การศึกษาความสามารถในการเปลี่ยนแปลงเป็นเซลล์ประสาทของเซลล์ต้นกำเนิดชนิดมีเซนไคม์

นางสาวกัณวัฒน์ ด่านวิเศษกาญจน

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THE STUDY OF NEURAL POTENTIAL IN MESENCHYMAL STEM CELLS

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เซลล์ต้นกำเนิดชนิดมีเซนไคม์ (Mesenchymal stem cells; MSCs) เป็นเซลล์ต้นกำเนิดที่พัฒนาไปเป็นเซลล์กระดูก กระดูกอ่อน เอ็น เซลล์ไขมัน และเซลล์กล้ามเนื้อ แต่เมื่อไม่นานมานี้ได้มีการศึกษาถึงความสามารถในการเปลี่ยน MSCs ไปเป็นเซลล์ชนิดอื่นๆ (transdifferentiation) ที่ต่างไปจากเนื้อเยื่อที่เป็นแหล่งกำเนิด เช่น เซลล์ประสาท เป็นต้น เพื่อประโยชน์ในการนำไปใช้ทดแทน หรือสร้างสารที่ช่วยซ่อมแซมเซลล์ประสาทที่เสียไปจากโรคทางระบบประสาท หรืออุบัติเหตุ ซึ่งในระบบประสาทพบว่ามีการซ่อมแซมตัวเองอยู่ในระดับต่ำ ดังนั้นผู้วิจัยจึงได้ทำการศึกษาความสามารถของเซลล์ต้นกำเนิดชนิดมีเซนไคม์ของหนู (mouse MSCs) ในการเปลี่ยนแปลงเป็นเซลล์ประสาท โดยศึกษาถึงกลไกที่เกี่ยวข้องกับการแสดงอออกของ neural gene โดยใช้ retinoic acid เป็นตัวเหนี่ยวนำ พบว่ามีกลไกที่เกี่ยวข้องคือ REST/NRSF ซึ่งเป็น repressor ของ neural gene และ *NRSE* dsRNA ซึ่งพบได้ใน mouse MSCs และจะมีการแสดงออกเพิ่มขึ้นเมื่อเหนี่ยวนำด้วย retinoic acid ซึ่งจากการศึกษาในครั้งนี้ทำให้ทราบถึงกลไกเบื้องต้นที่สามารถ นำไปสู่การพัฒนาวิธีที่ทำให้สามารถพัฒนาเซลล์ต้นกำเนิดชนิดมีเซนไคม์ ให้ใกล้เคียงกับเซลล์ประสาทที่แท้จริงได้

สาขาวิชาวิทยาศาสตร์การแพทย์.....ลายมือชื่อนิสิต.....

ปีการศึกษา 2550.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

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Mesenchymal stem cell (MSC) is tissue-specific stem cells that can differentiat into bone cartilage, chrodocyte, adipocyte, and myoblast. Recently, there have been many reports that MSC have a capacity to differentiate into cells in other cell lineage, so called transdifferentiation, such as neural cells. Using either stem cell-based therapy or creating trophic factor is benefit for treatment of neural injury because the rebuilding neuron is an inefficient process in nature. Thus, understanding on this mechanism will lead to an improvement of MSCs future application, while fibroblast growth factor 2 (FGF2) and retinoic acid (RA) are commonly used to induce neural differentiation of MSCs. Our results proposed that the mechanism neural gene regulation is associated with repressor element 1 (RE 1)-silencing transcription factor (REST) also called neuron-restrictive silencer factor (NRSF), REST/NRSF and *NRSE* dsRNA, found specifically in mouse MSCs which only found in neural tissue. *NRSE* dsRNA levels were up-regulated when mouse MSCs were treated with RA. In this study we found the basic understanding that will lead to improve the neural differentiation method of MSCs for future application.

Field of Study Medical Science.....Student's signature Kannawat Danwisetkanjana

Academic year 2007.....Advisor's signature

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ABBREVIATIONS

CNS	central nervous system
NSCs	neural stem cells
MSCs	mesenchymal stem cells
bp	base pair
cDNA	complementary deoxyribonucleic acid
°C	degree Celsius
dsDNA	double–stranded deoxyribonucleic acid
DNA	deoxyribonucleic acid
et al	et alia
FGF-2	fibroblast growth factor 2
IgG	immunoglobulin G
IGF	insulin-like growth factor I
NGF	nerve growth factor
RA	retinoic acid
Kbp	kilo Base pair
kDa	kilo Dalton
ml	milliliter
mM	millimolar
mRNA	massager ribonucleic acid

OD	optimal density
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
RT-PCR	reverses transcription PCR
µl	microlitter
µg	microgram
µM	micromolar
ng/ml	nanogram per millilitter

CHAPTER I

INTRODUCTION

Background and Rationale

Many common neurological diseases, such as Parkinson's disease, stroke and multiple sclerosis, are caused by a loss of neurons and glial cells. Today most treatments aim to relieve symptoms instead of stop the progression of the disease, due to the low efficiency of rebuilding in adult brains. However, the discoveries of tissue-specific stem cells, which have a unique self-renewal capacities a powerful tool to repair or replace cells and tissue that lost function in the central nervous system (CNS).

Neural stem cells (NSCs) are the tissue-specific stem cells, which are mostly found in subventricular zone (SVZ) of adult brain and at subgranular layer in hippocampus (Morshead et al. 1994). Although NSCs can differentiate directly into neuron, astrocyte, and oligodendrocyte but the use of NSCs is limited by the lack of cell service in addition to the cell resource, the limiting of the quantification of NSCs is not sufficient for therapy, and the immune response from the patients. Therefore, an alternative source of stem cells such as embryonic stem cells (ESCs), which are derived from the culture of the inner cell mass in the blastocyst (4- to 5- day of embryo) seems ES cells have to be high preferable potential is a source because is no limit of cell division and they can differentiate into every type of cells in the body. However, to date, no medical treatments derived from ESCs research has been approved because of many problems including the ethical issue, the generation of embryoid body (the cell cluster from germ layers of the embryo-endoderm, mesoderm, and ectoderm). However, the isolation of particular ectodermal cells is difficult. When these cells are injected into the body they establish they form teratomas (benign tumor). However, these problems of histocompatibity could be solved using tissue-specific stem cells from other tissues of patients.

Nowadays, there are many reports that tissue-specific stem cells are able to differentiate into specialized cell types, which different from its origin. This phenomenon is called transdiferentiation. Mesenchymal stem cells (MSCs) from bone marrow, have also commonly been reported to differentiate into various cell types, including osteocyte, chondrocyte, myocyte, and heamatosis-supporting cells, these cells powerful tools in the study of neural transdifferentiation because they are easy isolate from other cells in the bone marrow and can be expanded with high efficiency. In addition, they are able to differentiate into a variety of cell types when cultured under specific conditions (Woodbury et al. 2002).

There are many methods of *in vitro* induction of MSCs differentiation, Woodbury and colleague (Woodbury et al. 2000) reported that rat and human MSCs showed a neuronal morphology within 5 h and expressed the neuron-specific markers including nestin, neuron-specific enolase (NSE), Neuronal nuclei (NeuN), neurofilament-M, and tau, but no expression of the astroglial marker, glial fibrillary acidic protein (GFAP), when treated with media composed of Dulbecco's Modifed Eagle's Medium (DMEM), and different reducing agents such as β-mercaptoethanol (BME), dimethylsulfoxide (DMSO), and butylated hydroxyanisole (BHA). However, there has been an argument against since the morphological change and increase in immunolabeling for neuron-specific markers in MSCs was likely the result of cellular toxicity, cell shrinkage, disruption of the actin cytoskeleton, and a retraction of the cell edge (Lu et al. 2004; Neuhuber et al. 2004).

As Sanchez-Romos and colleague (Sanchez-Ramos et al. 2000) showed that co-culturing of human or mouse MSCs with rat fetal mesencephalic or striatal cells increased a neuron-like cells that expressed NeuN and GFAP. Consistent with these results, Aboulfetoh and colleague (Abolfetoh et al. 2004) reported that MSCs co-cultured with hippocampal brain slices can be differentiated into neuron-like cells. These reports suggested that cell-to-cell contact, trophic factors, and cytokines, play an important role in neural differentiation of MSCs. However, they have claimed that MSCs induction into neuron by co-culturing condition involved cell fusion. This experiment is a part of normal mammalian tissue development, such as the development of skeletal muscle cells and bone tissue osteoclasts and formation of giant cells from mononuclear phagocytes.

Report by Weimann and colleague (Weimann et al. 2003) showed that bone marrow-derived cells fused with the Purkinje neurons, which were obtaind from brain samples autopsied from female patients who received male bone marrow transplants. This might be caused either by regeneration of Purkinje neurons from bone marrow-derived cells or by fusion of marrow-derived cells with existing recipient Purkinje neurons. In addition, Terada and colleague (Terada et al. 2002) demonstrate that *in vitro* mouse MSCs fused spontaneously with ESCs in culture that contains interleukin-3 and these spontaneously fused cells can subsequently adopt the ESCs phenotype. However, cell fusion mechanisms cannot describe the results of the *in vitro* studies that MSCs transdifferentiation into neuron, which are not co-cultured with other cell types or tissues.

Following these studies an increasing number of independent groups have reported on MSCs transdifferentiation into neuron *in vitro*, including a study a demethylating agent, 5-azacytidine compound (5-azaC) (Kohyama et al. 2001), a physiological neural inducer, noggin (Kohyama et al. 2001), growth factors (alone or in combination with substrate) (Sanchez-Ramos et al. 2000; Kim et al. 2002; Jin et al. 2003; Qian et al. 2004), such as epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), retinoic acid (RA) etc. Substrates and extracellular matrix component were also used such as laminin gelatine, collagen, fibronectin, and polyornithine etc (Kim et al. 2002).

Although a variety of methods could be used to induce MSCs into neural-like cells, which express neural markers, the distribution of these neural markers in induced MSCs was different from mature neurons and is insufficient for neural differentiation (Jin et al. 2003). Besides, in these experiments, the functions of neuron from neural-like cells including electrophysiological parameters, such as resting membrane potentials, depolarizations in response to changes in membrane permeability to potassium and sodium, and induction of actions potentials, have been few tested. So far, most *in vitro* studies, with few researches has published reported for functional activity. Kohyama and colleague tested the ion channel in bone marrow-derived neural-like cells by the patch clamp method (Kohyama et al. 2001). Recently, report by Tropel and colleageue (Tropel et al. 2006) on the neural function of MSCs derived neural-like cells

demonstrated that FGF2-treated mouse MSCs expressed functional neural receptors and voltage-dependant channels. Although many methods have be used to study the neural function of MSCs-derived neural-like cells, there is no study the mechanism of neural induction of MSCs.

In developmental biology, several studies indicated that activation or suppression of master genes is essential for the differentiation of cells from one stage to another. As in the developing nervous system, the terminal differentiation stage of neuron express functional neural gene products, such as sodium channel type II, synapsin, glutamate receptor (GluR), and acetylcholine receptor, which are mostly direct targets of the repressor element1 (RE1)-silencing transcription factor (REST)/neuron-restrictive silencer factor (NRSF; Chong et al. 1995; Schoenherr and Anderson 1995).

REST/NRSF was found to be a transcriptional repressor of a large number of terminal neural differentiation genes in non-neural cells (Chen et al. 1998; Huang et al. 1999; Palm et al. 1998; Schoenherr et al. 1995). The regulation of neural genes expression of REST/NRSF by binding to a 23 bp consensus sequence (RE1 binding site/neuron-restrictive silencer element or RE1/NRSE), which is mostly present at the upstream promoter enhancer region of these genes (Immaneni et al. 2000). REST/NRSF requires interaction with several cofactors, including mSin3A and histone deacetylase complex (HDAC) which are bind to the N-terminal repressor domain and CoREST and HDAC which are bind to the C-terminal repressor domain. Based on the expression pattern of mSin3A, and CoREST, it has been proposed that mSin3A is constantly required for REST/NRSF repression, whereas CoREST is recruited for more specialized repressor functions (Grimes et al. 2000). Gene deletion studies with REST/NRSF$^{-/-}$ mice indicated that the absence of REST/NRSF *in vivo* cause expression of only one of the REST/NRSF target genes, the neuron-specific tubulin gene (Chen et al. 1998). This experiment suggested that the absence of REST/NRSF alone is not sufficient to activate other REST/NRSF target genes. In the study of Majumder's groups proposed that there are two classes of neural differentiation genes in animals (Immaneni et al. 200); one represented by the β-III tubulin gene, which depends primarily on the release of REST-

dependent direct repression and the other represented by synapsin and glutamate receptor genes, which require additional promoter/enhancer-specific positive activators (Majumder 2006). Immaneni et al. (Immaneni et al.2000) proposed that the recombinant transcription factor, REST-VP16, which containing the DNA binding domain of REST/NRSF and the activator domain of the herpes simplex virus protein VP16. This recombinant protein can directly activate the transcription of REST/NRSF target genes in multiple cell types. In addition, there is a report that direct activation of REST/NRSF target genes in NSCs with REST-VP16 is sufficient to neuronal differentiation, and this finding suggested that direct activation of the terminal differentiation genes may cause neural differentiation of NSCs (Su et al. 2004). Results from Watanabe et al. 2004 demonstrated that REST-VP16 could activate REST/NRSF target genes and other neural-differentiation genes in myoblasts cells and changed these cells which are normally differentiated into muscle cells into cells expressing physiologixall active neural phenotype. These works provide an efficient way of triggering neural differentiation in myoblasts and possibly other stem cells.

Beside the regulation of REST/NRSF target genes by REST/NRSF repressor complex, which relates to embryonic neurogenesis, in adult neurogenesis, there is another mechanism that regulates differentiation of adult neural stem cells. Kuwabara and colleague proposed that a 20 bp noncoding double strandsRNA (dsRNA) isolated from adult hippocampal neural stem cells, which resembles *RE-1/NRSE* sequences (Kuwabara et al. 2004) in a broad range of genes, involved in neural development and function (Johnson et al. 2007). They called this dsRNA was *NRSE* dsRNA. The *NRSE* dsRNA directly interact with the REST/NRSF transcriptional machinery present in these cells, preventing REST/NRSF from binding to its corepressors and converting REST/NRSF function from a repressor to an activator, which leading to neural differentiation (Kuwabara et al. 2004). As It is the interest to study the effect of *NRSE* dsRNA on mouse MSCs transdifferentiation. We have constructed a stable *NRSE* dsRNA expression system in mouse MSCs for this proposes.

In 1988, Green and Loewenstein. and Frankel et al. 1988 independently discovered trans-activator protein TAT, which was encoden by human

immunodeficiency virus 1 (HIV-1). This protein, also called HIV-TAT, is able to cross cell membrane and get translocate to nucleus in a transcriptionally active form (Green et al 1988; Frankel et al. 1988). In addition, HIV-TAT can interact with small and large macromolecular protein (Fittipaldi and Giacca. 2005). Therefore, in the last few years, many laboratories used this system as a tool for cellular transduction of proteins, based on the fusion of the protein transduction domain of TAT to protein, either at the N-terminus or at the C-terminus, followed by the addition of the recombinant fusion protein to the culture medium of the cells of interest.

The aim in this study to understand the mechanism of neural transdifferentiation involved in REST/NRSF regulation upon retinoic acid (RA) induction and REST-VP16 protein transduction in mouse MSCs cells.

Research Questions

Which mechanism is involved in growth factor induced neural differentiation in mouse mesenchymal stem cells (MSCs)?

Objectives

To study the mechanism of neural differentiation in mouse MSCs when induced with growth factors.

Hypothesis

Growth factor induce neural differentiation of mouse MSCs is involved the REST/NRSF repressor pathway.

CHAPTER II

LITERATURE REVIEW

1. Stem cells

Stem cells are cells which can go through both symmetric and asymmetric cell division, are self renewing and give rise to specialized cells. The self renewal capacity lead to at least one daughter cell that has the same potency as the mother cell (Figure 1A, B). The high degree of potency results in the ability to form a large number of different mature cell types.

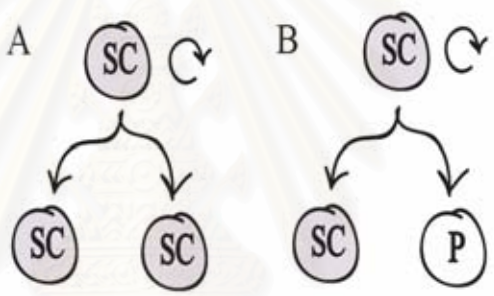


Figure 1. **Symmetric versus asymmetric cell division.** A mother cell is forming daughter cells with interchangeable fate (A) and different fates (B). SC, stem cell; P, Progenitor cell.

2. Totipotent and pluripotent stem cells

The ultimate stem cell is the fertilized egg. This cell is completely totipotent, which means that it can from the entire embryo and the extra embryonic tissues such as the placenta.

When the fertilized egg has undergone approximately twelve cell divisions, it arrives at a developmental stage called blastula. Inside the inner cell mass of this blastula, the so called embryonic stem cells (ES). These cells are completely undifferentiated, rapidly dividing with the potential to form the entire embryo, but not the extra embryonic tissues and are, therefore, called pluriotent.

3. Neural stem cells (NSCs)

In the developing embryonic brain, neural stem cells (NSCs) can be isolated from various regions such as the striatum, hippocampus and cortex. These NSCs can be cultured either as neurospheres or as monolayer cultures (Figure 2 A, B). In either case, NSCs require either fibroblast growth factor 2 (FGF2), or epidermal growth factor (EGF) to proliferate and to maintain their potency.

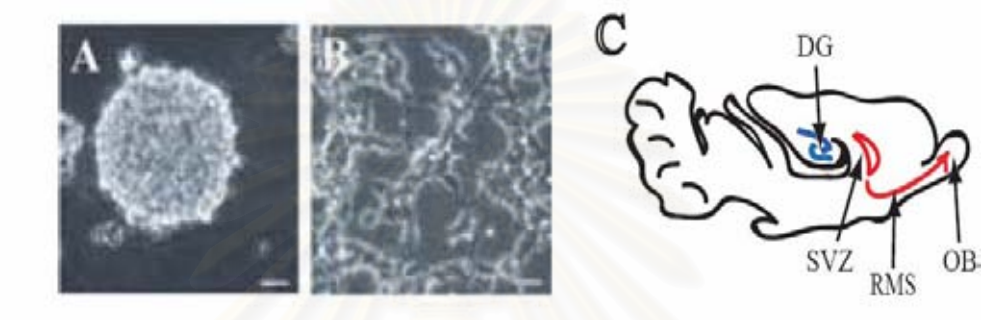


Figure 2. **Cultured NSCs and neurogenic regions of the adult brain.** Neurosphere (A), monolayer culture (B), dentate gyrus (DG); sub ventricular zone (SVZ); rostral migratory stream (RMS); olfactory bulb (OB) (C).

In the adult brain, neurogenesis occurs only in discrete areas, such as the subventricular zone (SVZ) or the dentate gyrus (DG) of the hippocampus (Altman and Das, 1965; Eriksson et al. 1998; Gould et al. 2001); these are the two regions from which adult NSCs can be isolated.

The identity of the progenitors found in the subventricular zone (SVZ) is, at the moment, under debate. One theory proposes that the nestin expressing ependymal cells present in the lining of the lateral ventricle are NSCs. Opposing evidence is suggesting that SVZ NSCs are multipotent astrocytes that can form neurons *in vivo* (Chiasson et al. 1999; Doetch et al. 1999; Laywell et al. 2000). These SVZ progenitors migrate as committed neuroblasts through the rostral migratory stream (RMS) to the olfactory bulb (OB), where they differentiate into interneurons (Figure 2C) (Garcia-Verdugo et al. 1998; Alvarez-Buylla and Lim, 2004). Since there are no radial glial cells present in the RMS, it is unknown how these neuroblasts know where to migrate (Garcia-Verdugo et al. 1998).

4. The life of a neural stem cell.

Neural stem cells (NSCs) are the most immature cells in the nervous system. These cells are going through both symmetric and asymmetric cell division, thus are both self renewing and multipotent (Temple et al. 2001). While symmetric stem cell division results in an exponential increase in cell number, asymmetric cell division maintains the stem cell pool and gives rise to more restricted progeny (Figure 3).

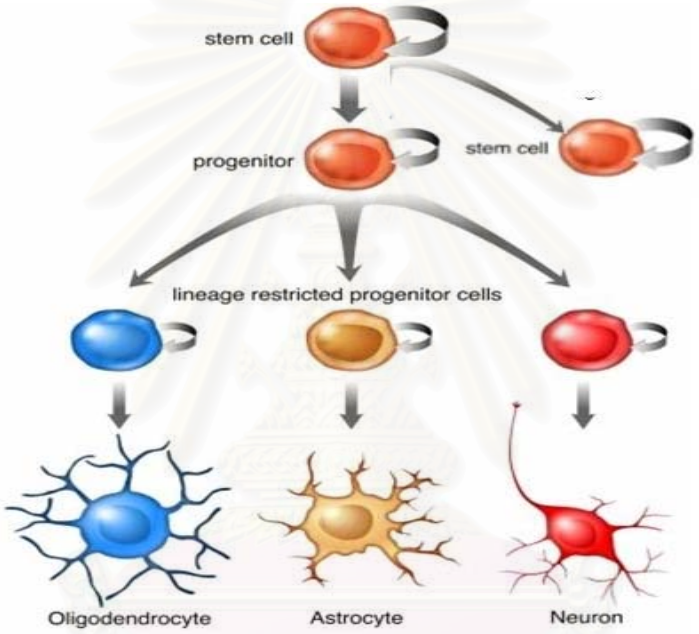


Figure 3. **The neural commitment.** (From http://www.gen.cam.ac.uk/Research/AMA/Tibor Kalmar.html)

While regulation of NSCs proliferation and fate determination is far from being completely understood, it is clear that developmental pathways such as the Notch pathway and cell fate specific transcription factors are involved.

5. Neural stem cells fate determination.

5.1 Notch pathway and cell fate specific transcription factors.

Notch is known to inhibit neuronal differentiation and maintain neural stem cells (Gaiano et al. 2002). Notch is a transmembrane protein that is activated by the ligands Delta and Jagged, which are also transmembrane proteins expressed by neighboring cells (Figure 4, step (1)). Upon activation, Notch is processed to release the intracellular domain (ICD), which is transferred into the nucleus and forms a complex with the DNA-binding protein RBP-J (Figure 4, step (2)) (Honjo et al. 1996; Selkoe et al. 2003). RBP-J itself is a transcriptional repressor and represses pro-neural bHLH protein; Hes1 and Hes5, expression by binding to their promoters (Figure 4, step (4)). However, when RBP-J forms a complex with Notch ICD, this complex becomes a transcriptional activator and induces Hes1 and Hes5 expression (Figure 4, step (2)). Thus, Notch activation leads to upregulation of Hes1 and Hes5 expression. In the absence of Hes1 and Hes5, however, Notch fails to inhibit neuronal differentiation, indicating that Hes1 and Hes5 are essential effectors of Notch signaling (Ohtsuka et al. 1999). In contrast to Hes1 and Hes5, there is no evidence that Hes3 expression is controlled by Notch signaling (Nishimura et al. 1998). In addition, Hes1 expression occurs at early stages when Notch and Delta are not expressed (see below). Thus, Notch signaling is not the sole regulator of Hes expression, although it remains to be determined which factors, in addition to Notch, directly regulate Hes expression.

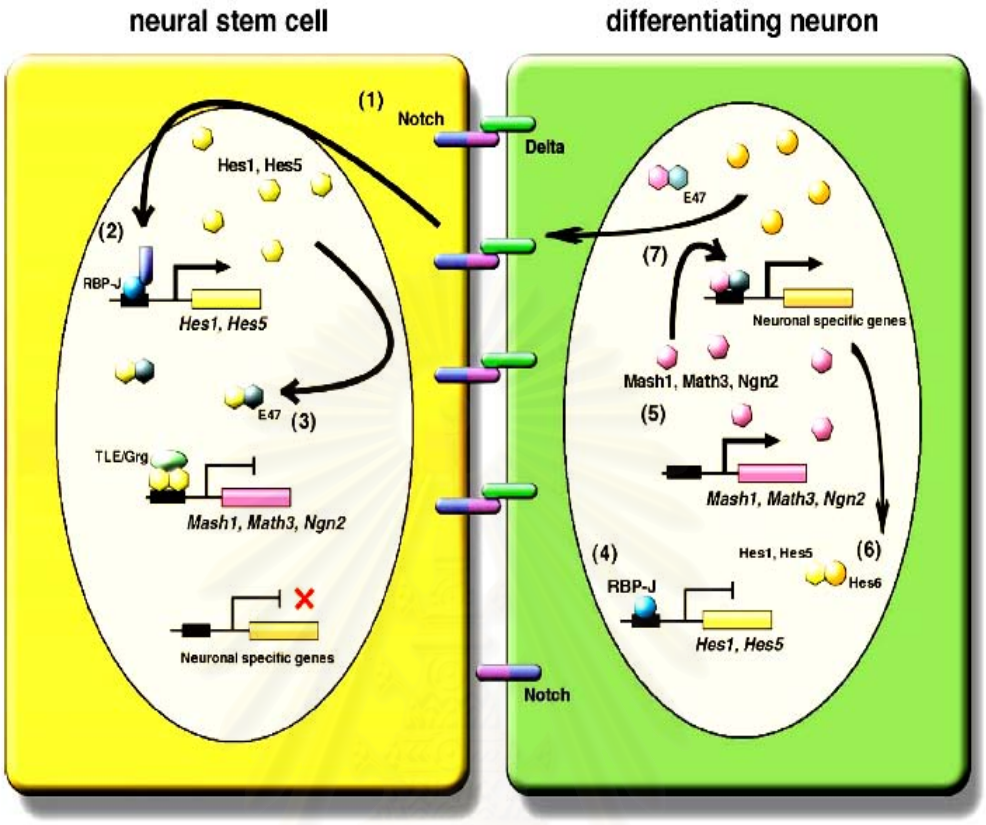


Figure 4. **Regulation of neural development by the repressor-type and activator-type bHLH genes.**

The family of transcription factors contains both pro-neural bHLH proteins such as Mash1, Ngn1 and 2, NeuroD and Math3, as well as transcription factors which direct progenitors into a gliogenic fate such as Hes1 and 5, olig1 and 2, and Ngn3 (Kintner et al. 2002).

In vitro, NSCs isolated from the early brain are dependent on bFGF for proliferation (Kalyani et al. 1997; Raballo et al. 2000), while at later stages NSCs require either bFGF or EGF for proliferation (Gritti et al. 1999; Tropepe et al. 1999). Upon withdrawal of bFGF or EGF, the NSCs will spontaneously differentiate into neurons and glial cells (Johe et al. 1996). A number of mitogenes have been identified that can influence the fate determination upon bFGF or EGF withdrawal, and some of the most potent ones are shown in Figure 5.

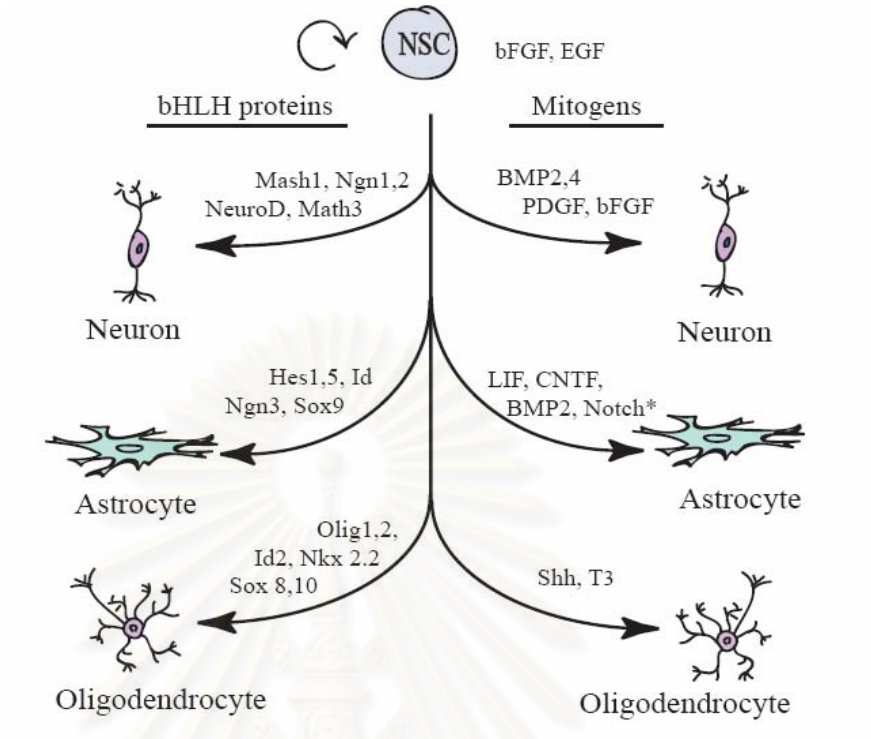


Figure 5. **bHLH and mitogens involved in NSC fate determination.**

6. Mature cell types within the central nervous system (CNS).

6.1 Neurons

Neurons are unique in the sense that they are highly specialized for inter-celular signaling. As a result, neurons have a special morphology, membrane formation and the ability to form synapses with connecting neurons. The axon is optimized for signal transduction, and its length can vary between neuronal types depending on how far the signal needs to be transmitted. While axons transmit information, dendrites are specialized for receiving information. As in the case of axons, the extension and branching of the dendrites vary between neuronal types.

6.2 Glial cells

The most abundant cell types in the nervous system are the glial cells. There are three types of glial cells in the CNS: astrocytes, oligodendrocytes and microglia. Glial

cells have important supportive roles, which include for example providing neurons with trophic support. Their functions have previously been underestimated. Recently, a study showed that factors secreted from astrocytes are able to instruct NSCs into forming neurons (Song et al. 2002). Also, Alvarez-Buylla and co-workers have identified a population of proliferating glial fibrillary acidic protein (GFAP) positive progenitors in the vicinity of the lateral ventricle of the adult brain (Seri et al. 2001). At the present moment, evidence is pointing towards that multipotent astrocytes are in fact the SVZ NSC which can form neurons *in vivo*.

7. Markers used in NSC research

Because studies of the nervous system depend on the ability to accurately distinguish between different cell types, biological markers are used. Some of the most commonly used markers in NSC research are summarized in Table 1.

Table 1. *Cell markers commonly used in NSC research.*

Cell type	Marker	Reference
NSCs	Nestin	Lendahl, 1990
"	Musashi	Yagita et al., 2002; Sakakibara et al., 2002
"	Sox1, 2	Pevny et al., 1998; Sasai, 2001
"	Bmi-1	Molofsky et al., 2003
"	Vimentin	Houle and Fedoroff, 1983
Immature Neurons	β-III-tubulin	Caccamo et al., 1989
"	MAP-2	Garner et al., 1988; Matus et al., 1988
Immature glial cells	A2B5	Hirano and Goldman, 1988
"	Olig 1	Zhou et al., 2000.
"	Sox 10	Kuhlbrodt et al.,1998
"	Nkx 2.2	Qi et al., 2001
"	Ngn 3	Liu et al., 2002
Neurons	NeuN	Mullen et al., 1992
"	NF (Neurofilament)	Debus et al., 1983
Cholinergic	ChAT	Haigh et al., 1994
Dopaminergic	TH	Nagatsu et al., 1964
GABAergic	DARPP-32	Lewis et al., 1983
Glutamatergic	VGLUT1, 2	Arriza et al., 1994
Seratonergic	SERT	Blakely et al., 1991
Astrocytes	GFAP	Eng et al., 2000
"	S100-β	Zimmer at al., 1995
Oligodendrocytes	CNPase	Staugatis et al., 1990
"	O4	Sommer and Schachner, 1981
"	MBP	Hartman et al., 1982

8. Mesenchymal stem cells (MSCs)

8.1 Sources of MSCs

Mesenchymal stem cells, also called bone marrow stromal cells and multipotent stromal cells (MSCs), are isolated from bone marrow (Caplan et al. 1991; Pittenger et al. 1999), adipose tissue (De Ugarte et al. 2003), skeletal muscle (Jankowski et al. 2002) and umbilical cord blood (Hou et al. 2003). MSCs are of mesoderm origin and can differentiate into mesenchymal derivatives, including osteocytes, chondrocytes, adipocytes, myocytes (Pittenger et al. 1999), and provide the stromal support system for haematopoietic stem cells (HSCs) (Figure 8). A number of studies have suggested that MSCs may also be capable of differentiating to neurons and glia under specific conditions *in vitro*.

To date, mesenchymal stem cells have been isolated from marrow, muscle, fat, skin, cartilage, and bone. Although, in bone marrow, MSCs are only found in low numbers (1 MSC per 10^5 mononuclear marrow cells), they are easy to purify and expand them (Pittenger et al. 1999). MSCs have also been isolated from tibial and femoral marrow compartments, after aspirating suspension cells from bone marrow and eliminate mononuclear cells by using density-gradient fractionation; MSCs can be separated from HSCs by their plastic-adherent propery. However, they remain a heterogeneous mixture of cells with varying proliferation and differentiation potentials.

8.2 Surface markers on MSCs

MSCs constitute a heterogeneous population of cells, in terms of their morphology, physiology and expression of surface antigens. Up to now, no single specific marker has been identified (Bobis et al. 2006). Many attempts have been made to develop a cell-surface antigen profile for better purification and identification of MSCs. Most studies focused on MSCs from human and mouse bone marrow. Negative markers of MSCs include CD11b (an immune cell marker), glycophorin-A (an erythroid lineage marker), CD45 or CD34 (marker of all hematopoietic cells), whereas positive MSCs markers are CD105, CD166, CD54, CD106, Stro-1, for instance. However, it has been

reported that CD106 singles out 1.4% of Stro-1-postive cells-expressing cells, increasing the CFC-F frequency to 1 in 3, which are all high Stro-1-expressing cells and are the show only Stro-1-positive cells that form colonies and show stem cells characteristics such as multipotentiality, expression of telomerase, and high proliferation *in vitro* (Gronthos et al. 2003). CD73, or lymphocyte-vascular adhesion protein 2, is a 5' nucleotidase . Although also expressed on many other cell types, two monoclonal antibodies (SH-3 and SH-4) against CD73 were developed with specificity for mesenchymal tissue-derived cells (Haynesworth et al. 1992). These antibodies do not react with HSCs, osteoblasts, or osteocytes, all of which could potentially contaminate plastic-adherent MSC cultures. The persistence of CD73 expression throughout culture also supports its utility as an MSC marker.

Stro-1, CD73, and CD106 have beenreported to be the most useful markers, although their functions remain to be determined (Kolf et al. 2007). Cell migration, cytoskeletal response, and signaling pathway stimulation assays currently used to analyze other MSC membrane proteins may prove to be helpful in studying these markers (Honczarenko et al. 2006).

9. Transdifferentiation of mesenchymal stem cells

9.1 Definition and concept of transdifferentiation

The term transdifferentiation was originally used by developmental biologists to describe the ability of apparently fully differentiated cells derived from a given tissue to change into cells with characteristics of a different tissue in response to either cell culture or surgical removal of adjacent tissue. Today the term ''transdifferentiation'' is commonly used to describe the plastic ability of tissue-specific stem cells to differentiate into cell lineages of tissues different from the one in which the somatic stem cell resides, and even into cells originating from other germ layers. Transdifferentiation *sensu stricto* must involve genetic reprogramming with turning off of some sets of genes and turning on of others. If such transformation occurred rapidly, transient coexpression of products of both sets of genes might be found in the cell. If, on the other hand, the process of transformation lasted some time, there might be intermediate stages of cells, where sets

of original cell type-characteristic genes were inactive, while new sets of genes had not yet been activated. Clearly true transdifferentiation can only be considered to have taken place when a new characteristic cellular phenotype is stably established (Slack et al. 2001).

9.2 Transdifferentiation of MSCs into neurons

Under various defined culture conditions and, stimulatory compounds, MSCs have been reported to exhibit transdifferentiation plasticity Woodbury and colleague (Woobury et al. 2002) found that populations and clonal lines of MSCs expressed ectodermal, endodermal and mesodermal genes, assuming a far broader differentiational potential or plasticity of MSCs than expected.

Several methods of *in vitro* Neuronal transdifferentiation of MSCs have been reported, including using compound (Woodbury et al. 2000; Sanchez-Ramos et al. 2000; Deng et al. 2001; Kohyama et al. 2001), co-culturing of MSCs with hippocampal brain slices (Aboulfetouh et al. 2004), growth factor (Jin et al. 2003) and combination of growth factor and substrate (Kim et al. 2002). So this data suggesting that cell-to-cell contact, trophic factors and cytokines, in addition to conditions that increase intracellular cAMP could induce plays an important role in neural differentiation of MSCs (Aboulfetouh et al. 2004; Krabbe et al. 2005).

In developmental biology several studies indicate that activation or suppression of so-called ‘‘master genes’’ is essential for the differentiation of cells from one stage to another. The differentiation of NSCs into neurons is generally believed to involve four steps characterized by the expression and action of specific gene products (Immaneni et al. 2000; Lawinger et al. 2000). In the first step, NSCs, which can multiply and make their own kind under one set of conditions and differentiate under other conditions, express p75 and nestin. The next step, the neuronal determination step, is characterized by the action of the basic helix-loop-helix proteins, such as MASH, MATH, and neuroD3/neurogenin. This is followed by the commitment step, in which gene products such as neuroD1/2, Myt1, and neurofilament 150 are expressed. The terminal differentiation step is characterized by the expression of gene products such as SCG10, sodium channel type II, synapsin, glutamate receptor, and acetylcholine receptor. It is

mostly the terminal differentiation genes that are direct targets of the REST/NRSF transcriptional repressor

10. The RE-1 silencing transcription factor or neuron-restrictive silencer factor (REST/NRSF)

The repressor element 1 (RE1)-silencing transcription factor (REST), also known as the neuron-restrictive silencer factor (NRSF) was first found in 1995 (Chong et al. 1995; Schoenherr and Anderson 1995). REST/NRSF is a 116-kDa zinc finger protein that contains a cluster of eight zinc-finger proteins as a DNA-binding domain, followed by a region rich in basic amino acids, a cluster of six proline-rich repeats, a single zinc finger near and two repressor domains: one at the N-terminal and the other at the C-terminal of the protein. The DNA-binding domain of REST/NRSF binds to a 21-bp consensus DNA regulatory element, consensus RE1-binding site/neuron-restrictive silencer element (*RE1/NRSE*) present in the genes' regulatory regions (Figure 6).

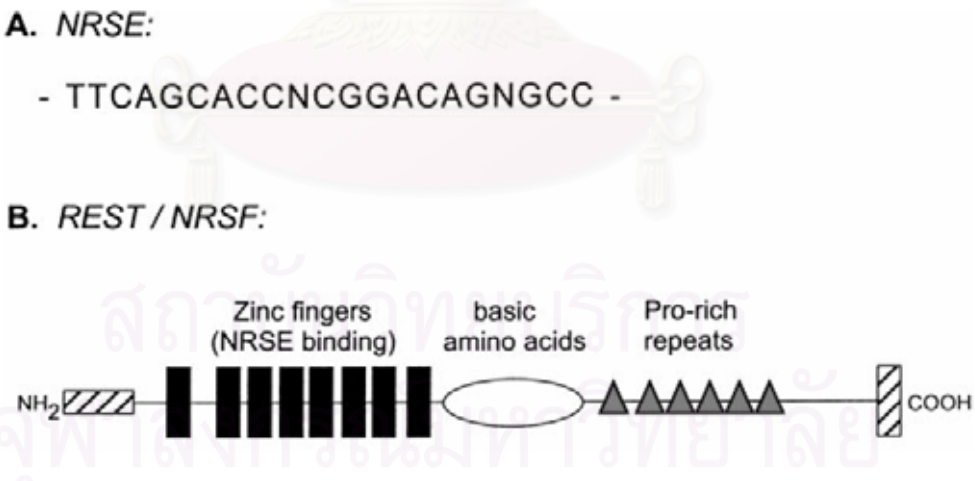


Figure 6. **A:** DNA sequence of the RE1-binding site/neuron-restrictive silencer element (*RE1/NRSE*) defined in a recent database search. **B:** Schematic representation of the repressor element 1 (RE1)-silencing transcription factor (REST), also known as the neuron-restrictive silencer factor (NRSF).

10.1 The epigenetic mechanisms to inactivate neural genes outside the nervous system.

The repressor domain of REST/NRSF can interact with several cellular cofactors to repress chromatin at its target promoters. The N-terminal repressor domain of REST/NRSF interacts with mSin3, a corepressor found in all eukaryoted that recruits histone deacetylases (HDACs) (Grimes et al., 2000; Naruse et al., 1999). The mSin3-HDAC complex, however, is associated primarily with a dynamic mode of repression that can alternate between repression and activation and, therefore, by itself, would probably be insufficient for long-term silencing of neural genes. The C-terminal repressor domain of REST/NRSF interacts with CoREST, the corepressor which is present only in organisms with a nervous system (Dallman et al., 1998), pointing to CoREST as a more specialized corepressor. Several recent studies indicate that the REST-CoREST complex recruits chromatin modifiers for long-term silencing of neural genes (Lanyak et al., 2002; Lanyak et al., 2004) (Figure 7a). Specifically, CoREST can form immuno-complexs not only with HDACs but also with the histone H3 lysine 9 (H3-K9) methyltransferase G9a and with the newly discovered histone H3 lysine 4 (H3-K4) demethylase LSD1, also known as KIAA0601 or BHC110 (Hakimi et al., 2002), both of which mediate modifications associated with gene silencing. Importantly, these histone-modifying enzynes are required for REST-CoREST silencing in non-neural cells (Shi et al., 2004). Furthermore, CoREST recruits to the REST-RE1 site other silencing machinery, including the methyl DNA-binding protein MeCP2 and the histone H3-H9 methyltransferase SUV39H1 (Lanyak et al., 2002). Heterchromatin protein 1 (HP1), which cause compaction of chromatin and is associated with histone H3-K9 methyltransferase, is also present on the neuronal gene chromatin (Lanyak et al., 2002), specifically on the RE1 region (Roopra et al., 2004). The effects of these modifications are manifested in histone deactylation, an absence of H3-K4 methylation, and presence of H3-K9 methylation, which creates binding sites for HP1 and condensation of the targeted chromatin (Figure 7b). In addition the recruitment of silencing machinery by REST-CoREST might result in the propagation of silencing across a large chromosomal interval containing several neuronal genes that do not have their own REST binding site (Lanyak et al., 2002), suggesting a relationship between higher order chromatin

structure and pattern of gene expression. In addition, The REST binding site (RE1) contains a CpG dinucleotide and the RE1 and surrounding region of neural genes is methylated in differentiated non-neural cells (Ballas et al. 2005). Furthermore, the DNA methyltransferase DNMT1, which interacts with histone H3-K9 methyltransferases is associated with the RE1 region of neural gene chromatin (J Chenoweth and G Mandel, unpublished). However, binding of REST to the RE1 site is independent of DNA methylation (Ballas et al. 2005). The repressor MeCP2 binds mehylated DNA and recruits HDAC and histone H3-K9 methyltransferase activity.

In embryonic stem cells (ES cells), REST/NRSF is bound to the RE1 motif and present corepressors, CoREST, mSin3, HDAC and MeCP2, unlike the situation in differentiated non-neural cells, the RE1 motif and surrounding sequences in neural genes are not methylated. In this case, MeCP2 is probably recruited to the RE1 by an mSin3-HDAC complex. Coincident with the hypomethylated DNA is the absence of histone H3-K9 methylation in the RE1 region and greatly reduced levels of the associated methylatransferase G9a (Ballas et al., 2005). Moreover, the repressed neural gene chromatin in ES cells is instead enriched in di- and tri- methylated K4 on histone H3 (Ballas et al., 2005), modifications associated normally with actively transcribed genes. In the case of ES cells, but not terminally differentiated non-neural cells, RNA polymerase II (Pol II) is present on RE1 sites in the 5' untranslated regions of several neural genes, accompanied by very low transcript levels (Ballas et al., 2005). Thus, the epigenetic modifications associated with the RE1 sites of neural genes in stem cells point to an inactive, but permissive, chromatin state that is poised for subsequent activation.

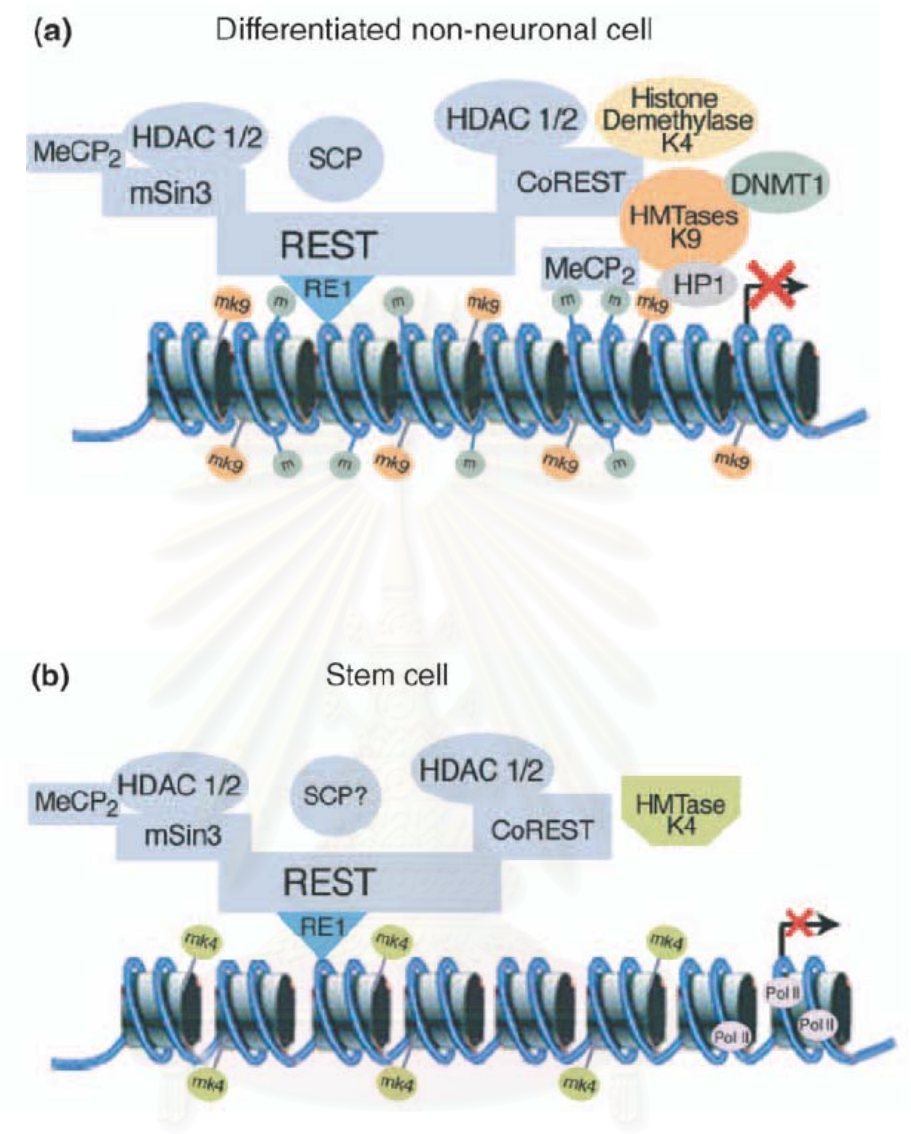


Figure 7. **REST-CoREST orchestrates differential epigenetic mechaisms to inactivate neural genes in non-neural cells (a) and embryonic stem cells (b).**

10.2. The regulation of neural genes by REST/NRSF during development and in mature neurons.

Ballas and colleagues (Ballas et al., 2005) described a dual regulatory mechanism of REST/NRSF during neural developing by using a model of neural differentiation from mouse ES cells (Figure 8). In ES cells neural gene expression is low as a result of maximal levels of REST/NRSF mRNA. When ES cells transition into neural

progenitors, REST/NRSF mRNA levels are still high but protein levels are low as REST/NRSF is post-translationally degraded as a result of neural gene expression. As progenitors exit the cell cycle and differentiate into mature neurons, the REST/NRSF gene is repressed via binding of the unliganded RA receptor (RAR) repressor complex to the retinoic acid receptor element (RARE) as a results of neural gene expression is high (Figure 8A). When neural progenitors transition into neuron, the neural genes can be divided into two classes on the basis of the different mechanisms of REST/NRSF-mediated repression Figure 8B). In neural progenitor cells, Class I neural genes are repressed by the REST/NRSF repressor complex. Upon neural differentiation, the REST/NRSF repressor complex were removes allowing derepression of neural genes. Class II neural genes, such as brain-derived neurotrophic factor (BDNF) and Calbindin, are repressed by both the REST/NRSF repressor complex and MeCP2/HDAC complex at nearby CpGs in neural progenitor cells. Upon neural differentiation, REST/NRSF is displaced (as for class I genes) but, neural gene expression is still repressed by MeCP2/HDAC. Upon a specific stimulus, such as membrane depolarization, CoREST remains bound to chromatin, while MeCP2/HDAC leave the CpG site, allowing neural gene activation.

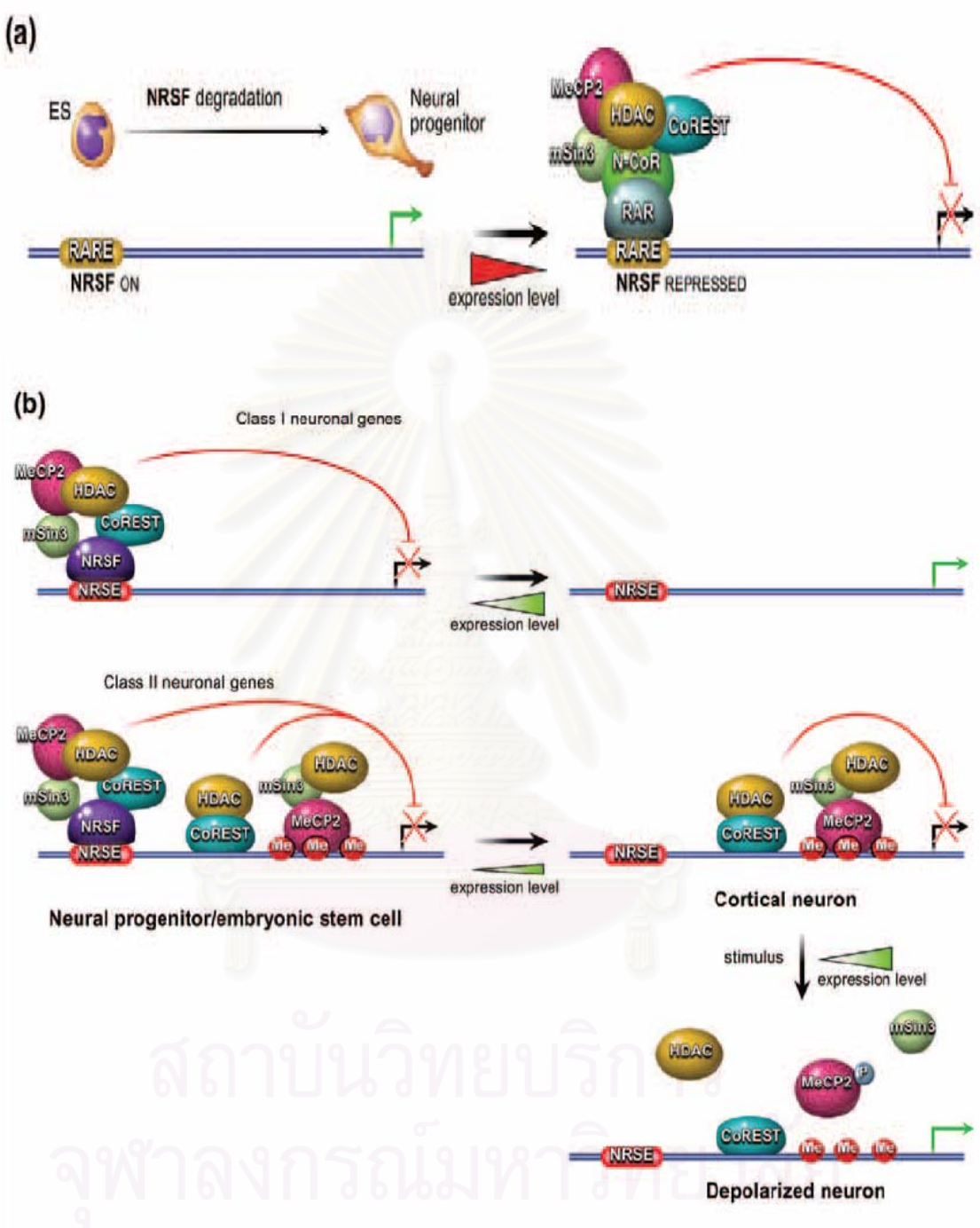


Figure 8. **Context–dependent gene regulation by the REST/NRSF complex.** (A) REST/NRSF is downregulated by two different mechanisms during neural development. (B) Two classed of RE1 containing genes are regulated differentially in postmitotic neurons (Ballas et al. 2005)

10.3. The regulation of neural genes by REST/NRSF during differentiation of adult neural stem cells.

Recently, Kuwabara and colleagues (Kuwabara et al., 2004) discovered the *NRSE* double-stranded RNA (*NRSE* dsRNA) in adult neural stem cells. REST/NRSF represses neural genes in adult neural stem cells. Neural induction triggers the production of *NRSE* dsRNA and REST/NRSF is converted form a repressor to an activator allowing of neural gene expression. The regulation of *NRSE* dsRNA to neural gene expression is able to explain with two models (Kuwabara et al., 2004). Model A, the *NRSE* dsRNA interacts directly with dsDNA-REST/NRSF machinery within the genome and triggers an organizational chage in ranscriptional activation (Figure 9, Model A). Another possibility is that the REST/NRSF protein acts as a homodimer. In this case, the *NRSE* dsRNA could bind one monomer of REST/NRSF while the other monomer remains physically associated with dsDNA/chromatin. In model B, through physical interactions with the REST/NRSF complex, *NRSE* dsRNA alter REST/NRSF function, possibly by inducing a conformation change in REST/NRSF and/or associated proteins (Figure 9, Model B). In both cases, after an interaction with *NRSE* dsRNA, the REST/NRSF complex can no longer associate with repressor proteins, such as HDAC, MeCP2.

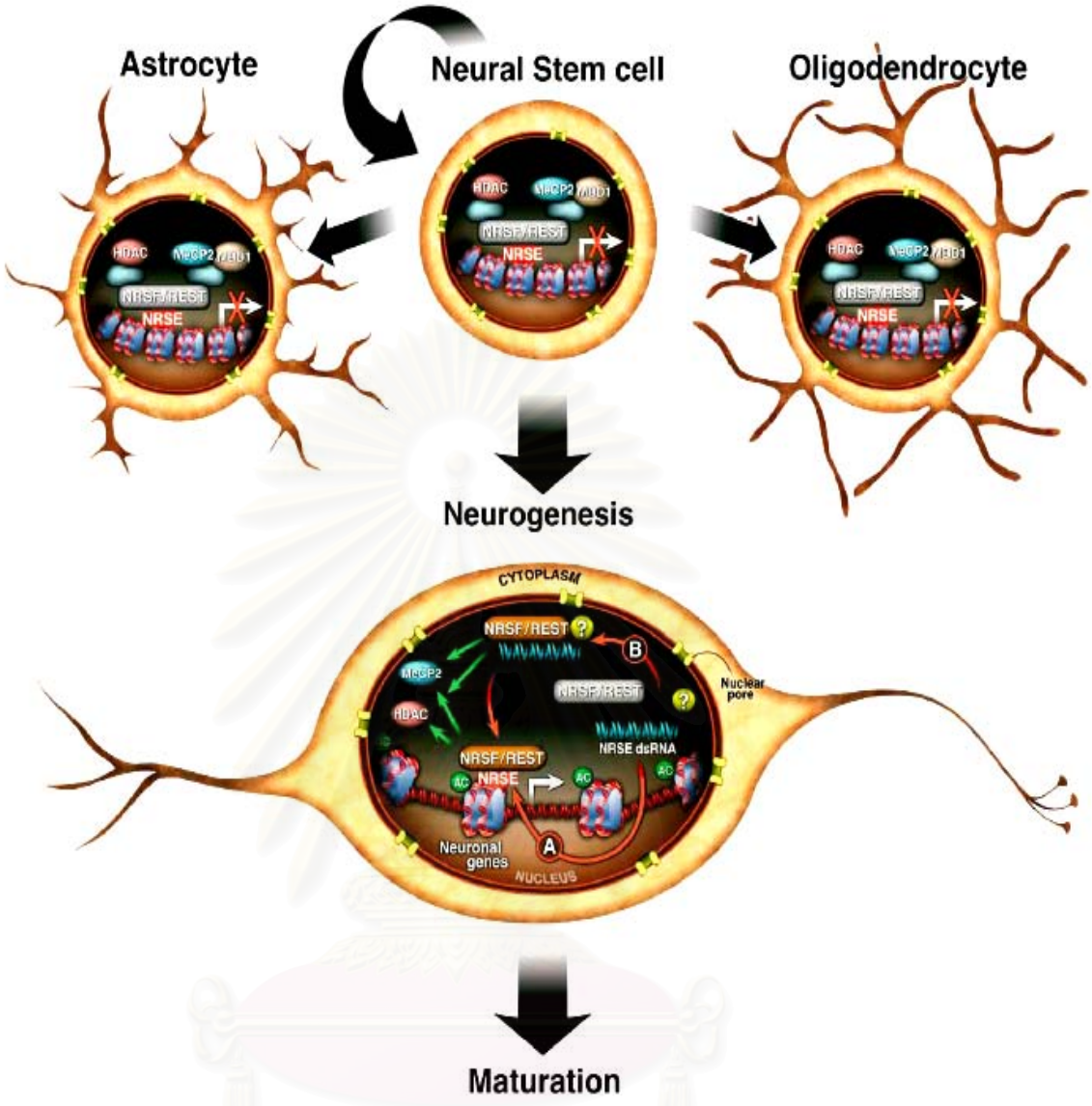


Figure 9. **Schematic Representation of activation events by *NRSE* dsRNA.** (Kuwabara et al. 2004)

11. REST-VP16

Gene deletion studies with REST/NRSF$^{-/-}$ mice indicate that the absence of REST/NRSF in vivo causes expression of only one of the REST/NRSF target genes, the neuron-specific tubulin gene, in a subset of non-neuronal tissue followed by embryonic lethality (Chen et al. 1998). This lack of REST/NRSF does not cause activation of other REST/NRSF target genes. This indicates that the absence of REST/NRSF-dependent repression alone is insufficient to activate multiple REST/NRSF target genes and that a process requires relief from other repression mechanisms and/or the presence of other promoter/enhancer-specific activators (Immaneni et al. 2000). Immannei and colleague constructed the recombinant protein, REST-VP16, by replacing repressor domains of REST/NRSF with the activation domain of a viral activator VP16 (Figure 10. REST-VP16 binds to the same DNA-binding site as REST/NRSF but functions as an activators instead of a repressor of neuronal genes cause expression of neural differentiation genes such as, *Synapsin*, Glutamate receptor (*GluR*) in NT2 cell (Immaneni et al. 2000)

Figure 10. **REST/NRSF and REST-VP16 construct.** RD : repressor domain, Zn-DBD : zinc finger DNA binding domain, Zn-RD : zinc finger repressor domain

12. Protein transduction

In 1988, Green M. (Green and Loewenstein, 1988) and Frankel (Frankel et al., 1988) independently discovered that human immunodeficiency virus type I (HIV-I) – encoded *trans*activator TAT protein , which is essential for viral gene expression and

replication , could rapidly translocate though the plasma membrane and accumulate in the nucleus when added into the culture medium.

“HIV-1-TAT” is small polypeptide of 101 amino acids, encoded by two exons.

In the first exon (aa 1-72)	:	an N-terminal acidic domain (aa 21)
	:	a domain containing 7 cysteins (aa 22-37)
	:	a core region (aa 38-48)
	:	a basic domain enriched in arginine and lysine amino acids , highly conserved among different strains **(HIV-1 TAT protein)** (aa 49-57)
In the second exon (aa 73-101)	:	variabie sequence

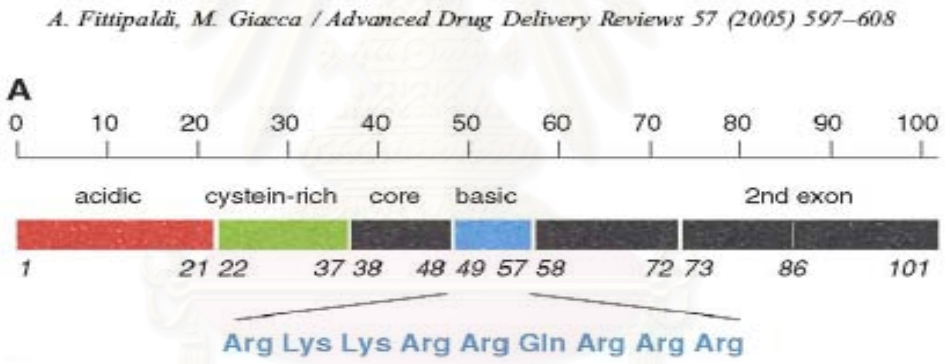


Fig 11. The amino acid sequence of basic domain of protein ,which is required for transcellular protein transduction

Previous studies have shown that the 11 amino acids TAT peptide YGRKKKQRRR (aa 49-57), the basic domain enriched amino acids (highlighted in bold), is sufficient for intracellular transduction and subcellular localization. (Vives et al. 1997; Nagahara et al. 1998) It is believed that this TAT motif can functionally be dissected into two parts: GRKKK can act as a potential nuclear localization signal (NLS), whereas RRR seems to be very critical for protein transduction. (Vives et al. 1997) Not only the protein

enters the cells, but also that it is translocated to the nucleus in a transcriptionally active from. After exogenous delivery, and found that, after 6 hours more than 80% of the internalized TAT was detectable in the nuclear compartment. (Frankel et al., 1988) Most of the applications are based on the fusion of the protein transduction domain of TAT to the protein of interest, either at the N-terminus or C-terminus, followed by the addition of the recombinant fusion protein to the culture medium of the cells of interest. Most notably, fusion or conjugation to TAT has been shown not only to mediate cells internalization of heterologous proteins (Kabouridis et al 2002; Wang et al. 2002), but also of the larger molecules or particles, including magnetic nanoparticle (Torchilin et al. 2001), phages and retroviral vector (Sandgren et al. 2002; Xio et al., 2001), liposomes and plasmid DNA. (Tasciotti et al. 2003).

CHAPTER III

MATERIALS AND METHODS

1. Cell Culture

The mouse Neuro2a cells were purchased from American Type Culture Collection (ATCC no. CCL-131). Neuro2a cells were cultured in Minimum Essential/Earles Balanced Salt Solution Medium (MEM/EBSS; HyClone) supplemented with 2 mM glutamine (Gibco), 1% non-essential amino acids (Gibco), 1 mM sodium pyruvate (Gibco), 1.5 g/L sodium bicarbonate (Gibco), 1% penicillin/streptomycin (Gibco) and 10% fetal bovine serum (FBS; HyClone). These cells were cultured at 37°C and 5% CO_2.

The Hive Five™ insect cell lines that perchased from Invitrogen were cultured in Express Five® SFM supplement with 18 mM L-glutamine (Gibco) and 1% penicillin/streptomycin (Gibco). These cells were maintained at 27°C.

2. Isolation and expansion of mouse mesenchymal stem cells (mouse MSCs)

Mouse MSCs were isolated from the bone marrow of BALB/cA (6-8 weeks) which were purchased from National Laboratory Animal Centre, Mahidol University. Under sterile conditions both ends of the long bones from femurs and tibias were cut with scissors so the bone marrow cells were flushed out with Dulbecco's Modified Eagle Media (DMEM; HyClone) using a syringe with a 21-to 25-gauge needle, followed by gentle pipetting several times. The aspirate is fractioned on a density gradient solution often Percoll or Ficoll (GE Healthcare) to eliminate some unwanted cell types and debris. The cells in the upper low-density fraction are then plated on cell culture dish that contained DMEM/high glucose (Hyclone) supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin/streptomycin. After 24 h, the non-adherent haematopoietic cells were removed by replacing the medium. The medium was added and replaced every 3 or 4 days for about 2 weeks. When the cells grew to confluent, they were harvested with

0.25% trypsin and 1 mM EDTA (Hyclone) for 2 min at 37°C, replated on 25 cm^2 culture flask, again cultured to next confluence, and harvested. Before being used in inducing neural differentiation, the MSCs had been expanded for about 20 passages.

3. Construction of plasmids and mouse MSC-NRSE stable cell lines

3.1 The construction of the *NRSE* dsRNA expressing vector

The *NRSE* dsRNA expressing vector were constructed according to the manufacturer's protocol from BLOCK-iT™ Pol II miR RNAi Expression Vector Kits (Invitrogen). The sense and antisense strand of *NRSE* DNA oligonucleotide (s*NRSE* and as*NRSE* DNA) used in these reactions had the following sequence: s*NRSE*, 5'-GGCGCTGTCCGTGGTGCTGAA-3'; as*NRSE*, 5'-'TTCAGCACCACGGACAGCGCC-3'. These sequence anneal for generating a double-stranded *NRSE* (*NRSE* dsDNA) for cloning into the linearized pcDNA™6.2-GW/EmGFP-miR vector (see appendix A). Then transform the ligation reaction into One Shot® TOP10 chemically competent *E. coli* and select for spectinomycin-resistant transformants.

3.2 The establish of mouse MSC-NRSE stable cell lines

One day before transfection, plate mouse MSCs at a density no more than 25% confluent per dish. Two milligrams of pcDNA6.2-GW/EmGFP-*NRSE* dsDNA was transfected with 8 µl of FuGene HD transfection reagent (Roche Diagnostics) according to the manufacture's instruction. 8 µg/mL blasticidine was added to the culture and refreshed every 2 days. For selection of stable cell lines, colonies were picked and expanded. After selection, clones were maintained in medium containing 8 µg/mL blasticidine.

4. Growth factor induction for neural differentiation

Before seeding cells, plates were previously coated overnight with a 10 µg/ml poly-D-lysine solution (Sigma) in distilled water sterile. After that Neuro2a and mouse MSCs were isolated and cultured as previously described. Cells were recovered from subconfluent culture by trypsinization (trypsine/EDTA solution from GIBCO), counted and plate to a 3,000 cells/cm^2 density on poly-D-lysine (Sigma) coated plates. Cells were subsequently incubated with Neurobasal medium (GIBCO) containing 1xB27 supplement, 1xglutaMAX, 1% penicillin/streptomycin (Gibco), 20 ng/ml recombinant human fibroblast growth factor (rhFGF-basic/FGF2), 20 ng/ml epidermal growth factor (both from R&D Systems). Medium was replaced every 3 to 4 days for 5 days. Then 0.5 µM all-*trans*-retinoic acid (RA; Sigma) were added to the medium and cells were cultured for 4 days, with medium changes every 2 days. Cells were fixed for immunocytochemistry or were extracted RNA for real-time PCR.

5. REST-VP16 protein transduction

5.1 The construction of the REST-VP16 expressing vector

The recombinant construct, REST-VP16, was constructed according to the manufacturer's protocol from InsectSelect™ System with pIZ/V5-His vector Kits (Invitrogen; see appendix A) by inserting the *Bam*HI-*Xba*I fragment of REST-VP16 (REST-VP16 sequence see appendix B) into the *Bam*HI-*Xba*I site of pIZ/V5-His-TAT (which inserting the *Kpn*I-*Bam*HI fragment of TAT into the *Kpn*I-*Bam*HI fragment of pIZ/V5-His). Then transform REST-VP16 into One Shot® TOP10 chemically competent *E. coli* and select for zeocin-resistant transformants.

5.2 Generation of the REST-VP16 protein

The Hive Five™ insect cell lines were seed on 60 mm culture dish at density approximately 50 – 60% confluent. One microgram of REST-VP16 in pIZ/V5-His-TAT was transfected with 20 µl of Cellfectin® transfection reagent (Invitrogen) according to the manufacture's instruction (Invitrogen). Harvest the cells 2 days posttransfection and

extract total protein according to the manufacture's instruction (M-PER; PIRECE). Then, measure the extracted protein concentration.

5.3 REST-VP16 protein transduction

One day before the REST-VP16 protein transduction, Neuro2a cells, mouse MSCs and mouse MSCs-*NRSE* stable cells were plated into 6 well culture dish at a density of $5x10^4$ cells/dish and were cultured in Neurbasal medium supplement with 1xB27 supplement, 1xglutaMAX, 1% penicillin/streptomycin. Approximately 30 µg/ml of total protein was added to the culture. Harvest these cells one day posttransduction and extract RNA according to the manufacture's instruction (TRI reagent; Molecular Research Center, Inc.).). Then, measure the RNA concentration for test gene expression.

6. Immunocytochemistry

The medium was removed and cells were washed twice with phosphate-buffered saline (PBS, pH 7.5) before fixation in 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were rinsed three times with PBS and incubated in a PBS solution containing Triton X-100 0.3% and bovine serum albumin (BSA) 4% for and then incubated with blocking buffer (2% goat serum, 1% bovine serum albumin, and 0.1% TritonX-100 in PBS, pH 7.5). Primary antibodies were added at 4°C overnight and included the following: mouse monoclonal anti-nestin (Chemicon; 1:200); mouse anti-β-III tubulin (Chemicon; 1:200); and mouse monoclonal anti-stro1 (Chemicon; 1:200). The secondary antibodies were added at room temperature for 1 h and included the following: Alexa Fluor goat anti-mouse or anti-rabbit IgG (Molecular probe: 1:200). Cells were finally incubated in 1 µg/ml DAPI (Sigma) to stain nuclear DNA. Then, cells were detected with a Nikon E300 microscope.

7. Gene expression

Total RNA from Neuro2a cells, mouse MSCs and mouse MSCs-*NRSE* stable cells was extracted by using the TRI reagent (Molecular Research Center, Inc.). For reverse transcription was performed by using RevertAid™H Minus M-MuLV Reverse Transcriptase (Fermentas) and identified genes expression by real-time PCR was performed by using Fast SYBR® Green Master Mix (Applied Biosystems). Partly, to study *NRSE* dsRNA expression. Reverse transcription was performed by using TaqMan®MicroRNA Reverse transcription Kit (Applied Biosystems) and identified genes expression by real-time PCR was performed by using LightCycler®Taqman®Master (Roche Molecular Biochemicals, Indianapolis, IN, USA). For the primer sets set for quantitative real-time PCR, see Appendix C.

8. Western blot analysis

Western blotting analyses were carried out according to the manufacture's instruction of Amplified Alkaline Phosphatase Goat Anti-Rabbit Immun-Blot® Assay Kit (BIO-RAD). Briefly, total proteins were electroporesed on 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 1 hour 30 minutes to detect REST/NRSF proteins. Blotted nitrocellulose membrane was blocked in 5% non-fat dry milk in 1% TBS ffor 1 hour at room temperature (RT) with constant agitation. To detect REST/NRSF, the blocked membranes were incubated with either antibody to the antibody used was anti-NRSF (Santa Cruz Biotechnology, INC.; 1:500 in TTBS) overnight at 4°C with constant agitation. The nitrocellulose membrane was washed two times with agitation in TTBS for 5-10 minutes and then incubated with secondary antibody, biotinylated goat anti-rabbit antibody (1:5000) for 1 hour at RT with agitation and washed the nitrocellulose membrane two times for 5-10 minutes each time with TTBS. The REST/NRSF were detected by using incubation with Streptavidin-biotinylated alkaline phosphatase complex for 1-2 hours with gentle agitation at RT and washed the nitrocellulose membrane three times for 5-10 minutes each time with TTBS. The AP color development buffer were used to visualize the REST/NRSF on the nitrocellulose membrane by

incubate for 30 minutes at RT with agitation and stop the development by washed the nitrocellulose membrane two times for 5-10 minutes with distilled water. Finally, The developed membrane will show purple bans, take photographs while the membrane is wet to enhance the purple color.

CHAPTER IV

RESULTS

1. Establish of MSC population from mouse bone marrow

Mouse MSCs could be isolated easily from bone marrow inside femurs and tibias by its adhesion property to plastic. 24 hour after plating , some cells attatched to culture plate. The primary cell cultures are most often maintained for 2 weeks. Culturing of this cells in DMEM high glucose supplement with 10% FBS and 1% penicillin-streptomycin ,these cells contain a morphologically heterogeneous population that included elongated, flat and round cells (Figure 12A) and stained with MSCs marker, Stro-1,(Figure 12B). Furthermore, this cell can differentiatiate into all of mesenchymal derivatives, including osteocytes, chondrocyte, adipocytes, and myocytes (data not show).

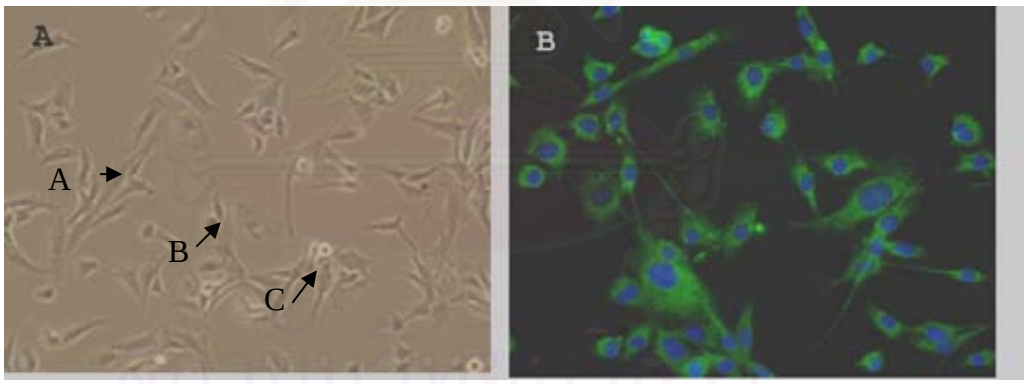


Figure 12. Phase-contrast of mouse MSCs. Left, morphological characterizatic of passage 33 mouse MSCs in cultures that included elongated cells (A), large flat cells (B) and round cells (C). Right, expression of MSC marker Stro-1. Stro-1: green; DAPI: blue. Magnification: x20.

2. The effect of growth factors induced mouse MSCs into neurons.

To study the effect of growth factor in neural differentiation of mouse MSCs. Initially, we cultured mouse MSCs in Neurobasal medium supplement with 1x B27 supplement, 1x glutaMAX, 1% penicillin-streptomycin , 20 ng/ml FGF-2 and 20 ng/ml EGF. Medium was replaced every 2 days for 5 days. Then remove and replace with medium that contain 0.5 μM all-*trans*-retinoic acid (RA) and cells were cultured for 4 days, with medium changes every 2 days. This cells has a long neural-like extension, and some of shorter (Figure 13). We used immunocytochemistry to determine the expression of neural markers of mouse MSCs (Figure 13B-C) including, the neuroepithlial precursor nestin (Figure 13B), the immature neural markers βIII-tubulin (Figure 13C).

Furthermore, we used quantitative real-time PCR analysis to determine neural genes expression level including, the proneural gene such as *Mash1* and *NeuroD* and terminal differentiation neural genes such as *TH* and *Nav1.2*. The result showed that only Synaptophysin mRNA level increased in 2-fold but the other genes showed a few increased. (Figure 14). Beside, we found the expression of myogenic genes ,*MyoD* and *Myf-5*, (Figure 15).

It is notable that, the neural genes expressed in mouse MSCs were REST/NRSF target genes. So it is interesting that whether RA induction in mouse MSCs involved REST/NRSF. Therefore, we used real-time PCR and western blot analysis to detect REST/NRSF mRNA and protein respectively. The result show that the level of REST/NTSF mRNA and protein were not changed (Figure 16A-B).

From this results, we proposed that RA induction might not be involved directly in REST/NRSF neural gene regulation pathway. Furthermore, RA induction is not sufficient for neural differentiation of mouse MSCs.

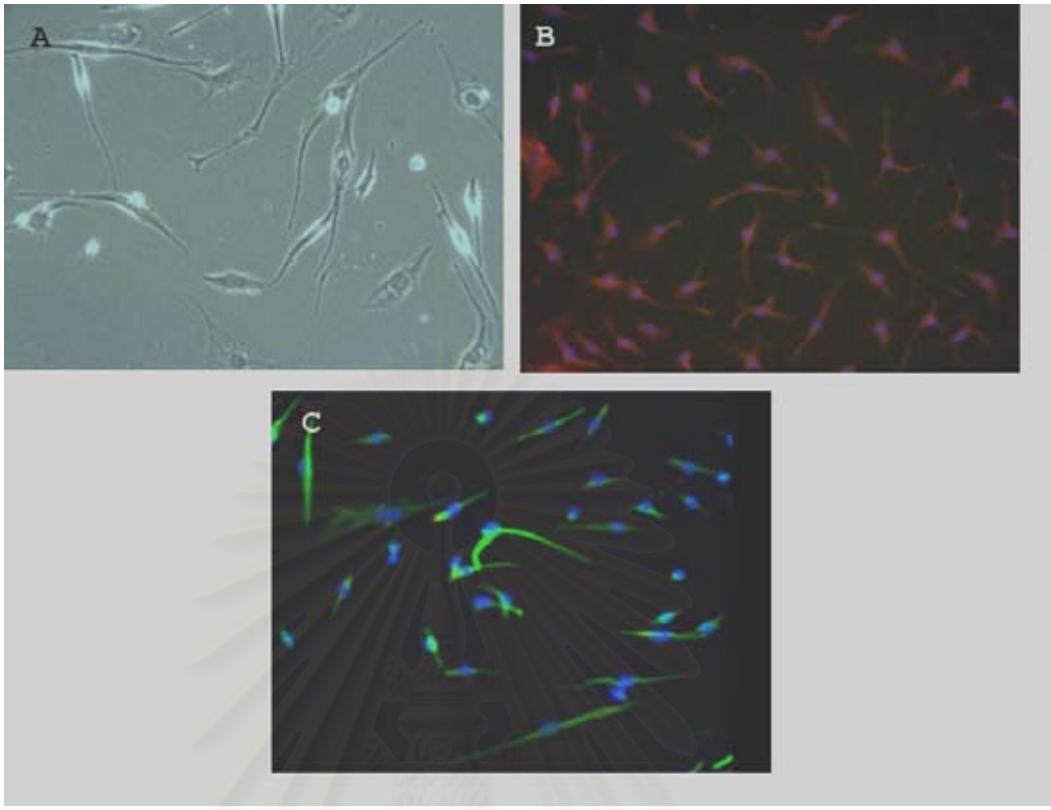


Figure 13. **Immunocytochemistry of neural markers in mouse MSCs.** (A) mouse MSCs plated on poly-D-lysine were treated with RA. These cells had long neural-like extension, sometimes a long extension and others shorter. (B) The expression of the neuroepithelial precursor nestin, (C) the immature neuronal marker βIII-tubulin. Nestin: red; βIII-tubulin: green; DAPI: blue. Images were collected on Nikon fluorescence microscope. Magnification: x20.

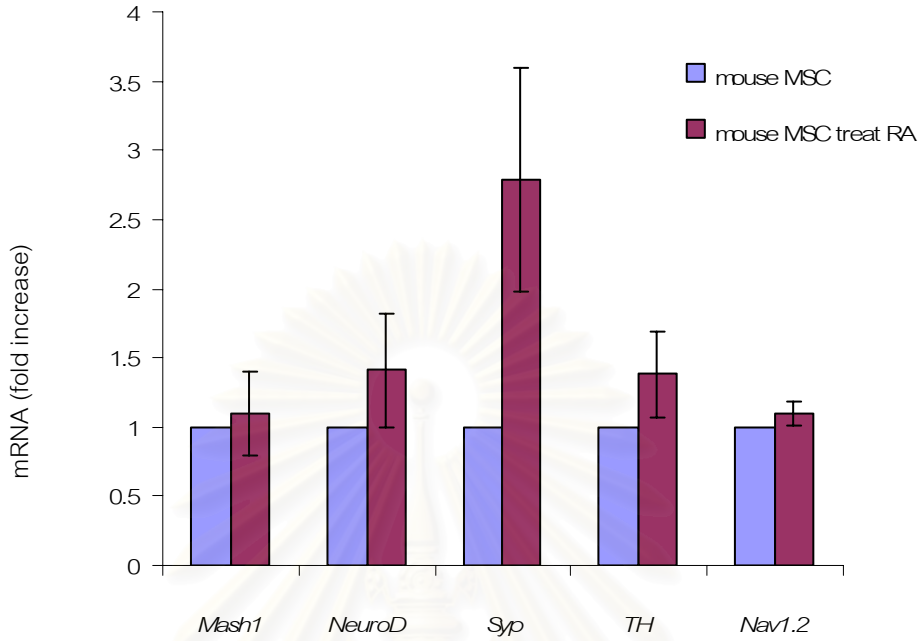


Figure 14. **Real-time RT-PCR measurements of neural genes in mouse MSCs.** Mouse MSCs were induced to neural differentiation by treatment with RA for 4 days (mMSC-RA).

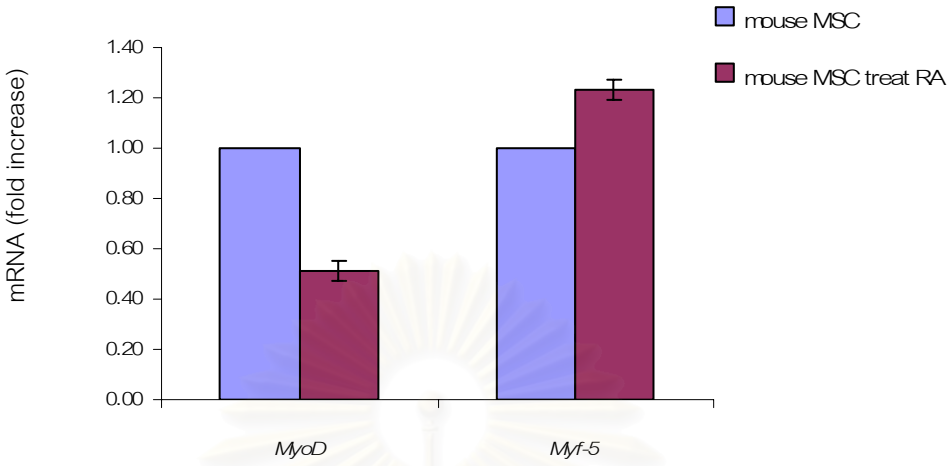
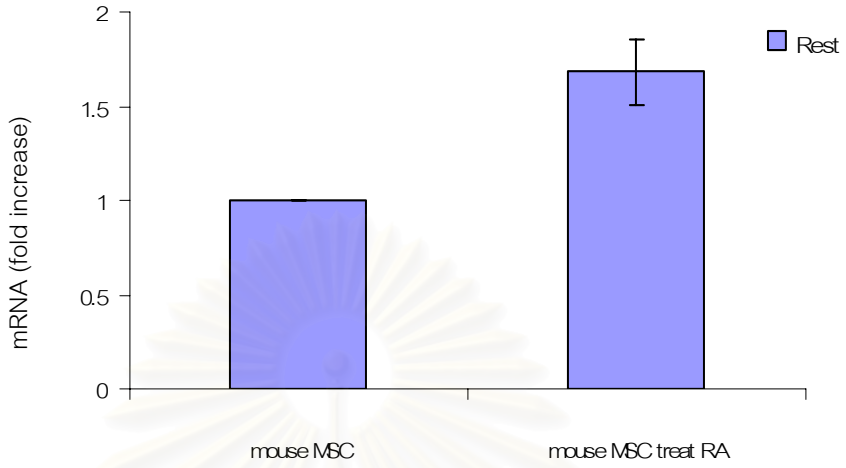


Figure 15. **Real-time RT-PCR measurements of myogenic genes in mouse MSCs.** Mouse MSCs were induced to neural differentiation by treated RA for 4 days (mMSC-RA).

A.



B.

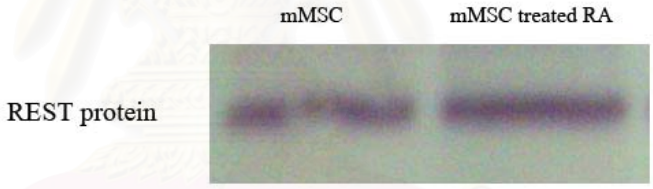
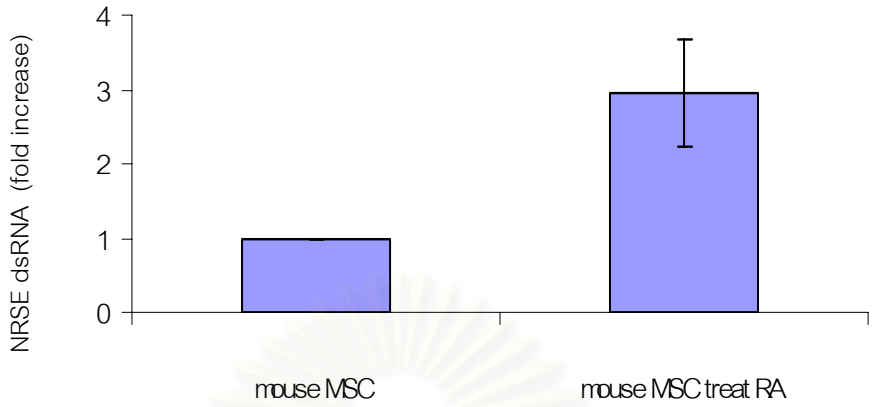


Figure 16. **The expression *REST/NRSF* in mouse MSCs.** Mouse MSCs were induced to neural differentiation by treated with RA for 4 days (mMSC-RA) . **(A)** real-time PCR for detecting REST/NRFF mRNA level. **(B)** Western blot ananlysis for detected REST/NRSF protein.

3. *NRSE* dsRNA expression in mouse MSCs

The *NRSE* dsRNA , fond in adult neural stem cells and were increased when using RA for neural differentiation, can function as an endogenous inducer of neural differentiation. So it is of interest to determine whether mouse MSCs contain the endogenous the *NRSE* dsRNA and can increase level of expression when using RA induction. We used real-time PCR for the detection of *NRSE* dsRNA before and after using RA induction in mouse MSCs. This result showed that the *NRSE* dsRNA were found in mouse MSCS and increase expression in 3-fold when treated with RA (Figure 17A-B) but reduced when treat with PDGF and GSK3 inhibitor. Therefore, we proposed that *NRSE* dsRNA that contain in mouse MSCs could be induced by RA.

A.



B.

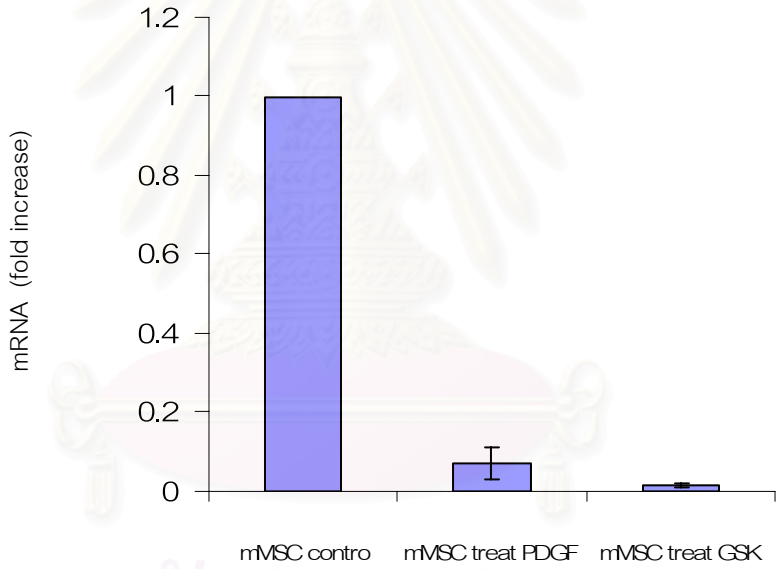


Figure 17. **The expression *NRSE* dsRNA in mouse MSCs.** Real-time PCR were used to detected *NRSE* dsRNA level in mouse MSCs before and after being induced to neural differentiation by treated with RA (A), PDGF and GSK3 inhibitor (B).

4. The expression on neural gene in mouse MSC-*NRSE* stable cell line upon RA induction.

To determine the neural genes expression in mouse MSC that contain *NRSE* dsRNA (mouse MSC-*NRSE*) after being treated with RA. We used real-time PCR to detected REST/NRSF target genes. This result shown that neural gene were increased in mouse MSC-NRSE cell (Figure 18). And after treated with RA, it is interesting that *Mash1*, *Syp* and *Nav1.2* gene were highly expressed. So we proposed that *NRSE* dsRNA might be involed in *Mash1*, *Synaptophysin* and *Nav1.2*gene regulation but not the other genes.

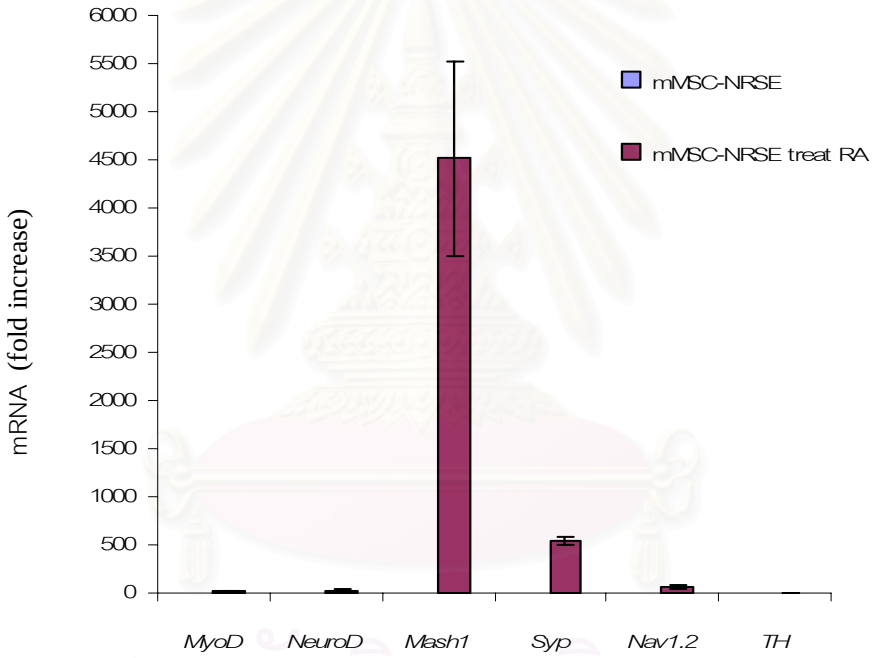


Figure 18. **The expression of neural genes in mouse MSC-*NRSE*.** Mouse MSC-*NRSE* cells were treated with RA and then detect mRNA neural gene level by using real-time PCR.

5. The REST/NRSF target genes expression by REST-VP16 protein transduction.

To determine whether REST-VP16 protein could be activated REST/NRSF target genes in Neuro2a, Initially, to determine the concentration of REST-VP16 proteins that sufficient to induce the expressions of neural genes. We constructed transient transfection of REST-VP16 plasmid from Hive Five™ insect cell lines and extracted total protein from those cells and then added 10 and 100 µl of total proteins into culture medium of Neuro2a cells. We used the total protein that extracted from normal cells as a control. The result showed that 10 µl of REST-VP16 proteins is sufficient to activate the REST/NRSF target gene (Figure 19). The level of neural gene expression is increase in dose dependent of REST-VP16 proteins. This result shown that protein transduction technique, REST-VP16 proteins, could be use in neural differentiation of mouse MSCs.

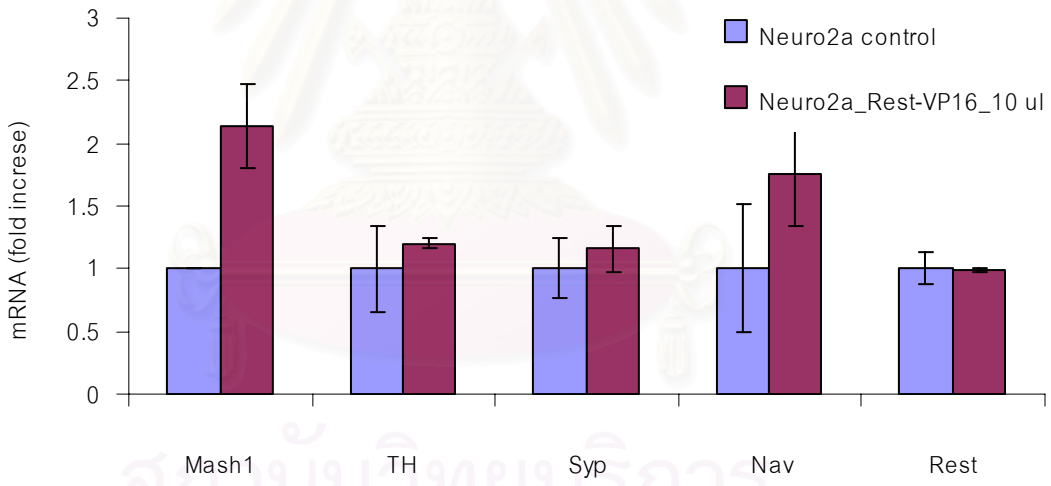


Figure 19. **Comparative the neural gene expression.** We added the concentration of REST-VP16 protein at 10 µl and 100 µl in Neuro2a cells for 1 day. Then using real-time PCR to detected the mRNA level of REST/NRSF target gene.

6. The effect of REST-VP16 protein in mouse MSCs to neural genes.

To determine whether the REST-VP16 protein could induced the expression of neural genes in mouse MSCs. We added REST-VP16 protein into mouse MSCs for 1 day and then used real-time PCR to neural genes expression. The result showed that mRNA level of *Mash1* and *Syp* genes were increased but mRNA level of *REST* and TH gene was not changed (Figure 20). There have we proposed that REST-VP16 protein could activate some of the neural genes in mouse MSCs.

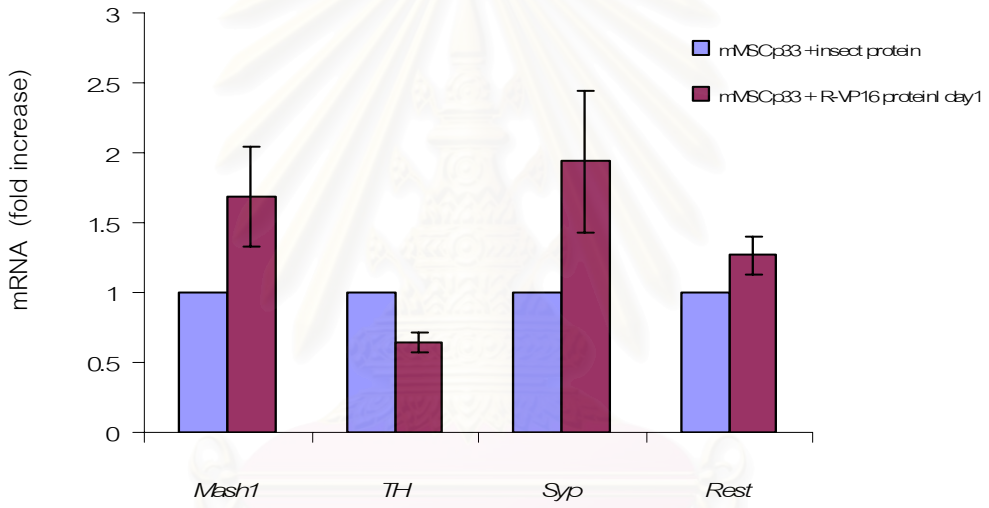


Figure 20. **The expression of neural gene in mouse MSCs**. Mouse MSCs were treated with REST-VP16 protein and the detect mRNA neural gene level by using real-time PCR. Using total protein that extracted from Hive Five™ insect cell lines as a control.

7. The expression on neural gene in mouse MSC-*NRSE* stable cell line upon REST-VP16 protein induction.

To study and understand the mechanism of neural gene regulation by *NRSE* dsRNA may be involved with REST/NRSF binding site. We use REST-VP16 protein to solve this problem. We determine whether the REST-VP16 protein could be induced the expression of neural genes in mouse MSC-*NRSE* cells. We added REST-VP16 protein into mouse MSC-*NRSE* cells for 1 day and then used real-time PCR for detected neural genes expression. The result showed that the mRNA level of neural genes and REST gene were not changed (Figure 21). It is proposed that REST-VP16 protein is not affect into neural genes in mouse MSC-*NRSE* cells.

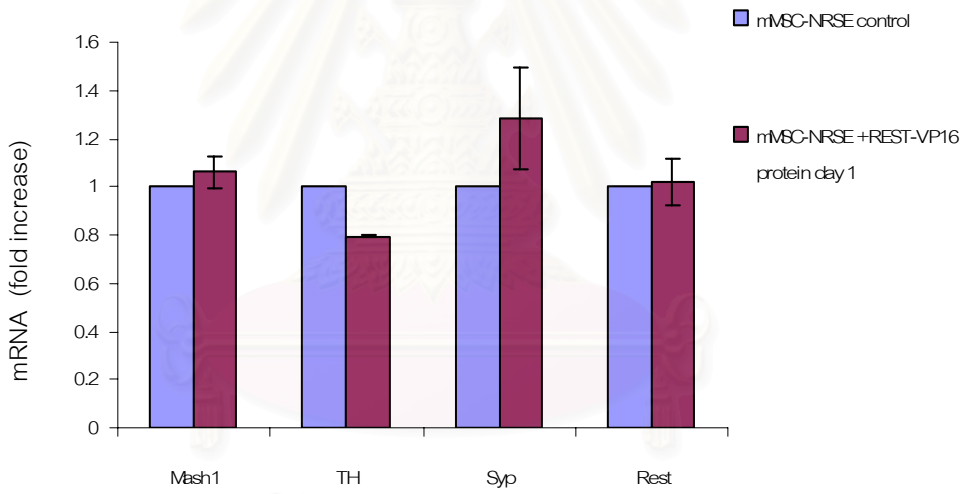


Figure 21. **The level of neural genes expression of mouse MSC-NRSE cells.** Mouse MSCs were treated with REST-VP16 protein and the detect mRNA neural gene level by using real-time PCR. Using total protein that extracted from Hive Five™ insect cell lines is control.

CHAPTER V

DISCUSSION AND CONCLUSION

Although it was shown that MSCs can express subset of neural specific genes when exposed to growth factors (Jin et al., 2003; Kohyama et al., 2001; Reyes and Verfaillie, 2001; Sanchez-Ramos et al., 2000; Tropel et al., 2004; Tropel et al., 2006), it is still debatable whether these neural-like cells can truly function as normal neurons. Moreover, it is indefinite whether the neural gene expression has an important role in physiological manner and what the mechanism of neural gene activation is. Thus the study and understanding about this mechanism will lead to an improvement of MSCs future application.

REST/NRSF is known as a key repressor protein that regulates the large network of neural genes essential for neural differentiation and function (Bruce et al., 2004). So the aim in this study is to determine the involvement of REST/NRSF in neural differentiation of mouse MSCs.

Most neural differentiation protocols of MSCs mainly used FGF2 and RA. So it is interesting thing to determine how RA affect the function of REST/NRSF. We found that REST/NRSF mRNA and protein levels untreated and RA treated mouse MSCs were similar. Therefore, we decided to study another component, *NRSE* dsRNA. *NRSE* dsRNA was found in adult hippocampal neural stem cells and involved with neural differentiation. After induction with RA, *NRSE* dsRNA was over expressed (Ballas et al. 2004). In our experiment, we can detect significant expression of *NRSE* dsRNA in mouse MSCs which found in neural tissue (Ballas et al. 2004). Morover, in mouse MSCs treatd with RA, *NRSE* dsRNA level were up-regulated more than 3 folds. Next, we tested whether *NRSE* dsRNA by itself is sufficient to activate neural genes. We found that while the resting levels of REST/NRSF target genes were similar to the levels is found in control group. The level of neural specific genes such as *Mash1*, *Syp* and *Nav1.2* were many folds higher than a control cells when mouse MSC-NRSE cells were treated with FGF2 and RA.

According to the results, to study and understand involving *NRSE* dsRNA with REST/NRSF target genes, we use REST-VP16 protein to solve this problem. We found that neural genes expression in mouse MSC-NRSE cells untreated and treated with REST-VP16 protein were similar. Results showed that REST-VP16 protein could not activate the neural genes in the condition that contained *NRSE* dsRNA. Therefore, the mechanism of neural gene regulation by *NRSE* dsRNA may be involved with REST/NRSF binding site. However, the result did not show the direct activation of neural genes by *NRSE* dsRNA; significant up-regulation of neural genes in mouse MSC-NRSE cells (the result showed in Appendix A). These results are different from Ballas's results (Ballas et al. 2004) which demonstrated REST/NRSF was converted from a transcriptional repressor to an activator in the presence of *NRSE* dsRNA, thereby up regulating Kuwabara et al., 2004).

Conclusion

We detected significant expression of *NRSE* dsRNA in mouse MSCs. Moreover, when mouse MSCs were treated with RA, *NRSE* dsRNA level was up-regulated more than 3-fold and this up-regulation also can be observed in mouse MSCs. The level of neural specific genes such as *Mash1*, *Syp* and *Nav1.2* were many folds higher than in a control cells when mouse MSC-NRSE cells were treated with FGF2 and RA. We used REST-VP16 protein to study the association between *NRSE* dsRNA to REST/NRSF target genes. We propose that the mechanism of neural gene regulation by *NRSE* dsRNA may be involved with REST/NRSF binding site. However, the results did not show the direct activation the to neural genes by *NRSE* dsRNA because the up-regulation of neural genes was not seen in mouse MSC-NRSE cells.

Further studies should be study the mechanism of *NRSE* dsRNA that involve the REST/NRSF binding site by using chromatin immunoprecipitaion technique. Moreover, it should be to study the epigenetic mechanism including histone modification and DNA methylation in RE1 region of REST/NRSF target genes such as *Mash1* and *Syp* etc .

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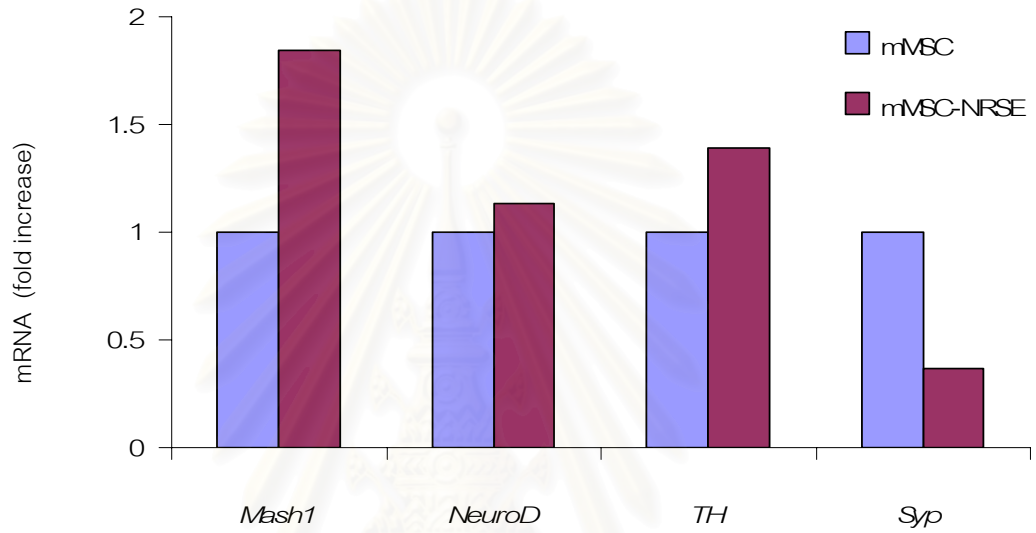
APPENDICES

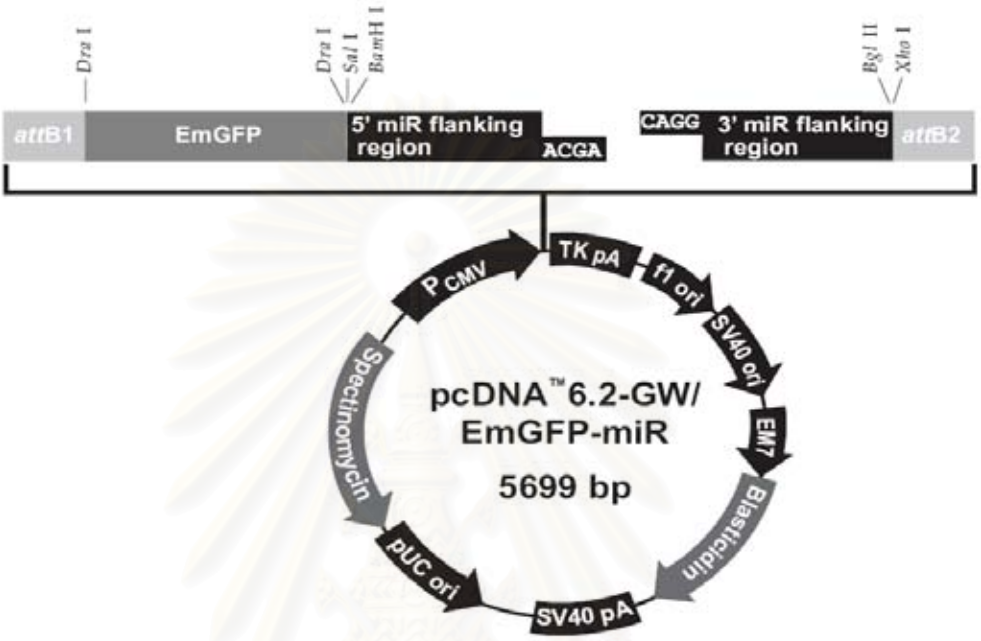
APPENDIX A

The expression of neural genes in mouse MSC and mouse MSC-NRSE cells.



APPENDIX B

1. Map and Features of pcDNATM6.2-GW/ EmGFP-miR

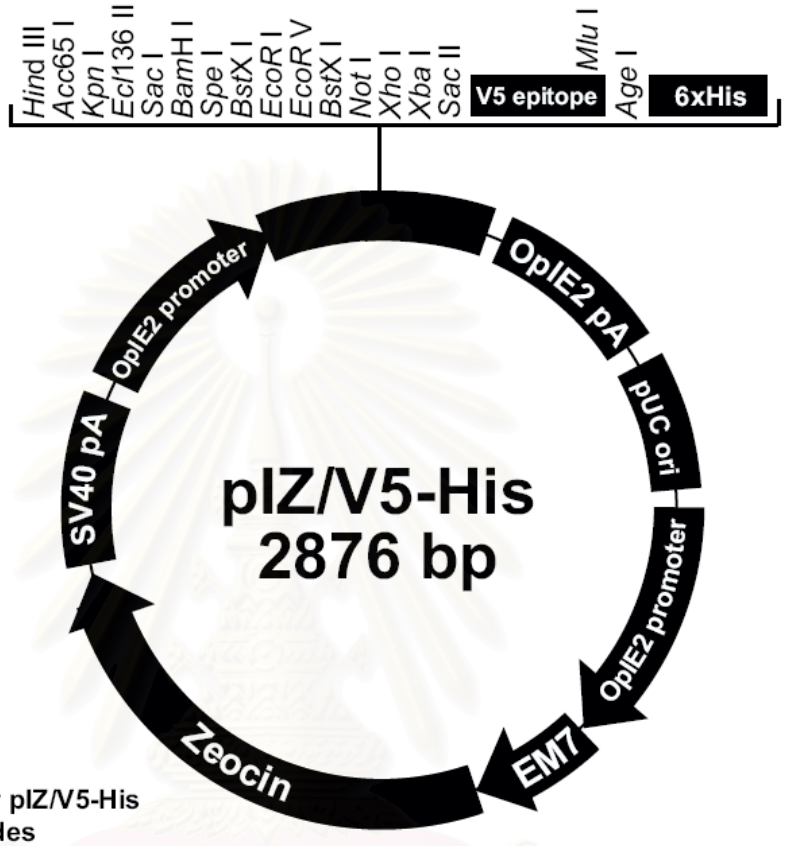


Comments for pcDNA™ 6.2-GW/EmGFP-miR
5699 nucleotides

CMV promoter: bases 1-588
*att*B1 site: bases 680 - 704
EmGFP: bases 713-1432
EmGFP forward sequencing primer site: bases 1409-1428
5' miR flanking region: bases 1492-1518
5' overhang (C): bases 1515-1518
5'overhang: bases 1519-1522
3' miR flanking region: bases 1519-1563
*att*B2 site (C): bases 1592-1616
miRNA reverse sequencing primer site (C): bases 1607-1626
TK polyadenylation signal: bases 1645-1916
f1 origin: bases 2028-2456
SV40 early promoter and origin: bases 2483-2791
EM7 promoter: bases 2846-2912
Blasticidin resistance gene: bases 2913-3311
SV40 polyadenylation signal: bases 3469-3599
pUC origin (C): bases 3737-4410
Spectinomycin resistance gene (C): bases 4480-5490
Spectinomycin promoter (C): bases 5491-5624

(C) = Complementary strand

2. Map and Features of pIZ/V5-His



Comments for pIZ/V5-His
2876 nucleotides

OpIE2 promoter: bases 4-552
Multiple cloning site: bases 561-656
V5 epitope: bases 663-704
6xHis tag: bases 714-731
OpIE2 Reverse priming site: bases 741-766
OpIE2 polyadenylation sequence: bases 749-878
pUC origin: bases 947-1620
OpIE2 promoter: bases 1665-2213
EM7 promoter: bases 2231-2308
Zeocin™ resistance marker (ORF): bases 2309-2683
SV40 early polyadenylation sequence: bases 2747-2876

APPENDIX C

Table 2. Representative examples of putative REST/NRSF target genes with a response element adjacent to the promoter based on informatics.

Table 1. Representative examples of putative REST/NRSF target genes with a response element adjacent to the promoter based on informatics.

Receptors		**DNA remodeling**	
Somatostatin rec 4	(SSTR4)	DNMT 3A (embryonic)	(DNMT3A)
Seratonin rec 3	(HTR3)	•–SMARCe	(130482)
Seratonin rec 6	(HTR6)		
Neuronal pentraxin rec	(NPXR)	**Nerve terminals/signaling**	
Histimine rec 3	(HR3)	Regulator of G-protein 10	(RGS10)
Dopamine D 3	(DRD3)		
GABA rec	(RG2)	**Channels**	
Glutamate rec	(GRIK3)	Na Chll	
Glutamate rec, kinate2	(GRIK2)	–Voltage gated Na+ channel	(33591)
Tyrosin phosphatase rec, typeH	(PTPRH)	K+ Ch.NAB2	(KCNAB2)
Glutamate rec, M4	(GRM4)	Hyperpolariz activated Chrom K+	(HCN4)
Glutamate rec	(GRIK4)	Ether-a-go-go	
Neurotropic tyrosine kinase rec	(NTRK3)	HCN2	
Glutamate rec	(GRIN2A)	–Ether-a-go-go-like K+ channel	(165681)
CRH rec 2	(CRHR2)	Task 3 (K+) channel KCNK9	
Growth hormone rel factor rec	(GHRHR)	Volt depend Ca+ channel	(CANA1H)
–Ephrin rec EphA7	(163918)	Voltage-gated sodium chb-3 sub1	(HSA243396)
Amyloid b (A4) PLP1	(APLP1)	KCNJ6	
Melatonin rec 1B	(MTHR1B)		
Olf-rel receptor-4F3	(168119)	**RNA binding proteins**	
–Olf-rel receptor	(123278)	Bruno-like 4	(BRUNOL4)
Receptor-associated		**Guidance/migration**	
Dvl 1		Adhesion/cytoskeletal	
G-protein signaling 1	(135997)	Sema domain/7 thromospondin repeats	(SEMA5A)
Tetraspan TM4SF	(TSPAN-2)		
Sweet-taste rec T1R3	(126788)	Neurofilament 3	(NEF3)
		–Ankyrin-like p. 1	(169415)
Ligands/neutrophils		Complexin 2	(CPLX2)
Urocortin	(UCN)	Carrier/transport	
Neural pentraxin 2	(NTPX2)	Sodium CA+ exchanger	(Sic2A8)
Proenkcphalin	(PENK)	ATPase, Cu++ trans, b -subunit	(ATP7B)
Orexin	(HCRT)	–Similar to SNARE Vtilla-b-protein	(143187)
		ATPase, Ca++ trans plas membr 2	(ATP2B2)
Angiogenesis		Putative Na-coupled cotransporter	(RKST1)
ADAMTS2	(Disintegrin)		
–ADAMTS-9 (disintegrin)	(125614)	**Secretion**	
–ADAMTS-9	(160783)	CPLX2	
Angiopoietin 4	ANGPT4	SIC2A8	
Brain-spec angiogenesis inhibitor 2	(BAI2)	AP3B2	
		Solute carrier, member 11	(SLC6A11)
Adhesion 1		Solute carrier family 12	(SLC12A5)
Cadherin-like	(CDH22)	Solute carrier family 6	(SLC6A11)
–Tubulin b 2 chain	(138099)	Solute carrier family 23	(SLC23A1)
Vitronectin		Chromagranin B	(CHB B)
SYT5	(SYT5)		
Myosin VB	MY05B	**Others**	
Teneurin 2	(134424)	Calneuris	(136832)
–Ankyrin binding protein	(169415)	Neurexophilin	
Neurofilament 3	(NEF3)	NRXN3	
Centaurin gamma 1	CENTG1	MEGF11	
		Cerebellin	(CBLN1)
Enzymes		SPIR2	
Cdk 5 Reg subunit 2	CDK5R2	–A Rhodopsin 1	(163535)
GAD1		OSBP2	
MGAT3		–Psoriasin1	(149017)
MMp24		BCAN	
Phenylalanine hydroxylase	PAH	•Otoferlin	(OTOF)
PAK7		CAC RR35	
Serine protease	P22	CARPX	
Binding proteins		RES4	
Oxysterol BP5	OSBP5	BCAN	
Oxysterol BP2 (retina)	OSBP2	Parvin beta	(PARVB)
Transcription factors/cofactors		**Placental**	
POU5P2		PP13	
POU4P3		PP13-like	(56891)
Med-6		PP13-like	(FCGBP)
DDX30		Ubiquitin-associated 3A	(UBASH3A)
RNF30			
Nuclear rec coactivator 5	(NCoA5)		

APPENDIX D

Table 3. The sequences of primers

Primer name	Primer sequences
GAPDH	Forward: 5'-tca acg acc cct tca ttg ac-3' Reverse: 5'-atg cag gga tga tgt tct gg-3'
Mash1	Forward: 5'-caa gtt ggt caa cct ggg tt-3' Reverse: 5'-gct ctt gtt cct ctg ggc ta-3'
Myf-5	Forward: 5'-tga agg atg gac atg acg gac-3' Reverse:5'-ttg tgt gct ccg aag gct gct-3'
MyoD	Forward: 5'-tac cca agg tgg aga tcc tg-3' Reverse: 5'-ctc atg cca tca gag cag t-3'
Nav1.2	Forward: 5'-ggg cat gga gta agg aat ga-3' Reverse: 5'-tac tcc tgg ttg gct tgc tt-3'
NeuroD1	Forward: 5'-gca tgc acg ggc tga acg c-3' Reverse: 5'-ggg atg cac cgg gaa gga ag-3'
REST	Forward: 5'-cgc gga tcc atg ccc gtg gga gac aac ca-3' Reverse: 5'-cgc gaa ttc ctc gtt gct gac ggc gtt at-3'
Real time-REST	Forward: 5'-acc ggg tca gga tct tct ca-3' Reverse: 5'-gcc ctg tta ggg aaa cct cc-3'
Syp	Forward: 5'-tcc cga gag aac aac aaa ggg c-3' Reverse: 5'-gcc tgt ctc ctt gaa cac gaa c-3'
TH	Forward: 5'-tgt cag agg agc ccg agg tc-3' Reverse: 5'-cca aga gca gcc cat caa ag-3'
VP16	Forward: 5'-cgc gaa ttc gcg tac agc cgc gcg cg-3' Reverse: 5'-ccg ctc gag cta ccc acc gta ctc gtc-3'

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1988-1991	Surasakmontri School, Bankok, Thailand
1992-1996	Srinakarintarawirot University, Bangkok, Thailand

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Namdaung U, Aroonrerk N, Sukamrarn S, **Danwisetkanjana K**, Saenboonrueng J, Arjchomphu W, Suksamrarn A. Bioactive constituents of the root bark of Artocarpus rigidus subsp. rigidus. Chem Pharm Bull (Tokyo). 2006 Oct;54(10):1433-6.

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