Neurogenesis versus gliogenesis : study on neuronal-glial differentiation switch

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**NEUROGENESIS VERSUS GLIOGENESIS:
STUDY ON NEURONAL-GLIAL
DIFFERENTIATION SWITCH**

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ABBREVIATIONS

AHiPM	Amygdalohippocampal area
AuD	Dorsal area
bHLH	basic Helix-Loop-Helix
cc	Corpus callosum
cDNA	Complementary DNA
cg	cingulum
CGT	Ceramide galactosyltransferase
CNP	2',3' cyclic nucleotide 3' phosphodiesterase
CNS	Central nervous system
DAPI	4',6-diamino-2-phenylindole dihydrochloride
DG	dentate gyrus
dhc	commissure of fornix
DMEM	Dulbecco's modified eagle's medium
ec	external capsule
ERM	Enhanced retroviral mutagen
FBS	Fetal bovine serum
FCS	Fetal Calf serum
fi	fimbria of hippocampus
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GrDG	Granular layer of dentate gyrus

GSK-3 β	Glycogen synthase kinase 3 beta
HA	Hemagglutinin
hf	hippocampal sulcus
IGF-I	Insulin growth factor-I
iN cells	Induced neuronal cells
InWh	layers of superior colliculus
iO cells	Induced oligodendrocyte cells
MAP2	Microtubule-associated protein 2
MBP	Myelin basic protein
ML	lateral part of medial mammillary nucleus
MOG	Myelin oligodendrocyte glycoprotein
NDPK	Nucleoside diphosphate kinase protein
NeuN	Neuronal Nuclei
NeuroD	Neurogenic differentiation 1
NF200	Neurofilament 200
Nm23	Non-metastatic cell 23 gene
NME1	Non-metastatic protein 1
NPCs	Neuronal precursor cells
NSCs	Neural stem cells
OPCs	Oligodendrocyte precursor cells
PCR	Polymerase chain reaction
p-GSK-3 β	phospho-Glycogen synthase kinase 3 beta
PLP	Proteolipid protein

PNS	Peripheral nerves system
PoDG	Polymorph layer, dentate gyrus
P/S	Penicillin/streptomycin
RACE	Rapid Amplification of cDNA Ends
RF	Reading frame
RSA	Retrosplenial agranular cortex
RT-PCR	Reverse transcription polymerase chain reaction
S	Secondary auditory cortex, subiculum
SNR	Substantia nigra, lateral part
SuM	Supramammillary nucleus
TCF/LEF	lymphoid enhancer-binding factor 1/T cell-specific transcription factor
Tuj-1	Neuron-specific class III beta-tubulin

SUMMARY

The adult glial precursor cells were recently shown to be able to produce neurons in central nervous system (CNS) and to obtain multipotential ability *in vitro*. These findings suggest that glial precursors are not irreversibly committed into one distinct fate and still can be converted to other lineages. This is of great significance for neuronal replacement during various neurological disorders by reprogramming of glial progenitors into neuronal differentiation. Although the fate determination of glial precursors was studied extensively, the signals and factors, which could redirect their fate to become neurons, still remained unknown. To elucidate the mechanisms underlying this remarkable ability of glial precursor cells, we modified the gene expression profile in NG2⁺ glial precursor cells using enhanced retroviral mutagen (ERM) technique followed by phenotype screening to identify possible gene(s) responsible for glial and neuronal cell fate determination. Among the identified molecules, we found the gene named non-metastatic cell 1 which encodes a nucleoside diphosphate kinase protein A (Nm23-M1 or NME1). So far, the Nm23 members have been shown to be involved in various molecular processes including tumor metastasis, cell proliferation, differentiation and cell fate determination. In the present study, we provide evidence suggesting the role of NME1 in glial and neuronal cell fate determination *in vitro*. We showed that NME1 is widely expressed in neuronal structures throughout adult mouse CNS. Our immunohistochemical results revealed that NME1 is strongly colocalized with mature neuronal marker through

white matter of spinal cord and brain. Interestingly, NME1 overexpression in oligodendrocyte precursor OLN-93 cells potently induced the acquisition of neuronal phenotype, while its silencing was shown to promote oligodendrocyte differentiation. Here we further demonstrate that NME1 overexpression promotes neuronal differentiation of oligodendrocyte precursor OLN-93 cells through β -catenin signaling pathway. We found that Wnt agonist (LiCl) treatment inhibits the proliferation and accelerates the neuronal differentiation of induced neuronal cells (iN cells). Moreover, we demonstrate that NME1 overexpression increases the phosphorylation of glycogen synthase kinase 3β (GSK- 3β) and causes to the β -catenin stabilisation in a manner similar to Wnt canonical signaling pathway. This effect was further confirmed by dominant negative TCF4 (DN-TCF4) transfection. Since NME1 overexpression does not affect the expression of Wnt molecules and their receptors, our co-immunoprecipitation experiments reveal that NME1 is able to interact with GSK- 3β directly. Therefore, our findings indicate a novel regulatory role of NME1 in driving glial precursor cells to neurons through β -catenin signaling pathway.

CHAPTER 1: INTRODUCTION

1.1. Cellular composition of the central nervous system (CNS)

The central nervous system is composed of two major cell types in vertebrates; neurons and glial cells. These cells are connected together and are able to communicate with each other in order to perform accurate development in the nervous system. Glial cells have several important roles related to neuronal function and protection such as carry oxygen and nutrients to neurons, surround neurons and protect them in place, insulate one neuron from another, and destroy and remove dead neurons (clean up).

Glial cells are divided into three major groups based on microscopic features and especially using metallic impregnation technique (1-3):

- 1- Oligodendrocytes
- 2- Microglia
- 3- Astrocytes

These cells can be recognized by different morphological shapes, sizes and expression of specific cell surface antigens (Figure 1).

1.1.1. Oligodendrocytes

Oligodendrocyte cells were first described by Rio Hortega as neuroglial cell types in the CNS. They are divided into three major types according to their morphology and the size or thickness of the myelin sheath (4):

1. Type I: distinguished with thin myelin sheaths and small diameter axons
2. Type II: intermediate types
3. Type III: largest cells with thick and long myelin sheaths

In the other classification system based on cytoplasmic densities (at the electron microscopy level), they are also distinguished in three different groups (5):

- 1- Dark group: oligodendrocytes with the dense cytoplasmic contents
- 2- Medium group: oligodendrocytes with the medium cytoplasmic contents
- 3- Light group: oligodendrocytes with the lightest cytoplasmic contents

It has been shown that light oligodendrocytes are actively dividing cells whereas dark groups are considered as mature oligodendrocytes (5). During maturation, oligodendrocyte cells undergo several stages of development including proliferation, migration, morphological changes, specific markers expression and myelination. Myelination is a multi-steps process for oligodendrocytes lineage due to final maturation (6). The main task of oligodendrocytes in the nervous system is to form myelin and for this reason they are called myelin-forming cells

(7, 8). They are in contact with axons in the adult vertebrate CNS and produce myelin sheath around them.

1.1.2. Microglia

Microglia are the smallest of the glial cell populations which reside in CNS. They are believed to generate from hematopoietic stem cells in the developing bone marrow. During microglia development, some cells differentiate into monocytes and further move to the brain, where they settle down and finally differentiate into microglia (9). Thus, most neurobiologists believe that they are members of the "mononuclear phagocyte system". Because they can protect brain from invading microorganisms microglia are thought to have similar function with macrophage cells in blood, including phagocytosis, chemokines, Antigen presentation and production of various cytokines (10, 11)

1.1.3. Astrocytes

Astrocytes are star shaped glial cells which are found in gray and white matter of CNS with variety of roles including, forming a physical support to neurons and dead neurons cleaning up in the brain (12-15). In addition, they can also provide growth factors (16) and finally, astrocytes play a role in providing nourishment to neurons. However, the most important role of them in the central nervous system is to provide a micro-environment for neuronal cells (15). Astrocyte development

requires a series of events including proliferation, specification, migration and terminal differentiation (Figure 1).

Figure 1. A schematic diagram of neurogenesis and gliogenesis (4, 15, 17).

The diversity of cell types (neurons, oligodendrocytes, astrocytes), which generate the adult central nervous system, is produced by a series of precursor cells during development. The multipotential neuron stem cells (NSCs) can give rise to lineage restricted populations with a more restricted glial (glial restricted precursor; GRP) or neuronal (neuronal restricted precursor; NRP) subtypes (18, 19). Glial cells, including astrocytes and oligodendrocytes, derive from glial lineage restricted populations; whereas neurons are raised from neuronal restricted precursors. Although the mechanisms of neurogenesis and gliogenesis are studied well during development, neuronal differentiation of glial precursor cells is still largely unknown.

1.1.4. Neurons

Neurons are the functional units of the nervous tissue which basically consist of three major parts:

- 1- Cell body: consists of the mitochondria, nucleus and other organelles.
- 2- Axons: are specialized extensions of cells that conduct messages away from the cell body.
- 3- Dendrites: which receive the informations from other cells and transfer it to the cell body.

Neurons are classified into three distinct types by the direction that they send information:

- 1- Sensory neurons: they typically have a long dendrite and short axon, and are able to carry messages from sensory receptors to the central nervous system.
- 2- Motor neurons: they have a long axon and short dendrites and can transmit messages from the central nervous system to the muscles or to glands.
- 3- Interneurons: they are found only in the central nervous system where they can connect neurons to each other.

Neuron cells in adult vertebrate nervous system are highly differentiated so that they can not basically undergo division and replacement after being lost or injured (20). They are originated from neuronal stem cells through multiple processes in CNS (17) (Figure 1).

1.2. Neurogenesis and Gliogenesis

The generation of mature neurons from multipotential neuronal stem cells in central nervous system is called neurogenesis (Figure 1 and 2). In fact, neurogenesis is a multistep process which includes cell proliferation, cell mitotic exit, cell migration, cell differentiation and subsequently cell fate determination. In the mammalian adult brain, neurogenesis takes place in two discrete areas:

subventricular zone (SVZ) and dentate gyrus (DG) of hippocampus (Figure 2). Generation of new born neurons can only be found in these two restricted brain regions. In order to make new neurons, NSCs first migrate from SVZ to olfactory bulb (OB) through rostral migratory stream (RMS), and subsequently further differentiate and become mature neurons (21). Beyond these two restricted regions, most precursor cells differentiate into glial cells but not neurons (22). Gliogenesis therefore is an alternative fate of NSC populations which is accompanied by formation of non-neuronal glial cells (23-25). In developing CNS, neurogenesis is processed earlier than glial cells (oligodendrocyte and astrocyte) generation. Therefore, the mechanisms which can control glial and neuronal cell fate decision are highly determined by time and space during development. Because the complexities of regulatory steps which can control cell fate, our knowledge about glial and neuronal cell fate determination is largely limited. However, it is clear that when neuronal fate decision is determined, the alternative fate (gliogenesis) will be blocked.

Figure 2. Neurogenesis in adult brain.

Neurogenesis occurs in two distinct regions of adult brain. The figure illustrates the two well-known neurogenic sites in the brain: Subventricular Zone (SVZ) and dentate gyrus (DG) of hippocampus. Neural Stem Cells (NSCs) give rise to migrating neuroblasts which are finally moved to olfactory bulb (OB) through rostral migratory stream (RMS) (26).

Studies of oligodendrogenesis versus neurogenesis relationship started when it was shown that ventricular zone (VZ) of the ventral PNS is subdivided into five distinct precursor types (27). These precursor types are called: progenitor 0, progenitor 1, progenitor 2, progenitor 3, and progenitor MN. These different precursor domains can generate distinct neural subtypes, which then characterized by expression of various homeodomain transcription factors (27, 28). It has been shown that different helices and homeodomains are produced by these progenitors during development. For example progenitors 0 and 3 generate somatic motoneurons, whereas progenitor MN produces four types of interneurons in VZ (27).

After neuron production, the ventricular zone generates glial cells. It has been demonstrated that small fractions of oligodendrocyte precursor cells were first produced in the VZ area during mouse embryonic development. Subsequently after migration to gray and white matter, they finally differentiate to mature oligodendrocytes (29-31). These studies suggest that neuroglial progenitor cells first produce the neuronal progenitors and after that they will be able to generate the oligodendrocyte progenitor cells (32). The finding of other studies has further supported this idea. For example two basic helix-loop-helix proteins (Olig1 and 2), which exist in pMN domain, are able to generate both oligodendrocyte precursor cells and motoneurons (33-35).

1.3. Neuronal and glial cell differentiation

1.3.1. Neuronal cell differentiation

Neuronal differentiation is a multi-process event which neuronal stem cell (NSCs) give rise and further adopt specific cell fate to become mature neurons. It has been well documented that NSCs have a high capacity for self-renewal and are able to differentiate into distinct type of cells in the CNS and during development. Firstly, the multipotent NSCs give rise to two types of precursor cells termed, neuron-restricted precursor (NRPs) and glial-restricted precursors (GRPs) (Figure 1). Subsequently, NRPs further differentiate into neuronal cells (36).

Several factor and signals are involved during neuronal differentiation in central nervous sytem such as Wnt, Retinoic Acid (RA), Sonic hedgehog (Shh), and fibroblast growth factor (FGF) (28, 37-40). For example it has been shown that wnt signaling pathway regulates neuronal differentiation of adult hippocampal through central nervous system (41). In addition, Wnt signaling was shown to stimulate the neuronal differentiation of peripheral olfactory stem cells during postnatal development (42). Similarly, shh signaling was also shown to regulate the generation of ventral neuronal subtypes in the spinal cord by modulating the Gli family transcriptin factors (43). Mutations in shh spinal cord is accopmined by accumulation of ventral neural precursor populations and subsequently cause delay in neuronal differentiation (43).

1.3.2. Glial cell differentiation

Glial cells (astrocytes and oligodendrocytes) are differentiated from glial-restricted precursors during CNS development. During early neural development when glial specification was occurred in the CNS, glial precursor populations first proliferate and subsequently migrate to their final position where they eventually undergo terminal differentiation (44, 45). Terminal differentiation of glial cells is accompanied by expression of specific antigenic markers and it is controlled by some distinct genes such as reverse polarity (*repo*), Repo (*repo*), locomotion defects (*loco-cl*), and pointedP1 (*pntP1*) which was shown to finalize the glial differentiation program (46, 49). For example, a homeobox gene *repo* controls the last step of glial differentiation by modulating ETS and BTB transcription factors (49). In different type of glial cells, Repo can use two independent mechanisms to regulate glial differentiation and diversification:

- 1- It can behave like a transcription activator (thorugh CCAATTA motif)
- 2- It interacts with TTK69 and PNTP transcriptional factors to regulate different target genes

Co-operation of different target genes expression through these two mechanisms modulate the diversity of glial cells (46, 49). Furthermore, *repo* not only regulates the glial differentiation program but also inhibits the neuronal development via TTK69 interaction (49).

1.3.3. Neuronal differentiation of glial precursor cells

Glial precursor cells exhibit several unique features which make them an attractive choice for study of differentiation, reprogramming and cell-based therapy. They have the capacities to self-renew and differentiate into distinct cell types during development (50-53). Although it is well documented that glial precursor cells can give rise to oligodendrocytes and astrocytes, recent findings suggested that they can also be involved in neuronal cells generation. However, the molecular mechanism underlying neuronal differentiation of glial precursor cells is poorly understood.

Previously, it has been demonstrated that in the presence of certain growth factors, oligodendrocyte precursor cells (OPCs) are able to produce floating neurosphere-like bodies which subsequently can generate neurons *in vitro* (54). It was also shown that a defined sequence of extracellular signals could reprogram OPCs to acquire unique characteristics and to become multipotential neural stem cells (54). Moreover, *in vivo* study revealed that reprogrammed OPCs exhibit a neuronal network property in the postnatal rat cortex (55).

These findings suggest that glial precursors are not irreversibly committed into one distinct fate and still can be converted to other lineages. This is of great significance for neuronal replacement during various neurological disorders by reprogramming of glial precursors into neuronal differentiation.

1.4. Glial-neuronal cell fate decision

As stated earlier, in developing CNS, glial and neuronal cell fate determination is a tightly regulated multi-step process in which neurogenesis proceeds earlier than the glial cells production. During development of central nervous system, neural and glial cell types are generated at appropriate time and place (27). Although studies on the biology of stem and glial precursor cells have been carried out predominantly on their time of appearance during development, little is known about the intrinsic/extrinsic signals and factors which can differentiate and/or reprogram them into alternative fates.

1.4.1. *Glial and neuronal cell fate modulators*

It is clear that genetic programs that can link neural versus glial differentiation are modulated by the functions of distinct transcription factors (56-59). During development, these transcription factors have been found to be able to promote the activation of neural and glial specific genes (60, 61). For example, Ngn1 protein can negatively regulate astrocyte differentiation and promote neuronal formation through independent mechanisms (62). It can regulate neuronal differentiation by acting as a transcriptional activator and alternatively inhibit astrocyte differentiation by inhibiting STAT and CBP-Smad1 transcription factors. Recently, it has been found that combination expression of *Dlx1*, *Dlx2* and *Mash1* transcription factors can regulate the neuronal versus oligodendroglial cell fate decision (63). While *Dlx1* and *Dlx2* can negatively regulate Olig2-

dependant OPCs formation, Mash1 promotes this formation via limiting the Dlx^+ precursor numbers. Therefore, it was concluded that Dlx genes act as a switcher or modulator of oligodendrocyte versus neuron cell fate determination in the ventral embryonic forebrain (63).

In addition, NeuroD has been shown to have similar activity in rodent (64). Furthermore, gain-of-function studies showed that NeuroD promotes neuronal differentiation of P19 cells *in vitro* (65). This basic helix-loop-helix protein seemed to be essential for the specification of neuronal production in the developing neural retina in rodent. Loss-of-function experiments have also indicated that proneural bHLH factor Mash1 can directly induce the neuronal fate decision in the embryonic telencephalon.

Taken together, all above studies support the idea that neurogenesis is accompanied by the suppression of glial fate determination.

1.4.2. Glial and neuronal cell fate mechanism

One of the important and initial decisions that neuronal stem cells must make in developing central nervous system is either to differentiate to neurons or generate glial cells. It has been well documented that they first proliferate and give rise to neuronal restricted precursors and after that are able to produce glial cells (66, 67). Therefore, neurogenesis takes place earlier than gliogenesis during brain development.

Recent studies have been implicated the involvement of several intrinsic factors and extracellular signaling molecules in the determination of neuronal and glial cell fate decision. For example, Notch signaling pathway has been shown to influence the neuronal and glial cell fate determination in the peripheral nervous system (68). In addition, it can stimulate the glial production of precursor cells in adult hippocampus (69). Notch signaling similarly promotes the glial fate decision in the embryonic mouse forebrain (70).

It has been shown that Notch signaling pathway influences the neuronal and glial cell fate determination by modulating *Hairy/Enhancer of Split (Hes)* gene function. *Hes* genes are downstream targets of Notch signaling which produce the nuclear protein to suppress the transcription (71). In fact, *Hes* genes encode the transcriptional repressors to block the gene expression. They can inhibit the expression of proneuronal proteins (bHLH factors) in order to promote gliogenesis and suppress the neuronal differentiation (72, 73).

It has been shown that several extracellular factors such as fibroblast growth factor 2 (FGF2), BMP2 and leukemia inhibitory factor (LIF) are also able to control the glial and neuronal cell fate decision during brain development (74, 75). Furthermore, some of the downstream targets of these signaling molecules have been shown to play dual role during glial and neuronal development. They have ability to activate the generation of glial cells and simultaneously can suppress the neurogenesis. For example, BMP2 and its downstream modulator Smad1

were previously shown to be involved in astrocyte differentiation. They are able to interact with LIF/STAT3 effectors in order to form a gliogenic transcriptional complex (BMP2/Smad1-LIF/STAT3) (76). Simultaneously, BMP2 and Smads elevate the expression of *Hes* gene family which is known to suppress the neuronal differentiation by inhibiting the bHLH factor Ngn activity (Figure 3) (76). Similarly, proneural Ngn1 protein was also shown to have double functions, stimulating the neuronal production by its transcriptional activity and inhibiting the glial differentiation by competing for the formation of the gliogenic LIF/STAT3-BMP2/Smad complex (62).

Figure 3. Promotion of neurogenesis is accompanied by the inhibition of the glial cell generation.

The ternary gliogenic complex (BMP2/Smad1-lif/STAT3) both stimulates gliogenesis and suppresses the neuronal differentiation pathways (74, 75). The first signal of gliogenesis pathway induced by BMP and LIF signaling molecules is still unknown. BMP2 can also negatively regulate the expression of *Hes* genes (75). Proneural genes (*Mash1* and *Ngn2*) subsequently can be inhibited by the *Hes* genes. The natures of the extracellular signals which stimulate neurogenesis and activate the proneural gene factors are still unknown.

Some intrinsic factors such as Olig1 and Olig2 were also shown to be involved in the specification of oligodendrocyte precursor cells *in vivo* (77, 78). Coexpression of basic helix-loop-helix factor Olig2 and homeodomain protein Nkx2.2 induces precocious oligodendrocyte differentiation in the spinal cord (34). In the telencephalon, *Olig1* retrovirus injection does suppress the neurogenesis, indicating that *Olig* genes not only promote oligodendrocyte generation but also inhibit the neuronal specification in this region of the brain (33). Moreover, ectopic expression of *Id2*, a negative regulator of b-HLH factor, significantly impaired the oligodendrocyte differentiation (79), suggesting the dual functional role of *Id2* in neuronal versus oligodendrocyte cell fate choice.

Although the precision mechanisms which can control glial and neuronal decision are still not completely understood, it is revealed that apart from transcription factors, some distinct microRNAs and protein kinases are able to regulate this machinery. Two kinds of microRNAs can control the cell fate determination in *Caenorhabditis elegans* taste receptor neurons (ASEL and ASER). These microRNAs are able to control the stability and irreversibility of the terminal differentiation step via double-negative feedback loop (80). A tyrosine kinase can modulate the photoreceptor cell fate decision in *Drosophila* (81). This kinase which is encoded by sevenless gene (*sev*) controls the critical step in developmental decision in the eye of the fly (81, 82).

1.5. Non-metastatic clone 23 (Nm23) family members

Non-metastatic cell 23 gene (*Nm23*) encodes a nucleoside diphosphate kinase (NDPK) including of 152 amino acids (~17 KD) which was first identified in several tumors and because of its ability to suppress the metastasis it so called "metastasis inhibition factor" (83-85). So far, two mouse Nm23; Nm23-M1 (83) and Nm23-M2 (86), two rat Nm23; Nm23-R1 (87) and Nm23-R2 (88) and seven human Nm23 (Nm23-H1 to H-7) (89-92) members have been well identified. These family members are almost conserved across all organisms, from prokaryote to human (86, 93, 94). For example, Nm23-M1 and Nm23-M2 have 95% identity in their amino acid sequences while Nm23-H1 and Nm23-H2 share lower homology of 87% (95).

Nm23 proteins composed of two different subunits which are termed NDPK A (NME1) and NDPK B (NME2) (96). These two subunits are regulated separately in rat and it was shown that level of NME1 is particularly high in the brain (97, 98).

Figure 4. Nucleoside diphosphate kinase reaction.

NM23 proteins are nucleoside transphosphorylase which are able to catalyze the transferring of γ -phosphate from a nucleoside triphosphate (NTP) to a nucleoside diphosphate (NDP) (99, 100). These family members can also act as histidine-dependent protein kinases and phosphorylate proteins (101, 102).

Nucleoside diphosphate kinase catalyses the phosphorylation of nucleoside diphosphates (NDP) into nucleoside triphosphates (NTP) required for the nucleic acids biosynthesis (Figure 4) (103, 104). In addition to binding nucleotide, Nm23 proteins are also able to interact with single and double-stranded DNAs and proteins. Through their binding to polymeric DNA they can act as transcriptional factors to regulate the expression of c-myc (105), gelatinase (106) and platelet-derived growth factor-A promoter (107). Furthermore, they also exhibit the exonuclease activity by binding to single-stranded DNA or polypyrimidine-rich RNAs (108, 109). Previous findings indicated that Nm23 family members regulate the endocytosis through interaction with cytoskeleton components (110, 111). Moreover, NM23 proteins were shown to play some important roles which

are unrelated to its catalytic properties such as apoptosis process, development, proliferation, differentiation and cell fate determination (112-115).

1.5.1. Expression and Function of Nm23 proteins in CNS

Although the roles of NM23 proteins are well-recognized in oncogenesis, their functions during normal development of CNS have not been studied largely. Because they interact with numerous molecules, which are involved in development, Nm23 members are considered as potential mediator or regulator during CNS development. For example, it has been documented that Nm23 proteins have an important role in neural patterning because these members can regulate the function of microtubules via interaction with some GTPases (116, 117). Bodies of evidence also suggest that NM23 proteins are involved in neuron functions. For example, it has been shown that mutations in NM23 can induce abnormal development in *Drosophila* neuronal tissue (118). They are also detected to accumulate in the mouse nervous system on embryonic day E10.5 undergoing differentiation (114). In addition, different expression levels of *Nm23* genes and their cellular distributions were detected in mouse dorsal root ganglia through peripheral nervous system (119). Few studies have been performed concerning the tissue distribution of *Nm23* through central nervous system. Pervious study was investigated the *Nm23-M1* and *Nm23-M2* mRNAs distribution in mouse central nervous system and provided detailed analysis of *Nm23* mRNA expression pattern (83). Results from Northern blot analysis and in

situ hybridization revealed the wide expression of *Nm23-M2* mRNAs in CNS. Moreover, similar patterns of expression were found for *Nm23-M1* transcripts (83).

1.5.2. The role and molecular mechanism of Nm23 family in glial and neuronal cell fate determination

All members of the *Nm23* family are expressed in vertebrate developing central nervous system and some members seem to have roles in mature glial and neuronal cells through regulation of synaptic function. In addition, some members play important roles in distinct aspect of neuronal development and during glial and neuronal cell fate determination. For example, *Nm23-M1* is mainly expressed in post-mitotic cells in mouse brain cortex, while *Nm23-M4* is highly expressed in proliferating cells (120). In addition, *Nm23-X2* (a member of the *Xenopus* *Nm23* family) which is widely expressed in the organizer, is able to stimulate neuronal cell fate decision in the overlaying tissues (121). Furthermore, in *Xenopus* retinogenesis, *Nm23-X4* is shown to regulate gliogenesis and neurogenesis via interaction with specific shRNAs named p27Xic1 (122).

Although the role and function of these proteins in cell fate decision have been reported in several systems such as *Xenopus* retinogenesis (122), bone marrow cells (123) and hematopoietic cells (124), the molecular mechanisms by which *Nm23* family regulate glial and neuronal cell fate determination are still largely unknown. In the only study of *Nm23* members in this case, it has been revealed

that a NDP kinase named Nm23-X4 can control neurogenesis and gliogenesis during *Xenopus* retinal development (122). This NDP kinase has a particular ability to delay or inhibit gliogenesis through interaction with a distinct shRNA, p27Xic1, during retinogenesis. In addition, p27Xic1 shRNA has neural-inducing activity which can promote neurogenesis of retinal precursor cells. Therefore, it has been proposed a genetic model of both *Xenopus* NDPK and shRNA to control retinal neuron and glial cell fate determination (122). Although little is yet known about their function in CNS development, it seems that Nm23 proteins play a crucial role in neuronal and glial cell fate determination.

1.6. Wnt signaling pathway

1.6.1. *Wnt gene family*

The Wnt gene family belongs to a family of structurally related genes which encode secreted signaling proteins. Wnt genes were originally identified by the studies of Roeland Nusse and Harold Varmus in 1982. It was first identified for its role in carcinogenesis, but has since been recognized for its function in embryonic development. The origin of the name Wnt comes from a hybrid of Wg and Int (wingless) in *Drosophila*, which is the best characterized Wnt gene (41, 125, 126). So far, several Wnt genes have been discovered (Table 1). Most of the genes from this family are conserved during evolution and have been implicated in oncogenesis and in several developmental events, such as cell fate determination and patterning during embryogenesis. Table 1 shows the list of Wnt genes that encode Wnt signaling proteins together with their function during development.

Table 1. List of human Wnt genes, proteins and their functions (127)

Wnt Family		
Gene	Protein	Function
<i>Wnt1</i>	Wnt1	Cell fate determination and patterning during embryogenesis
<i>Wnt2</i>	Wnt2	Oncogenesis Cell fate determination
<i>Wnt2B</i>	Wnt2B	Cell growth Differentiation
<i>Wnt3</i>	Wnt3	Primary axis formation in the mouse
<i>Wnt3A</i>	Wnt3A	Cell fate determination and patterning during embryogenesis
<i>Wnt4</i>	Wnt4	Female fetal genital development
<i>Wnt5A</i>	Wnt5A	Skin development
<i>Wnt5B</i>	Wnt5B	Cell fate determination and patterning during embryogenesis
<i>Wnt6</i>	Wnt6	Cell fate determination and patterning during embryogenesis
<i>Wnt7A</i>	Wnt7A	Uterine smooth muscle patterning Maintenance of adult uterine function Development of the anterior-posterior axis in the female reproductive tract
<i>Wnt7B</i>	Wnt7B	Gastric development
<i>Wnt8A</i>	Wnt8A	Cell fate determination and patterning during embryogenesis
<i>Wnt8B</i>	Wnt8B	Brain development
<i>Wnt9A</i>	Wnt9A	Gastric development
<i>Wnt9B</i>	Wnt9B	Cell fate determination and patterning during embryogenesis
<i>Wnt10A</i>	Wnt10A	Cell fate determination and patterning during embryogenesis
<i>Wnt10B</i>	Wnt10B	Adipogenesis
<i>Wnt11</i>	Wnt11	Skeleton development Kidney development Lung development
<i>Wnt16</i>	Wnt16	Pancreas Development

1.6.2. Wnt molecular mechanism

Wnt signaling pathways are pleiotropic and can regulate diverse biological processes, such as cell proliferation, cell differentiation and cell fate determination. It has been well documented that Wnt proteins interact with specific receptors of signaling cells such as Frizzled (Fz) and low density lipoprotein (LDL) receptor-related protein (LRP) families. After interaction, Wnt signal will be activated and through some cytoplasmic components including glycogen synthase kinase-3 β (GSK-3), Axin, and Adenomatous Polyposis Coli (APC), the signal will be transduced to a transcriptional regulator termed β -catenin (β -catenin). β -catenin then enters the nucleus and forms a complex with transcription factors such as lymphoid enhancer-binding factor 1/T cell-specific transcription factor (LEF/TCF) to activate transcription of Wnt target genes (128-130) (Figure 5).

Figure 5. A schematic diagram of the Wnt signaling pathway (130).

(A) In the absence of a Wnt ligand, Axin recruits GSK-3 β to the β -catenin destruction complex (adenomatous polyposis coli; APC). Phosphorylation of β -catenin by GSK-3 β results in β -catenin inhibition. Phosphorylated β -catenin is then recognized and degraded by proteasome complex resulting in decreased level of cytosolic β -catenin. (B) In the presence of a Wnt ligand, Wnt molecule first binds to the Frizzled (Fz) and low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6). This results in activation of Dishevelled protein (Dsh). Dsh protein then inhibits the APC complex and phosphorylates the GSK-3 β . Subsequently β -catenin will be stabilized and translocated to the nucleus, where it can activate the transcriptional activity of the lymphoid enhancer factor/T cell factor (LEF/TCF) family.

1.6.3. The role of Wnt signals in neuronal differentiation

A body of evidence suggests that Wnt/ β -catenin signaling pathway plays crucial roles in cell fate determination during mammalian neural development. In the central nervous system, Wnt-3 and Wnt-3A were largely expressed across the neural tube (131) and it was shown that β -catenin-mediated transcriptional activity is responsible for fate decision of precursor cells to differentiate into neurons in cortical ventricular zone (132). Overexpression of stabilized β -catenin or Wnt-7 blocks the self-renewal ability and induces the neuronal differentiation of mouse cortical neural precursor cells (133). Moreover, β -catenin amplification was shown to be sufficient to promote neurogenesis of embryonic stem cells (134). It has also been demonstrated that β -catenin signaling induces neuronal differentiation from somatic and neural stem cells (135, 136). Recently, it was shown that β -catenin expression not only promotes neurogenesis but also inhibits oligodendrocyte differentiation of neural stem/precursor cells in knockout mice model (137).

Following these noteworthy findings, in this study we found the new function of protein kinase NME1 that is involved in neuronal and glial cell fate determination by modulating β -catenin signaling pathway.

CHAPTER 2: AIMS AND SIGNIFICANCE

One of the main questions during neuronal development is to understand how a single precursor cell can give rise to different classes of glial and neuronal cells. Recently, the adult glial precursor cells were shown to be able to produce neurons in the central nervous system and *in vitro*. Although the fate determination of glial precursors was studied extensively, the programs that could redirect their fate to become neurons still remained unknown.

The main goal of this study is to understand the role and molecular mechanism of NME1 during glial and neuronal cell fate decision. As NME1 is up-regulated during neuronal differentiation and down-regulated after oligodendrocyte differentiation of precursor cells, we proposed a regulatory role of NME1 during glial and neuronal cell differentiation program.

Using gene amplification and silencing approaches, the potential role of NME1 in driving neurons from oligodendrocyte precursor OLN-93 cells was determined. Since, NME1 cellular distribution was identified by immunohistochemistry analysis and it was shown to be colocalized with neuronal marker NF200, we focus our studies to elucidate the molecular mechanism underlying NME1-mediated activity.

Understanding the role of the NME1 in precursor cells may help us to visualize the critical insight on the molecular mechanism of neurogenesis and gliogenesis.

Taken together, our study may address following aims:

- 1- The general aspects of glial and neuronal cell differentiation from precursor cells *in vitro*.
- 2- Neuronal cell fate specification of oligodendrocyte glial precursors.
- 3- The role and molecular mechanism of NME1 in driving neuronal cells from oligodendrocyte precursor cells *in vitro*. Understanding these mechanisms will help to represent a method for studying cell reprogramming, cellular plasticity and neuronal regenerative treatment for neurological diseases.

CHAPTER 3: MATERIALS AND METHODS

3.1. Plasmids, DNA constructs and transfection

3.1.1. Construction of plasmids

Rat NG2 cell (138) cDNAs and a pair of primers corresponding to the coding region of *Nme1* were used to amplify the gene. Primer sequences were as follows: forward (5'-3'): CGGAATTCACCATGGCCAACAGCGAGCGTACCTT and reverse (5'-3'): CGGGATCCTCACTCATAGATCCAGTTCTG (Sigma-Aldrich).

The cDNA encoding rat NME1 was then subcloned into pIRES2-EGFP (Figure 6) (BD Biosciences, USA). In order to silencing the gene, oligos were designed using the siRNA target-finder algorithm (www.ambicon.com). Forward and reverse 63- and 55-nucleotide fragments were ligated to pSilencer™ 3.1-H1 puro and pSilencer™ 4.1-CMV siRNA expression vectors (Ambion), respectively (Figure 7). Sequences for siRNA oligos were as follows: forward 55-nucleotide primer (5'-3'): (GATCC

GTTGGCAGGAACATCATTCTTCAAGAGAGAATGATGTTTCCTGCCAACT
TA), reverse 55-nucleotide primer (5'-3'): (AGCTT
AAGTTGGCAGGAACATCATTCTCTCTTGAAGAATGATGTTTCCTGCCAA
CG), forward 63-nucleotide primer (5'-3'):
(GATCCATTTATACAGGCTTCAGAGTTCAAGAGACTCTGAAGCCTGTA
TAAATTTTTTTTGAAA) and reverse 63-nucleotide primer (5'-3'):

(AGCTTTTCCAAAAAATTTATACAGGCTTCAGAGTCTCTTGAACCTCTG AAGCCTGTATAAATG). In order to select pIRES2-EGFP-*NmeI* positive cells, flow cytometry (FACSariaIII, Becton-Dickinson) was used. The dominant negative TCF4 plasmid was a gift from Dr. Alman, University of Toronto and plasmids of wild type and dominant negative beta-catenin were described previously (139). Transfection of wild type and dominant negative beta-catenin and TCF4 were also performed using Lipofectamine 2000 (Invitrogen).

Figure 6. The restriction map of pIRES2-EGFP.

pIRES2-EGFP contains the internal ribosome entry site (IRES; 1, 2) of the encephalomyocarditis virus (ECMV) between the MCS and the enhanced green fluorescent protein (EGFP) coding region (www.clontech.com). This permits both the gene of interest (cloned into the MCS) and the EGFP gene to be translated from a single bicistronic mRNA. Unique restriction sites are in bold.

NME1 was inserted between EcoRI (631) and BamHI (661) in the pIRES2-EGFP plasmid (BD Biosciences, USA).

3.1.2. ERM Plasmids, NG2⁺ cells electroporation and clonal selection

To construct MAP2 promoter-driven reporter plasmid, mouse MAP2 promoter of 1,472 bp was cloned into EGFP reporter vector. A set of ERM plasmids (pMSCV-tTA-neo, pBabe-HA-puro) (140-142), gifts from Zhou Songyang (Baylor College of Medicine), was transfected into NG2⁺ cells (143) by electroporation. The transfected NG2⁺ cells were subsequently selected with G418 (500µg/ml) and flow cytometry. The selected cells were then transfected with MAP2 promoter-driven EGFP reporter vector and sorted by FACS technique. Finally, EGFP-positive cells which exhibited neuron-like morphology were further analyzed by the 3' RACE and RT-PCR to identify the gene(s) targeted by ERM cassette.

Figure 7. pSilencer™ 3.1-H1 puro and 4.1-CMV vector maps.

The pSilencer vectors employ RNA polymerase III (pol III) promoters which generate large amounts of small RNA using relatively simple promoter and terminator sequences (www.ambion.com). They also include antibiotic resistance genes (Ampicillin and puromycin) that provide a mechanism to select for transfected cells that express the introduced DNA (144,

145). This vector also includes the negative control siRNA template (66 base pairs) between the BamH I and Hind III sites.

3.1.3. OLN-93 cells transfection, G418 selection and flow cytometry

For transfection, 5µg of DNA construct was introduced into OLN-93 cells cultured in 60-mm dish (70-80% confluent) by electroporation using Neon™ Transfection System (Invitrogen). G418-resistant cells (500µg/ml) were then harvested and used for further experiments. In order to select pIRES-*NmeI* positive cells, flow cytometry (FACS Aria III, Becton-Dickinson) was used.

3.1.4. Plasmid isolation from cells

In order to isolate the plasmid, the cultured cells were first trypsinized and washed twice with PBS by centrifugation at 4,000 rpm for 10 minutes. The pellet was then re-suspended in 500 µl of S-Buffer (10 mM EDTA, 50 mM β-mercaptoethanol, 10 mM KPO₄ pH=7.2, and 100 U/ml zymolase) and incubated at 37°C for 40 minutes followed by adding 50 µl of lysing solution (25 mM EDTA, 2.5 % SDS, 0.25 M Tris-HCL pH=7.5). Subsequently, 80 µl potassium acetate (3 M) was added and samples were incubated on ice for 15 minutes. After centrifugation at 14,000 rpm for 15 minutes, the pellet was removed and the aqueous phase was transferred to a fresh tube followed by adding 1 ml of 100% ethanol. The samples were then incubated at -20°C for 1 hour and centrifuged at

14.000 for 30 minutes. Finally, pellet was transferred to a new 1.5 ml tube and re-suspend in 50 µl of distilled water.

3.2. Antibody preparation

3.2.1. Preparation of NME1 polyclonal antibody

Human *nme1* cDNA was cloned into pGEX-4T-2 at *Bam*HI and *Eco*RI sites. Primers targeting the whole reading frame of NME1 were designed as follows: forward (5'-3'): CGGGATCCGCCAACAGCGAGCGTACCT and reverse (5'-3'): GGAATTCTCACTCATAGATCCAGTTC (Sigma-Aldrich). For antibody preparation, *E.coli* culture containing the recombinant plasmid pGEX-4T-2-NME1 was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hours. The induced culture was spun down, washed with PBS and then was solubilized by sonication on ice. After centrifugation, the supernatant was mixed with glutathione agarose beads (Sigma-Aldrich) and incubated for 2 hours. The beads were washed 3 times with PBS and the fusion protein GST-NME1 was obtained using glutathione elution buffer. The purified GST-NME1 fusion protein was used as immunogen and injected in mice intradermally. Four days after the last injection, mice were exsanguinated and the antisera were collected. Finally, IgG antibodies against GST-NME1 were purified with protein A-coupled beads.

3.3. Cell culture

Rat oligodendroglial OLN-93 and neuroblastoma B104 cell lines were gifts from Dr. Fengyi Liang (Department of Anatomy, National University of Singapore) and maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), supplemented with 10% fetal calf serum (FCS, HyClone), 100U/ml streptomycin and 100U/ml penicillin. For differentiation, cells were cultured on poly-L-lysine (PLL) coated dishes for 12 hours after attachment and the medium was exchanged for a low-serum DMEM (0.5% FCS) containing forskolin (5 μ M) (146) or IGF-I (100ng/ml) (147, 148) to promote neuronal and oligodendroglial differentiation respectively. Lithium chloride was purchased from Sigma and Lipofectamine 2000 was from Invitrogen.

3.4. Protein binding assays and Western Blotting

3.4.1. Antibodies and Western Blotting

Antibodies against β -actin, MBP, CNPase, PLP Beta-catenin (sc-29210) was purchased from Santa Cruz Biotechnology, antibody against GSK-3 β was from Cell Signaling Technology and antibodies against MAP2, NF200 and Tuj1 was from Chemicon. For Western blot analyses, cells were first lysed in RIPA buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% DOC, 1 mM PMSF, 25 mM MgCl₂, and supplemented with a phosphatase inhibitor cocktail). Protein concentration was then determined by the BCA assay (Bio-Rad

Laboratories, Hercules, CA). Equivalent amounts of protein were electrophoresed on SDS–polyacrylamide gels. Protein bands were visualized with substrate system from Millipore and PageRuler™ Prestained Protein Ladder (Fermentas, USA) were used to determine molecular weight.

3.4.2. Immunoprecipitation

Immunoprecipitation is a method that enables the purification of a protein. An antibody for the protein of interest is incubated with a cell extract so that the antibody will bind the protein in solution. The antibody/antigen complex will then be pulled out of the sample using protein A/G-coupled agarose beads. This physically isolates the protein of interest from the rest of the sample. The sample can then be separated by SDS-PAGE for Western blot analysis. For immunoprecipitation experiments cells were first cultured in DMEM-HG medium (Invitrogen) supplemented with 10% fetal calf serum (FCS, HyClone), and 1% penicillin/streptomycin (Invitrogen). An equal amount of cell lysates (300µg) were then incubated with 5µg GSK-3β (Cell Signaling) and NME1 antibodies overnight at 4°C. Samples were pelleted with protein A+G-agarose beads (Calbiochem) by centrifugation at 10,000g for 1 minute followed by boiling in 30 µl sample buffer (2X) for 5 minute. Immunoprecipitated samples were finally analyzed for the presence of NME1 and/or GSK-3β.

3.5. Quantitative reverse transcriptase-PCR (RT-PCR) and real-time PCR

3.5.1. RNA extraction and RT-PCR

Reverse transcription-polymerase chain reaction (RT-PCR) is a sensitive method for the detection of mRNA expression levels. Traditionally RT-PCR involves two steps: the RT reaction and PCR amplification. RNA is first reverse transcribed into cDNA using a reverse transcriptase as described here, the resulting cDNA is used as templates for subsequent PCR amplification using primers specific for one or more genes. RT-PCR can also be carried out as one-step RT-PCR in which all reaction components are mixed in one tube prior to starting the reactions. Although one-step RT-PCR offers simplicity and convenience and minimizes the possibility for contamination, the resulting cDNA cannot be repeated used as in two step RT-PCR. In order to perform RT-PCR Trizol Reagent (Invitrogen) was first used to isolate total RNA from harvested cells and cDNA was synthesized with Superscript III reverse transcriptase (Invitrogen) from One microgram total RNA. Using random hexamer primers, reverse-transcription reactions incubated at 50°C for 90 minutes. Each sample was RNase H treated (30 minutes at 37°C) followed by 30 minutes incubation at 65°C. Each template was also used in subsequent real-time PCR reactions. Primer sequences are shown in Table 2.

Table 2. Reverse-transcriptase PCR primer sequences (5'-3')

GAPDH	F: TATCCGTTGTGGATCTGACAT R: CATGTAGGCCATGAGGTCCACCAC
MOG	F: TGCCTCTAGAAAACACCCCA R: AGCACCTAGCTTGTTTGTGT
CNPase	F: AGAGCTGCAGTTCCCTTTCCTTCA R: TGTCATCGAGCACAAGAACCCTGA
MBP	F: GGATGTGATGGCATCACAGAAGAGA R: TTTAGCCAGTCAGGGTGCCGTGGG
CGT	F: CGTACTCCTAGAACACAGACT R: AGTTACTGAAGGGGAGCTGTA
PLP	F: TTGAGACTTGTTGTTGGGCC R: ATGAAGGAAACACTTGCAGTTG
NF200	F: ACCTATACCCGAATGCCTTCTT R: AGAAGCACTTGGTTTTATTGCAC
Reelin	F: CAGGCCTCAGAAACACACAA R: AGCACCAGTAGAAATGGATGA
Synapsin	F: ACCACAAGAGGTGCAAGATAGG R: TAGTTCATGGTGGCAGCTTGGG
Tuj-1	F: GAACATGATGGCTGCCTGTGA R: CCCGTGTACCAGTGGAGGAA
NeuroD	F: GGAGTAGGGATGCACCGGGAA R: CTTGGCCAAGAACTATATCTGG
Tau	F: TACAGCAACTGCACAGGCTACA R: GCAGTGGGTAAGACCACTTCCT

3.5.2. Real-time PCR

Real-time PCR, also known as kinetic PCR, qPCR, qRT-PCR and RT-qPCR, is quantitative PCR method for the determination of cope number of PCR templates such as DNA or cDNA in a PCR reaction. There are two flavors of real-time PCR: probe-based and intercalator-based. Both methods require a special thermocycler equipped with a sensitive camera that monitors the fluorescence in each well of the 96-well plate at frequent intervals during the PCR Reaction.

Probe-based real-time PCR, also known as TaqMan PCR, requires a pair of PCR primers as regular PCR does, an additional fluorogenic probe which is an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached. Intercalator-based method, also known as SYBR Green method, requires a double-stranded DNA dye in the PCR reaction which binds to newly synthesized double-stranded DNA and gives fluorescence. TaqMan method is more accurate and reliable than SYBR green method, but also more expensive. In our study real-time PCR was performed with the KAPA SYBR® qPCR kit (KAPA Biosystems). SYBR-Green PCR master mix was first used to monitor each amplicon and ROX served as the passive dye to control for pipette errors. Each primer set was subsequently validated and showed a single peak in the dissociation curves. Relative changes in expression levels for each amplicon was finally normalized against β -actin expression. Stated changes were quantified as changes in threshold cycle (ΔC_t) compared to controls ($\Delta\Delta C_t$) and reported as fold changes ($2^{-\Delta\Delta C_t}$). Samples were obtained from three independent experiments, and each sample was used for three reaction of real-time PCR. Primer sequences are shown in Table 3.

Table 3. Real-time PCR primer sequences (5'-3')

β -actin	F: GAGACCTTCAACACCCCAGCC R: AATGTCACGCACGATTTCCC
Wnt3A	F: CTCCTCTCGGATACCTCTTAGTG R: GCATGATCTCCACGTAGTTCCTG
Wnt5A	F: CTTCGCCCAGGTTGTTATAGAAGC R: CTGCCAAAGACAGAAGTATTGTCC
LRP5	F: AAGGGTGCTGTGTACTGGAC R: AGAAGAGAACCTTACGGGACG
LRP6	F: TTGTTGCTTTATGCAAACAGACG R: GTTCGTTTAATGGCTTCTTCGC
Frizzled7	F: CGGGGCCTCAAGGAGAGAA R: GTCCCCTAAACCGAGCCAG
Frizzled8	F: ATGGAGTGGGGTTACCTGTTG R: CACCGTGATCTCTTGGCAC
Axin2	F: CAATGACACCACTCCAGATGAG R: GGCCAAAGAAGTCGTTGCG
MITF	F: ACTTTCCCTTATCCCATCCACC R: TGAGATCCAGAGTTGTCGTACA
Cyclin D1	F: AAGCTGTGCATCTACACCGA R: CTTGAGCTTGTTCAACAGGA
NG2	F: GAACGCATCAGCCACCGTAA R: GGACGCTTCTTCCTGGTTTC
Nestin	F: CTGCAGGCCACTGAAAAGTT R: GACCCTGCTTCTCCTGCTC

3.6. Cell proliferation and differentiation assays

3.6.1. Cell proliferation analysis

3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay is an established method of determining viable cell number in proliferation studies. This assay is based on the cleavage of the yellow tetrazolium salt, MTT, to form a soluble blue formazan product by mitochondrial

enzymes, and the amount of formazan produced is directly proportional to the number of living, not dead cells, present during MTT exposure. Cells were first cultured in 96-well plates at 2000 cells per well and incubated for 2 days. The yellow tetrazolium MTT (Roche Diagnostics GmbH, Mannheim, Germany) working solution was then added into wells being assayed at 4ul/100ul. Cells were then Incubate at 37°C for 3hours. Absorbance was measured at 570 nm with the reference filter of 660 nm by Tecan microplate reader (Tecan Group Ltd). All results were from three independent experiments.

3.6.2. Cell cycle analysis

For analysis of DNA content, cells were first harvested by trypsin and washed twice with PBS buffer. Isolated cells were subsequently treated with 70% ethanol for one hour at 4°C. The fixed cells were then labeled with propidium iodide (50µg/ml, Sigma) followed by incubation with RNase A (50µg/ml) at 37°C for one hour. Samples were finally run on FACSCalibur flow cytometer (BD Biosciences) at a low flow rate and analyzed with ModFIT software (Vantage Software, Topsham). All results were from three independent experiments.

3.6.3. BrdU labeling of cultured cells

The thymidine analog, 5-bromo-2-deoxyuridine (BrdU), is a common reagent used for cell proliferation assays and for the detection of apoptotic cells. BrdU is a uridine derivative and a structural analog of thymidine, and it can be

incorporated into DNA during the synthesis-phase of the cell cycle as a substitute for thymidine, thereby serving as a marker for proliferation. Cells marked by BrdU incorporation may be detected by multiple detection methods using fluorescently-labeled or enzyme-linked anti-BrdU antibodies. In order to BrdU labeling of cultures, OLN-93 cells were treated with BrdU (Invitrogen) at final concentrations ranging from 10 nM to 10 μ M, for overnight. After labeling, cells were fixed with 70-80% alcohol or acid alcohol for 30 minutes followed by immunocytochemical staining.

3.7. Brain sections preparation

Adult Sprague-Dawley rats were purchased from the Laboratory Animal Center of National University of Singapore. Animals were scarified by overdose of pentobarbital sodium and followed by cardiac perfusion with cold heparinized saline and 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Fixed brain were then dissected and immersed in PBS containing 30% sucrose. Frozen coronal sections (30 μ m) were then cut with microtome (Hyrax c20, Zeiss).

3.8. Immunofluorescence and Immunohistochemistry staining

Cultured cells were first rinsed in PBS and fixed with 4% paraformaldehyde for 20 minutes at room temperature and then permeabilized with PBS containing 0.2% Triton X-100 for 20 minutes. Cells were then treated with 4% bovine serum albumin (BSA) at 4°C for 1 hour and incubated with primary antibodies against

NME1, β -catenin, MAP2, O4, GSK-3 β , p-GSK-3 β and CyclinD1 in PBS containing 1% BSA/Triton X-100 overnight at 4°C. After 3 times washing, cell incubated for 2 hours at room temperature with Alexa Fluor 568 rabbit anti-mouse IgG, Alexa Fluor 488 rabbit anti-mouse IgG, Alexa Fluor 568 goat anti-rabbit IgG, Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 647 rabbit anti-mouse IgG (Invitrogen). Cells were finally stained with 4', 6-Diamidino-2-Phenylindole (DAPI, Invitrogen) in PBS containing 4% BSA for 5 minutes. Samples were mounted with FluorSave™ reagent (Calbiochem) in order to observe with a confocal microscope (LSM510, Carl Zeiss). For morphological observations and immunofluorescence analysis, samples were observed under light or fluorescence microscope (Olympus 1X71) and confocal microscope (Zeiss 510, Zeiss) was used to observe immunohistochemistry staining.

3.9. PKA activity assay

Protein kinase inhibitors, including Akti and H-89 were purchased from Merck. PKA activity assay (Promega) was performed in order to measure the enzyme activity (149, 150). Total protein extracts were obtained from vehicle, iO and iN cells and 20 μ g of lysates for each sample were used for assay. At the same time, standard cAMP-dependent protein kinase, PKA positive and negative control (no PKA added) assays were prepared. The reactions were blocked by heating (95°C) for 10 minutes and sample were loaded in 0.8% agarose gel. Non-phosphorylated peptide migrated toward the anode (–) while phosphorylated one migrated toward

the cathode (+). The gel was then photographed (Syngene) and enzyme activity was measured by Magellan software (version 6.5) using Tecan Genios plus plate Reader. Finally, enzyme activity was calculated by these formulas: $C = A/\epsilon B$, enzyme activity (U/ml) = $C \times V \times \text{dilution factor}/\text{reaction time}$ while A = absorbance of the sample, ϵ = the molar absorptivity of the peptide in $\text{L/mol} \cdot \text{cm}^{-1}$, B = the width of the light cell, C = the concentration of the peptide in mol/L of the sample read and V is volume of the sample in the well.

3.10. TCF/LEF report assay

Cells were first cultured in 96-well plates with 80% confluence 12 hours before transfection. Cultured cells were then transiently transfected with TCF/LEF4 vector using Lipofectamine 2000 following the manufacture's instructions and treated with serum free medium for 24 hours. Finally, cell lysates that obtained from Lysis Buffer (Promega) were used to measure the luciferase activity using Tecan Genios plus plate Reader. All results were from three independent experiments and expressed as fold induction relative to basal activity.

3.11. Statistical data analysis

Statistical significances were determined using Student's t-test or one way analysis of variance. The results were expressed as mean, standard deviation ($\pm$ SD). All results were from three independent experiments.

CHAPTER 4: RESULTS

4.1. Expression and cellular distribution of NME1 in adult mouse central nervous system

As stated earlier, Nm23 proteins composed of two different subunits (NDPK-A and NDPK-B). These two subunits are expressed in different regions of vertebrate CNS. A cDNA for NDPK-A was isolated from mouse embryonic stem cells and the tissue distribution of *Nm23-M1* mRNA was investigated in CNS by using Northern blot analysis and in situ hybridization (83). Here, we determined the expression pattern and cellular distribution of NME1 in the adult mouse CNS. Western blot analysis against NME1 was first carried out to demonstrate the presence of protein in the adult brain and spinal cord. As shown in Figure 8 NME1 is highly expressed in adult mouse brain.

Figure 8. NME1 expression in mouse central nervous system.

Western blot analysis of NME1 in mouse brain and spinal cord. As shown in the figure, NME1 expresses much higher in adult brain in comparison to spinal cord.

Immunohistochemical analysis was further performed to identify the general expression of NME1 in central nervous system. Figure 9 shows the expression of NME1 in both spinal cord (Figure 9A) and brain sections (Figure 9B).

Figure 9. NME1 expression pattern in mouse central nervous system.

(A) Distribution of NME1 in coronal section of brain and in sagittal section of the spinal cord (B). NME1 highly expresses in corpus callosum (cc), hippocampus (hi), caudate–putamen (cp), heart (ht) and amygdala (am) and in gray matter (GM) of spinal cord. NME1 (green), DAPI (blue). Scale bars: 400 μm (b–b'') and 500 μm (a–a'').

Moreover, fine cellular screening, we could observe the cellular distribution of NME1 in different regions of gray and white matter of the adult mouse brain. (Figure 10).

Figure 10. Cellular distribution of NME1 in adult mouse brain.

NME1 is highly expressed in both gray and white matter of brain. Immunohistochemical staining of NME1 (green) in (A) RSA: Retrosplenial agranular cortex. (B) AuD: dorsal area. (C) AHiPM: amygdalohippocampal area. (D) SNR: substantia nigra, lateral part. (E) InWh: layers of superior colliculus. (F) S: subiculum. (G) GrDG: granular layer of dentate gyrus, PoDG: polymorph layer, DG: dentate gyrus and dentate gyrus. (H) ML: lateral part of medial mammillary nucleus and pm: principal mammillary tract. (I) cc: Corpus callosum. (J) ec: external capsule. (K) bsc: brachium of superior colliculus. (L) cg: cingulum. (M) ml: medial lemniscus. (N) dhc: commissure of fornix.

(O) fi: fimbria of hippocampus and (P) hf: hippocampal sulcus. Scale bars: 100 μm (D, E, F and L–P) and 200 μm (A–C and G–K).

To elucidate its expression pattern in neurons and oligodendrocytes, double immunohistochemical staining was performed in brain and spinal cord sections. Figure 11A and B compare the NME1 expression of myelin basic protein positive (MBP⁺) oligodendrocytes in retrosplenial agranular cortex (RSA) and commissure of fornix (dhc) respectively. No colocalization was found between MBP and NME1 in both white and gray matter of brain coronal sections. We also compared the NME1 expression pattern in neurofilament-200 positive (NF200⁺) neurons in gray and white matter (Figure 11C and D respectively). Interestingly, strong NME1-NF200 colocalization was observed in principal mammillary tract (Figure 11D) and in different regions of white matter (Figure 12). Similarly, using double immunohistochemical staining, NME1 was also found to colocalize with NF200 only in white matter through sagittal sections of spinal cord (Figure 11E and F). These observations suggest that NME1 is mainly expressed in neuronal structures throughout white matter of adult mouse CNS.

Figure 11. Cellular distribution of NME1 through white and gray matter of CNS.

(A–D) Differential expression of NME1 in neurons and oligodendrocytes through brain coronal sections. (A and B) MBP-NME1 double immunohistochemical labeling in retrosplenial agranular cortex (RSA) and commissure of fornix (dhc) respectively. NME1 (green), MBP (red) and DAPI (blue). (C and D) NF200-NME1 double staining in ectorhinal cortex (Ect) and principal mammillary tract (pm) respectively. NME1 (green), NF200 (red) and DAPI (blue). (E and F) NF200-NME1 double immunohistochemical labeling in gray and white matter of the spinal cord respectively. NME1 (green), NF200 (red) and DAPI (blue). Scale bars: 20 μ m.

Figure 12. NME-NF200 colocalization in white matter of the mouse brain.

(A) external capsule. (B) cingulum. (C) commissure of fornix. (D) hippocampal sulcus. (E) rhinal fissure and (F) external capsule. NME1 (green), NF200 (red) and DAPI (blue). Scale bars: 20 μ m.

Taken together our results indicated that NME1 not only distributed in mouse central nervous system but also is able to colocalize with mature neuronal marker NF200.

4.2. Expression of NME1 during neuronal and oligodendrocyte differentiation

As stated earlier, Nm23 proteins play several important roles in various aspect of cell function including proliferation and differentiation; however, there are no direct evidences whether they can regulate glial and neuronal cell differentiation. In order to elucidate the potential role of NME1 during differentiation, we used two different cell lines OLN-93 and B104 as standard models of oligodendrocyte and neuronal differentiation, respectively. For oligodendrocyte differentiation, OLN-93 cells were subjected to differentiation protocol (Material and Methods) for one week, followed by the examination of mature glial cell markers expression. In comparison with untreated cells (Figure 13A), after induction, cells clearly exhibit oligodendrocyte-like morphology and express the myelin basic protein (This protein encoded by the classic MBP gene which is a major constituent of the myelin sheath of oligodendrocytes in the nervous system and therefore can be used as a standard marker of Oligodendrocytes) (Figure 13B and C). Results from Western blot analysis showed that NME1 is obviously decreased during oligodendrocyte differentiation, indicated by distinct expression of oligodendrocyte and neuronal markers (Figure 13D and E).

Figure 13. NME1 expression decreases during oligodendrocyte differentiation.

NME1 down-regulation during Oligodendrocyte differentiation of OLN-93 cells. (A) Untreated cells. (B and C) Light and fluorescence image of cells 7 days after differentiation. MBP (red) and DAPI (blue). (D) Western blot analysis of PLP (28kDa), CNP (48kDa), MBP (14kDa), NME1 (18kDa), Tuj1 (50kDa), MAP2 (280kDa), Tau (45kDa) and β-actin (42kDa) during oligodendrocyte differentiation. (E) Quantitative analysis of standard oligodendrocyte and neuronal markers expression. Data are representative of three independent experiments and values are means ± S.D. * $P<0.05$ and ** $P<0.005$. Scale bars: 50 μm.

In contrast, rat neuroblastoma B104 cell line was analyzed to examine the expression of NME1 during neuronal differentiation (Material and Methods). The differentiated cells exhibited neuron-like morphology (Figure 14B) and strongly expressed microtubule associated protein-2 (MAP2) (Figure 14C). Correspondingly, the expression of NME1 was found to be up-regulated during

neuronal differentiation (Figure 14D and E), indicating that NME1 may have a role in regulating of neuronal activity.

Figure 14. NME1 expression increases during neuronal differentiation of B104 cells.

(A) Untreated cells. (B and C) Light and fluorescence image of cells 7 days after differentiation. MAP2 (green) and DAPI (blue). (E) Western blot analysis of Tuj1, MAP2, Tau, NME1, PLP, CNP, MBP and β -actin during neuronal differentiation. (F) Quantitative analysis of markers expression. Data are representative of three independent experiments and values are means $\pm$ S.D. * P <0.05 and ** P <0.005. Scale bars: 50 μ m.

4.3. The role of NME1 in oligodendroglial and neuronal cell fate determination

Our observation suggests either that *Nme1* gene function in neurons and not in oligodendrocyte fate decision, or alternatively that it can play a dual role in both glial and neuronal cell fate choice in vitro. To further clarify the role of NME1 in oligodendrocyte versus neuronal cell differentiation we used the gene silencing and amplification approaches.

4.3.1. *NME1* silencing in OLN-93 cells

In order to understand the role of NME1 during oligodendrocyte differentiation, *Nme1* gene silencing was first performed by transfection of two siRNA vectors (targeting two different region of NME1 mRNA) in OLN-93 cells (Materials and Methods). Interestingly, cells with oligodendrocyte-like morphology were observed on day 7 after gene silencing (Figure 15B compared with Figure 15A). The remarkable reduction of NME1 was confirmed by Western blot analysis in these induced oligodendroglial (iO) cells (Figure 15C and D). The oligodendrocyte phenotypes were further exhibited by the expression of mature oligodendrocyte markers on day 7 of transfection by immunostaining. Figure 15E-E''' is an example of iO cell which clearly expressed 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP) and proteolipid protein (PLP). Additionally, NME1 down-regulation not only facilitates OPCs differentiation but also improves the

characteristics of oligodendrocyte generation (compare Figure 15B and E with Figure 13B and C).

Figure 15. NME1 knock-down promotes oligodendrocyte differentiation.

(A) Vehicle-treated OLN-93 cells. (B) iO cells 7 days after NME1 down-regulation. (C) Western blot result of OLN-93, iO and vehicle-treated cells against NME1. (D) Quantification of NME1 expression. (E–E''') CNP-PLP double immunofluorescence staining of iO cells. (E) CNP (green), (E') PLP (red), (E'') DAPI (blue) and (E''') merge Figure. Data are representative of three independent experiments and values are means $\pm$ S.D. $**P<0.005$. Scale bars: 10 μ m (E–E'''), 20 μ m (B) and 25 μ m (A).

We then examined for standard glial and neuronal markers expression of iO cells on day 7 after gene silencing by RT-PCR. The results showed that NME1 silencing not only promotes the up-regulation of mature oligodendrocyte markers

but also decreased the expression of Tuj1 and MAP2 (Figure 16A and B). Next, we examined the kinetics of iO cells differentiation. In iO populations, immature oligodendrocyte-like morphology was observed on day 3 after gene silencing. The morphology further changed toward multi-branched cell appearances on day 5 and became mature status on day 7 (Figure 15B and E-E"). To approximate how many of iO cells divided after NME1 down-regulation, we treated the cells with 5-bromodeoxyuridine (BrdU) at day 0 and 1 after gene silencing. The result showed that about 41% of iO cells became post mitotic one day after gene silencing ($65\% \pm 3.62\%$ versus $27\% \pm 7.8\%$, $n = 10$; Figure 16C and D).

Figure 16. NME1 down-regulation promotes mature neuronal markers expression.

(A) RT-PCR results of oligodendrocyte and neuronal markers expression 7 days after NME1 down-regulation in iO cells. (B) Quantitation of markers expression of vehicle-treated and iO cells. MOG (422 bp), MBP (354 bp), PLP (687 bp), CNP (346 bp), CGT (479 bp), Tuj1 (300 bp), MAP2 (249bp), Tau (470 bp), GAPDH (269 bp). (C) Comparison of BrdU positive iO cells on day 0 and 1 after gene silencing. (D) Example of MBP⁺ cell not labelled with BrdU. MBP (red) and BrdU (green). Data are representative of three independent experiments and values are means $\pm$ S.D. * P <0.05 and ** P <0.005. Scale bars: 20 μ m (D).

4.3.2. NME1 overexpression in OLN-93 cells

In order to determine the proneural activity of NME1 in neuronal differentiation, *Nme1* was cloned into the pIRES2-EGFP vector and introduced into OLN-93

cells. As stated earlier, OLN-93 cells are derived from glial cultures therefore, they can not normally undergo neuronal differentiation. However, surprisingly, after NME1 transfection, the OLN-93 cells exhibit neuronal phenotype (compare Figure 17A and B). Western blot analysis was performed to confirm the overexpression of NME1 protein level in these induced neuronal (iN) cells (Figure 17C and D). We then determined the percentage of iN cells which exhibited bi- ($32\% \pm 1.5\%$, $n = 10$) or multi-polar ($9\% \pm 3.6\%$, $n = 10$) branches emerged from the cell body by NME1 overexpression. Moreover, we next analyzed the expression of NF-200 and NME1 in iN cells on day 10 after gene overexpression by double immunocytochemistry. Figure 17E–E''' is an example of NME1⁺-NF200⁺ iN cell. In addition, NME1 overexpression not only increases the neuronal markers expression but also improves the features of neuronal phenotype (compare Figure 17B and E with Figure 14B and C respectively).

Figure 17. NME1 overexpression induces neuronal-like morphology of OLN-93 cells.

(A) Vehicle-treated OLN-93 cells. (B) iN cells 10 days after NME1 induction. (C and D) Western blot and quantification result of OLN-93, iN and vehicle-treated cells against NME1. (E–E''') NME1-NF200 double immunofluorescence staining of iN cell. (E) NME1, (E') NF200, (E'') DAPI and (E''') merge figure. Data are representative of three independent experiments and values are means $\pm$ S.D. * $P < 0.05$ and ** $P < 0.005$. Scale bars: 10 μ m (E–E'''), 20 μ m (A) and 50 μ m (B).

We next analyzed the neuronal markers expression of iN cells on day 10 after gene overexpression. As shown in Figure 18A–C, NME1 overexpression effectively induces the up-regulation of mature neuronal markers. Interestingly, iN cells also exhibit decreased expression of MBP compared with control (Figure 18B and C). Similar to iO cells, we determined the kinetics and efficiency of iN cell generation. In iN populations, NF-200⁺ cells with immature neuron-like morphology were detected on day 3 after gene overexpression. The branching processes were further extended on day 5-7 and completed on day 10 (Figure

17E–E"). To approximate the efficiency of iN cell generation, we first treated the cells with BrdU at day 0 and 1 after gene overexpression to determine how many of iN cells divided after NME1 overexpression. The result showed that nearly half of iN cells became post mitotic one day after gene induction ($67\% \pm 2.08\%$ versus $34\% \pm 3.51\%$, $n = 10$; Figure 5D and E). To calculate the efficiency of iN cell generation total numbers of NF200 positive cells with neuronal appearances were quantified 10 days after NME1 induction. We then divided the average number of NF200 positive cells (present in 10 randomly selected $\times 20$ visual fields) by the total number of cells. With this method, the generation efficiency was ranged from 77.7 % to 86.1 % (Figure 18F).

Figure 18. NME1 amplification induces neuronal differentiation of OLN-93 cells.

NME1 overexpression not only induces the mature neuronal markers expression but also decrease the expression of oligodendrocyte marker MBP. (A) RT-PCR results of neuronal markers expression 10 days after NME1 induction in vehicle-treated and iN cells. (B) Western blot analysis of mature neuronal and oligodendrocyte markers in Vehicle and iN cells. (C) Quantification of markers expression. (D) Comparison of BrdU positive iN cells on day 0 and 1 after gene induction. (E) Example of NF200⁺ cell not labelled with BrdU. NF200 (red) and BrdU (green). (F) Efficiency estimates for iN cells generation 10 days after gene induction. Data are representative of three independent experiments and values are means $\pm$ S.D. * P <0.05 and ** P <0.005. Scale bars: 20 μ m (E).

4.4. β -catenin signaling pathway contributes to NME1-mediated activity

4.4.1. Inhibition of GSK-3 β by NME1 overexpression

Previously, it was shown that inactivation of GSK-3 β , by its phosphorylation, is involved in increased neuronal differentiation of ventral midbrain precursors *in vivo* (151), while *in vitro*, increased proliferation and enhanced neurogenesis have been found upon inhibition of GSK-3 β in neural progenitor cells (152-153). Moreover, recent study indicated that GSK-3 β is able to inhibit oligodendrocyte differentiation of oligodendrocyte precursor cells *in vivo* (154). Therefore, inhibition of GSK-3 β can play dual role, both promoting neurogenesis and inhibiting oligodendrogenesis in different cell lines. In order to gain insight into the involvement of GSK3- β in neuronal differentiation of glial precursors, we examined the effect of NME1 overexpression on GSK-3 β phosphorylation in oligodendrocyte precursor OLN-93 cells. We previously showed that NME1 overexpression induces neuronal differentiation of OLN-93 cells (155). Therefore, we used iN cells to analyze the expression and phosphorylation of GSK-3 β during neuronal differentiation. The results showed that GSK-3 β phosphorylation is significantly increased by NME1 overexpression (6.24 ± 0.4 -fold vs 1.6 ± 0.15 -fold, $n=3$; Figure 19A and B). To further confirm whether increase in NME1 induces the inhibition of GSK-3 β , immunohistochemical analysis was performed. Notably, no significant difference in GSK-3 β expression was found between vehicle-treated and iN cells (Figure 19C). However p-GSK-3 β is markedly

overexpressed in iN and not in control cells (Figure 19D), suggesting that NME1 overexpression increases the phosphorylation of glycogen synthase kinase-3 in OLN-93 cells.

Figure19. NME1 overexpression promotes the phosphorylation of GSK-3β in OLN-93 cells.

(A) Inhibition of GSK-3β by NME1 overexpression. Western blot analysis of OLN-93, Vehicle-treated and iN cells against phospho-GSK-3β, total GSK-3β (47kDa) and β-actin (42kDa). (B) Quantitative of phospho-GSK 3β/total-GSK-3β in cells. (C and D) NME1 overexpression induces the phosphorylation of phospho- GSK-3β. Immunostaining results of Vehicle-treated and iN cells against total GSK-3β and phospho- GSK-3β. Data are representative of three independent experiments and values are means ± S.D. **P*<0.05 and ***P*<0.005. Scale bars: 20 μm.

4.4.2. Inhibition of GSK-3 β by LiCl elevates neuronal differentiation of OLN-93 cells

To evaluate the effects of GSK-3 β inhibition on OLN-93 neuronal differentiation, we used lithium chloride (a potent inhibitor of GSK-3 β) which was shown to be involved in Wnt signaling pathway (156). It is well known that lithium is able to inhibit the GSK-3 β by its phosphorylation (157). Cells were therefore incubated with LiCl (25mM) for 48 hours followed by MTT cell proliferation assay to measure and compare the cell proliferation rates. The results revealed that treatment of LiCl and overexpression of NME1, decreased cell proliferation to 0.79 ± 0.06 -fold and 0.63 ± 0.02 -fold respectively (Figure 20A). To further elucidate this effect, we sought to compare the mature neuronal markers expression of iN cells 48 hours after LiCl (5, 15, 25 mM) treatment by RT-PCR. Our results showed that LiCl not only elevates neuronal markers expression in a dose-dependent manner (Figure 20B and C), but also induces neuron-like morphology in iN cells 48 hours after induction (Figure 20D). In addition, we also compared the growth capacity of iN and vehicle-treated cells 48 hours after LiCl treatment. Our cell cycle analysis revealed that LiCl induction resulted in a decrease of S-phase fraction in iN cells (Figure 20E). Furthermore, western blot (Figure 20F) and Immunocytochemistry analyses (Figure 20G) of iN and iN/LiCl cells confirmed that LiCl stimulation led to increased level of mature neuronal marker Tau. Thus, LiCl not only inhibits proliferation, but also accelerates neuronal differentiation of iN cells.

Figure20. LiCl treatment inhibits the proliferation and accelerates the neuronal differentiation of OLN-93 cells.

(A) MMT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay compares the cell proliferation of OLN-93, vehicle-treated, iN, LiCl (25mM) treated and iN/LiCl treated cells. (B and C) Dose-dependent effect of LiCl on neuronal differentiation of iN cells. (B) Cells were treated with different concentration of LiCl (5, 10, 25mM) for 48 hrs. Mature neuronal markers were analyzed by RT-PCR 48 hrs after LiCl treatment. (C) Quantitative of neuronal marker expression. (D) neuron-like morphology of iN cells 48 hrs after LiCl (25mM) treatment. (E) Cell cycle analysis of vehicle/LiCl and iN/LiCl treated cells. (F and G) Western blot analysis and

immunostaining results of iN and iN/LiCl treated cells against neuronal marker Tau. Data are representative of three independent experiments and values are means $\pm$ S.D. * P <0.05 and ** P <0.005. Scale bars: 20 μ m.

4.4.3. The role of β -catenin during neuronal differentiation of OLN-93 cells

Since GSK-3 β has been shown to phosphorylate and inactivate β -catenin (151), we next tested whether NME1 overexpression could influence the expression of β -catenin (via GSK-3 β phosphorylation) during neuronal differentiation. Interestingly, when NME1 was overexpressed in cells, the level of total β -catenin increased nearly two times compared with control cells (Figure 21A and B). To determine the role of β -catenin on neuronal differentiation, cells were transfected with either β -catenin siRNA (si- β -catenin) or control siRNA (si-Control), followed by Western blot analysis against NME1 and β -catenin. Protein lysate obtained from the iN cells after β -catenin silencing (iN/si- β -catenin cells) showed a significant decrease in total β -catenin protein compared to controls (Figure 21C and D). Next, we examined the effect of β -catenin loss on neuronal cells differentiation. Our immunochemical analysis showed that β -catenin silencing markedly abolished the neuron-like morphology of the iN cells (Figure 21E).

Figure 21. The role of β-catenin during neuronal differentiation of OLN-93 cells.

(A and B) Induction of β-catenin by NME1 overexpression. (A) Western blot analysis of OLN-93, vehicle-treated and iN cells against phospho-β-catenin and total-β-catenin (92kDa). (B) Quantitative analysis of phospho-β-catenin and total-β-catenin expression in cells. (C–E) β-catenin silencing inhibits the neuronal differentiation of iN cells. (C) Western blot analysis of vehicle-treated, iN/si-Control and iN/si-β-catenin against NME1, β-catenin and β-actin. (D) Quantitative analysis of β-catenin and NME1 expression in cells. (E) si-β-catenin transfection inhibits the neuronal characteristics of iN cells. Upper panel: iN/si-control cells; NME1 (red), β-catenin (green), DAPI (blue) and MAP2 (pink). Lower panel: iN/si-β-catenin cells; NME1 (red), β-catenin (green), DAPI (blue) and O4 (pink). Data are representative of three independent experiments and values are means ± S.D. * $P < 0.05$ and ** $P < 0.005$. Scale bars: 20 μm.

In addition, β -catenin silencing resulted in a significant down-regulation of mature neuronal marker Tau, NeuroD and TUJ1 in iN cells (0.57 ± 0.05 -fold, 0.63 ± 0.08 -fold and 0.84 ± 0.04 -fold and respectively, $n=3$; Figure 22). Interestingly, this effect was accompanied by up-regulation of neural stem cell marker Nestin (1.08 ± 0.09 -fold, $n=3$; Figure 22) and glial precursor marker NG2 (1.13 ± 0.04 -fold, $n=3$; Figure 22).

Figure 22. β -catenin silencing inhibits neuronal markers expression and induces oligodendrocyte precursor marker expression of iN cells.

β -catenin silencing not only suppresses the mature neuronal markers expression of iN cells but also increases the oligodendrocyte precursor marker expression NG2. Real-time PCR analysis of control, iN and iN/si- β -catenin cells against NME1, Tau, NeuroD, Tuj1, NG2 and neural stem cell

marker Nestin. mRNA levels of NeuroD, Tau and Tuj1 were significantly decreased after β -catenin silencing. Data are representative of three independent experiments and values are means $\pm$ S.D. * $P < 0.05$ and ** $P < 0.005$.

Similar results were also obtained using dominant-negative beta-catenin (DN- β -catenin) construct. To solidate whether loss of beta-catenin is involved in a decreased neuronal differentiation of cells, vehicle-treated and iN cells were transfected with wild-type and dominant-negative beta-catenin (WT / DN) constructs. Interestingly, inhibition of beta-catenin signaling through transfection with a dominant-negative construct abolished the neuronal morphology of iN cells largely (Figure 23A).

Moreover, we compared the expression of cell differentiation markers following the application of DN- β -catenin. We used glial precursor marker NG2 to detect oligodendrocyte precursor cells and Neuron-specific class III beta-tubulin (TUBJ1), Neuronal Nuclei (NeuN) and Neurofilament 200 (NF200) to detect neurons. DN- β -catenin transfection increased the fraction of cells expressing NG2 (48.5–80.1%; Figure 23B) and did not change NeuN expression ($P < 0.05$). Other neuronal markers such as TUBJ1 and NF-200 showed significant down-regulation (50.9–19.3% and 52.6–0.8% respectively) in iN/DN- β -catenin cells (Figure 23B), suggesting the participation of beta-catenin in neuronal differentiation of OLN-93 cells by NME1 overexpression.

Figure 23. Inhibition of β-catenin suppresses the characteristics of iN cells.

(A) Fluorescence microscopy results indicated that dominant negative β-catenin transfection could abolish the iN cell phenotype. (B) In the FACS-like scattergrams, the Tuj1 and NF200 fluorescence intensity are depicted on the x-axis, and the NG2 and NeuN fluorescence intensity on the y-axis. Detection thresholds are indicated by black lines. Single cell fluorescent signals result in single dots. After β-catenin inhibition, the fraction of Tuj1 and NF200-positive cells were significantly decreased (50.9-19.3% and 52.6-0.8% respectively) while NeuN marker did not affected. Scale bars: 20μm (vehicle) and 50 μm (iN cells).

4.4.4. Involvement of TCF/LEF transcriptional factor in NME1-mediated activity

Previously, it was shown that beta-catenin is able to interact with TCF/LEF family transcription factors to promote transcription of Wnt-responsive genes involved in cell proliferation and differentiation (158-160). Since the role of beta-catenin in neuronal differentiation is documented, we performed further experiments to determine whether NME1 overexpression is able to alter the TCF/LEF activation. As shown in Figure 24A, overexpression of NME1 significantly increased the TCF/LEF reporter activity (1.7 ± 0.16 -fold, $n=3$; $*p < 0.05$, $**p < 0.005$). We then carried out further experiments whether inhibition of TCF4 activity contributes to the reduction of mature neuronal markers expression. We found that transfection of the dominant-negative TCF4 (DN-TCF4) plasmid markedly decreased the expression TUJ1, NF200 and Synapsin (0.61 ± 0.04 , 0.44 ± 0.02 and 0.73 ± 0.09 -fold respectively, $n=3$, Figure 24B and C), suggesting the role of TCF/LEF activity in NME1-mediated neuronal activity. Thus NME1 could promote neuronal differentiation of oligodendrocyte precursor cells by modulating TCF/LEF transcriptional activity.

Figure 24. TCF/LEF transcription factor is involved in MNE1-mediated activity.

(A) NME1 overexpression increases TCF/LEF reporter activity. The OLN-93 cells were transfected with *nme1*-pIRES2-EGFP Plasmid. After transfection, cells were cultured in serum-free medium for 24 hours and then the cell lysates were obtained to determine the luciferase activity as described in the Materials and Methods Section. (B) Dominant-negative TCF4 transfection reduces the expression of mature neuronal markers expression. (C) Quantification of markers expression. Data are representative of three independent experiments and values are means $\pm$ S.D. * P <0.05 and ** P <0.005.

4.4.5. Wnt molecules and their receptors are not involved in NME1-mediated activity

Because NME1 induction was found to elevate the phosphorylation of GSK-3 β and increase the total amount of beta-catenin, we then set to determine the contribution of Wnt molecules and their receptors (up-stream targets of GSK-3 β)

during NME1 overexpression in OLN-93 cells. Since activation of Wnt molecules and their receptors was shown to regulate different aspects of neuronal differentiation during development and *in vitro* (40, 161-165), we therefore examined the mRNA expression levels of Wnt3A, Wnt5A, LRP5, LRP6, Frizzled7 and Frizzled8 during NME1 overexpression by real-time PCR. As shown in Figure 25 NME1 overexpression does not affect the expression of Wnt molecules and their receptors, suggesting that they are not involved in NME1-mediated activity.

Figure 25. NME1 overexpression dose not affect the expression of Wnt molecules and their receptors.

Real-time PCR analysis of OLN-93, vehicle-treated and iN cells against Wnt3A, Wnt5A, LRP5, LRP6, Frizzled7 and Frizzled8. mRNA levels of Wnt molecules and their receptors showed no changes after NME1 overexpression. Data are representative of three independent experiments and values are means $\pm$ S.D.

4.4.6. NME1 promotes neuronal differentiation of OLN-93 cells via interaction with GSK-3 β

Although our results indicated the role of beta-catenin signaling during neuronal differentiation by NME1 overexpression, they raised the question of how NME1 regulates this pathway. Because NME1 was demonstrated to regulate GSK-3 β activity in our study, we wondered whether it could bind to GSK-3 β directly. Interestingly, our co-immunoprecipitation experiments show that NME1 is capable of interacting with beta-catenin in oligodendrocyte precursor OLN-93 cells (Figure 26A). To verify these results, immunocytochemical analysis was performed in iN cells and as expected, NME1–GSK-3 β interaction was confirmed by their co-localization (Figure 26B). These findings clearly demonstrated that NME1 could cooperate with GSK-3 β through its role during neuronal differentiation of OLN-93 cells.

Figure 26. NME1 is able to interact with GSK-3β.

(A) Cell lysates were immunoprecipitated (IP) with anti-NME1 antibody or unrelated isotype IgG and analyzed for the presence of NME1 and GSK-3β by Western blot (IB). (B) Cell lysates were immunoprecipitated with anti-GSK-3β antibody or unrelated isotype IgG and analyzed for the presence of NME1 and GSK-3β by Western blot. (C) Immunocytochemistry results showed the expression (merge, yellow) of NME1 (red) with GSK-3β (green) in the same cells. Data are representative of three independent experiments. Scale bars: 50 μm.

CHAPTER 5: DISCUSSION

As stated earlier, the adult glial precursor cells were recently shown to be able to produce neurons in central nervous system (CNS) and to obtain multipotential ability *in vitro*. These findings suggest that glial precursors are not irreversibly committed into one distinct fate and still can be converted to other lineages. This is of great significance for neuronal replacement during various neurological disorders by reprogramming of glial progenitors into neuronal differentiation. Although the fate determination of glial precursors was studied extensively, the signals and factors, which could redirect their fate to become neurons, still remained unknown. In the present study, we provide evidence suggesting the role of NME1 in glial and neuronal cell fate determination *in vitro*. We showed that NME1 is widely expressed in neuronal cells throughout adult mouse CNS. Interestingly, NME1 overexpression in oligodendrocyte precursor OLN-93 cells potently induced the acquisition of neuronal phenotype, while its silencing was shown to promote oligodendrocyte differentiation. Here we further demonstrate that NME1 overexpression promotes neuronal differentiation of oligodendrocyte precursor OLN-93 cells through β -catenin signaling pathway. Since NME1 overexpression does not affect the expression of Wnt molecules and their receptors, our co-immunoprecipitation experiments reveal that NME1 is able to interact with GSK-3 β directly. Therefore, our findings indicate a new regulatory role of NME1 in driving glial precursor cells to neurons through β -catenin signaling pathway.

During central nervous system development, fine regulation of pathways and mechanisms are necessary to generate mature neurons, oligodendrocytes and astrocytes from stem/precursor cell populations. Although previous studies have indicated the importance of some distinct pathways in driving stem/precursor cells into neuronal or glial cell fate determination, they were unable to explain how cell fate decision can be regulated by distinct signals or how a gene can control glial versus neuronal cell fate choice. Here, we discovered the regulatory role of NME1 in driving oligodendrocyte precursor OLN-93 cells to neuronal cell fate decision.

Previous works have shown that glial precursor cells can be an interesting candidate for study of neuronal differentiation and reprogramming. For example, the adult committed glial progenitor pools were shown to be able to generate neurons in certain areas of the brain (166) and acquire neuronal phenotypes *in vitro* by various strategies (167). Moreover, oligodendrocyte precursor cells are reprogrammed into multipotent neuronal stem cells after sequential exposure to Fetal Calf Serum (FCS) and basic Fibroblast Growth factor (bFGF) (54). These findings suggest that glial precursors are not irreversibly committed into one distinct fate and still can be converted to other lineages. This is of great significance for neuronal replacement during various neurological disorders by reprogramming of glial progenitors into neuronal differentiation.

In the present study, we characterized the role of NME1 in driving neuronal fate specification from oligodendrocyte precursor OLN-93 cells. We found that NME1 overexpression induces neuronal differentiation of glial precursors while, its down-regulation leads to an increased number of oligodendrocytes. The proneural effects of NME1 were further shown to be dependent on the activities of β -catenin pathway.

5.1. The role of nucleoside diphosphate kinase in glial and neuronal cell fate determination

NME1 belongs to the non-metastatic cell 23 gene family which has been first identified in some tumors with the effect of metastasis suppression (168-170). It has been shown that all members of Nm23 family are highly expressed in the human brain as well as other tissues (171, 172), but their functions in nervous system are still obscure. Previous studies have investigated the expression and distribution pattern of Nm23 family in *xenopus* (173) and *zebrafish* (174) during early development. However, all members of this family are expressed in vertebrate central nervous system and some members display particular properties generally linked to developmental or to differentiation processes. For example, It has been postulated that ectopic expression of NDP kinase DR-Nm23 is required for neuronal and schwannocytic differentiation via regulation of integrins expression (112, 175). In the presence of nerve growth factor, overexpression of nm23 delays cell cycle transition, rapidly promotes neurite

outgrowth, and significantly elevates the expression of neuronal markers in PC12 cells (176). In addition, the presence of a serine residue in position 61 in the catalytic domain seems essential for the ability of DR-Nm23 to trigger differentiation and for protein-protein interaction (175).

Here, our western blot analysis confirmed the abundance of NME1 protein in brain (Figure 8). Furthermore, our immunohistochemical experiments provided evidence for a wide expression of NME1 throughout the adult central nervous system (Figure 9 and 10). In the present study we demonstrated that NME1 protein levels vary from one region to another. Strong expression of NME1 has been observed in the brain while in spinal cord, the expression of protein was much lower (Figure 8). We found that NME1 particularly expressed in neurons in grey matters and in myelinated axons throughout the white matter of the brain and spinal cord (Figure 11). These results suggest the involvement of NME1 in neuronal development and function through adult central nervous system. Additionally, our *in vitro* experiments confirmed the potential role of NME1 in neuronal differentiation of neuroblastoma cell line (Figure 14) and its proneural effect was further demonstrated by driving oligodendrocyte precursors to neuronal fate (figure 17 and 18).

5.1.1. The role of NME1 in inhibition of gliogenesis and stimulation of neuronal differentiation

Because of the complex regulatory systems, our knowledge about glial and neuronal cell fate determination is largely limited. However, it has been postulated that determination of one's fate results in the suppression of alternative fate (177-179). As stated earlier, glial versus neuronal cell fate determination is mutually exclusive process in which initiation of proneural machinery is accompanied by suppression of gliogenesis and vice versa. For example, in the central nervous system injury, inhibition of gliogenesis was shown to promote neuronal production in mouse brain and in *zebrafish* (180-182). Moreover, the proneural basic helix-loop-helix (bHLH) transcription factor neurogenin (Ngn1) inhibits the glial differentiation of neural stem cells via independent mechanisms while it can promote neurogenesis (178, 183). It has been previously shown that Dlx 1 and 2 transcriptional factors repress the oligodendrocyte precursor cell formation and they are therefore necessary for GABAergic interneuron production (177). The transcription factor, Pax6 was also found to regulate neuronal differentiation from glial cells during central nervous development (184-185).

Similarly, like most of the proneural genes, NME1 was shown to suppress oligodendrocyte differentiation. This finding was supported by acquiring oligodendrocyte phenotype of OPCs by NME1 silencing (Figure 15 and 16). In contrast, NME1 overexpression leads to an increased number of neurons, up-regulates the mature neuronal markers expression and decreases the expression of

oligodendrocyte marker myelin basic protein (Figure 17 and 18). Therefore, our data granted the strong proneural activity of NME1 in regulating glial and neuronal cell fate switch *in vitro*.

Although little is yet known about their function in central nervous system, it is proposed that NM23 proteins play crucial roles in glial and neuronal cell fate determination. A NDP kinase named NM23-X4, a family member of NM23 proteins, could delay or inhibit gliogenesis through interaction with a distinct shRNA named p27Xic1 during retinogenesis of *Xenopus*. This shRNA was shown to promote neurogenesis of retinal progenitor cells (122). The same fundamental role in cell fate determination of the NM23 members was also reported in other systems such as bone marrow cells (123) and hematopoietic cells (124). Here, for the first time, we demonstrated that NME1 behaves like a bHLH factor to regulate glial and neuronal cell fate decision in OLN-93 cells. However, it is still not clear whether *Nme1* shares the similar functional role with bHLH genes and therefore the relation between neurogenesis and NME1 needs to be more studied. Our findings shed light on the neuronal replacement therapy for various nerve diseases where the local glial precursors could serve as an alternative cell source for neurons.

5.2. NME1 promotes neuronal differentiation of oligodendrocyte precursor OLN-93 cells through modulating β -catenin signaling pathway

As stated earlier, the diversity of cell types (neurons, oligodendrocytes, astrocytes) which are present in the adult central nervous system, is produced by a series of precursor cells during development. These precursor cells can give rise to lineage restricted populations with a more restricted glial or neuronal progeny subtypes (Figure 1) (18, 19). Most glial cells, including astrocytes and oligodendrocytes, derive from glial lineage restricted populations; whereas neurons are raised from neuronal precursor cells (Figure 1). Although the mechanisms of neurogenesis and gliogenesis are studied well during development, neuronal differentiation of glial precursor cells is still largely unknown. However, recent studies have substantially expanded this conception that glial precursor cells can be another source to generate neurons both *in vitro* and *in vivo* (54, 55, 155). Following these noteworthy findings, in this study we demonstrated that nucleoside diphosphate kinase NME1 promotes the neuronal differentiation of oligodendrocyte precursor OLN-93 cells by activation of β -catenin signaling pathway. Furthermore, we found that NME1 is able to interact with GSK-3 β in order to induce neuronal differentiation. These findings may enrich our understanding of the role of NME1-mediated pathways in glial and neuronal cell fate determination.

5.2.1. Inhibition of GSK-3 β by NME1 overexpression

It has been well studied that glycogen synthase kinase 3 is implicated in multiple biological processes including cell proliferation, cell survival, metabolism, development and cell fate determination (186-188). In mammalian cells and in the presence of certain factors, GSK-3 β can rapidly phosphorylated at serine 9, resulting in inhibition of its kinase activity (189-190). Inactivation of GSK-3 β was shown in the regulation of cell fate decision in *Xenopus*, *Dictyosteliu* and *Drosophila* (188, 191, 192). Recent studies revealed that inhibition of GSK-3 β is also able to induce neurogenesis from different precursor subtypes (151, 193, 194). We previously showed that NME1 is involved in the regulation of glial and neuronal cell fate decision (Figure 15–18) (155). Here, we further reported that GSK-3 β phosphorylation is markedly increased by NME1 overexpression (Figure 19). Because lithium has been well documented to phosphorylate and inactivate the glycogen synthase kinase 3 β (195-198), we therefore examine whether lithium is involved in the decreased proliferation and increased neuronal differentiation of iN cells. Our results clearly showed that treatment of LiCl on iN cells could markedly decrease the cell proliferation rate (Figure 20A), indicating the synergistic antiproliferation effect of LiCl and NME1 in oligodendrocyte precursor cells. Lithium was also shown to enhance the neuronal differentiation of neuronal progenies both *in vitro* and *in vivo* (199, 200). In the current work we further reported that LiCl strongly induces neuronal differentiation of iN cells (Figure 20B–G), suggesting the synergic effect of NME1 and LiCl to induce neuronal differentiation of glial precursor cells.

5.2.2. The expression and the role of β -catenin during neuronal differentiation of OLN-93 cells by NME1 overexpression

Upon activation of Wnt signaling pathway, β -catenin phosphorylation by GSK-3 β will be inhibited and therefore β -catenin protein will be stabilized. As β -catenin signaling pathway was shown to participate in multiple biological functions such as cell proliferation, differentiation and cell fate determination, we examined the expression and the role of β -catenin during neuronal differentiation by NME1 overexpression. Notably, our experiments revealed that β -catenin expression is significantly increased by NME1 overexpression (Figure 21 A and B). More interestingly, we found that β -catenin silencing not only abolished the neuron-like morphology of the iN cells but also down-regulated the expression of mature neuronal markers (Figure 21 C–E and Figure 22). This effect could also be supported by transfection of dominant-negative beta-catenin construction on iN cells (Figure 23). Taken together, our results showed that beta-catenin is increased during neuronal differentiation of OLN-93 cells by NME1 overexpression.

5.2.3. Elevation of TCF/LEF transcription activity by NME1 overexpression

Since the unphosphorylated and stabilized β -catenin enhanced the neuronal differentiation of oligodendrocyte precursor cells, there might be a relation between the β -catenin and specific transcriptional factors. As demonstrated by previous studies, the activity of TCF/LEF transcription factors was affected by β -

catenin protein during neuronal proliferation and differentiation (153, 201). TCF/LEF transcription factors also known as an essential component in regulating cell proliferation and differentiation in different precursor subtypes (153, 202, 203).

In the present study, we show that TCF/LEF transcription activity is significantly elevated during NME1 overexpression (Figure 24 A). The importance of TCF4 activity in neuronal differentiation of OLN-93 cells was also supported by comparison of neuronal markers expression in iN and iN/DN-TCF4 cells (Figure 24B and C). These findings strongly suggested that β -catenin-mediated signaling pathway is employed by NME1 overexpression during neuronal differentiation of oligodendrocyte precursor cells.

5.2.4. NME1 is able to interact with glycogen synthase kinase 3 β

Because β -catenin pathway was shown to be employed by NME-1 during neuronal differentiation of oligodendrocyte precursor cells, this motivates us to investigate whether NME1 is able to interact with any component of Wnt/ β -catenin signaling pathway. Once Wnt molecules bind to their receptors, cell surface Frizzled proteins (FRZs) and low-density lipoprotein receptor-related proteins (LRPs), the inhibitory effect of glycogen synthase kinase 3 β will be removed, and β -catenin protein subsequently will translocate into nucleus where it can activate the transcription factors (204). Here we found that Wnt molecules

are not affected by NME1 overexpression in OLN-93 cells (Figure 25). Not surprisingly, we also found no change in mRNA levels of LRP5/6 and FRZ7/8 (Figure 25), indicating that Wnt and their receptors are not involved in NME1 mediated activity. Notably, the inactivation of GSK-3 β protein was shown to be regulated by its phosphorylation at Ser-9 or through its interaction with other protein factors (204-206). However, in the present study we found that phosphorylation of GSK-3 β at Ser-9 is important for its inactivation in iN cells. Based on this finding we next investigated the possible interaction between GSK-3 β and NME1 in oligodendrocyte precursor OLN-93 cells. Interestingly, our results indicated that NME1 is able to interact with GSK-3 β through its role during neuronal differentiation (Figure 26A). Our immunocytochemistry results further verified the co-localization of NME1 and GSK-3 β in OLN-93 cells (Figure 26B). Thus, we proposed a novel model which NME1 could enhance the neuronal differentiation of oligodendrocyte precursor cells via β -catenin signaling pathway and through interaction with GSK-3 β .

CHAPTER 6: CONCLUSION

As stated earlier, although most oligodendrocyte precursor cells differentiate into oligodendrocytes by default, it is known that fetal calf serum and specific cytokines treatment induce them to differentiate into astrocytes (54). Moreover, oligodendrocyte precursor cells cultured sequentially in basic fibroblast growth factor, platelet-derived growth factor and fetal calf serum produced floating neurosphere-like bodies like those produced by central nervous system stem cells under similar condition (54). Furthermore experiments revealed that such OPCs could give rise to neuronal cells (54). A similar scenario was also shown by our finding that overexpression of NME1 could derive the oligodendrocyte precursor OLN-93 cells in culture to become neurons (155). Although it has been shown that NME1 could influence the proliferation and neuronal differentiation of other cell lines such as PC12 and PC12D cells (176, 207, 208) the molecular mechanism underlying NME1-mediated activity is still largely unknown.

In this study, our results suggest that β -catenin pathway is involved in glial and neuronal cell fate decision of oligodendrocyte precursor OLN-93 cells. Moreover, we further demonstrated that activation of β -catenin signaling pathway by NME1 overexpression leads to the differentiation of oligodendrocyte precursor cells into neuronal fate *in vitro*. However, there is no direct evidence supporting the role and function of NME1 during glial and neuronal cell fate decision *in vivo*. Gene

deletion studies have shown that NME1 knock-out mice displayed a manifest delayed in mammary gland development and high rate of neonatal mortality (209, 210). Double knockout NME1 (-/-) and NME2 (-/-) mice model also suggests a critical role for NM23 family in erythroid development (211). However, none of the above studies identified the role of NME1 in glial or neuronal development through central nervous system.

In the present study, we revealed the molecular basis of NME1-mediated activity *in vitro*. Here, we demonstrated that overexpression of NME1 could markedly enhance the phosphorylation of glycogen synthase kinase 3 β in Oligodendrocyte precursor OLN-93 cells through its direct interaction with GSK-3 β . This results in stabilization of β -catenin protein and stabilized β -catenin subsequently induces the activity of TCF/LEF transcription factor.

Taken together, our study suggests a novel regulatory role of NME1, through β -catenin signaling pathway, to induce the neuronal differentiation from oligodendrocyte precursor cells in culture.

In conclusion, our study suggested a new role of NME1 in neural differentiation of glial precursor cells and this work could represent a method for studying cell reprogramming, cellular plasticity and neuronal regenerative treatment for neurological diseases.

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CHAPTER 8: APPENDICES

8.1. List of oligonucleotides used

8.1.1. Primers used for NME1 overexpression in OLN-93 cells

Primer name	Sequence	Description
Nme1 F	5'CGGAATTCACCATGGCCAACAGCGAGCGTACCTT3'	Forward primer for PCR of NME1
Nme1 R	5'CGGGATCCTCACTCATAGATCCAGTTCTG3'	Reverse primer for PCR of NME1

8.1.2. Primers used for NME1 silencing in OLN-93 cells

Forward and reverse 63- and 55-nucleotide fragments were ligated to pSilencer™

3.1 H1 neo and pSilencer™ 4.1-CMV siRNA expression vectors (Ambion).

Primer name	Sequence	Description
NSP4 F	5' GATCC GTTGGCAGGAACATCATTCTTCAAG AGAGAATGATGTTCTGCCAACTTA 3'	Forward primer for NME1 silencing (pSilencer™ 4.1-CMV)
NSP4 R	5' AGCTT AAGTTGGCAGGAACATCATTCTCT CTTGAAGAATGATGTTCTGCCAACG 3'	Reverse primer for NME1 silencing (pSilencer™ 4.1-CMV)
NSP3 R	5' GATCCATTTATACAGGCTTCAGAG TTCAAGAGACTCTGAAGCCTGTATAAATTTTTTGGAAA 3'	Forward primer for NME1 silencing (pSilencer™ 3.1 H1 puro)
NSP3 R	5' AGCTTTTCCAAAAAATTTATACAGGCTTCAGAG TCTCTTGAACCTCTGAAGCCTGTATAAATG 3'	Reverse primer for NME1 silencing (pSilencer™ 3.1 H1 puro)

8.2. List and sequence of c-DNA used

8.2.1. NME1 c-DNA sequence

ATGGCCAAC AGCGAGCGTA CCTTCATCGC CATCAAGCCT GATGGGGTCC AGCGGGGGCT
TGTGGGAGAG ATCATCAAGC GATTTCGAGCA GAAGGGATTG CGCCTGGTTG GTTTGAAATT
TATACAGGCT TCAGAGGATC TTCTCAAGGA GCACTACATT GACCTGAAGG ACCGCCCCCTT
CTTTTCTGGC CTGGTGAAGT ACATGCACTC AGGACCGGTG GTTGCTATGG TCTGGGAGGG
ACTGAATGTT GTGAAGACAG GCCGGGTGAT GCTTGGAGAG ACCAACCCCG CAGACTCTAA
GCCTGGGACC ATACGAGGAG ACTTTTGCAT TCAAGTTGGC AGGAACATCA TTCATGGCAG
CGATTCTGTG GAGAGTGCGG AGAAGGAGAT CAGTTTGTGG TTTCAGCCTG AGGAGCTGGT
GGACTACAAG AGCTGTGCGC AGAACTGGAT CTATGAGTGA

8.3. List and sequence of plasmids used

8.3.1. Plasmid used for NME1 overexpresion

8.3.1.1. pIRES2-EGFP sequence

1 TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT AGCCCATATA TGGAGTTCCG
61 CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC CCCGCCCAT
121 GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA GGGACTTTCC ATTGACGTCA
181 ATGGGTGGAG TATTTACGGT AAACGCCCCA CTTGGCAGTA CATCAAGTGT ATCATATGCC
241 AAGTACGCCC CCTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT ATGCCCAGTA
301 CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA TCGCTATTAC
361 CATGGTGATG CGGTTTTGGC AGTACATCAA TGGGCGTGGA TAGCGGTTTG ACTCACGGGG
421 ATTTCCAAGT CTCCACCCCA TTGACGTCAA TGGGAGTTTG TTTTGGCACC AAAATCAACG
481 GGACTTTCCA AAATGTCGTA ACAACTCCGC CCCATTGACG CAAATGGGCG GTAGGCGTGT
541 ACGGTGGGAG GTCTATATAA GCAGAGCTGG TTTAGTGAAC CGTCAGATCC GCTAGCGCTA
601 CCGGACTCAG ATCTCGAGCT CAAGCTCGA ATTCTGCAGT CGACGGTACC GCGGGCCCCG
661 GGAACAAAAA CTCATCTCAG AAGAGGATCT GTGAGGATCC GCCCCCTCTCC CTCCCCCCCC
721 CCTAACGTTA CTGGCCGAAG CCGCTTGGA TAAGGCCGGT GTGCGTTTGT CTATATGTTA
781 TTTTCCACCA TATTGCCGTC TTTTGGCAAT GTGAGGGCCC GGAAACCTGG CCCTGTCTTC
841 TTGACGAGCA TTCCTAGGGG TCTTTCCCTC CTCGCCAAAG GAATGCAAGG TCTGTTGAAT
901 GTCGTGAAGG AAGCAGTTCC TCTGGAAGCT TCTTGAAGAC AAACAACGTC TGTAGCGACC
961 CTTTGACAGG AGCGGAACCC CCCACCTGGC GACAGGTGCC TCTGCGGCCA AAAGCCACGT
1021 GTATAAGATA CACCTGCAAA GGCGGCACAA CCCCAGTGCC ACGTTGTGAG TTGGATAGTT
1081 GTGGAAAGAG TCAAATGGCT CTCCTCAAGC GTATTCAACA AGGGGCTGAA GGATGCCCCA
1141 AAGGTACCCC ATTGTATGGG ATCTGTCTG GGGCCTCGGT GCACATGCTT TACATGTGTT
1201 TAGTCGAGGT TAAAAAACG TCTAGGCCCC CCGAACCACG GGGACGTGGT TTTCTTTTGA
1261 AAAACACGAT GATAATATGG CCACAACCAT GGTGAGCAAG GGCGAGGAGC TGTTCACCGG
1321 GGTGGTGCCC ATCCTGGTCG AGCTGGACGG CGACGTAAAC GGCCACAAGT TCAGCGTGTC


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1381 CGGCGAGGGC GAGGGCGATG CCACCTACGG CAAGCTGACC CTGAAGTTCA TCTGCACCAC
1441 CGGCAAGCTG CCCGTGCCCT GGCCCCACCT CGTGACCACC CTGACCTACG GCGTGCACTG
1501 CTTCAGCCGC TACCCCGACC ACATGAAGCA GCACGACTTC TTCAAGTCCG CCATGCCCGA
1561 AGGCTACGTC CAGGAGCGCA CCATCTTCTT CAAGGACGAC GGCAACTACA AGACCCGCGC
1621 CGAGGTGAAG TTCGAGGGCG ACACCCTGGT GAACCGCATC GAGCTGAAGG GCATCGACTT
1681 CAAGGAGGAC GGCAACATCC TGGGGCACAA GCTGGAGTAC AACTACAACA GCCACAACGT
1741 CTATATCATG GCCGACAAGC AGAAGAACGG CATCAAGGTG AACTTCAAGA TCCGCCACAA
1801 CATCGAGGAC GGCAGCGTGC AGCTCGCCGA CCACTACCAG CAGAACACCC CCATCGGCGA
1861 CGGCCCCGTG CTGCTGCCCG ACAACCACTA CCTGAGCACC CAGTCCGCCC TGAGCAAAGA
1921 CCCCACGAG AAGCGCGATC ACATGGTCCT GCTGGAGTTC GTGACCGCCG CCGGGATCAC
1981 TCTCGGCATG GACGAGCTGT ACAAGTAAAG CGGCCGCGAC TCTAGATCAT AATCAGCAT
2041 ACCACATTTG TAGAGGTTTT ACTTGCTTTA AAAAACCTCC CACACCTCCC CCTGAACCTG
2101 AAACATAAAA TGAATGCAAT TGTTGTGTGTT AACTGTGTTA TGCAGCTTA TAATGGTTAC
2161 AAATAAAGCA ATAGCATCAC AAATTCACA AATAAAGCAT TTTTTCACAT GCATTCTAGT
2221 TGTGGTTTGT CCAAATCAT CAATGTATCT TAAGGCGTAA ATTGTAAGCG TTAATATTTT
2281 GTTAAAAATC GCGTTAAATT TTTGTTAAAT CAGCTCATTT TTTAACCAAT AGGCCGAAT
2341 CGGCAAAATC CTTTATAAAT CAAAAGAATA GACCGAGATA GGGTTGAGTG TTGTTCCAGT
2401 TTGGAACAAG AGTCCACTAT TAAAGAACGT GGACTCCAAC GTCAAAGGGC GAAAAACCGT
2461 CTATCAGGGC GATGGCCAC TACGTGAACC ATACCCTAA TCAAGTTTTT TGGGGTCGAG
2521 GTGCCGTAAA GCACTAAATC GGAACCCATA AGGGAGCCCC CGATTTAGAG CTTGACGGGG
2581 AAAGCCGGCG AACGTGGCGA GAAAGGAAGG GAAGAAAGCG AAAGGAGCGG GCGCTAGGGC
2641 GCTGGCAAGT GTAGCGGTCA CGCTGCGCGT AACCACCACA CCCGCCGCGC TTAATGCGCC
2701 GCTACAGGGC GCGTCAGGTG GCACTTTTCG GGGAAATGTG CGCGGAACCC CTATTTGTTT
2761 ATTTTCTAA ATACATTCAA ATATGTATCC GCTCATGAGA CAATAACCCCT GATAAATGCT
2821 TCAATAATAT TGAAAAAGGA AGAGTCTGA GCGGAAAGA ACCAGCTGTG GAATGTGTGT
2881 CAGTTAGGGT GTGGAAGTC CCCAGGCTCC CCAGCAGGCA GAAGTATGCA AAGCATGCAT
2941 CTCAATTAGT CAGCAACCAG GTGTGGAAG TCCCCAGGCT CCCCAGCAGG CAGAAGTATG
3001 CAAAGCATGC ATCTCAATTA GTCAGCAACC ATAGTCCCGC CCCTAACTCC GCCCATCCCG
3061 CCCCTAACTC CGCCCAGTTC CGCCCATTCT CCGCCCCATG GCTGACTAAT TTTTTTTATT
3121 TATGCAGAGG CCGAGGCCGC CTCGGCCTCT GAGCTATTCC AGAAGTAGTG AGGAGGCTTT
3181 TTTGGAGGCC TAGGCTTTTG CAAAGATCGA TCAAGAGACA GGATGAGGAT CGTTTCGCAT
3241 GATTGAACAA GATGGATTGC ACGCAGGTTT TCCGGCCGCT TGGGTGGAGA GGCTATTCGG
3301 CTATGACTGG GCACAACAGA CAATCGGCTG CTCTGATGCC GCCGTGTTC GGCTGTACG
3361 GCAGGGGCGC CCGGTTCTTT TTGTCAAGAC CGACCTGTCC GGTGCCCTGA ATGAAGTGA
3421 AGACGAGGCA GCGCGGCTAT CGTGGCTGGC CACGACGGGC GTTCCTTGCG CAGCTGTGCT
3481 CGACGTTGTC ACTGAAGCGG GAAGGGACTG GCTGCTATTG GGCGAAGTGC CGGGGCAGGA
3541 TCTCCTGTCA TCTCACCTTG CTCCTGCCGA GAAAGTATCC ATCATGGCTG ATGCAATGCG
3601 GCGGCTGCAT ACGCTTGATC CGGCTACCTG CCCATTCGAC CACCAAGCGA AACATCGCAT
3661 CGAGCGAGCA CGTACTCGGA TGGAAGCCGG TCTTGTCGAT CAGGATGATC TGGACGAAGA
3721 GCATCAGGGG CTCGCGCCAG CCGAAGTGTG CGCCAGGCTC AAGGCAGACA TGCCCGACGG
3781 CGAGGATCTC GTCGTGACCC ATGGCGATGC CTGCTTGCCG AATATCATGG TGGAAAATGG
3841 CCGCTTTTCT GGATTCATCG ACTGTGCGCG GCTGGGTGTG GCGGACCGCT ATCAGGACAT
3901 AGCGTTGGCT ACCCGTGATA TTGCTGAAGA GCTTGGCGGC GAATGGGCTG ACCGCTTCCT
3961 CGTGCTTTAC GGTATCGCCG CTCCCATTTC GCAGCGCATC GCCTTCTATC GCCTTCTTGA
4021 CGAGTTCTTC TGAGCGGGAC TCTGGGGTTC GAAATGACCG ACCAAGCGAC GCCCACTGT
4081 CCATCACGAG ATTTTCGATC CACCGCCGCC TTCTATGAAA GGTTGGGCTT CGGAATCGTT
4141 TTCCGGGACG CCGGCTGGAT GATCCTCCAG CGCGGGGATC TCATGCTGGA GTTCTTCGCC
4201 CACCCTAGGG GGAGGCTAAC TGAAACACGG AAGGAGACAA TACCGGAAGG AACC CGCGCT
4261 ATGACGGCAA TAAAAAGACA GAATAAAACG CACGGTGTTG GGTCGTTTGT TCATAAACGC
4321 GGGGTTCCGT CCCAGGGCTG GCACTCTGTG GATACCCAC CGAGACCCCA TTGGGGCCAA
4381 TACGCCCGCG TTTCTTCCTT TTCCCACCC CACCCCCCAA GTTCGGGTGA AGGCCAGGG
4441 CTCGCAGCCA ACGTCGGGGC GGCAGGCCCT GCCATAGCCT CAGGTTACTC ATATATACTT
4501 TAGATTGATT TAAAACTTCA TTTTAAATTT AAAAGGATCT AGGTGAAGAT CCTTTTGTAT
4561 AATCTCATGA CCAAAATCCC TTAACGTGAG TTTTCGTTCC ACTGAGCGTC AGACCCGTA
4621 GAAAAGATCA AAGGATCTTC TTGAGATCCT TTTTCTCTGC GCGTAATCTG CTGCTTGCAA
4681 ACAAAAAAAC CACCGCTACC AGCGGTGGTT TGTTTGCCGG ATCAAGAGCT ACCAAGTCTT
4741 TTTCCGAAGG TAACTGGCTT CAGCAGAGCG CAGATACCAA ATACTGTCTT TCTAGTGTAG
4801 CCGTAGTTAG GCCACCACTT CAAGAAGTCT GTAGCACCGC CTACATACCT CGCTCTGCTA
4861 ATCCTGTTAC CAGTGGCTGC TGCCAGTGGC GATAAGTCGT GTCTTACCGG GTTGGACTCA
4921 AGACGATAGT TACCGGATAA GGCGCAGCGG TCGGGCTGAA CGGGGGGTTT GTGCACACAG
4981 CCCAGCTTGG AGCGAACGAC CTACACCGAA CTGAGATACC TACAGCGTGA GCTATGAGAA

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5041 AGCGCCACGC TTCCCGAAGG GAGAAAGGCG GACAGGTATC CGGTAAGCGG CAGGGTCGGA
5101 ACAGGAGAGC GCACGAGGGA GCTTCCAGGG GGAAACGCCT GGTATCTTTA TAGTCTGTGTC
5161 GGGTTTCGCC ACCTCTGACT TGAGCGTCGA TTTTGTGTGAT GCTCGTCAGG GGGCGGAGC
5221 CTATGGAAAA ACGCCAGCAA CGCGGCCTTT TTACGGTTCC TGGCCTTTTG CTGGCCTTTT
5281 GCTCACATGT TCTTTCCTGC GTTATCCCTT GATTCTGTGG ATAACCGTAT TACCGCCATG
5341 CAT

8.3.2. List and sequence of plasmids used for NME1 silencing

8.3.2.1. pSilencer™ 3.1-H1 Puro

1 TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG GAGACGGTCA
61 CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG TCAGGGCGCG TCAGCGGGTG
121 TTGGCGGGTG TCGGGGCTGG CTTAACTATG CGGCATCAGA GCAGATTGTA CTGAGAGTGC
181 ACCATATGCG GTGTGAAATA CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGGCGCC
241 ATTCCGCATT CAGGCTGCGC AACTGTTGGG AAGGGCGATC GGTGCGGGCC TCTTCGCTAT
301 TACGCCAGCT GGCGAAAAGG GGTGTGCTG CAAGGCGATT AAGTTGGGTA ACGCCAGGGT
361 TTTCCCAGTC ACGACGTTGT AAAACGACGG CCAGTGAATT CATATTTGCA TGTCGCTATG
421 TGTTCTGGGA AATCACCATA AACGTGAAAT GTCTTTGGAT TTGGGAATCT TATAAGTTCT
481 GTATGAGACC ACTCGGATCC ACTACCGTTG TTATAGGTGT TCAAGAGACA CCTATAACAA
541 CGGTAGTTTT TTGGAAGAGC TTGGCGTAAT CATGGTCATA GCTGTTTCCT GTGTGAAATT
601 GTTATCCGCT CACAATTCCA CACAACATAC GAGCCGGAAG CATAAAGTGT AAAGCCTGGG
661 GTGCCTAATG AGTGAGCTAA CTCACATTAA TTGCGTTGCG CTCACTGCCC GCTTTCAGT
721 CGGGAACCTT GTCGTGCCAG CTCGATTAAT GAATCGGCCA ACGCGCGGGG AGAGGCGGTT
781 TGCGTATTGG GCGCTCTTCC GCTTCCTCGC TCACTGACTC GCTGCGCTCG GTCGTTCGGC
841 TGCGGCGAGC GGTATCAGCT CACTCAAAGG CGGTAATACG GTTATCCACA GAATCAGGGG
901 ATAACGCAGG AAAGAACATG TGAGCAAAAG GCCAGCAAAA GGCCAGGAAC CGTAAAAAGG
961 CCGCGTTGCT GCGCTTTTTC CATAGGCTCC GCCCCCCTGA CGAGCATCAC AAAAATCGAC
1021 GCTCAAGTCA GAGGTGGCGA AACCCGACAG GACTATAAAG ATACCAGGCG TTTCCCCCTG
1081 GAAGCTCCCT CGTGCGCTCT CCTGTTCCGA CCCTGCCGCT TACCGGATAC CTGTCCGCTT
1141 TTCTCCCTTC GGGAAAGCGTG GCGCTTTCTC ATAGCTCACG CTGTAGGTAT CTCAGTTTCG
1201 TGTAGGTCGT TCGCTCCAAG CTGGGCTGTG TGCACGAACC CCCCCTTCAG CCCGACCGCT
1261 GCGCCTTATC CGGTAACTAT CGTCTTGAGT CCAACCCGGT AAGACACGAC TTATCGCCAC
1321 TGGCAGCAGC CACTGGTAAC AGGATTAGCA GAGCGAGGTA TGTAGGCGGT GCTACAGAGT
1381 TCTTGAAGTG GTGGCCTAAC TACGGCTACA CTAGAAGAAC AGTATTTGGT ATCTGCGCTC
1441 TGCTGAAGCC AGTTACCTTC GGAAAAAGAG TTGGTAGCTC TTGATCCGGC AAACAAACCA
1501 CCGCTGGTAG CCGTGGTTTT TTTGTTTGCA AGCAGCAGAT TACGCGCAGA AAAAAAGGAT
1561 CTCAAGAAGA TCCTTTGATC TTTTCTACGG GGTCTGACGC TCAGTGGAAC GAAAACTCAC
1621 GTTAAGGGAT TTTGGTCATG AGATTATCAA AAAGGATCTT CACCTAGATC CTTTTAAATT
1681 AAAAAATGAAG TTTTAAATCA ATCTAAAGTA TATATGAGTA AACTTGGTCT GACAGTTACC
1741 AATGCTTAAT CAGTGAGGCA CCTATCTCAG CGATCTGTCT ATTTCTGTCA TCCATAGTTG
1801 CCGTCAATCC CGTCGTGTAG ATAACATCGA TACGGGAGGG CTTACCATCT GGCCCCAGTG
1861 CTGCAATGAT ACCGCGAGAC CCACGCTCAC CGGCTCCAGA TTTATCAGCA ATAAACAGC
1921 CAGCCGGAAG GGCCGAGCGC AGAAGTGGTC CTGCAACTTT ATCCGCCCTC ATCCAGTCTA
1981 TTAATTGTTG CCGGGAAGCT AGAGTAAGTA GTTCGCCAGT TAATAGTTTG CGCAACGTTG
2041 TTGCCATTGC TACAGGCATC GTGGTGTAC GCTCGTCGTT TGGTATGGCT TCATTCAGCT
2101 CCGGTTCCCA ACGATCAAGG CGAGTTACAT GATCCCCCAT GTTGTGCAAA AAAGCGGTTA
2161 GCTCCTTCGG TCCTCCGATC GTTGTGAGAA GTAAGTTGGC CGCAGTGTTA TCACTCATGG
2221 TTATGGCAGC ACTGCATAAT TCTCTTACTG TCATGCCATC CGTAAGATGC TTTTCTGTGA
2281 CTGGTGAGTA CTCAACCAAG TCATTCTGAG AATAGTGTAT GCGGCGACCG AGTTGCTCTT
2341 GCCCGGCGTC AATACGGGAT AATACGCGC CACATAGCAG AACTTTAAAA GTGTCATCA
2401 TTGGAACACG TTCTTCGGGG CGAAACTCT CAAGGATCTT ACCGCTGTTG AGATCCAGTT
2461 CGATGTAACC CACTCGTGCA CCCAACTGAT CTTCAGCATC TTTTACTTTC ACCAGCGTTT


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2521 CTGGGTGAGC AAAACAGGA AGGCAAAATG CCGCAAAAAA GGAATAAGG GCGACACGGA
2581 AATGTGAAT ACTCATACTC TTCCTTTTTC AATATTATTG AAGCATTTAT CAGGGTTATT
2641 GTCTCATGAG CGGATACATA TTTGAATGTA TTTAGAAAAA TAAACAAATA GGGGTTCGCG
2701 GCACATTTCC CCGAAAAGTG CCACCTATTG GTGTGGAAG TCCCAGGCT CCCCAGCAGG
2761 CAGAAGTATG CAAAGCATGC ATCTCAATTA GTCAGCAACC AGGTGTGGA AGTCCCCAGG
2821 CTCCCCAGCA GGCAGAAAGTA TGCAAAGCAT GCATCTCAAT TAGTCAGCAA CCATAGTCCC
2881 GCCCCTAAC CCGCCCATCC CGCCCCTAAC TCCGCCAGT TCCGCCATT CTCCGCCCA
2941 TGGCTGACTA ATTTTTTTTA TTTATGCAGA GGCCGAGGCC GCTCGGCCT CTGAGCTATT
3001 CCAGAAGTAG TGAGGAGGCT TTTTGGAGG CCTAGGCTTT TGCAAAAAGC TAGCTTGCAT
3061 GCCTGCAGGT CGGCCGCCAC GACCGTGCC GCCACCATCC CTGACCCAC GCCCCTGACC
3121 CCTCACAAGG AGACGACCTT CCATGACCGA GTACAAGCCC ACGGTGCGCC TCGCCACCCG
3181 CGACGACGTC CCCCAGGCCG TACGCACCTT CGCCGCGCG TTCGCCACT ACCCCGCCAC
3241 GCGCCACACC GTCGACCCGG ACCGCCACAT CGAGCGGGTC ACCGAGCTGC AAGAACTCTT
3301 CCTCAGCGCG GTCGGGCTCG ACATCGGCAA GGTGTGGGTC GCGACGACG GCGCCGCGGT
3361 GCGGTCTTGG ACCACGCCGG AGAGCGTCGA AGCGGGGCG GTGTTCGCG AGATCGGCC
3421 GCGCATGGCC GAGTTGAGCG GTTCCCGGCT GGCCGCGCAG CAACAGATGG AAGGCCTCCT
3481 GCGCGCGCAC CGGCCCAAGG AGCCCGCGTG GTTCCCTGCC ACCGTGCGCG TCTCGCCCGA
3541 CCACCAGGGC AAGGGTCTGG GCAGCGCCGT CGTGCTCCCC GGAGTGAGG CGGCCGAGCG
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3661 GCTCGGCTTC ACCGTACCCG CCGACGTCTA GGTGCCGAA GGACCGCGCA CCTGGTGCAT
3721 GACCCGCAAG CCCGGTGCCT GACGCCGCC CCACGACCCG CAGCGCCCGA CCGAAAGGAG
3781 CGCACGACCC CATGGCTCCG ACCGAAGCCA CCCGGGGCGG CCCCGCCGAC CCCGCACCCG
3841 CCCCCGAGGC CCACCGACTC TAGAGGATCA TAATCAGCCA TACCACATTT GTAGAGGTTT
3901 TACTTGCTTT AAAAAACCTC CCACACCTCC CCCTGAACCT GAAACATAAA ATGAATGCAA
3961 TTGTTGTTGT TAACTTGTTT ATTGCAGCTT ATAATGGTTA CAAATAAAGC AATAGCATCA
4021 CAAATTTTAC AAATAAAGCA TTTTTTTCAC TGCAATCTAA GAAACCATTA TTATCATGAC
4081 ATTAACCTAT AAAAATAGGC GTATCACGAG GCCCTTTCGT C

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8.3.2.2. *pSilencer™ 4.1-CMV*

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1 TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGTCCCG GAGACGGTCA
61 CAGCTTGCT GTAAAGCGGAT GCCGGGAGCA GACAAGCCCG TCAGGGCGCG TCAGCGGGTG
121 TTGGCGGGTG TCGGGGCTGG CTTAACTATG CGGCATCAGA GCAGATTGTA CTGAGAGTGC
181 ACCATATGCG GTGTGAAATA CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGGCGCC
241 ATTCGCCATT CAGGCTGCGC AACTGTTGGG AAGGGCGATC GGTGCGGGCC TCTTCGCTAT
301 TACGCCAGCT GCGGAAAGGG GGATGTGCTG CAAGGCGATT AAGTTGGGTA ACGCCAGGGT
361 TTTCCAGTC ACGACGTGTG AAAACGACGG CCAGTGCCAA GCTAGCGGCC GCATACAAAA
421 AACCACACA CAGATCCAAT GAAATAAAAA GATCCTTTAT TAAGCTTACT ACCGTTGTTA
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601 TGTTACGACA TTTTGAAAG TCCCGTTGAT TTTGGTGCCA AAACAACTC CCATTGACGT
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901 TTTACCGTAA ATACTCCACC CATTGACGTC AATGGAAAGT CCCTATTGGC GTTACTATGG
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1261 TATCCGCTCA CAATTCCACA CAACATACGA GCCGGAAGCA TAAAGTGTA AGCCTGGGGT
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1561 AACGCAGGAA AGAACATGTG AGCAAAAGGC CAGCAAAAGG CCAGGAACCC TAAAAAGGCC
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1681 TCAAGTCAGA GGTGGCGAAA CCCGACAGGA CTATAAAGAT ACCAGGCGTT TCCCCCTGGA
1741 AGCTCCCTCG TCGCTCTCC TGTTCCGACC CTGCCGCTTA CCGGATACCT GTCCGCTTTT

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2161 GCTGGTAGCG GTGGTTTTTT TGTTTGCAAG CAGCAGATTA CGCGCAGAAA AAAAGGATCT
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2461 TGACTCCCCG TCGTGTAGAT AACTACGATA CGGGAGGGCT TACCATCTGG CCCCAGTGCT
2521 GCAATGATAC CGCGAGACCC ACGCTCACCG GCTCCAGATT TATCAGCAAT AAACCAGCCA
2581 GCCGGAAGGG CCGAGCGCAG AAGTGGTCTT GCAACTTTAT CCGCTCCAT CCAGTCTATT
2641 AATTGTTGCC GGGGAAGCTAG AGTAAGTAGT TCGCCAGTTA ATAGTTTGCG CAACGTTGTT
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2761 GGTTCCTAAC GATCAAGGCG AGTTACATGA TCCCCCATGT TGTGCAAAAA AGCGGTTAGC
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4201 CATTAGGGC ACCGGACAGG TCGGTCTTGA CAAAAAGAAC CGGGCGCCCC TCGCTGACA
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4321 GCCTCTCCAC CCAAGCGGCC GGAGAACCTG CGTGCAATCC ATCTTGTTCA ATCATGCGAA
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4501 CAGAGGCGGC CTCGGCTCT GCATAAATAA AAAAAATTAG TCAGCCATGG GCGGAGAAT
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4861 GAAAAGTGCC ACCTGACGTC TAAGAAACCA TTATTATCAT GACATTAACC TATAAAAAATA
4921 GGCGTATCAC GAGGCCCTTT CGTC

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8.4. My publications

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