throughout 8 days of suspension period. Following disaggregate the day-8 EB into single cells, we further cultured of these cells in the presence of sonic hedgehog (SHH) and RA. These two factors function to ventralize and caudalize MN progenitors, respectively (Wichterle et al., 2002; Li et al., 2005). We then investigated differentiated cells on day 10 after EB dissociation or 18 days after differentiation. The neuroepithelial cells detected by Pax6 have been found in EBs derived from both groups. The expression of Pax6 has been showed to be essential for early neurogenesis *in vivo*, cells survival and differentiation during *in vitro* neuronal

induction (Quinn et al., 2007; Quinn et al., 2010). Besides, the neuronal progenitors expressed Pax6, Olig2 and NKX6.1 has been reported their capability to further differentiate into motor neurons (Briscoe et al., 2000). The quantitative RT-PCR analysis demonstrated that Olig2-specific motor neuron progenitors can be generated from A83-01-treated EBs which indicated to be higher than untreated group. Conversely, the Hoc8-positive caudal motor neurons were showed to be lower when compared to those from control. Since several transcriptional and growth factors involve the organization of spinal motor neuron identities along the axis including Isl-1, Lim3 and HB9 (Pfaff et al., 1996; Arber et al., 1999; Thor et al., 1999), modulating the TGF-β1 signaling pathway by its inhibitor therefore probably drive the neuronal progenitor to other motor neuron identities rather than Hoxc8-specific caudal motor column type. Furthermore, small number of these differentiated cells exhibited ChAT and Isl-1 were observed. It is therefore importance to determine other factors that improve the efficacy of motor neuron differentiation from embryonic stem cells.
Further investigations are required to provide more understanding in the role of selective TGF-β1 inhibitor on cell fate identity during spinal cord development.

6.2 Conclusion and further studies

The potentiality represents of ES cells facilitate as a promising source to overcome many incurable diseases by providing an unlimited supply of all cell types, including neuronal cells and specific subtypes of NPCs including motor neurons, dopaminergic (DA) and glial cells, for future cell-based therapies for neurodegenerative and neurological disorders. ES cells have been used for studying biological and molecular events underlying organogenesis of embryos (Xian and Gottlieb, 2001; Lee et al., 2006). ES cells-derived specific cells/tissues inspire excellent regenerative potential, but the researches on these cells need more understanding of many obscure underlying mechanisms. Furthermore, to avoid the ethical and practical limitations of therapeutic application of ES cells, it would be useful to reprogram somatic cells directly into pluripotent state which resemble to ES cells properties without the use of oocytes and embryos. Then iPS cells become an alternative options for enforce regenerative medicine become reality (Chapter II). As we know, ES cells are now derived rather easily, and they can grow unlimited in

culture. ES cells can be manipulated genetically by either heterozygous or homologous recombination to correct a genetic defect (as we demonstrated in Chapter III). Even though prior the clinical applications of those cells, ideally, they are needed to optimize the differentiation of pluripotent stem cells and the development of a protocol to obtain uniform populations of cell precursors from ES cells. Therefore several techniques, chemicals, and growth factors-related neuronal development have been intensely investigated (Bibel et al., 2007; Robertson et al., 2008; Chuang et al., 2011) as we also have done in Chapter IV. ES cells can become any type of cells through the use of specific culture conditions such as NPCs, neurons, motor neuron progenitors and motor neurons. However, the further optimization are continually necessitated (Chapter V).

Summarily, study of mouse ES cells as a fundamental research model provides our understanding in complex molecular and cellular events during early development and also aims to improve the appropriated differentiation methods for future tissue/cell transplantation.

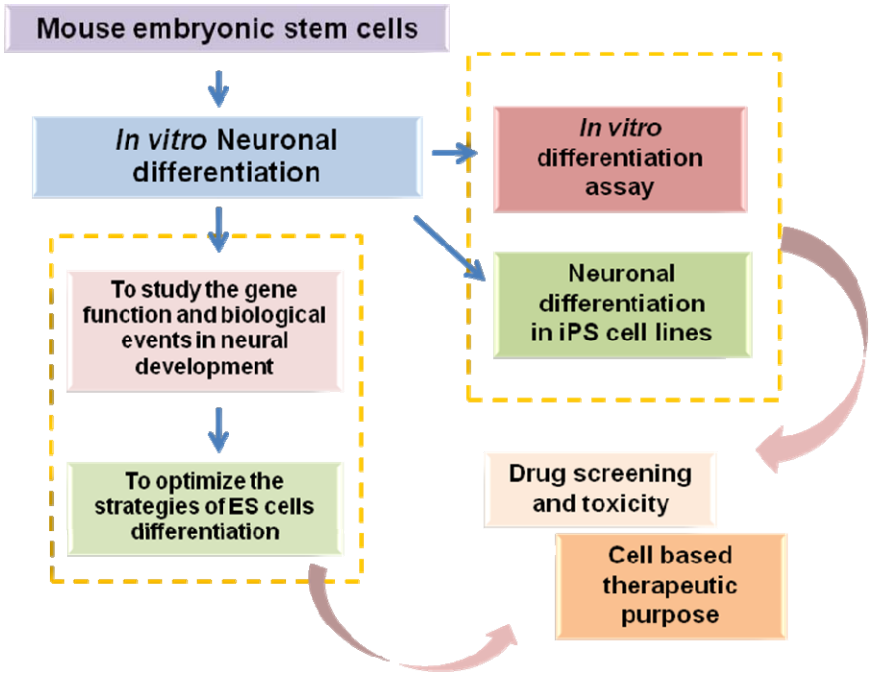

Figure 26. Potential of neuronal differentiation of mouse ES cells for valuable study model in neurogenesis

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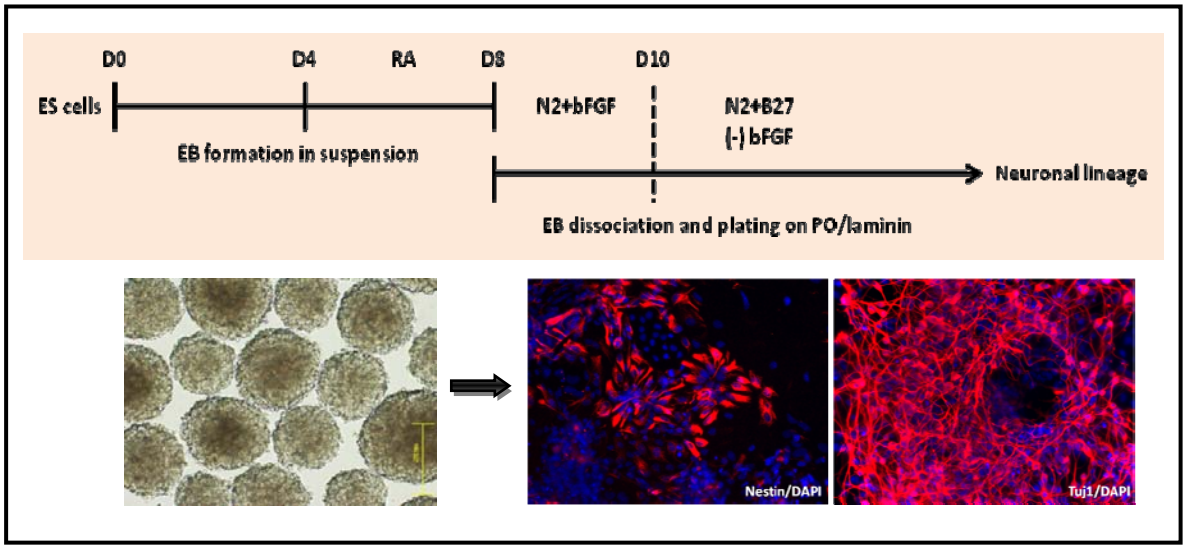
APPENDICES

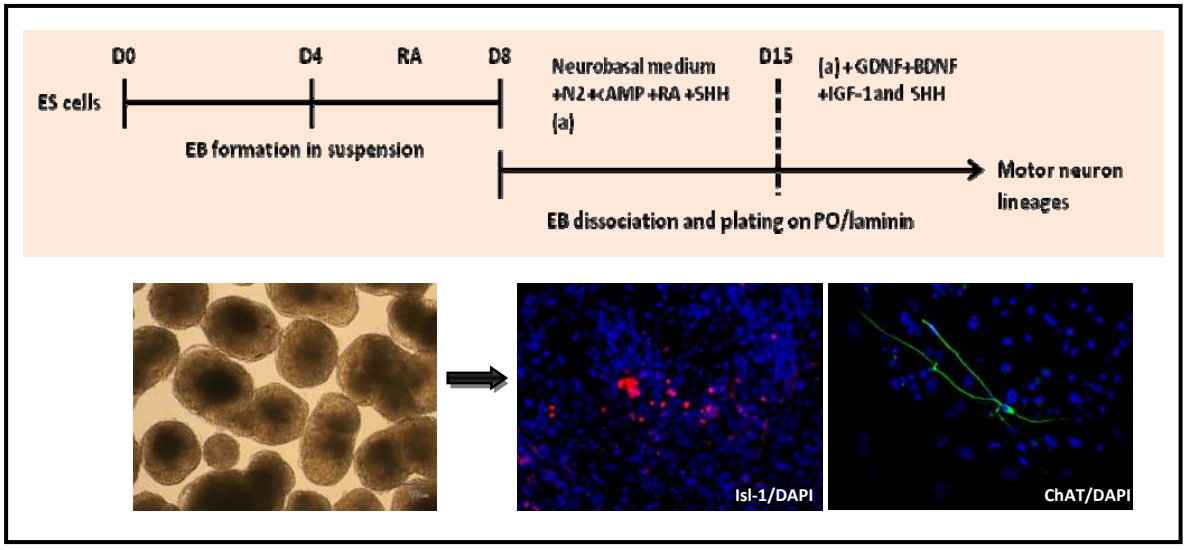
APPENDIX A

1. Neuronal differentiation of mouse pluripotent stem cells into NPCs and neurons



Supplementary figure 1. Schematic of pluripotent stem cells differentiation toward NPCs and neurons using EB formation method.

2. Neuronal differentiation of mouse ES cells into motor neurons



Supplementary figure 2. Schematic of pluripotent stem cells differentiation toward motor neuron lineage using EB formation method.

APPENDIX B

Paper publications

1. Klincumhom, N., Thongkittidilok, C., Tiptanavattana, N., Dinnyes, A., Tharasanit, T., Techakumphu, M. The effect TGF-β1 inhibitor on differentiation potential of embryoid bodies-derived from mouse ES cells toward neuronal lineage. Manuscript preparation.
2. Klincumhom, N., Thongkittidilok, C., Tiptanavattana, N., Phakdeedindan, P., Dinnyes, A., Tharasanit, T., Techakumphu, M. The effect of TGF-β1 inhibitor on the differentiation of mouse embryonic stem cells toward motor neurons. Manuscript preparation.
3. Klincumhom, N., Pirity, M.K., Berzsenyi, S., Ujhelly, O., Muenthaisong, S., Rungarunlert, S., Tharasanit, T., Techakumphu, M., Dinnyes, A. 2012. Generation of neuronal progenitor cells (NPCs) and neurons from mouse Sleeping Beauty transposon-generated induced pluripotent stem (iPS) cells. Cell Reprogram. In press.
4. Klincumhom, N., Thongphakdee, A., Techakumphu, M., Chatdarong, K. 2012. Time of the First Embryonic Cleavage Indicates Cat Blastocyst Quality. Thai J Vet med. 42 (1): 67-72.
5. Rungarunlert, S., Klincumhom, N., Bock, I., Nemes, C., Techakumphu, M., Pirity, M., Dinnyes, A. 2011. Enhanced cardiac differentiation of mouse embryonic stem cells by use of the slow-turning, lateral vessel (STLV) bioreactor. Biotechnol Lett. 33 (8): 1565-1573.
6. Thongphakdee, A., Siriaroonrat, B., Manee-in, S., Klincumhom, N., Kamolnorranath, S., Chatdarong, K., Techakumphu, M. 2010. Intergeneric somatic cell nucleus transfer in marbled cat and flat-headed cat. Theriogenology 73 (1): 120-128.

Proceeding and poster presentations

1. Dinnyes A., Ujhelly, O., Nemes, C., Muenthaisong S., Rungarunlert, S., Klincumhom, N., Varga, E., Lauko, A., Tosoki, R., Jakus, M., Polgar, Z., Feher, A., Pirity, M.K., Kovacs, K. Pluripotent stem cell-derived cardiac and neural cells for toxicity testing and regenerative medicine. Abstract in IV congress of polish biotechnology, IV EUROBIOTECH, Krakow, Poland, Oct 12-15th, 2011.
2. Klincumhom, N., Rungarunlert, S., Pirity, M.K., Ujhelly, O., Muenthaisong S., Chatdarong, K., Techakumphu, M., Dinnyes A. Generation of Neuronal Progenitor Cells (NPC) and Neurons Derived from Mouse Embryonic Stem (ES) and Induced Pluripotent (iPS) cells *in vitro.* Poster in 1st "Joint Symposium of Thai and Japanese Societies for Animal Reproduction". Chulalongkorn University, Bangkok, Thailand, Sep 29 – 30th, 2011.
3. Varga, E., Nemes, C., Klincumhom, N., Polgar, Z., Muenthaisong, S., Ujhelly, O., Pirity, M. K., Dinnyes A. Generation of mouse induced pluripotent stem cells from different genetic backgrounds by excisable lentiviral system. Abstract in ISSCR 9th Annual Meeting, Toronto, Ontario, Canada, June 15 – 18th, 2011.
4. Rungarunlert, S., Klincumhom, N., Nemes, C., Techakumphu, M., Pirity, M., Dinnyes, A. Mass Production of NKX2.5 Positive Cardiac Progenitor Cells Derived from Mouse Embryonic Stem (ES) Cells in Slow Turning Lateral Vessel (STLV) for Cell Transplantation and Drug Testing. Poster in 37th Annual conference of the IETS, Orlando, Florida, USA, Jan 8-12th, 2011.
5. Rungarunlert, S., Klincumhom, N., Tar, K, Muenthaisong, S., Techakumphu, M., Pirity, M., Dinnyes, A. Differentiation of Mouse Embryonic Stem (ES) Cells into Cardiomyocytes by using Slow Turning Lateral Vessel (STLV/BIOREACTOR). Poster in 36th Annual conference of the IETS, Cordoba, Argentina, Jan 9-12th, 2010.
6. Klincumhom, N., Thongphakdee, A., Chatdarong, K., Techakumphu, M., Comparison of the Developmental Ability and Quality of Cat IVF Embryos Produced from Fresh and Frozen-thawed Semen. Poster in 8th Chulalongkorn University Veterinary Annual Conference, Bangkok, Thailand, April 3rd, 2009.

7. Thongphakdee, A., Manee-in, S., Klincumhom, N., Siriaroonrat, B., Kamolnorranath, S., Chatdarong, K., Techakumphu, M., In vitro development of flat-headed cat (*Prionailurus planiceps*) cloned embryos. Abstract in 35th Annual conference of the IETS, California, USA, January 3rd-7th, 2009.

Training courses, conferences and oral presentations

1. Chulalongkorn-ASEAN-EU-PartnErsMeeting: Human stem cell research and animal models for therapeutic cloning. Bangkok, Thailand. February 8th, 2012. (Oral presentation)
2. Third Bi-regional EU-SEA Stakeholders Conference of the SEAEU-NET Project. "PartnErS": an EU FP7 IAPP project on pluripotent stem cell research for biomedical applications. Budapest, Hungary. November 24-25th, 2010. (Oral presentation)
3. Industem Yearly Open Workshop of Stem cells imaging technologies and cardiac differentiation. October 26th, 2010.
4. PartnErS and InduStem FP7 project yearly Open Workshop of Pluripotent Stem Cells in Animals and Humans. Eger, Hungary. September 15th, 2010.
5. Comparative embryonic stem cell research in mammalians (PartnErS) MidTerm Review Meeting. Eger, Hungary. September 14th, 2010.
6. "Bioimaging of Stem Cells" and "Workshop on Bioethics" at Department of Basic Animal and Veterinary Sciences, LIFE, Copenhagen, Denmark. April 12-17th, 2010.
7. PartnErS and InduStem FP7 project yearly open workshop and training of Nuclear Transfer and Embryonic Stem Cell Research in Animal Models. Gödöllő, Hungary. September 22th, 2009.
8. International Society for Stem Cell Research (ISSCR) 7th annual meeting. Center , Conventions Internacional Barcelona, Barcelona, Spain. July 8-11th, 2009.
9. Participated in the VPAT Regional Veterinary Congress held by the Veterinary Practitioner Association of Thailand (VPAT)

VITAE

Miss Nuttha Klincumhom was born on July 13th 1983 in Bangkok province, Thailand. She has been finished her degree of Doctor of Veterinary Science (DVM) with the 2nd honor from Faculty of Veterinary Science, Chulalongkorn University, Thailand on 2007. Before staring the stem cell researches, she has experiences about sperm preservation, *in vitro* fertilization and *in vitro* embryo production in feline. She has began her Ph.D. program after changed from Master's degree program on 2008, at the department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University. She has been supporting by grant under the program Strategic Scholarships for Frontier Research Network for the Joint Ph.D. Program Thai Doctoral degree from the Office of the Higher Education Commission, Thailand, and the PartnErS project which she was trained in research area of stem cells culture for 2 years at BioTalentum Ltd., Gödöllö, Hungary. She also participated in training programs established by the PartnErS project such as training course of "Bioimaging of Stem Cells and Workshop on Bioethics" at Department of Basic Animal and Veterinary Sciences, LIFE, Copenhagen, Denmark. Her thesis is focus primarily on differentiation of mouse ES/iPS cells into neuronal lineages, stem cell application-related neuronal development and the neuronal cell types-specific differentiation.