

The Proliferation and Differentiation Responses of Adult Mouse Neural Stem Cells *in vitro*

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Declaration

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Robert Chang-Chih Chou

Abstract

Recent studies have shown that the adult brain retains a population of stem cells in specific locations. Adult neural stem cells (NSCs) from the subventricular zone of the lateral ventricle have self-renewing and differentiation capability. These NSCs are capable of differentiating into astrocytes, oligodendrocytes and neurons *in vitro*. Study the proliferation and differentiation capability of the adult NSCs would provide a resource for developing new treatments for neurodegenerative diseases in the future. Retinoic acid has been described to promote neuron formation from the several different types of stem cell *in vitro*. Recently, a novel synthetic analogue EC23 has been released. However, the impact of EC23 on neuron formation and cell growth remain unclear. This project focuses on comparing the pro-neurogenic capability and impact on cell growth and cell cycle of EC23 with Retinoic acid and preliminary evaluating the character of PPARγ in Retinoid-induced neuron formation by using PPARγ agonist Rosiglitazone.

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Abbreviations

AGE	Advanced glycation end products
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BDNF	Brain-derived neurotrophic factor
BDNF-CREB	Brain-derived neurotrophic factor - cAMP response element-binding protein
bFGF	Basic fibroblast growth factor
BLBP	Brain lipid-binding protein
BMP	Bone morphogenic protein
BNM	Basic neuronal medium
BNM+EC23	Basic neural differentiation medium with EC23
BNM+RA	Basic neural differentiation medium with Retinoic acid
BNM+Z	Basic neural differentiation medium with Rosiglitazone
BNM+ZE	Basic neural differentiation medium with Rosiglitazone with EC23
CNS	Central nervous system
CRABP	Cellular RA binding protein
DMSO	Dimethyl sulfoxide
Dppa	Developmental pluripotency associated
E	Embryonic day
EGF	Epidermal growth factor
Erk	Extracellular signal-regulated kinase
FCS	Fatal calf serum
FGF	Fibroblast growth factor
GAD	Glutamic acid decarboxylase
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GLUT-1	Glucose transporter protein 1
hESC	Human embryonic stem cell
hPNC	Human neural progenitor cells
iGluR	Ionotropic glutamate receptor

Abbreviations

iNOS	Inducible nitric oxide synthase
JAK	Janus kinase
JNK	Jun N-terminal kinase
Kcnq	Voltage-gated K^+ channel protein
Lefty	Left-right determination
LIF	Leukaemia inhibitory factor
LRRK	Leucine-rich repeat receptor kinase
MAP	Microtubule associated protein
mGluR	Metabotropic glutamate receptor
mEC	Mouse embryonal carcinoma
mESC	Mouse embryonic stem cell
miPSC	Mouse induced pluripotent stem cell
mNSC	Mouse neural stem cell
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NB	Neural base medium
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NSAIDs	Non-steroids anti-inflammatory drugs
NSC	Neural stem cell
PBS	Phosphate buffered saline
PBT	0.1% Tween-20 in PBS
PFA	Parafomaldehyde
PGC-1α	PPARγ cofactor 1α
PI3K	Phosphoinositide 3-Kinas
PKA	cAMP-dependent protein kinase A
PKC	Protein kinase C
PMA	Phorbol myristyl acetate
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator response element
PSA-NCAM	Polysialylated neural adhesion cell molecule
RAR	Retinoic acid receptor
RARE	Retinoids response element

Abbreviations

RMS	Rostral migratory stream
RXR	Retinoic X receptor
Scn	Voltage-gated Na^+ channel protein
Sfrp	Secreted frizzled-related protein
STAT	Signal transduers and activators of transcription
SV	Synaptic vesicle protein
trk	Signal transducing receptor
TZDs	Antidiabetic thiazolidindiones

Chapter 1. Introduction

1.1. Neural stem cells (NSCs) generated from Subventricular zone of lateral ventricle

During the development of the central nervous system (CNS), neurons are born in the ventricular and subventricular zones. In the adult brain, neurogenesis and gliogenesis have been discovered in the subventricular zone (Smart, 1961; Blakemore, 1969; Privat and Leblond, 1972; Reynolds and Weiss, 1992; Lois and Alvarez-Bulla, 1993; Lois and Alvarez-BuyIIa, 1994; Morshead et al., 1994; Alvarez-Buylla et al., 2002; Episkopou, 2005; Goffredo et al., 2008) and also in the subgranular zone of hippocampal dentate gyrus (Cameron et al., 1993; Gage et al., 1998; Alvarez-Buylla et al., 2002; Venere et al., 2012). For example, the subventricular zone lining the lateral ventricle has long been known to harbour neurons and glia. These cells migrate along rostral migratory stream (RMS) to the olfactory bulb (Smart, 1961; Altman, 1969; Privat and Leblond, 1972; Doetsch and Alvarez-Buylla, 1996). From embryogenesis to adult life, these cells differentiate into a range of cell types, such as interneurons, granular neurons, periglomerular neurons and neuroglia in the olfactory bulb (Smart, 1961; Altman, 1969; Luskin, 1993; Doetsch and Alvarez-Buylla, 1996; Weiss et al., 1996; Doetsch et al., 1997; Doetsch et al., 1999). Studies carried out more recently on adult brains have also highlighted the presence of progenitors (Smart, 1961; Privat and Leblond, 1972; Lois and Alvarez-Bulla, 1993; Weiss et al., 1996; Doetsch et al., 1997; Martens et al., 2000; Rietze et al., 2001). These multipotent neural stem cells (NSCs) are polysialylated neural adhesion cell molecule (PSA-NCAM) negative, TuJ1 (□-tubulin) negative, glial fibrillary acidic protein (GFAP) negative, vimentin negative and Nesin positive (Reynolds and Weiss, 1992; Lois and Alvarez-Bulla, 1993; Gritti et al., 1995; Weiss et al., 1996; Doetsch et al., 1997; Doetsch et al., 1999; Imura et al., 2003). They retain the capability to self-renew, and undergo *de novo* neurogenesis and gliogenesis. *In vitro* evidence confirmed that these NSCs, which can be maintained using epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), are capable of differentiating into neurons and glia when treated with appropriate media (Reynolds et al., 1992; Reynolds and Weiss, 1992; Morshead et al., 1994; Gritti et al., 1995; Weiss et al., 1996; Martens et al., 2000; Rietze et al., 2001). However, the precise molecular mechanisms of bFGF and EFG in these NSCs are not fully

understood. The NSCs are easy to propagate in floating aggregation as neurosphere formation or monolayer culture when they are cultured in the medium containing those growth factors. However, withdrawing EGF resulted in markedly reduction in tyrosine phosphorylation of signal transducers and activators of transcription 3 (p-STAT3-Y705) (Natarajan et al., 2014). These NSCs are capable of differentiating into neurons, glia and oligodendrocytes when treated with appropriate media. By *in vitro* treatments, it has been possible to generate neurons associated with $GABA_A$, Kainate, α-amino-3-hydroxy-5- methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA), Substance P receptors and voltage-gated Na^+ channel (Reynolds and Weiss, 1992; Goffredo et al., 2008). Furthermore, it raises the possibility that the subventricular zone in the adult brain could act as a resource of precursor cells to generate neurons for transplantation and neural replacement caused by neural injury or neural degeneration diseases. It will also be helpful to provide a new treatment of neurodegenerative diseases in the future.

1.2. Brain-derived neurotrophic factor (BDNF)

Neurotrophins are a family of growth factors which influence neurogenesis (Ahmed et al., 1995). Members of this family are brain-derived neurotrophic factor (BDNF), never growth fact (NGF), neurotrophin-3, neurotrophin4/5 and neurotrophin 6. These small proteins share around 50% homology. They are involved in the proliferation of NSCs and enhance the survival and differentiation of postmitotic neurons. The signalling of neurotrophins is conducted by a group of proteins, signal transducing receptors, trk, belonging to tyrosine kinase receptors. Each receptor has a different affinity binding to a respective ligand. For example, trkB has high affinity to BDNF. The expression of neurotrophins and trks in different regions in embryonic and adult CNS suggested the neurotrophin/trk signalling pathways play specific roles during the development and maturation of the brain.

The pro-neurogenic capability of BDNF has been studied in a range of embryonic or adult stem cells or immature neurons (Table 1-1). It has been reported that BNDF enhance morphological differentiation and the expression of neural markers, such as □-III tubulin, MAP2 and neurofilament (Benoit et al., 2001; Cheng et al., 2003; Ito et al., 2003; Catapano et al., 2004; Babu et al., 2007; Shim et al., 2007; Choi et al., 2008; Maciaczyk et al., 2008),

especially in EGF-responsive neural stem cell systems (Ahmed et al., 1995; Shetty and Turner, 1998). Under BDNF treatments, the NSCs are capable of differentiating into neurons showing the characters of the brain regions where the NSCs were generated. For example, BDNF induced the neural stem cells from hippocampus to differentiate into granular cell-like cells (Babu et al., 2007). It showed that BDNF contributed to neuron formation from NSCs.

Table 1-1 The reported pro-neurogenic effect of BDNF on ESCs and NSCs.

Cell type	Condition	Reference
EGF-responsive neuro-progenitors from adult rat striatum	After a five to seven day treatment of BDNF, the spheres of EGF-responsive neural progenitors from adult rat striatum starts to express □-III tubulin and the most level is at day ten which is no difference between one BDNF treatment for ten days and one treatment every 48 hrs treatments. The expression of □-III tubulin decreased a lot at day twenty one which shows no difference from comparing with EGF-treated control group at day twenty five.	(Ahmed et al., 1995)
EGF-responsive NSCs from P1-P2 mouse	After a three-day BDNF treatment, the NSCs showed neuronal morphology and pyramidal neuron-like cells at day eleven. There are two different types of neurons observed under a BDNF treatment. The larger neurons only increase arborisation whilst the small neurons increase arborisation and the size of cell body. The length of axon increases between day four and day fourteen and no more extension after day fourteen.	(Shetty and Turner, 1998)
Mouse ES cells (E15)	Increase the expression of neurofilament and GFAP after a fourteen-day treatment	(Benoit et al., 2001)
Mouse ES cells (E12)	After a two-day BDNF treatment, the expression of nNOS increase in the cells which is a positive feedback loop regulating the capability of BDNF in cell proliferation and differentiation.	(Cheng et al., 2003)
Mouse ES cells (E14)	Increase the expression level of mRNA of choline acetyltransferase, tyrosine hydroxylase, and dopamine □-hydroxylase after a seven-day treatment. It suggested that BDNF induced the expression of neurotransmitters before the morpgological change.	(Ito et al., 2003)
Collosal projection neurons from mouse E19, P2-3, P6-7	Increase the arborisation of dendrites and soma size but not the length of axon after a three-day treatment in P2-3 and P6-7 collosal projection neurons.	(Catapano et al., 2004)
NSCs from adult mouse hippocampus	After a four to five day treatment, the cells started to show aoutward K^+ current. After a ten to twelve day treatment, the cells started to express GABA synthesis enzyme GAD67 which suggested that the cells differentiated into GABAergic granular-like cells. After a twenty to thirty day treatment, the cells showed K^+ current as a mature granular cell.	(Babu et al., 2007)
Nurrl over-expressed NSCs from adult rat SVZ	Induce the expression of □-III tubulin and differentiated the cells into mid-brain-like dopaminergic neurons after a seven-day treatment.	(Shim et al., 2007)
NSCs from adult rat hippocampus	Induce the expression of T□-III tubulin and Neugranin after a fourteen-day treatment	(Choi et al., 2008)
NSCs from human fatal midbrain and neocortex	Induce the expression of Map2 and tyrosine hydroxylase in a low oxygen and ascorbic acid condition after a treatment	(Maciaczyk et al., 2008)

	up to twenty one days which is driven by TrkB signalling. BDNF is more efficient in neural differentiation than the combined treatment of Shh and FGF8.	
Mouse NSC (E14)	BDNF promoted gilial and neural differentiation via activating MAP kinase, Akt and STAT-3. The BDNF receptor, truncated TrkB highly expressed in the embryonic mNSCs and differentiated glia whilst full length TrkB expressed in differentiated neurons. In the presence of EGF, BDNF increased the expression of Nestin and Oct-3B which promoted the embryonic mNSCs proliferation in a does-dependent manner. Without EGF, BDNF promoted the expression of GAD-65/67 and GFAP.	(Islam et al., 2009)

1.3. Signal transducers and activators of transcription

1.3.1 The mechanism of STAT3

The signal transducers and activators of transcription is a family of transcription factor (De-Fraja et al., 1998; Campbell, 2005; Cheng et al., 2011). There are 7 members (STAT 1,2,3,4,5A,5B and 6) in the STAT family participating in signalling pathways mediated by a range of growth factors and cytokines (Matsuda et al., 1999; Rajasingh and Bright, 2006; Asano et al., 2009; Islam et al., 2009; Mo et al., 2010b; Shen et al., 2010). For example, STAT3 regularly presents as monomers in cytoplasm (Figure 1-1). When the extracellular ligands bound to their cell-surface receptors, it led to activation of associated receptor tyrosine kinas, such as Janus kinases (JAKs), and subsequently phosphorylated tyrosine residue(s) in the receptor for STAT3 activation. The tyrosine residue (Y759) of glycoprotein (gp)130 is a widely known mechanism of STAT3 activation (Bonni et al., 1997). The phosphorylated- STAT3 (p-STAT3) interacts with the SH2 domain in STAT3 polypeptide to generate stable cytoplasmic homodimers (Darnell, 1997; Cheng et al., 2011) or heterodimers with STAT1 (Darnell, 1997). Subsequently, these dimers are translocated into the cell nuclei. There are two forms of p-STAT3: one is phosphorylated at tyrosine (Y705) via Janus kinase (JAK) and the other one is at serine (S727) via mitogen-activated protein (MAP) kinase (Darnell, 1997; van Puijenbroek et al., 1999). The p-Y705-STAT3 in conjunction of other cofactors binds sequence-specific DNA response elements in the promoters of target genes leading to regulate transcription of target genes. The p-S727-STAT3 enhances the transcriptional activity of STAT3.

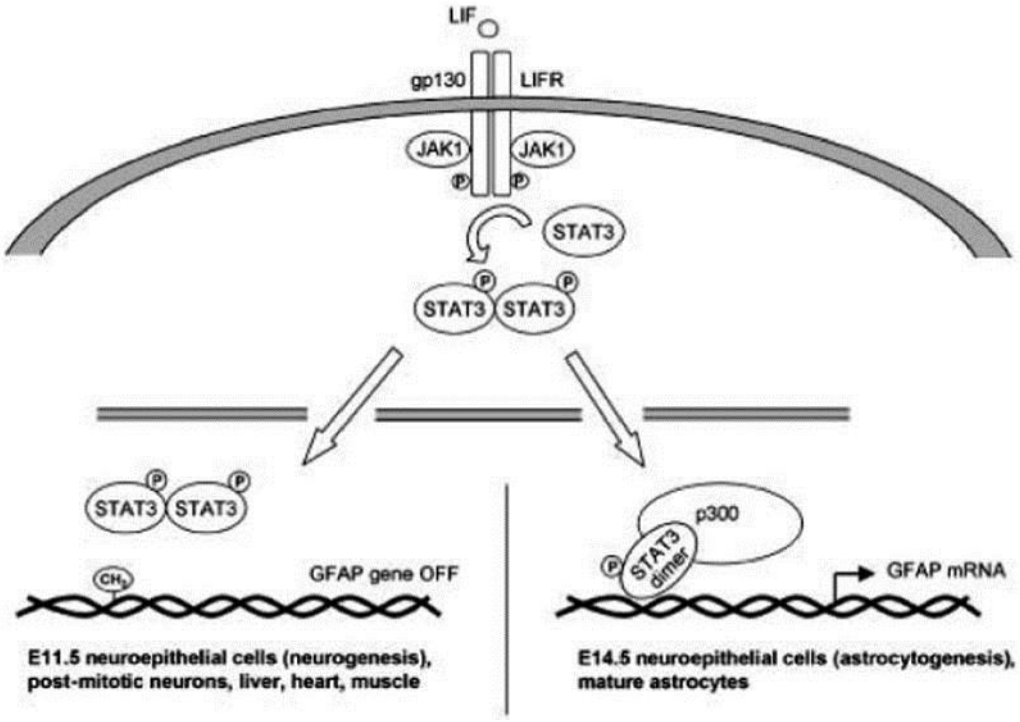


Figure 1-1 The mechanism of LIF induced *gfap* gene expression.
When embryonic day (E) 11.5- or E14.5-derived neuroepithelial cells are treated with LIF, signal transducer and activator of transcription (STAT) 3 in both cells is activated in response to the stimuli. In E14.5 neuroepithelial cells, activated STAT3 translocates into the nucleus and binds to its recognition sequence in the GFAP promoter. The STAT3/p300 transcriptional complex activates the GFAP gene, thus cells differentiates into astrocytes. In contrast, in E11.5 neuroepithelial cells, phosphorylation of STAT3 occurs, but it cannot bind to the GFAP promoter because the methylated cytosine residue interferes with the binding of STAT3 to its recognition element. As a result, E11.5 neuroepithelial cells do not differentiate into GFAP-positive astrocytes. (Fukuda and Taga, 2005)

STAT3 is expressed constantly in developing and mature CNS whilst the expression level decreases in aging brains (Bonni et al., 1997; De-Fraja et al., 1998; Niwa et al., 1998; De-Fraja et al., 2000). STAT3 is activated by extracellular signals, such as a range of growth factors and cytokines (Bonni et al., 1997; De-Fraja et al., 2000; Paillaud et al., 2002; Li, 2010; Shen et al., 2010; Moore and Goldberg, 2011; Natarajan et al., 2014). It has been known that STAT3-binding sites are cell-type specific so that STAT3 is involved in a variety of cell responses, such as, maintaining undifferentiated and proliferating status of ESCs, blocking apoptosis, neurogenesis and gliogenesis (Takeda et al., 1997; Matsuda et al., 1999; Cheng et al., 2011; Hutchins et al., 2013; Natarajan et al., 2014). For example, EFG is important for the proliferation of NSCs and EGF induced STAT3 activation leading high level of p-STAT3Y705 (Yu et al., 2009). STAT3 is abundantly and constantly expressed in a rat brain from embryonic day 14 (E14) to 18 (De-Fraja et al., 1998). Moreover, targeted disruption of

mouse *Stat3* gene during embryogenesis leads to embryonic lethality (Takeda et al., 1997). It suggested that STAT3 also plays an important role in embryogenesis and brain maturation. STAT3 and p-STAT3 also expressed in mouse embryonic motor neurons (Lee et al., 2013). P-STAT3 (Y705 form) cooperated with the motor neuron-hexamer to activate the motor neuron gene enhancers. It indicated that STAT3 played a significant role in the development of embryonic spinal cord. In E14 mouse ESCs, STAT3 locally assembles with other transcription factors and co-factors to form distinct transcriptional regulatory modules (Figure 1-2) (Hutchins et al., 2013). However, it has not been identified how this system works or if there is a regulator controlling the formation of these modules.

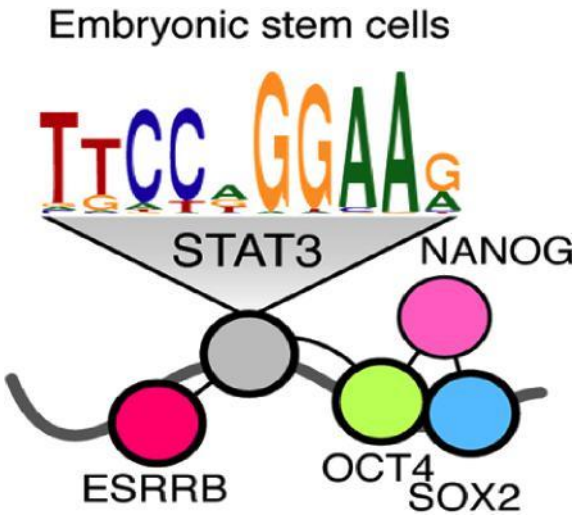


Figure 1-2 STAT3 formed transcriptional regulatory models with other transcription factors and co-factors. Transcriptional regulatory modules include both cell type-specific and general transcription factors, and are largely responsible for the various functions of STAT3 in different cell types. (Hutchins et al., 2013)

The effect of STAT3 in neural stem cell, however, remains controversial. In a CNTF-induced STAT3 activation study, the embryonic rat NSCs differentiated into GFAP-positive astrocytes and inhibited neurogenesis via JAK1-STAT3 activation and Ras/MAPK inhibition (Bonni et al., 1997). This effect was stronger in the embryonic rat NSCs derived from E17 embryos than E14. In another embryonic rat NSC study, STAT3 suppression, by overexpressing domain negative- modified STAT3 (Y705F), promoted neurogenesis and inhibited gliogenesis (Gu et al., 2005). Under a combination treatment of LIF and BMP2, the mNSCs from embryonic telencephalon differentiated into astrocytes (Fukuda and Taga,

2005). It was a synergic effect between LIF activated STAT3 and BMP2 activated Sma-MAD homolog (Smad) protein 1. In the cell nuclei, transcription co-activator p300 bound to p-STAT3 at N-terminal and Smad1/4 at C- terminal. This complex recognised sequences of promoter of *gfap* and subsequently regulated the expression of GFAP leading to astrocytogenesis (Figure 1-3). Neurotrophin-4 promoted neurogenesis in embryonic mNSC through inhibiting STAT3, modulating of the activity of Akt-mediated PKB and suppressing IL-6 and Notch signalling pathway (Shen et al., 2010). In an adult rat NSC study, the p-STAT3 protein level increased in MAP-2-positive neurons on differentiation time point day three and seven whilst it increased in GFAP-positive astrocytes from day eleven to day twenty one (Cheng et al., 2011). It suggested that STAT3 activation is involved in the differentiation of NSCs in a time- and cell-type- specific manner.

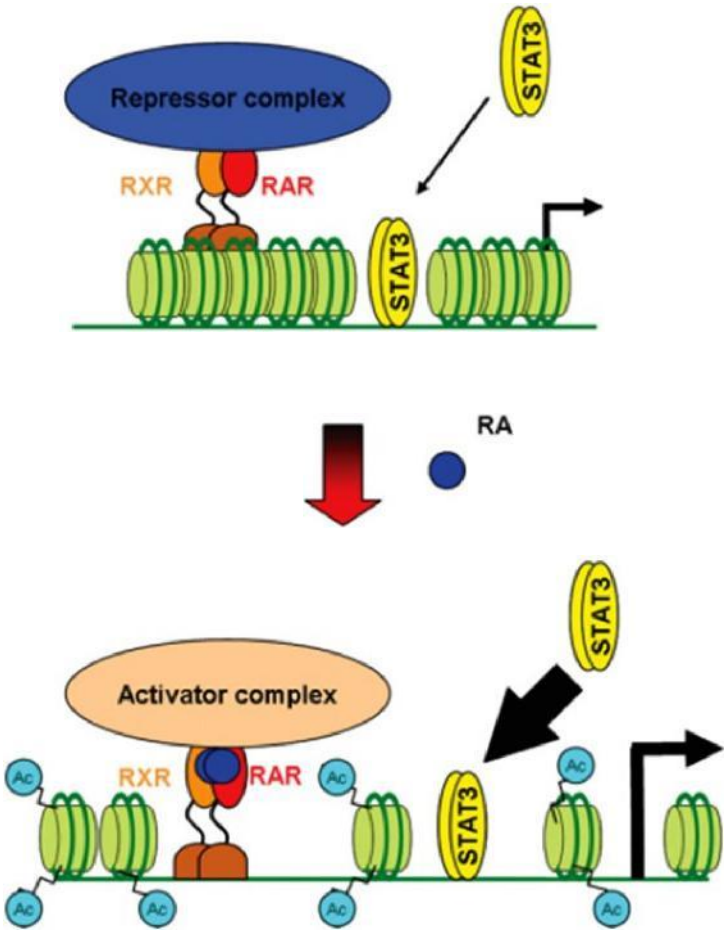


Figure 1-3 A model for synergic activation of *gfap* promoter by using Retinoic acid and LIF.
In the absence of RA, RAR/RXR complexes are transcriptional repressors, leading to a closed chromatin structure. Upon ligand (RA) binding, the repressor complex is replaced by an activator complex, inducing a relaxed chromatin structure through histone H3 acetylation. This acetylation facilitates LIF-induced binding of STAT to its cognate sequence. Abbreviation: Ac, acetyl group. (Asano et al., 2009)

1.3.2 STAT3 activator, Colivelin

Colivelin, a synthesised peptide, is composed of activity-dependent neurotropic factor (ADNF) -9 C-terminally fused to a potent Humanin derivative AGA-(C8R)HNG17 (Chiba et al., 2005; Chiba et al., 2007; Ciesler and Sari, 2013) (Figure 1-4) . Colivelin is able to suppress neuronal death caused by familial Alzheimer's disease-causative gene and f3-amyloid protein 1-43 overexpression (Figure 1-5) at a concentration of 100 fM (Chiba et al., 2005) that is more potent than Humanin (10 μM) and AGA-(C8R)HNG17 (10 pM) (Matsuoka et al., 2006). Colivelin does not lose its neuroprotecctive activity at higher concentration as ADNF(10 nM) does (Chiba et al., 2005; Ciesler and Sari, 2013). This neuroprotection of Colivelin was considered to occur via two pathways: one is Humanin mediated STAT3 activation; the other is ADNF triggered Ca^{2+}/ calmodulin-dependent protein kinase IV .I*n vivo,* intracerebroventricular (i.c.v.) administration Colivelin not only abolished impaired spatial working memory but also antagonised neuronal loss in CA1 region of hippocampus in Alzheimer's disease animal models (Chiba et al., 2005) which had also been observed to ameliorate amyloid f3 –induced choline acetyltransferase- positive neuron loss in medial spetum after intranasal (i.n.) administration (Yamada et al., 2008). Additionally, intraperitoneal (i.p.) administration of Colivelin supressed the impaired memory caused by muscarinic acetylcholine receptor antagonist, 3-quinuclidinyl benzilate, indicating that Colivelin passed through the blood-brain barrier and then regulated neuronal function in the brain (Chiba et al., 2005; Yamada et al., 2008). In a G93A-SOD1 transgenic amyotrophic lateral sclerosis (ALS) mouse model, i.c.v. Colivelin also improved motor neuron performance in a dose-dependent manners (100fmol ~ 1nmol) and attenuated the loss of acetylcholine tranferase-positive motor neuron in ventral horns of spinal cord (Chiba et al., 2006). This prolonged survival of the ALS mice whilst ANDF-9 did not. It indicates that Colivelin is a potent neuroprotecctive agent. Colivelin is being developed as a new treatment for a range of neurodegenerative diseases (Ciesler and Sari, 2013).

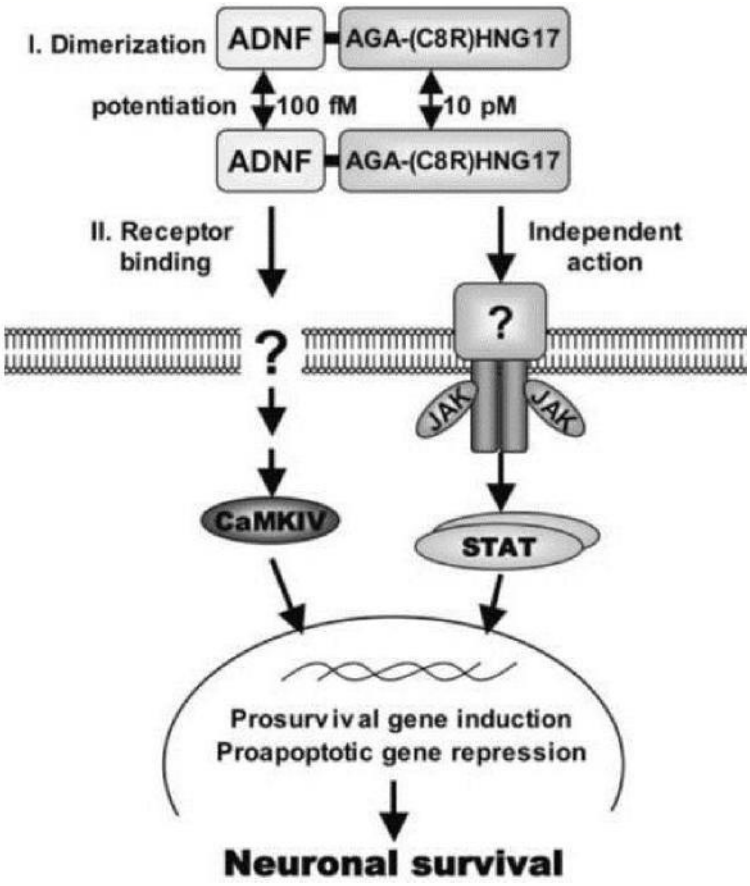


Figure 1-4 The structure and neuroprotection of Colivelin.
(Chiba et al., 2007)

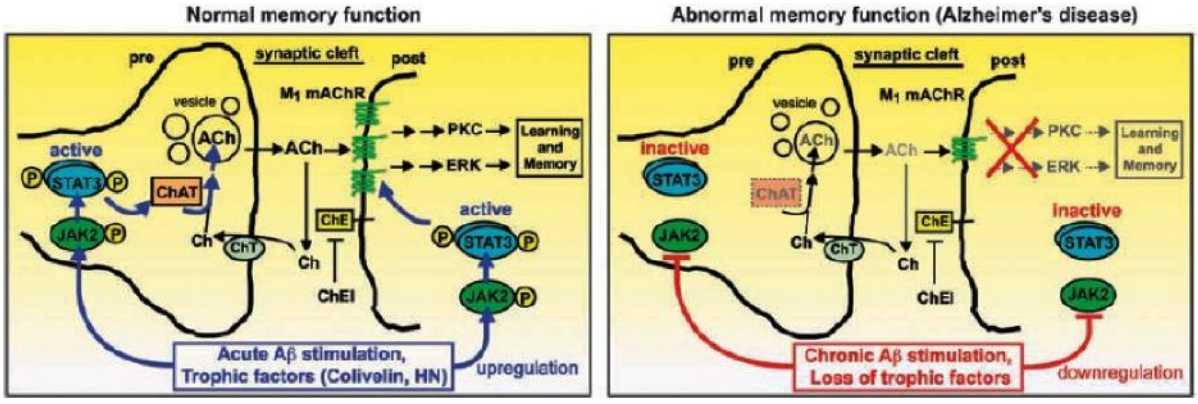


Figure 1-5 A proposed model of cognitive function related to Alzheimer's disease.
In a normal condition, the acute amyloid β stimilation and trophic factors activate STAT3 and then activates the signalling pathways maintaining learning and memory function, such as PKC and ERK. In an Alzheimer's disease animal model, the chronic amyloid β-III acumulation led to neuron death which was the cause of losing learing and memory functions due to STAT3 inactivation. Exogenous application of Colivelin may alter this phenoninon by activating STAT3. (Chiba et al., 2009)

1.4. The pro-neurogenic capability of Retinoids

Experimental evidence showed that BDNF alone or in combination with Retinoid treatments promoted neuron formation from NSCs *in vitro* (Weiss et al., 1996; Goffredo et al., 2008; Kim et al., 2009; Lu et al., 2009; Maltman et al., 2009; Christie et al., 2010; Lee et al., 2012b). Under Retinoic acid treatment, cells with neural-like character were 7.8 times higher than the untreated ESCs (Bain et al., 1995). Retinoic acid is one of the important morphogens during embryogenesis, especially in neuronal patterning, neural differentiation and axon outgrowth (Bain et al., 1995; Kastner et al., 1995; Maden, 2007; Lu et al., 2009; Elizalde et al., 2010).

Retinoic acid has been characterised to bind to two families of Retinoid nuclear receptors (Figure 1-6) (Leid et al., 1992; Kastner et al., 1995; Cullingford et al., 1998). One is the Retinoic acid receptors (RARs); the other is the Retinoic acid X receptors (RXRs). There are three receptors (RARα, J3 and y) each with multiple isoforms (α1 and 2; J31 to J34; y1 and 2). RARs are activated by the Retinoids active metabolites, all trans and 9-cis Retinoic acid. RARs binds to cognate response elements as heterodimers with RXRs. The RXRα, J3 and y are activated by 9-cis Retinoic acid and other natural or synthetic retinoid-like compounds. The isoforms of both Retinoid receptor families share functional redundancy within each family. The RARs and RXRs are coded by separated genes (Bastien and Rochette-Egly, 2004). The proteins of these receptors only differ in N-terminal regions, whilst the highly conserved C-terminal harbours the DNA binding domain. One RXR binds to another RXR as homodimers or to RARs as heterodimers in vitro (Figure 1-6). In the absence of Retinoids, the RXR/RAR heterodimer binds to specific DNA sequence or retinoids response element (RAREs) (Bastien and Rochette-Egly, 2004; Asano et al., 2009). These homodimers or heterodimers subsequently recruit co-repressor complexes and maintain target gene repression (Rhinn and Dollé, 2012). Besides RARs, there are other co-partners for RXRs, including peroxisome proliferator-activated receptor (PPAR) y (Rodriguez et al., 1994; Cullingford et al., 1998). The RXR/ PPARy heterodimer transduce signalling effects of Retinoids and upregulate the transcription of peroxisome proliferator response element (PPRE) *in vitro*.

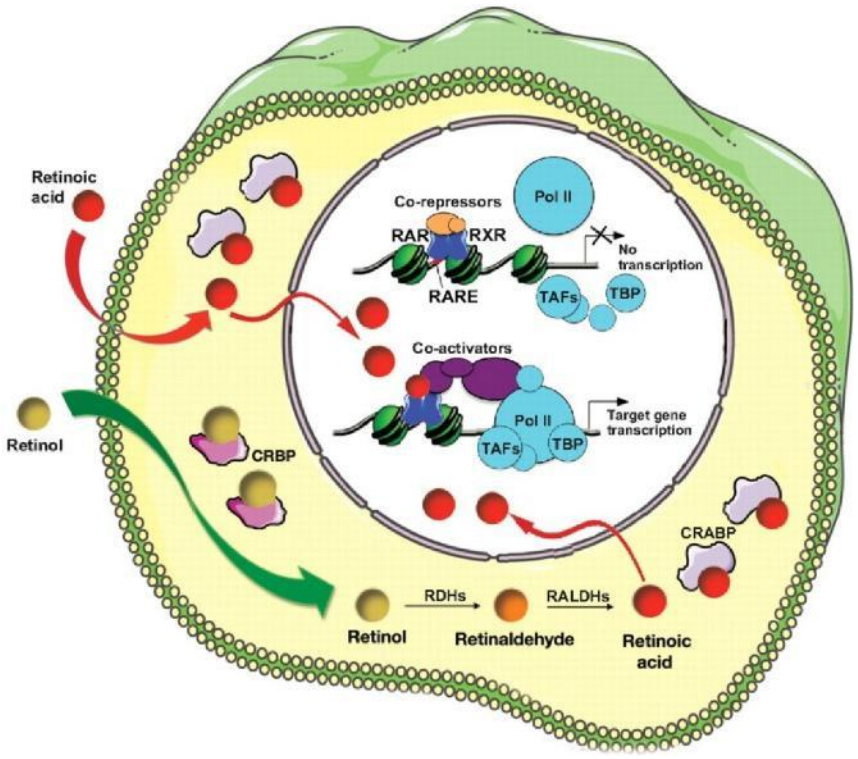


Figure 1-6 The metabolism and signalling pathway of Retinoid.
(Rhinn and Dollé, 2012)

During embryogenesis, Retinoic acid acts as one of the morphogens guiding the development of the eye, forebrain and hindbrain (Shearer et al., 2012). The functions of Retinoic acid in the developing CNS are inducing neurogenesis and directing neuronal patterning (Bain et al., 1995; Kastner et al., 1995; Maden, 2007; Lu et al., 2009; Elizalde et al., 2010; Shearer et al., 2012). RARs are involved in the development of neural crest cell structure, anterior-posterior axis patterning, limb morphogenesis, development of the eye, spermatogenesis and epidermal morphogenesis (Lohnes et al., 1993; Lohnes et al., 1994; Kastner et al., 1995; Rhinn and Dollé, 2012). RXRs are involved in ventricular wall development, eye development and spermatogenesis (Lohnes et al., 1993; Lohnes et al., 1994; Kastner et al., 1995). Retinoic acid is involved in the development of the neural network at synapses in a dynamic balance (Sadeghian et al., 2012). When the neuron activity or postsynaptic calcium re-entry was blocked, the synaptic transmission decreased. It required

more Retinoic acid to increase the synaptic transmission suggesting that Retinoic acid plays a role in the development of neural circuit during embryogenesis.

The pro-neurogenic capability of Retinoic acid has been demonstrated by using several cell models (Appendix 1). Under Retinoic acid treatment, mESCs differentiated into neurons and showed the activities of NMDA, AMPA, Kainate, GABA and Glycine receptors and voltage-gated Na^{+}, K^{+} and Ca^{2+} channels (Bain et al., 1995; Strübing et al., 1995; Rohwedel et al., 1999; Kim et al., 2009; Schulz et al., 2011). The evidence from mouse brain slice culture suggested that Retinoic acid doubled the number of neurons produced in the embryonic subventricular zone and tripled the amount in the adult hippocampus via RAR α/ RXR α signalling (Rodriguez et al., 1994; Bain et al., 1995; Maden, 2007).

Retinoic acid has been also showed to play an important role in the mature CNS (Maden, 2007; Shearer et al., 2012). Cognitive studies showed that the dominant negative RARα expressed in the brain was linked to impaired long-term potentiation which interrupted the AMPA receptor-mediated synaptic transmission in the hippocampus and resulted in reducing social recognition and spatial memory. The impairment of spatial memory was reversible by Retinoic acid treatment in adult rats which showed recovery of long-term potentiation. It suggested that Retinoids play an important role in age-related neuron loss, cognitive decline or even the onset of neurodegenerative diseases through Retinoids signalling disruption.

Because Retinoic acid is photo-sensitive, stable analogues of Retinoic acid had been synthesised (Maltman et al., 2009; Gluyas et al., 2012). EC23, a stable synthetic analogue of Retinoic acid, has been used in few neural differentiation studies (Appendix 1) (Maltman et al., 2009; Christie et al., 2010; Gluyas et al., 2012). In a ligand-receptor assay, EC23 bonded intracellular Retinoic acid receptor RARα, RAR□ and RAR □ in a concentration dependent manner, from 0.1 nM to 1 □M (Gluyas et al., 2012) in contrast to the 3 to 100 nM of Retinoic

acid required for the same effect (Allenby et al., 1993). However, the impact of EC23 on neuron formation and the growth of cells remain unclear.

1.5. Peroxisome proliferator-activated receptor (PPAR) y

The peroxisome proliferator-activated receptors (PPARs) are ligand-dependent transcription factors and belong to a member of the steroid hormone receptor superfamily (Green and Wahli, 1994; Green, 1995; Schoonjans et al., 1996; Kliewer et al., 1997). There are three mammalian members in this superfamily, PPARα, ·/δ and y (y1 and y2). The three isoforms are coded by separated genes (Cullingford et al., 1998). During embryogenesis, the PPARs mRNA are differentially expressed in CNS at different embryonic days (Table 1-2 modified from (Braissant and Wahli, 1997). PPARα mRNA displays moderate expression in forebrain, midbrain and hindbrain at E13.5. PPARβ mRNA was detectable from E11.5 to E18.5 in forebrain, midbrain and hindbrain. PPARγ mRNA expression at E13.5 was high in hindbrain, moderate in midbrain and weak in forebrain. In neonatal rat brains the mRNA of the three PPARs were detectable in Primary culture from cerebellum, cortex, midbrain, meningeal fibroblast cortical astrocytes and cerebellar astrocytes (Cullingford et al., 1998).

Table 1-2 Differential mRNA expression of PPARs during embryogenesis in rats.
(Braissant and Wahli, 1997) - absent, +/- barely detectable, + weak expression, ++ moderate expression, +++ high expression and ++++ very high expression.

	E8.5	E11.5	E13.5	E15.5	E18.5
PPARα					
Embryonic Ectoderm	-				
Forebrain		-	++	+	+/-
Midbrain		-	++	+	+/-
Hindbrain		-	++	+	+/-
PPARβ/δ					
Embryonic Ectoderm	++				
Forebrain		+	++++	++++	++
Midbrain		+	++++	+++	++
Hindbrain		+	++++	+++	++
PPARγ					
Embryonic Ectoderm	-				
Forebrain		-	+	+/-	-
Midbrain		-	++	+/-	-
Hindbrain		-	+++	+/-	-

Introduction

The PPAR□ protein shows a remarkable conservation cross species with 95% identity at amino acid level between human and rodents (Bernardo and Minghetti, 2008). PPAR□ is expressed in embryonic rodent brain and embryonic NSCs (Wada et al., 2006; Wang et al., 2009). The PPARγ null mouse embryos died at E11 and the size was smaller than the wild-type embryos. It suggested that PPARγ played an important role during embryogenesis. In a postnatal seven-day-old rat brain, PPAR□ is expressed at low levels in cerebellar granule neurons, astrocyte and meningeal fibroblasts (Cullingford et al., 1998). PPAR□ is also expressed in adult neural stem cells (Wada et al., 2006; Miglio et al., 2009).

PPARs are activated by a variety of fatty acids, their acyl CoA derivative esters, eicosanoids, arachidonic acid-derived prostaglandin metabolites (e.g. 15-deoxy-$\Delta^{12,14}$-prostaglandin J2), antidiabetic thiazolidindiones (TZDs), anti- hyperlipidemic fibrates and non-steroids anti-inflammatory drugs (NSAIDs) (Figure 1-7) (Green and Wahli, 1994; Tontonoz et al., 1995; Schoonjans et al., 1996; Kliewer et al., 1997; Cullingford et al., 1998; Spiegelman, 1998; Heneka et al., 1999; Berger and Moller, 2002; Cimini et al., 2005). PPARs form heterodimers with RXRs and the heterodimer subsequently binds to a peroxisome proliferator-activated receptor response element (PPRE) and then regulate the function of targeted genes. In the absence of PPAR ligand or Retinoids, the PPAR/RXR heterodimer occupied PPRE inactively as a gene repressor (Green and Wahli, 1994; Tontonoz et al., 1995; Cullingford et al., 1998; Berger and Moller, 2002; Cimini et al., 2005). In the presence of PPAR ligand or Retinoids, the heterodimer is activated and subsequently stimulates target gene expression which is enhanced by the presence of both PPAR ligand and Retinoid. All nine possible PPAR/RXR heterodimers have been shown to bind the PPRE of target genes (Cullingford et al., 1998).

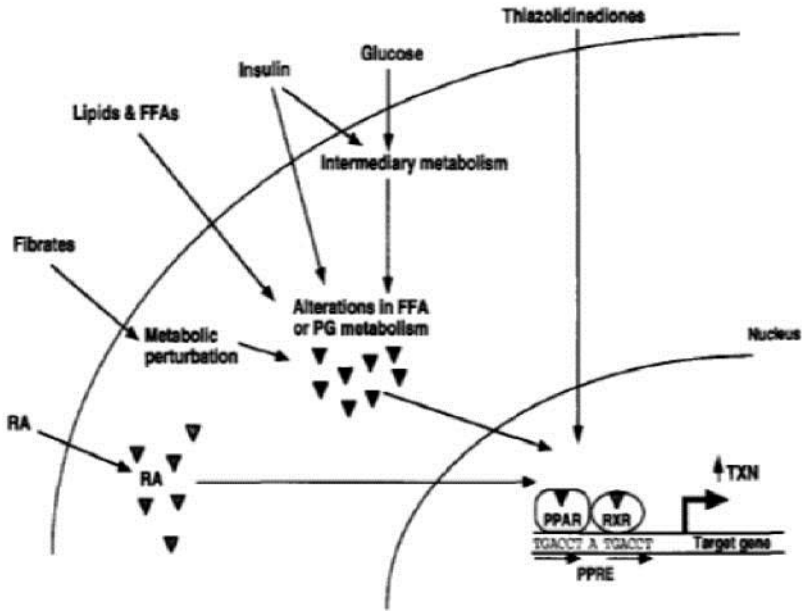


Figure 1-7 The activation of PPAR signalling pathway by multiple physiological stimuli and describing its cross-talk with Retinoic acid.
(Schoonjans et al., 1996)

The impact of PPARγ in neural stem cell models has been studied (Rajasingh and Bright, 2006; Wada et al., 2006; Mo et al., 2010a; Morales-Garcia et al., 2011). The embryonic mNSCs generated from PPARγ $^{+/-}$showed lower growth rate than wild-type that was reversible by treating PPARγ agonist (Wada et al., 2006). PPARγ activation stimulated the growth rate of the wild-type mNSCs via inducing rapid activation of the Erk pathway with subsequent increase in EGF receptor and cyclin B protein levels. PPARγ activation also inhibited the neural differentiation capability of the embryonic mNSCs which was associated with activation of STAT3. The adult mNSCs generated from rat subventricular zone showed that PPAR agonist increased the amount of dividing cells in seven days and increased the numbers and size of neurosphere in ten days (Morales-Garcia et al., 2011). PPARγ antagonist induced the protein level of activated caspase-3 leading to apoptosis (Wada et al., 2006).

PPARγ has been studied by using disease models (Kitamura et al., 1999; Breidert et al., 2002; Brodbeck et al., 2008; Pancani et al., 2009; Escribano et al., 2010b; Yoon et al., 2010; Meng et al., 2011; Lee et al., 2012a; Sadeghian et al., 2012). *In vivo* study showed that the PPARγ agonist reversed the impaired locomotor activity in an adult rat spinal injury model and increased the proliferation of the neural progenitors generated from the spinal injury model after a seven-day treatment of PPARγ agonist (Meng et al., 2011). In Parkinson's

disease animal models, PPARγ activation protected against the degeneration of dopaminergic neurons in striatum induced by 6-OHA (Lee et al., 2012a; Sadeghian et al., 2012) and loss of dopaminergic neurons in the substantia nigra induced by MPTP (Breidert et al., 2002). In Alzheimer's disease animal models, PPARγ agonist rescued the loss of dendritic spine density in hippocampal neurons induced by apolipoprotein E4 (Brodbeck et al., 2008). In amyloid protein over- expressed transgenic mice, PPARγ agonist highly decreased amyloid plaques accumulation in the brain and ameliorated memory loss (Escribano et al., 2010b). Furthermore, it has been discovered that the level of PPARγ protein is increased in the cytosolic fraction of brain from patients suffering from Alzheimer's disease (Kitamura et al., 1999; Pancani et al., 2009; Yoon et al., 2010). Therefore, PPARγ is considered to be a target of potential neuroprotective treatments for neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and multiple sclerosis (Heneka et al., 1999; Breidert et al., 2002; Feinstein et al., 2002; Russo et al., 2003; Brodbeck et al., 2008; Leisewitz et al., 2008; Paintlia et al., 2010; Wang et al., 2011; Sadeghian et al., 2012).

1.6. Aims and Objectives

This project aims to optimise a monolayer system for further study on the proliferation and differentiation capabilities of the adult mNSC primary culture generated from murine lateral ventricle. In the proliferation part, STAT3 activator Colivelin will be used to study the impact of STAT3 activation in the metabolic activity and growth rate of the purified adult mNSCs. It will contribute to determine if STAT3 activation promotes the growth rate of primary adult mNSCs. In the differentiation part, Retinoic acid and a novel synthetic Retinoic acid analogue EC23 will be used to study their effects on neuronal and glial differentiation in a time course dependent manner. It presents an optimised protocol for promoting neuron formation using EC23. Comparing the efficiency of EC23 with Retinoic acid will be able to clarify if EC23 is a better Retinoid to be used in neurogenesis study. In order to increase the percentage of neurons generated in Retinoid containing neural differentiation treatment, PPARγ agonist Rosiglitazone will be used to study if PPARγ activation enhances neurogenesis when adult mNSCs were cultured in combined Rosiglitazone with EC23 neural differentiation medium. It will contribute to determine the

mechanism regulating pro- neurogenic capability of adult mNSC, providing new information to support the development of future treatments for neurodegenerative disease.

Chapter 2. Methods and Materials

2.1. Adult mNSC culture

2.1.1 Adult mNSC culture medium

The adult mNSCs were grown in a serum-free medium, as previously described (Alcock and Sottile, 2009). The medium contained 25 mL Neurobasal medium (no Glutamine and Phenol Red, Gibco®), 25 mL Dulbecco's Modified Eagle Medium (DMEM)-F12 Nutrient Mixture (no Glutamine, Gibco®), 1 mL B27 supplement (50x) (Gibco®), 0.5 mL N2 supplement (100x) (Gibco®), 0.5% Penicillin/Streptomycin (Sigma®), 0.2 % Heparin solution (5 μg/mL) (Stemcell Technologies®) as neural base medium (NB) and freshly added recombinant human FGFb (AA10-155) Lyophilised (10 ng/mL) (Invitrogin®) and recombinant mouse EGF Lyophilised (20 ng/mL) (Invitrogin®) right before applying to adult mNSCs.

2.1.2 Isolation of adult mNSC

The wild type or SOX1 GFP +/- adult mNSC (Aubert et al., 2003) was isolated from the lining of the lateral ventricle of adult mice (Figure 2-1) (Clarke, 2003; Hutton and Pevny, 2008) and the mice were older than 2 months. The brain was extracted from the scull and placed into a 90mm Petri dish filled with phosphate buffered saline (PBS) (Dulbecco A, OXOID®). After the exposure of the lateral ventricle (Figure 2-1) (Hutton and Pevny, 2008), the tissue lining the ventricle was harvested and incubated with 5mL Accumax (Innovate Cell Technologies®) at 37°C for 30 minutes with intermittent agitation. Subsequently, 5mL PBS were added and the suspension was centrifuged for 4 minutes at 200 G. After centrifugation, the cells were resuspended in 5mL PBS and allowed larger fragments to settle. The suspension was transferred to a fresh centrifuge tube and centrifuged for 4 minutes at 200 G. Finally, the cell pellet was resuspended in fresh adult mNSC culture medium and transferred to a T25 cell culture flask.

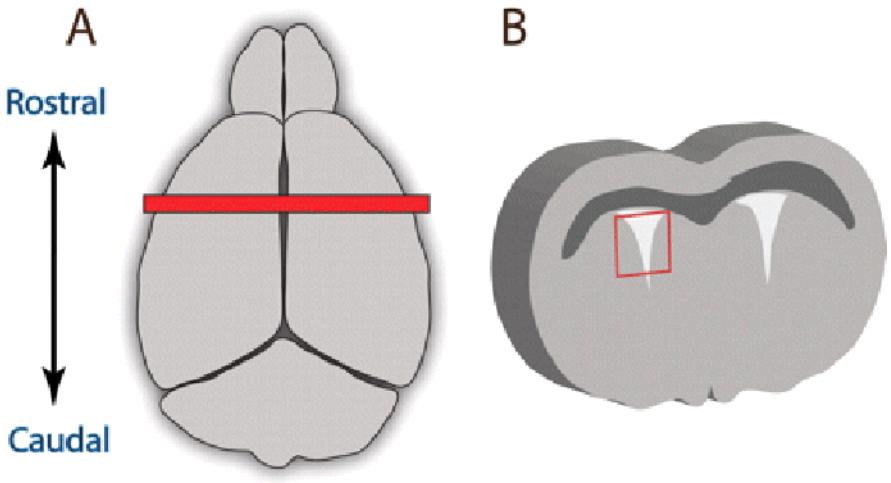


Figure 2-1 Isolating adult mNSC from the lining of lateral ventricle.
(A) Dorsal view of the adult mouse brain. The red bar indicates the location of the cut used to isolate periventricular tissue. (***B***) The resulting cross-section of tissue with lateral ventricles exposed. Periventricular tissue should be dissected where indicated by the red box.(Hutton and Pevny, 2008)

2.1.3 Passaging of adult mNSC

Adult mNSCs were routinely passaged once every 2 or 3 days. The cells were treated with 500µl Accutase (Sigma®) for up to 5 minutes in an cell culture incubator and the cell supernatant were collected and centrifuged for 5 minutes at 200 G. The cell pellet was resuspended in fresh adult mNSC culture medium (see section 1.1 in this chapter above) and transferred to new cell culture flasks.

2.1.4 Cryopreservation of adult mNSC

The adult mNSCs were accutased and collected in PBS and centrifuged for 5 minutes at 200 G. After aspirating the supernatant, the cells were resuspended in ice cold freezing mix (90% NSC medium and 10% Dimethyl sulfoxide (DMSO Sigma®)). The cells were kept at -80°C. For reanimation, the cells were quickly thawed at 37°C and transferred to a centrifuge tube containing 5ml PBS. After centrifugation at 200 G for 5 minutes, the cells were resuspended in 5ml of mNSC culture medium (see section 1.1 in this chapter above) and transferred to a T25 cell culture flask.

2.1.5 Coating Vessels

Three coating agent had been ysed for generating monolayer culture system in this study. Firstly, Gelatin (111M6002, Sigma®) 2.5 mL diluted in 47.5 mL PBS as 0.1% solution. The diluted Gelatin solution then sterilised by using sterile filter. Add enough amount of diluted Gelatin solution into a vessel (e.g. 3mL per T25 or 500 µL per well of a 24-well-plate) and then incubate at room temperature for 10 to 30 minutes. Aspirate the Gelatin solution and then put medium or seed cells in the Gelatin-coated vessel. Secondly, mouse laminin-I (PathClear®) 10 µL diluted in 1 mL cold DMEM F-12. Add enough diluted laminin to coat all the vessels (e.g. 3mL per T25 or 400 µL per well of a 24-well-plate). Checking the shelf of the incubator is level then places the vessels in a 37°C incubator for 4 to 24 hours. Aspirate the laminin solution and wash with warm DMEM F-12 (e.g. 5 mL per T25 or 1 mL per well of a 24-well-plate) at 37°C for at least 5 minutes. The laminin-coated vessels were ready for seeding cells. Thirdly, vitronectin 2 µg diluted in 5 mL PBS. Add enough vitronectin solution into vessels (e.g. 3mL per T25 or 500 µL per well of a 24-well-plate) and incubate at room temperature for 1.5 hour. Aspirate the vitronectin solution and then wash once in PBS at room temperature. The PBS wash would keep until the cells being ready to be seeded.

2.1.6 Colivelin treatments

Four concentrations (100 nM, 100 pM, 100 fM and 1 fM) Colivelin (Tocris Bioscience®) in adult mNSC culture medium (see section 1.1 in this chapter above) were used in the study understanding the impact of STAT 3 activation in the proliferation of adult mNSCs. The SOX1GFP +/- adult mNSCs cultured in adult mNSC culture medium were untreated control group and EGF withdrawal group (NB+bFGF) was proliferation negative control. The cells were resuspended in each treatment and then seeded onto a laminin coated-24-well plate for two days. The density of the cells was $6X10^5$/mL. The cells were ready for further up experiments.

2.1.7 Neural differentiation treatments

There were five different differentiation treatments using in this project (Table 2-1) including basic neural differentiation medium (BNM), Retinoic acid (BNM+RA) (Sigma®), EC23 (BNM+EC23) (Stable Synthetic Retinoid EC23, Reinnervate®), Rosiglitazone (BNM+Z) (Enzo Life Science® ALX-350-125-M025), and Rosiglitazone with EC23 (BNM+ZE) in the BNM. The adult mNSCs were seeded on chemical-coated containers as a monolayer condition and the density of cell is $2X10^4$/mL. After three days settled down, the cells were treated by adult mNSC culture medium (see section 1.1 in this chapter above) for an undifferentiated group or the five different neural differentiation treatments. The media were changed every two days and fixed with cold 4% parafomaldehyde (PFA) (VWR International®) or cold 70% Ethanol (Ethanol Absolute, Analytical reagent grade, Fisher Scientific®) in PBS at the studied time point.

Table 2-1 The formulars of neural differentiation treatments.

Control	Adult mNSC culture medium	NB[a], EGF (10 ng/mL), bFGF (10 ng/mL)
BNM	Basic neural differentiation medium	NB[a], 0.5% FCS[b] (5µL/mL), BDNF[c] (10 µg/mL),
BNM+RA	BNM with Retinoic acid	NB[a], 0.5% FCS[b] (5µL/mL), BDNF[c] (10 µg/mL), Retinoic acid 1 µM (1µL/mL)
BNM+EC23	BNM with EC23	NB[a], 0.5% FCS[b] (5µL/mL), BDNF[c] (10 µg/mL), EC23 1 µM (1µL/mL)
BNM+ ZE	BNM with Rosiglitazone and EC23	NB[a], 0.5% FCS[b] (5µL/mL), BDNF[c] (10 µg/mL), EC23 1 µM (1µL/mL) + Rosiglitazone 0.5 µM (0.5 µL/mL)
BNM+Z	BNM with Rosiglitazone	NB[a], 0.5% FCS[b] (5µL/mL), BDNF[c] (10 µg/mL), Rosiglitazone 0.5 µM(0.5 µL/mL)

a. NB: see section 1.1 in this chapter above

b. FCS: Fatal calf serum, Life Technologies®

c. BDNF human, Source Bioscience®

In a series of serum free experiments, fetal calf serum was removed from the formulas described above.

2.2. Immunocytochemical staining

Adult mNSCs were seeded on gelatin- (0.1% in PBS), laminin- (10 □g/mL DMEM-F12) or vitronectin- coated glass coverslips for immunohistochemical staining and the density of cells was $2X10^4$/mL. The cells were fixed with cold 4% PFA for 30 minutes at room temperature and then preserved in PBS. The cells were washed for 10 min in 0.1% Tween-20 (Sigma-Aldrich®) in PBS (PBT). And then, the cells were incubated in blocking solution (0.1% Tween-20 in PBS, 0.2% FCS) for 30 min at room temperature for attenuating non-specific binding of antibodies. Afterwards, the cells were incubated in the primary antibody diluted in 0.02% FCS blocking solution for 1.5 hour at room temperature. Subsequently, the cells were washed thrice with PBT for 15 min at room temperature and then incubated in the secondary antibody diluted in 0.02% FCS blocking solution for 1 hour in the dark. After washing thrice with PBT for 15 min at room temperature, the glass coverslips were mounted on glass slides with Vectrashield® Mounting Medium containing DAPI (H-1200, Vectrolabs®). The slides were stored at room temperature in the dark at least four hours and fluorescent image were taken by a Nikon ECLIPE 90i Fluorescence Microscope, a Hamamatsu Digital Camera and Volocity® software (version 5.2.0, Improvision). The brands and concentrations of primary and secondary antibodies referred to Table 2-2.

Table 2-2 The source and concentration of primary and secondary antibodies.

Antibody	Product information	Concentration
Nestin	Rat-401®, Developmental Studies Hybridoma Bank	1:50
GFAP	Z0334®, Dako	1:200
B-III tubulin	D71G9 XP® mAb #5568, Cell Signaling	1:100
Fict rabbit	Fluorescein Goat Anti-Rabbit IgG Antibody FI-1000®, Vector Laboratories	1:200
Fict mouse	Fluorescein Horse Anti-Mouse IgG Antibody FI-2000®, Vector Laboratories	1:200
Texas red rabbit	Texas Red Goat Anti-Rabbit IgG Antibody TI-1000®, Vector Laboratories	1:200
Texas red mouse	Texas Red Horse Anti-Mouse IgG Antibody TI-2000®, Vector Laboratories	1:200

2.3. Flow cytometry

Adult mNSCs were seeded in a laminin-coated T25 cell culture flask for flow cytometry and the density of cells was $2X10^4$/mL. The cells were trypsinised (0.05% trypsin-EDTA, Life Technologies®) and then incubated at 37°C up to 5 minutes. Subsequently, the

cells were treated by 40% FCS in PBS in order to stop trypsinisation. The cells were quickly fixed with cold 4% PFA for 30 minutes at room temperature and then preserved in PBS. Subsequently, the cells were centrifuged for 5 minutes at 3500 rpm which centrifugation criteria was also used for cells collection in the following procedures. The cells were washed for 10 minutes in 0.1% Tween-20 in PBS (PBT). The cells were incubated in blocking solution (0.2% FCS in 0.1% Tween-20 in PBS) for 1 hour at room temperature for attenuating non-specific binding of antibodies. Afterwards, the cells were incubated in the primary antibody (Nestin see Table 2-2) diluted in blocking solution for 1.5 hour at room temperature. Subsequently, the cells were washed three times with PBT for 20 min at room temperature and then incubated in the secondary antibody (Fict mouse see Table 2-2) diluted in blocking solution (1:400) for 1 hour in the dark. After washing three times with PBT for 15 min at room temperature, the cells were analysed by GUAVA® flow cytometer. The results were analysed on biological duplicates using Weasel® software.

2.4. Cell counting

2.4.1 Dapi positive cell nuclei counting

The Dapi positive cell nucleus counting was manually calculated from 5 individual images taken from each sample to determine the number of Dapi positive cell nuclei in one field of view. The results of two identical experiments were presented in the result section.

2.4.2 Percentage of β-III tubulin positive neurons

The 3-III tubulin positive neuron counting was calculated from 5 individual images taken from each sample to determine the number of cells in one field of view from two identical experiments. The percentage of 3-III positive neuron was calculated by the numbers of 3-III tubulin positive neuron divided by the numbers of Dapi positive cell nucleus from the same image.

2.4.3 Cell counting for cell growth rate study

mNSCs were seeded on laminin coated-24-well plates at a density of $2X10^4$/mL. After three days, the cells were treated with adult mNSC culture medium (see section 1.1 in this chapter above) as undifferentiated control group or the 5 different neural differentiation treatments described in Table 2-3. The cells were trypsinised and then incubated at 37°C up to 5 minutes. Subsequently, the cells were treated by 40% FCS in PBS in order to stop trypsinisation. 10 □L supernatant of cells were counted using a Haemocytometer and cell numbers calculated in quadruplicates from the same batch at each condition and time point.

2.5. PrestoBlue cell viability assay

PrestoBlueTM Cell Viability Reagent (Invitrogen®) is a ready-to-use reagent for rapidly evaluating the viability and proliferation of a wide range of cell types. PrestoBlueTM reagent is quickly reduced by metabolically active cells, providing a quantitative measure of viability and cytotoxicity in as little as 10 minutes (Boncler et al., 2014). In high metabolic rate cells, the dye showed red fluorescent because of oxidation whilst the colour of the dye remains blue in low metabolic rate cells. Protect reagent from long term exposure to light and avoid contamination, as this can reduce sensitivity.

In a 24- well plate format, added 350 µL of warm PrestoBlue solution (10% PrestoBlue reagent diluted in Hank's Balanced Salt Solution (HBSS, Invitrogen®)) to each well and one well for blank. The samples incubated in a cell culture incubator up to 25 minutes. 100 µL extract from each sample well was transferred to a flat 96-well plate in triplicates. Fluorescence is measured by using 560 nm excitation and 590 nm emission filters on a fluorescence plate reader (FLx800 micro-plate fluorescent reader, Bio-TEK®).

2.6. Statistics

Results were analysed with GraphPad Prism 6®. Ddata are shown as mean ± the standard error of the mean and are compared with One-way ANOVA. A p value < 0.05 is considered statistically significant and is symbolised **, p value < 0.001 is *** and p value < 0.0001 is ****.

Chapter 3. Results

It has been reported that the NSCs generated from the lining of the lateral ventricle of neonatal mice can be maintained using EGF and bFGF. (Reynolds et al., 1992; Reynolds and Weiss, 1992; Morshead et al., 1994; Gritti et al., 1995; Weiss et al., 1996; Martens et al., 2000; Rietze et al., 2001). The experiments described in this study were performed using the primary culture of mNSCs generated from the lining of the lateral ventricle of adult mice, after purification as described in 2.1.2 Isolation of adult mNSC. After the optimisation of monolayer cultures, primary from the lining of the lateral ventricle of adult mice their proliferation and differentiation capabilities were studied.

3.1 The expansion of the primary culture of adult mNSCs in a monolayer system.

3.1.1 Optimisation of monolayer adult mNSC culture.

A range of substrates, such as gelatin, laminin and vitronectin, have been used as coating agents for attaching cells onto an experimental vessel. It has been known that the nature of the coating agent may affect the survival and differentiation of stem cells (Russo et al., 2003; Qian and Saltzman, 2004; Bigdeli et al., 2008; Chen et al., 2011). In order to generate a monolayer cell culture system, gelatin, laminin and vitronectin had been tested. Adult mNSCs showed different seeding patterns whilst cultured on gelatine- (Figure 3-1 (A) and (D)), laminin- (Figure 3-1 (B) and (E)) or vitronectin-coated (Figure 3-1(C) and (F)) glass coverslips. In gelatin-coated conditions, the cell bodies of adult mNSCs formed a three dimensional structure as a cluster (indicated with * in Figure 3-1 (A) and (D)). The cells formed several clusters on a single glass coverslip. It was hard to observe the morphology and processes of individual cells under a bright-field microscope (Figure 3-1 (A)) or fluorescent microscope after conducting immunocytochemical staining (Figure 3-1 (D)). The adult mNSCs on laminin coating were spread evenly and appeared as a monolayer culture on a single glass coverslip (Figure 3-1 (B) and (E)). The adult mNSCs showed homogenous spindle-shape cell bodies which made it possible to observe the distribution, morphology and

process of individual cells. In vitronectin-coated environment, the cell bodies of adult mNSCs overlapped (indicated with ↓ in Figure 3-1 (C) and (F)) which made it difficult to observe the morphology and process of a single cell. Comparing Figure 3-1 (F) with (D) and (E), GFAP-positive astrocytes were only detectable on vitronectin-coated environment. It suggested that the adult mNSCs might start to differentiate under these conditions. Also, the cells on vitronectn looked unhealthy, which would not be an ideal model for a long time course research. Therefore, laminin provided a more suitable environment for the adult mNSCs to form monolayer which helps in studying the cell morphology changes in further differentiation studies.

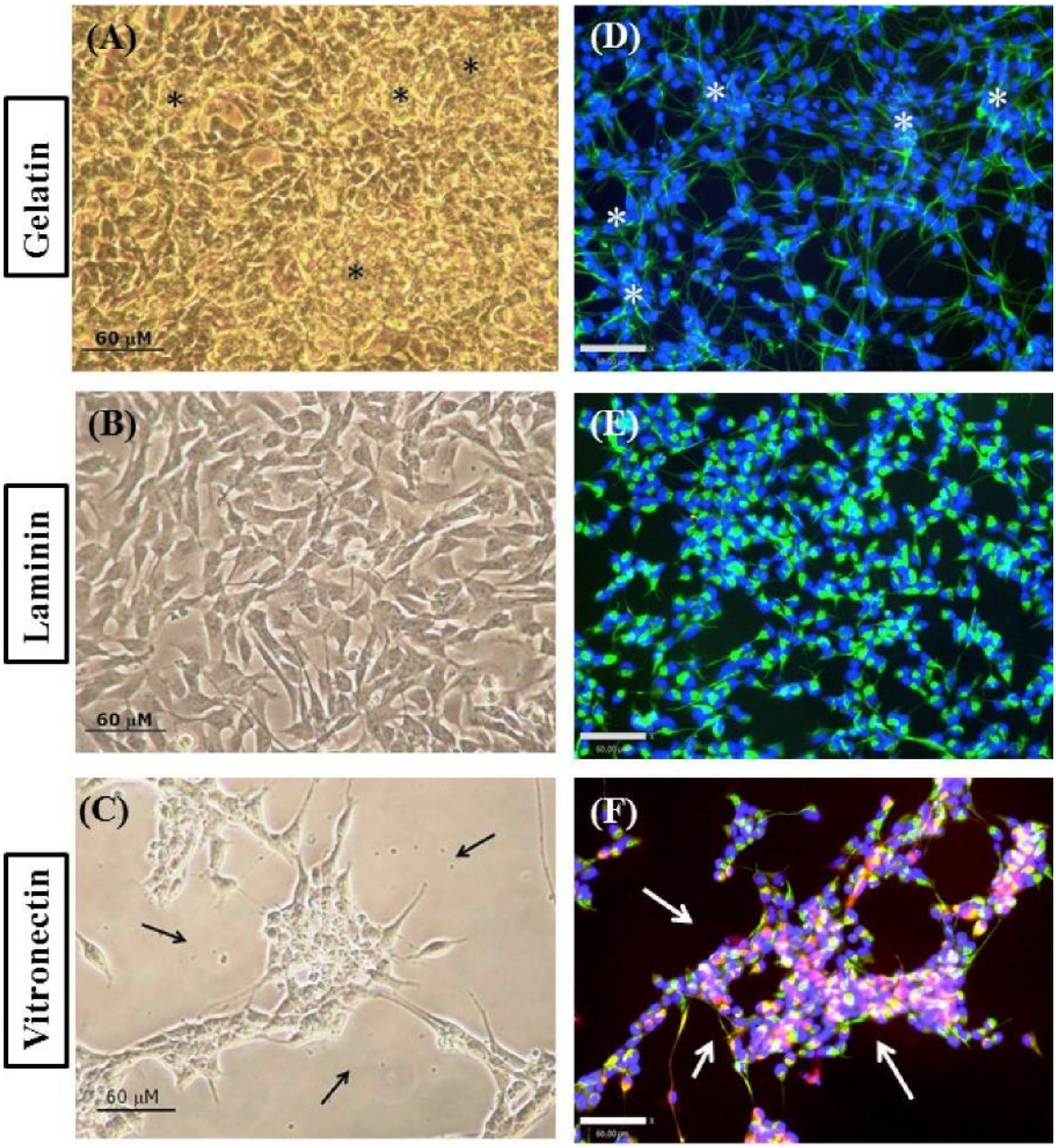


Figure 3-1 Adult mNSCs showed different patterns on different coating agents.
(A), (B), and (C) were taken under a bright-field microscope after the adult mNSCs were seeded for three days. (D), (E), and (F) represent the immunocytochemical staining of Nestin (green), GFAP (red), and Dapi counterstain (blue). The scare bars were 60 µm. * showed the clusters of cell bodies on a gelatine-coated environment, and ↓ showed the cell bodies overlapped with each other on a vitronectin-coated environment. This experiment has been done twice.

Stem cells are able to proliferate when they were cultured in suitable media. The mNSCs generated from the lining of the lateral ventricle have been known to be maintained in EGF and bFGF containing condition (Reynolds et al., 1992; Reynolds and Weiss, 1992; Morshead et al., 1994; Gritti et al., 1995; Weiss et al., 1996; Martens et al., 2000; Rietze et al., 2001; Alcock and Sottile, 2009). This part of experiments studied the time point at which

adult mNSCs doubled in numbers and the impact of STAT3 activation in the growth rate of adult mNSCs primary culture.

3.1.2 The growth rate of adult mNSCs.

Adult mNSCs generated from SOX1GFP+/- mice (Aubert et al., 2003) were used in this part of experiments. In SOX1GFP+/- mice, the GFP positive region located SOX1-positive neural stem cells and SOX1 is one of the markers to identify neural stem cells (Wegner, 1999; Wood and Episkopou, 1999; Bylund et al., 2003; Graham et al., 2003; Zhao et al., 2004; Pevny and Placzek, 2005; Wegner and Stolt, 2005; Kiefer, 2007; Holmberg et al., 2008). The purpose of using SOX1GFP+/- adult mNSCs is to study the SOX1 expression dynamic during differentiation in the future. The results are shown by calculating cell numbers in 4 replicates of wells in 24-well plates. The numbers of cell at linear phase (24, 36, 48 and 60 hours) were plotted and the linear equation describing the data points distribution was calculated (Figure 3-2). The results indicated that SOX1GFP+/- adult mNSCs have a doubling time of 19.39 hours in linear phase. Thus, the primary culture generated from the lining of the lateral ventricle of SOX1GFP+/- adult mice could be expended to double the number in 19.39 hours when the cells were cultured in adult mNSC culture media (referred to section 2.1.1 Adult mNSC culture medium).

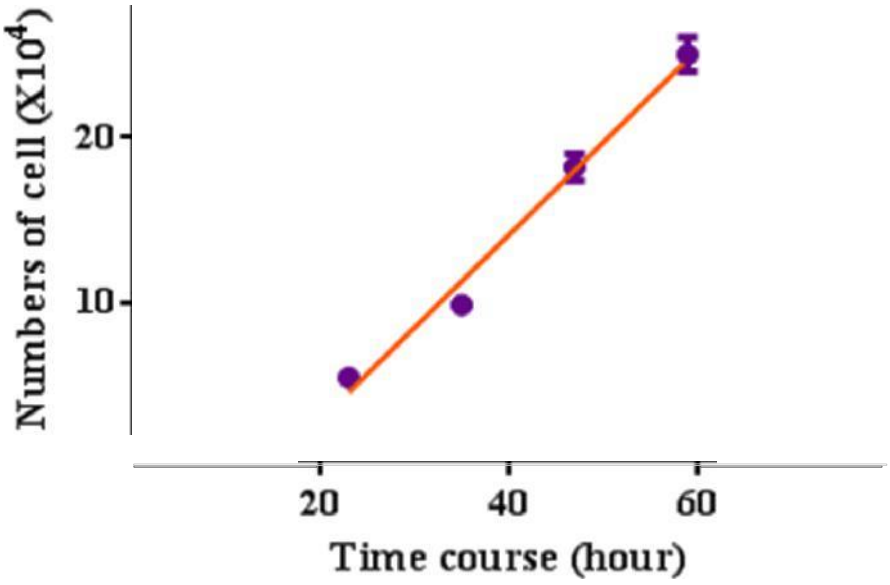


Figure 3-2 The growth rate of adult mNSCs generated from the lateral ventricle.
Adult mNSCs were seeded onto laminin-coated vessels. The cells were harvested and counted every 12 hours. The numbers of quadruplicates were used to fit a growth rate equation.The growth rate equation was Y = 0.5556*X - 8.772 (r^2 =0.99), which indicated that adult mNSCs doubling time is 19.39 hours. The data is represented as mean ± the standard error of the mean, and the equation was fitted by using GraphPad Prism 6®.

3.1.3 The impact of STAT3 activation on metabolic activity of adult mNSCs.

STAT3 is one of the well-known signalling pathways being involved in stem cell proliferation (Takeda et al., 1997; Matsuda et al., 1999; Cheng et al., 2011; Hutchins et al., 2013; Natarajan et al., 2014). Colivelin, a novel synthetic peptide which acts as a STAT3 activator, has been used to study the impact of STAT3 signalling in neurodegenerative disease models because it is potent at an fM range in neural protection studies (Chiba et al., 2005; Matsuoka et al., 2006; Yamada et al., 2008).The NB+FGF group is a negative control of adult mNSCs proliferation (Figure 3-3). In Colivelin- (100 nM, 100pM, 100fM and 1 fM) treatments, Colivelin did not increase the metabolic activity of the SOX1GFP+/- adult mNSCs. It suggested that the exogenous application of Colivelin did not promote the metabolic activity of the adult mNSCs. It hinted that Colivelin-induced STAT3 activation might not subsequently promote the proliferation of the SOX1GFP+/- adult mNSCs.

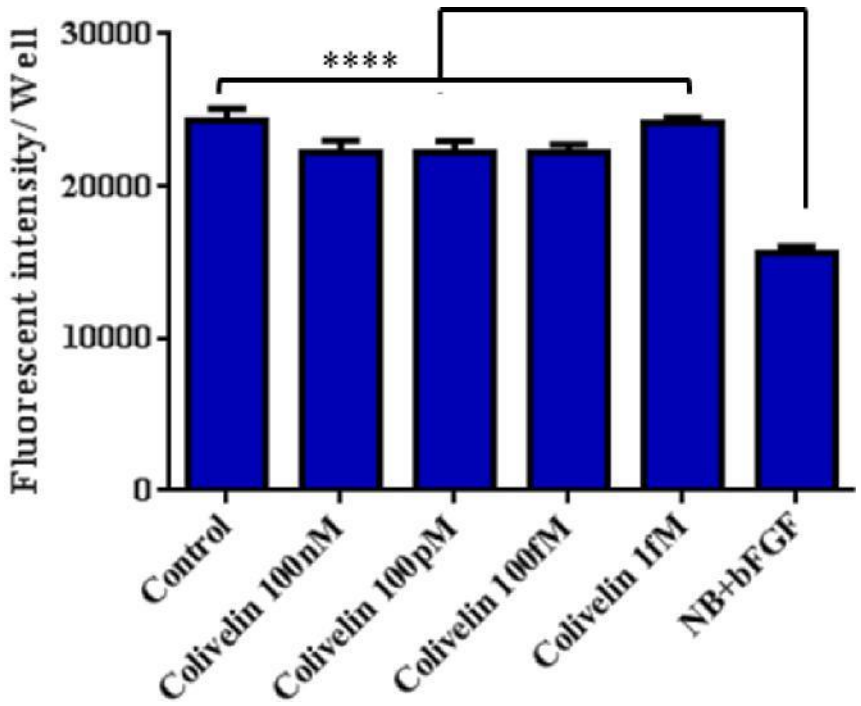


Figure 3-3 The metabolic viability of adult mNSCs under STAT3 activation.
This graph is showed the metabolic activity after a two-day treatment of a STAT3 agonist, Colivelin, from 100nM to 1fM. The results of three identical experiments with triplicates presented mean ± standard error of the mean. After running a one-way ANOVA analysis, there was no statistically significant difference from the control group and the Colivelin (100 nM ~ 1fM) treated groups.

3.2. Comparing the impact of EC23 on neurogenesis and cell growth with Retinoic acid.

The differentiation capability is another character to identify stem cell. This section illustrates that the primary culture of adult mNSCs were capable of differentiating into neurons and glia under differentiation treatments (refer to 2.1.7 Neural differentiation treatments) by conducting immunocytochemical staining. It also compared the expression level of Nestin, a NSC marker of untreated adult mNSCs in the differentiation treated-cells, by executing flow cytometry.

Retinoic acid as a morphogen plays a significant role during embryogenesis, especially in neuronal patterning, neural differentiation and axon outgrowth (Bain et al., 1995; Kastner et al., 1995; Maden, 2007; Lu et al., 2009; Elizalde et al., 2010). Retinoic acid has been used for promoting neurogenesis from NSCs *in vitro* (Weiss et al., 1996; Goffredo et al., 2008; Kim et al., 2009; Lu et al., 2009; Maltman et al., 2009; Christie et al., 2010; Lee et al., 2012b). However, Retinoic acid is photo-sensitive. A novel synthetic analogue, EC23, has

been released recently (Maltman et al., 2009; Gluyas et al., 2012). This part of the project focused on comparing the impact of EC23 on neurogenesis with Retinoic acid. Adult mNSCs were seeded at a density of $2X10^4$/mL on laminin-coated conditions. After three days, the cells were treated with NB, BNM, BNM+RA (1.iM) or BNM+EC23 (1.iM) as described in 2.1.7 Neural differentiation treatments (see Table 2-1).

3.2.1 Neurogenesis of adult mNSCs under Retinoic acid or EC23 treatment.

Under a BNM+RA treatment, some J3-III tubulin-expressing neurons were detectable from day four (data not show), day five (Figure 3-4 (A) green) and day seven (Figure 3-4 (B) green). However, the J3-III tubulin-expressing neurons became undetectable on day eleven (Figure 3-4 (C) green) and day thirteen (Figure 3-4 (D) green). In the BNM +EC23- treated cells, some J3-III tubulin-expressing neurons were detectable from day four (data not show), day five (Figure 3-4 (F) green), day seven (Figure 3-4 (G) green), day eleven (Figure 3-4 (H) green) and day thirteen (Figure 3-4 (I) green). Comparing the morphology of J3-III tubulin-positive neurons on day five and day seven with day eleven and thirteen, the J3-III tubulin-positive neurons showed long neurites on day five and seven but short neurites on day eleven and thirteen. In the untreated adult mNSC, only Nestin-expressing cells were detectable (Figure 3-4 (J) red) but not J3-III tubulin- positive cells. It suggested that BNM+RA or EC23 induced adult mNSCs to produce neurons from day four and the J3-III tubulin expressing neurons survived until day seven in BNM+RA but day thirteen in BNM+EC23.

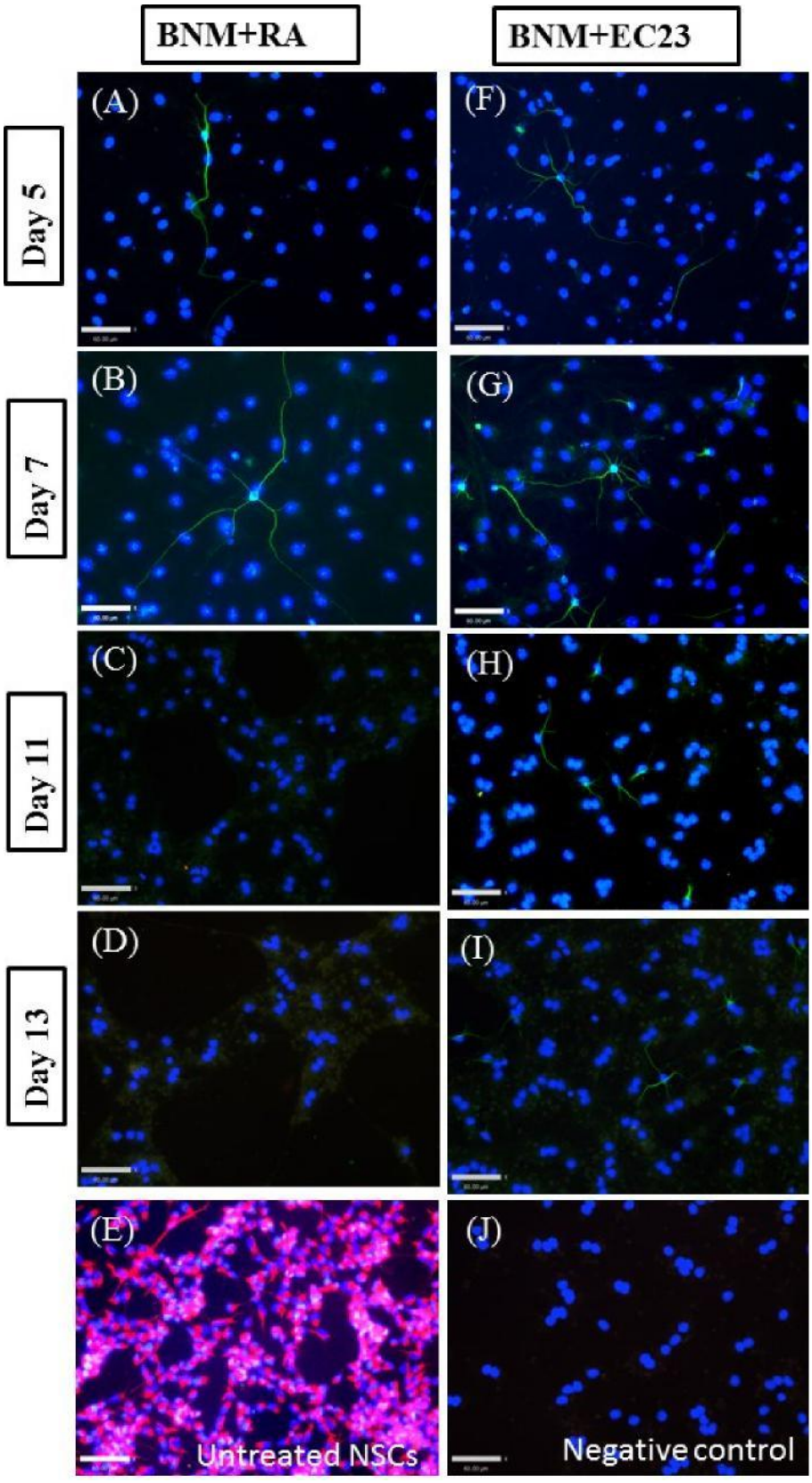


Figure 3-4 Retinoic acid and EC23 promoted neurogenesis in a differentiation time course.
(A)~(I) are expression of □-III tubulin (green), Nestin (red), and Dapi counterstain (blue) in a series of double immunocytochemical staining. (J) illustrated immunocytochemical staining without primary antibody, controlling for possible auto-fluorescence from the cells. The scare bars were 60 μm. The results constatntly showed in two identical experiments.

After counting the number of Dapi-stained nuclei and J3-III tubulin-positive neurons, it showed the percentage of J3-III tubulin-positive neurons under BNM+RA or BNM+EC23 on each differentiation time point (Figure 3-5). Under BNM+RA treatment, the percentage of J3-III tubulin-positive neurons slightly decreased from day five (Figure 3-5 BNM+RA blue) to day seven (Figure 3-5 BNM+RA red) without reaching statistically significance but disappeared on day eleven and thirteen (Figure 3-5 BNM+RA green and purple). In BNM+EC23, it showed a trend that J3-III tubulin-positive neurons decreased from day five to day eleven (Figure 3-5 BNM+EC23 blue, red and green) but increased on day thirteen (Figure 3-5 BNM+EC23 purple). Comparing the percentage of J3-III tubulin-positive neurons under BNM+RA on day five (Figure 3-5 blue) and day seven (Figure 3-5 red) with BNM+EC23, the BNM+EC23-treated cells showed slightly higher percentage of J3-III tubulin-positive neurons than BNM+RA on both days but no statistically significant difference. However, the J3-III tubulin-positive neurons survived longer when cultured in BNM+EC23 than in BNM+RA.

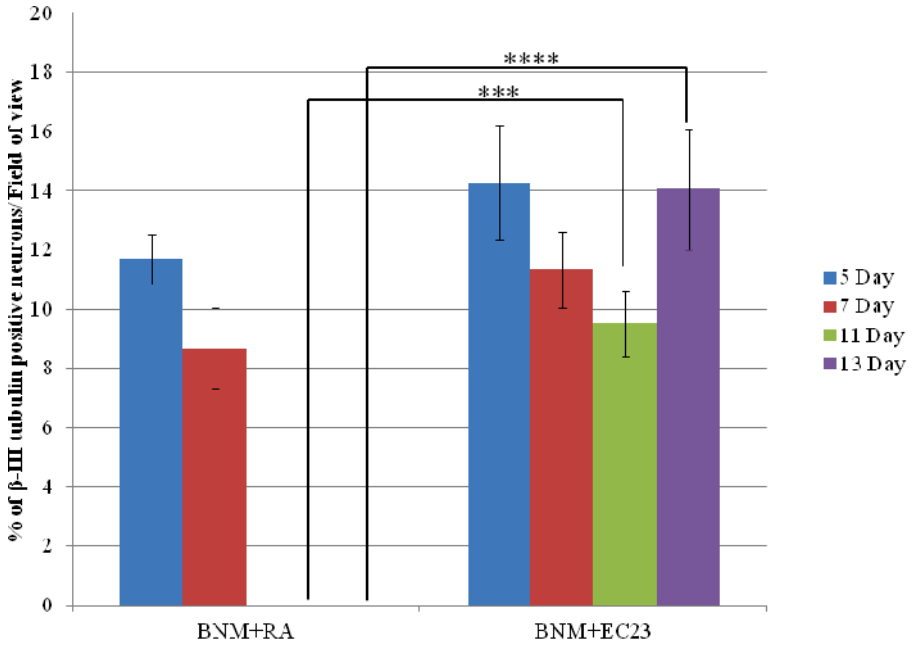


Figure 3-5 The percentage of β-III tubulin positive-neuron under BNM+RA or BNM+EC23 on day five, seven, eleven and thirteen.
The numbers are shown by mean ± standard error of the mean of ten different fields of view. The results of one way ANOVA analysis showed P value *** < 0.001 and **** < 0.0001. BNM+RA was basic neural differentiation medium with Retinoic acid, and BNM+EC23 was basic neural differentiation medium with EC23.

3.2.2 Gliogenesis of adult mNSCs under Retinoic acid or EC23 treatment.

GFAP is a frequently used marker in identifying astrocytes (Maunoury et al., 1977) so that GFAP staining was used to determine if gliogenesis had occurred when the differentiation conditions were tested. Some GFAP-positive astrocytes were detectable under BNM+RA treatment from day four (data not show) to day thirteen (Figure 3-6 red (A) day five, (B) seven, (C) eleven and (D) thirteen). It was also observed under BNM+EC23 from day four (data not show) to thirteen (Figure 3-6 red (F) day five, (G) seven, (H) eleven and (I) thirteen). However, only Nestin was detectable in untreated adult mNSCs (Figure 3-6 green (J)) but not GFAP. Thus, some adult mNSCs differentiated into astrocytes under Retinoic acid or EC23 treatment. It also suggested that the population of only Dapi-positive cells showed in Figure 3.4 (Figure 3-4 (A) ~ (D) and (F) ~ (I) blue) had very high tendency to be GFAP-positive astrocytes as shown in Figure 3.6 (Figure 3-6 (A) ~ (D) and (F) ~ (I) red). In order to proof that, it acquired to conduct a series of double immunohistochemical staining by using □-III tubulin and GFAP at the same time.

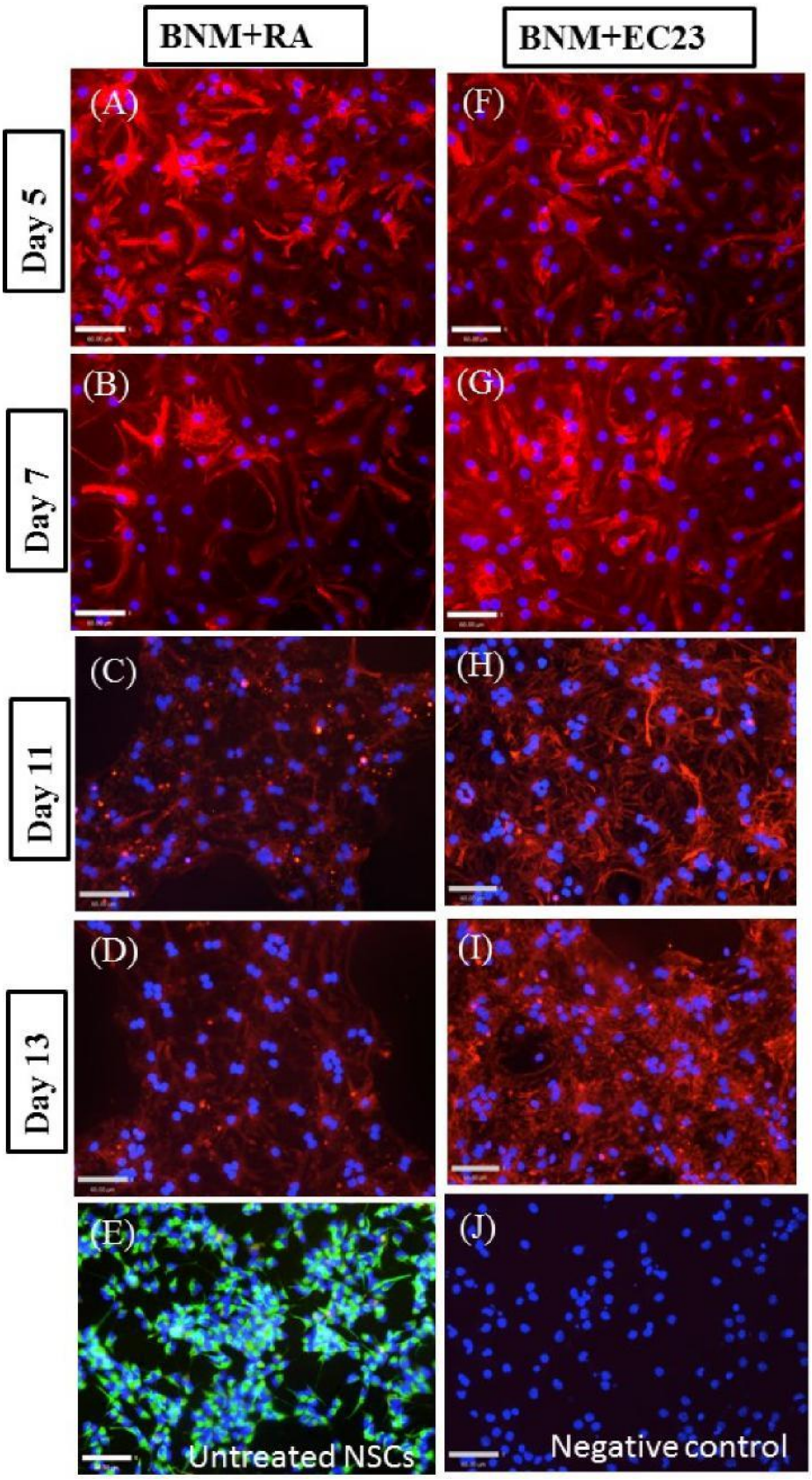


Figure 3-6 Retinoic acid and EC23 promoted glia formation in a time course differentiation.
(A)~(I) are double immunocytochemical staining images of GFAP, Nestin (green), and Dapi counterstain (blue). (J) illustrated staining without primary antibody, eliminating possible false-positive signals from auto-fluorescence of cells. The scare bars were 60 μm. The same results were observed in two identical experiments.

3.2.3 The expression of Nestin under Retinoic acid or EC23 treatment.

Nestin is a marker for undifferentiated NSCs (Lendahl et al., 1990). Upon differentiation, Nestin is downregulated and is replaced by cell type-specific intermediate filaments, such as neurofilaments and GFAP. Under BMN+RA or BNM+EC23 treatments, Nestin-positive cells were hardly observed after a five-day treatment (Figure 3-4 (A) and (F), Figure 3-6 (A) and (F)). Flow cytometry analysis would be able to show the alteration of Nestin expression under Retinoid treatments. In BNM+RA, the histogram of Nestin expression shifted toward unstained cells after day one, two and three of treatment (Figure 3-7 (A) compared green, red and blue with black). BNM+RA treatment altered the expression level of Nestin and decreased from 67.7% on day one to 35.4% on day three (Figure 3-7 (B) purple). The expression of Nestin in the untreated adult mNSCs slightly decreased in three days from 92.6% at day one to 79.0% at day three (Figure 3-7 (B) orange). However, this set of experiment was conducted with biological duplicates and no statistically significance was detected between the untreated adult mNSCs and BNM+RA-treated cells at each time point. It however suggested that Retinoic acid showed a tendency to decrease the expression level of Nestin at an early time point of differentiation.

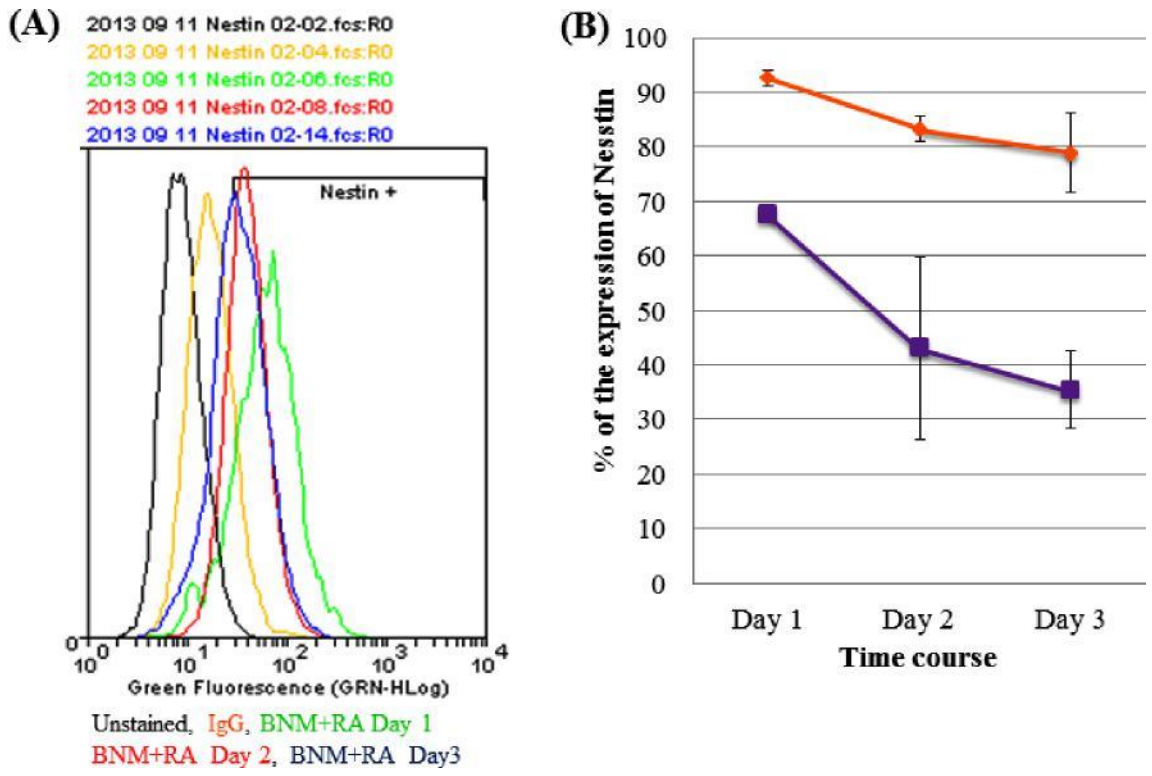


Figure 3-7 The expression level of Nestin under BNM+RA in an early time course.
(A) illustrated the flow cytometry histograms of Nestin expresion (% of Nestin positive cells after being stained by fluorescent) of one set of results, and green showed day one, red was day two and blue was day three after BNM+RA treatment. (B) illustrated the statistics of results of duplicates. The orange line illustrated the expression level of Nestin in the untreated adult mNSCs and the purple line was BNM+RA-treated-cells in a three day-time course. The statistic was presented by mean □ standard error of the mean. The one-way ANOVA analysis indicated that no statistically significant difference from the untreated and BNM+RA-treated cells at each time point (p value: Day 1 =0.361, Day 2 = 0.082 and Day 3 = 0.059).

In Figure 3-7, the expression level of Nestin started to decrease under BNM+RA and day two showed more severe decreased than day one. In an observation under a bright-field microscope, the neural differentiation treated-cells started to show different morphology than the untreated adult mNSC on day two (data not show). It indicated that day two would be a suitable early time point to analyse change in the expression level of Nestin under neural differentiation treatments. On day two, the histogram of Nestin expression under BNM (Figure 3-8 (A) blue), BNM+RA (Figure 3-8 (A) green) and BNM+EC23 (Figure 3-8 (A) purple) showed lesser Nestin-positive area than mNSCs (Figure 3-8 (A) red). The expression level of Nestin in the untreated adult mNSCs was 83.3% (Figure 3-8 (B) red). It decreased by 40%~50 % after a two-day treatment of BNM, (Figure 3-8 (B) blue) BNM+RA (Figure 3-8 (B) green) or BNM+EC23 (Figure 3-8 (B) purple). It showed that the expression level of Nestin had a tendency to decrease as early as day two when the cells were cultured in all three neural differentiation treatments. However, it represented the results of biological

duplicates, and that was no statistically significance between the untreated cells and the adult mNSCs under the three different neural differentiation treatments.

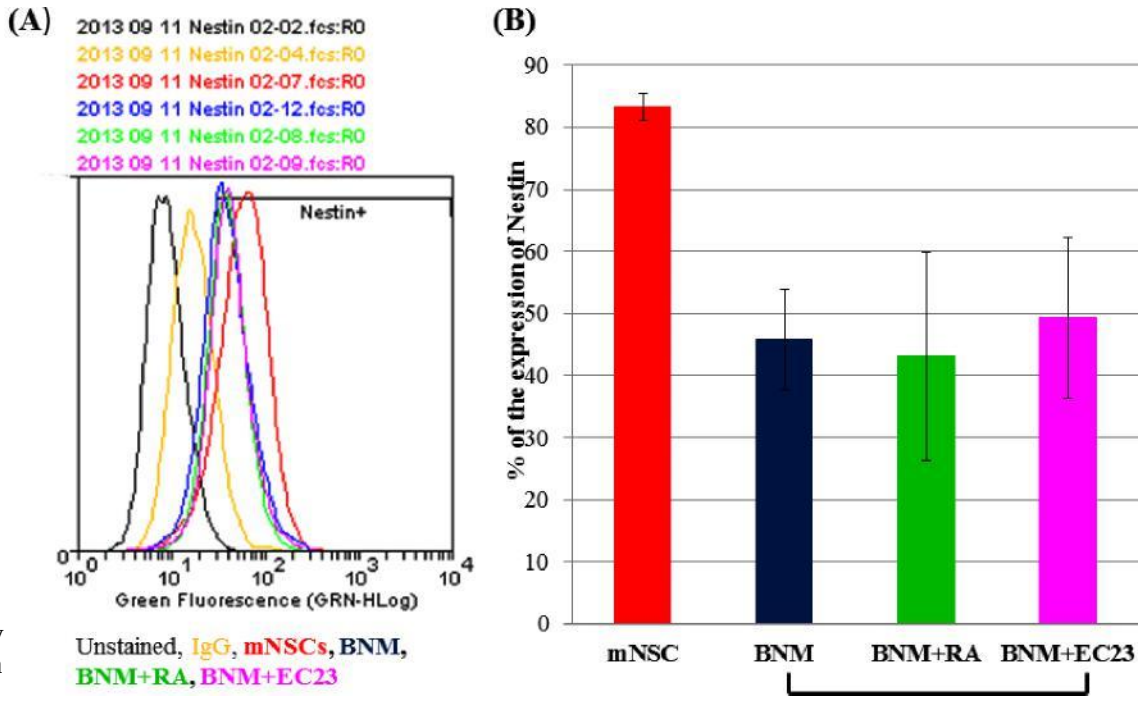


Figure 3-8 The expression level of Nestin in the untreated adult mNSCs or after a two-day treatment of BNM, BNM+RA or BNM+EC23. (A)illustrated the flow cytometry histograms of the expression level of Nestin (% of Nestin positive cells after being stained by fluorescent) from one set of data. Red was untreated adult mNSCs for setting up Nestin-positive scale. (B) illustrated the statistics of the results of duplicates. Red illustrated the expression level of Nestin in untreated adult mNSCs and blue illustrated the expression level of Nestin in BNM, green was BNM+RA, and purple was BNM+EC23.The statistic was presented by mean ± standard error of the mean. However, there was no statistically significance comparing untreated adult mNSCs to the three-treated groups. BNMwas basic neural differentiation medium, BNM+RA was basic neural differentiation medium with Retinoic acid and BNM+EC23 was basic neural differentiation medium with EC23(p value: mNSC vs BNM = 0.588, mNSC vs BNM+RA = 0.54, and mNSC vs BNM+EC23 = 0.615).

3.2. 4 The growth rate of the Retinoids-treated adult mNSCs.

The results of immunocytochemical staining (Figure 3-4 and Figure 3-6) showed that the density of the neural differentiation treated-cells was lower than the untreated adult mNSCs. A cell counting experiment was conducted to monitor the change in numbers of cell under a series of neural differentiation treatments. The neural differentiation treatments started at day zero. After a two-day culture in BNM, the number of treated cells remained to that of untreated adult mNSCs at day zero (Figure 3-9 red Day 2); the same effect was observed in BNM+RA- (Figure 3-9 green Day 2) and BNM+EC23-treatement (Figure 3-9

purple Day 2). The numbers of treated-cells showed a decrease on day three and day four. The numbers of cell treated by BNM+RA decreased the most between day two and day four (Figure 3-9 green). It indicated that Retinoic acid attenuated the growth of adult NSCs the most.

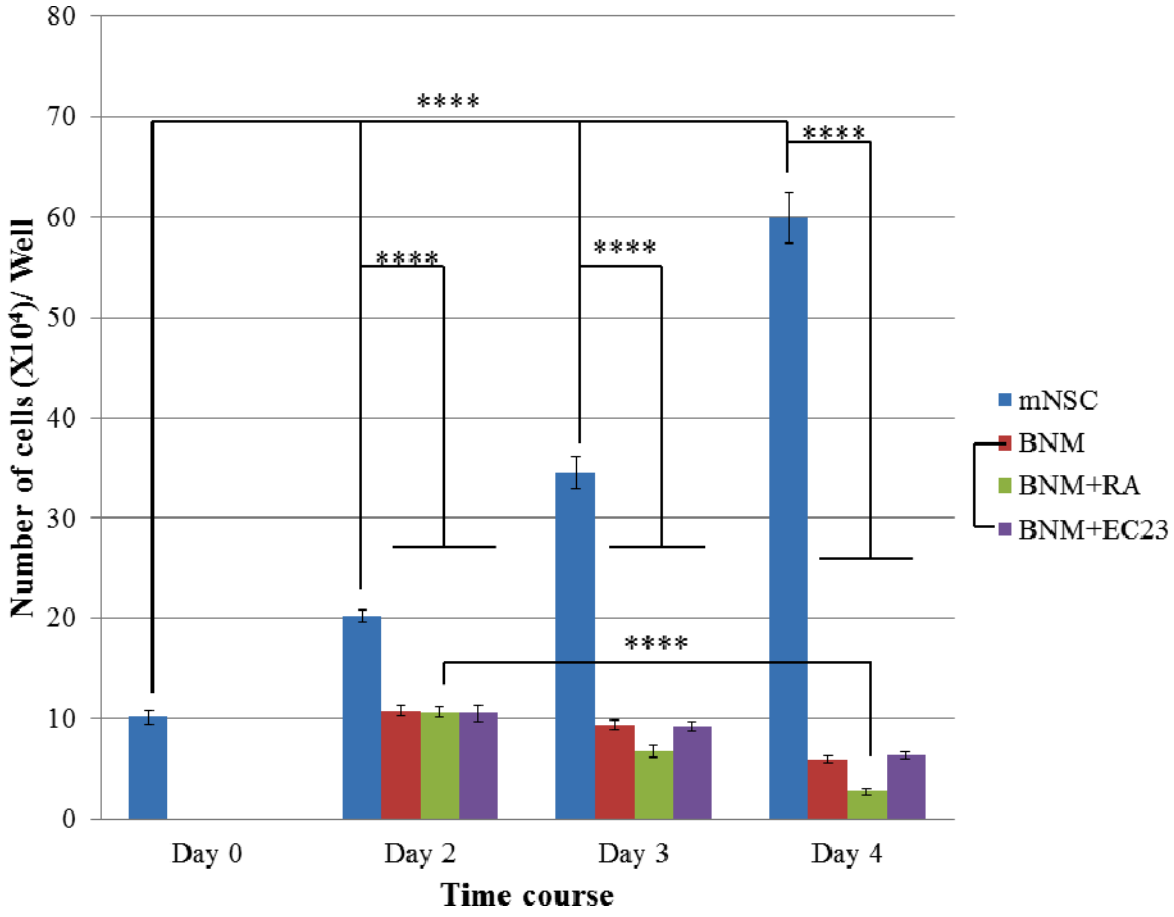


Figure 3-9 The numbers of the untreated adult mNSCs or treated by BNM, BNM+RA or BNM+EC23 from day two to day four. The results of quadruplicates are shown as mean ± standard error of the mean. **** indicated the p value < 0.0001 by a one-way ANOVA analysis. (P values: Day 3 BNM vs BNM+RA was 0.81and BNM+RA vs BNM+EC23 was 0.86. Day 4 BNM vs BNM+RA was 0.34 and BNM+RA vs BNM+EC23 was 0.54.)

3.2.5 The influence of serum in Retinoic acid-induced neurogenesis of adult mNSCs .

Serum has been reported to be used as a main element in Retinoic acid-induced neurogenesis (Weiss et al., 1996; Goffredo et al., 2008; Kim et al., 2009; Lu et al., 2009;

Maltman et al., 2009; Christie et al., 2010; Lee et al., 2012b). In the results of neural differentiation study above (refer to Figure 3-4 and Figure 3-10), f3-III tubulin-positive neurons were undetectable in BNM+RA conditions on day eleven (Figure 3-4 (C) and Figure 3-10 (A)) and thirteen (Figure 3-4 (D) and Figure 3-10 (B)).It suggested that the formula of BNM+RA might not support the survival of neurons on day eleven and thirteen. Therefore, a foetal calf serum free neural differentiation formula was tested. Surprisingly, some f3-III tubulin-positive neurons were detectable under serum free BNM+RA on day eleven (Figure 3-10 (C) green) and thirteen (Figure 3-10 (D) green) which was not seen in the untreated adult mNSCs (Figure 3-10 (I)). In BNM+EC23, there were more Dapi-positive nuclei and β-III tubulin-positive neurons being observed in serum free conditions than regular BNM+EC23 on day eleven and thirteen (Figure 3-10 compared (E) to (G) and (F) to (H)). It indicated that serum was not required in Retinoid-induced neurogenesis.

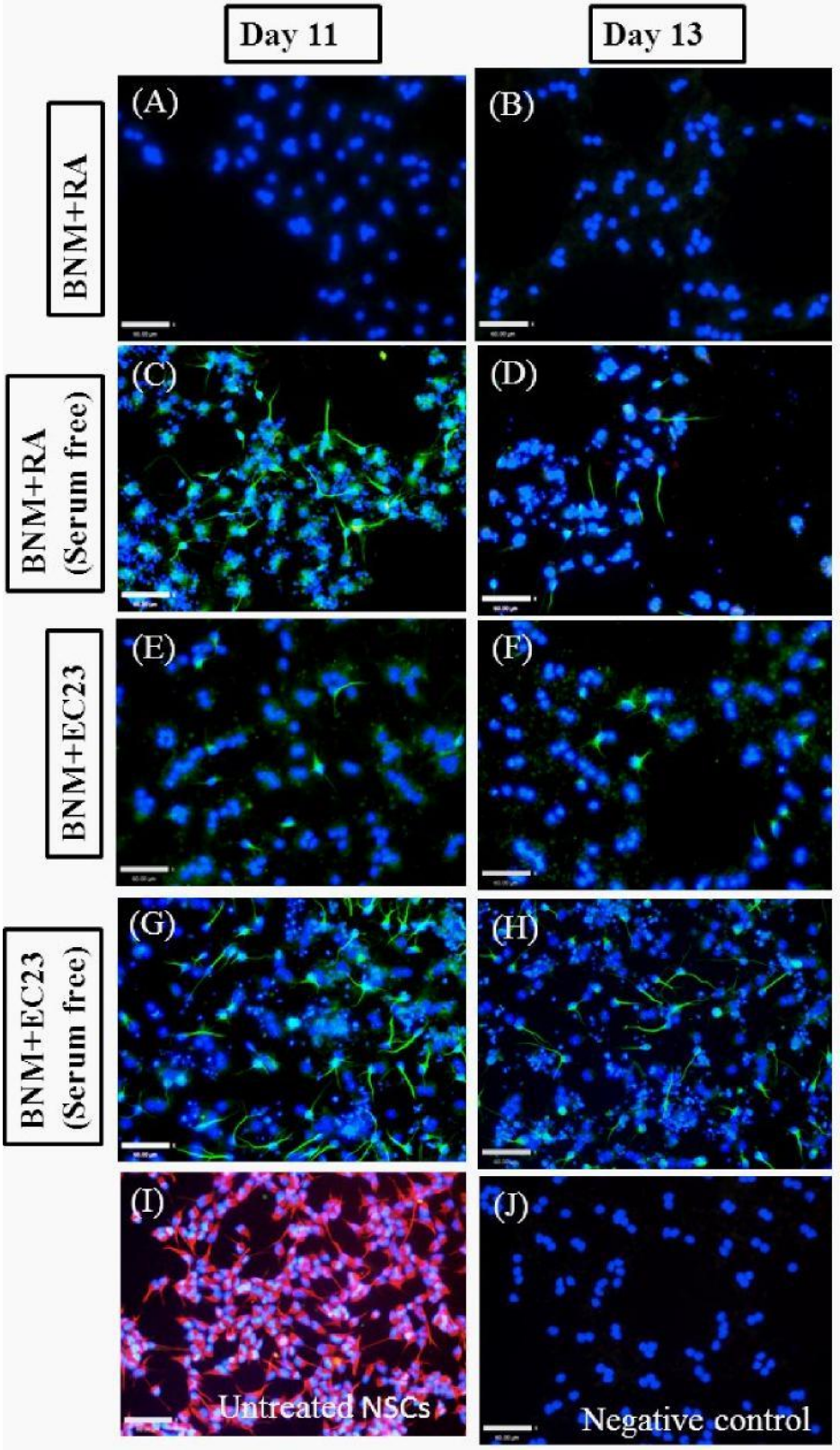


Figure 3-10 The expression of 3-III-tubulin under Retinoic acid or EC23 treatments with/without serum. (A)~(I) were double immunocytochemical staining of 3-III tubulin (green), Nestin (red) and Dapi counterstain (blue). (J) illustrated staining without primary antibody, eliminating possible false positive signals from auto-fluorescence of cells. The images were taken by a fluorescent microscope with Nikon i90 camera and analysed by Volocity® software. The scare bars were 60 μm. The same results were shown in two identical experiments.

After performing double immunocytochemical stainings (Figure 3-10), it seems that there are more Dapi-positive nuclei in serum-free condition than regular BNM in both RA and EC23 treatments on day eleven and thirteen. Calculating the numbers of Dapi-positive nuclei would represent the numbers of cells under an observed field. In BNM+RA-treated cells, the number of Dapi-positive nuclei slightly decreased comparing day eleven (Figure 3-11 (A) 11 day) with thirteen (Figure 3-11 (A) 13 day) without statistically significance. This trend was observed in serum free BNM+RA on day eleven (Figure 3-11 (A) serum free 11 day) and thirteen (Figure 3-11 (A) serum free 13 day). The numbers of Dapi-positive nuclei in BNM+EC23 remained stable on day eleven (Figure 3-11 (B) 11 day) and thirteen (Figure 3-11 (B) 13 day). However, the numbers of Dapi-positive nuclei under serum free BNM+EC23 were higher than under serum containing condition (Figure 3-11 (B) compared serum free 11 day with 11 day and serum free 13 day with 13 day). It indicated that higher numbers of Dapi-positive cell nuclei remained under serum free BNM+EC23 on day eleven and thirteen and serum was not required in EC23 treatment.

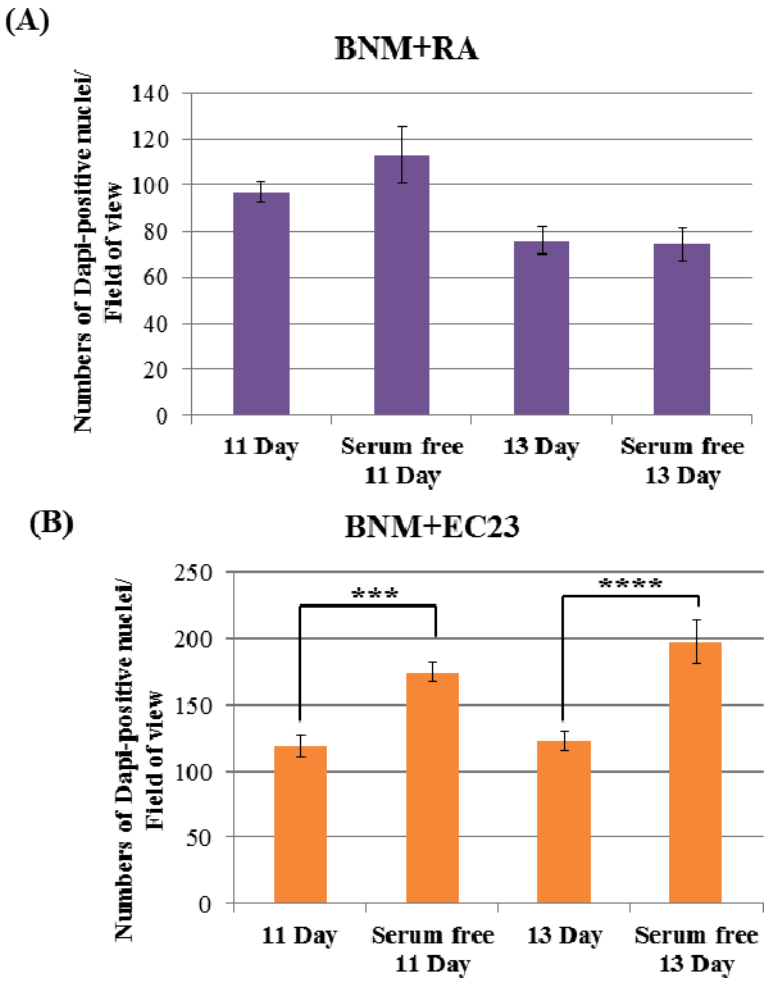


Figure 3-11 The total numbers of cell under Retinoids with or without fetal calf serum.
(A) illustrated the numbers of Dapi-positive nuclei in Retinoic acid treatment and (B) illustrated EC23. The resuts are presented by mean ± standard error of the mean of ten images. After one way ANOVA analysis, *** indicated the p value < 0.001 and **** < 0.0001.

In the images of double immunocytochemical staining (Figure 3-10), some f3-III tubulin-positive neurons was detectable under serum free BNM+RA on day eleven (Figure 3-10 (C) green) and thirteen (Figure 3-10 (D) green). It seems that more 13-III tubulin-positive neurons were observed in EC23 groups (Figure 3-10 compared (E) to (G) and (F) to (IT)). After calculating the percentage of 13-III tubulin-positive neurons, there was around 17% 13-III tubulin-positive neurons in serum free BNM+RA on day eleven (Figure 3-12 (A) serum free 11 day) and 15 % on day thirteen (Figure 3-12 (A) serum free 13 day). In BNM+EC23, the cells treated under serum-free formula also showed the percentage of f3-III tubulin-positive neurons was around 10% higher than regular BNM+EC23 on both days (Figure 3-12 (B) compared serum free 11 day with 11 day and serum free 13 day with 13 day). Under serum

free condition, BNM+EC23 showed slightly higher percentage of β-III tubulin-positive neurons then BNM+RA on day eleven (Figure 3-12 compared (B) serum free 11 day with (A) serum free 11 day) and significant higher on day thirteen (Figure 3-12 compare (B) serum free 13 day with (A) serum free 13 day ****). It indicated that foetal calf serum free BNM+EC23 would provide β-III tubulin-positive neurons to survive in a long term differentiation time course and EC23 would be a better chemical to use for neural differentiation study than Retinoic acid.

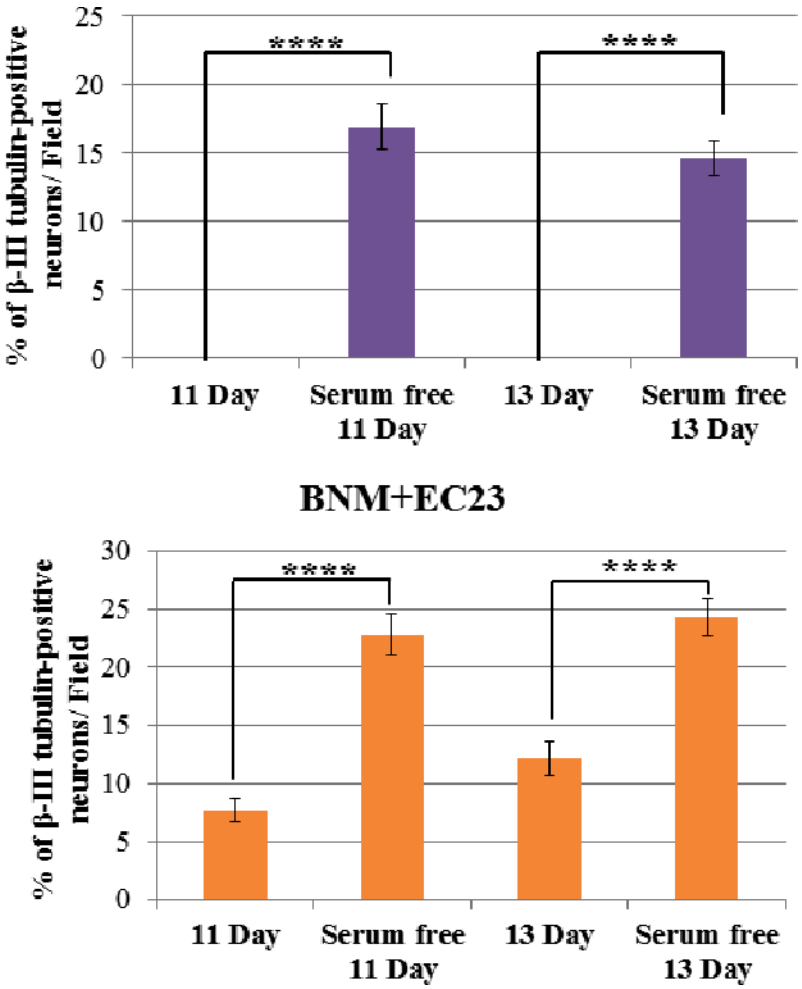


Figure 3-12 The percentages of β-III tubulin-positive neurons under eleven- or thirteen-day treatment of Retinoids with or without foetal calf serum.
(A) illustrated the percentage of β-III tubulin-positive neurons in Retinoic acid and (B) illustrated in EC23. The resuts are presented by mean ± standard error of the mean of technological duplicates (ten images). One way ANOVA analysis showed that p value was smaller than 0.0001 (****).

3.3. Preliminary study about the Rosiglitazone treatment on mNSC growth and differentiation.

In order to understand the mechanism of neurogenesis, using PPAR□ agonist Rosiglitazone would contribute to understand if PPAR□ activation promoted Retinoid-induced neuron formation via a series of time-course differentiation experiments.

3.3.1 The impact of Rosiglitazone on neuron formation from the adult mNSCs.

The first part of this section was about the impact of Rosiglitazone on neurogenesis and gliogenesis from adult mNSCs when the cells cultured in Rosiglitazone (BNM+Z), combined Rosiglitazone with EC23 (BNM+ZE) and EC23 (BNM+EC23). After BNM+Z incubation, some J3-III tubulin-expressing neurons were detectable by an immunocytochemical staining on day eleven (Figure 3-13 (A) green) and day thirteen (Figure 3-13 (E) green). Interestingly, some Nestin-expressing neural stem cells were detectable only on day eleven (↓ in Figure 3-13 (A) red) in BNM+Z which was also observed in untreated adult mNSCs (Figure 3-13 (D) red). That was not observed on day thirteen (Figure 3-13 (E)) or in BNM+ZE- (Figure 3-13 (B) and (F)) and BNM+EC23-treated (Figure 3-13 (C) and (G)) groups on day eleven and thirteen. Some J3-III tubulin-expressing neurons were also detectable under BNM+ZE (Figure 3-13 (B) day eleven and (F) day thirteen) and BNM+EC23 (Figure 3-13 (C) elevens day and (G) thirteen days). It resulted in Rosiglitazone maintaining some of undifferentiated adult mNSCs after an eleven day treatment and supported the survival of differentiated neurons on day eleven and thirteen.

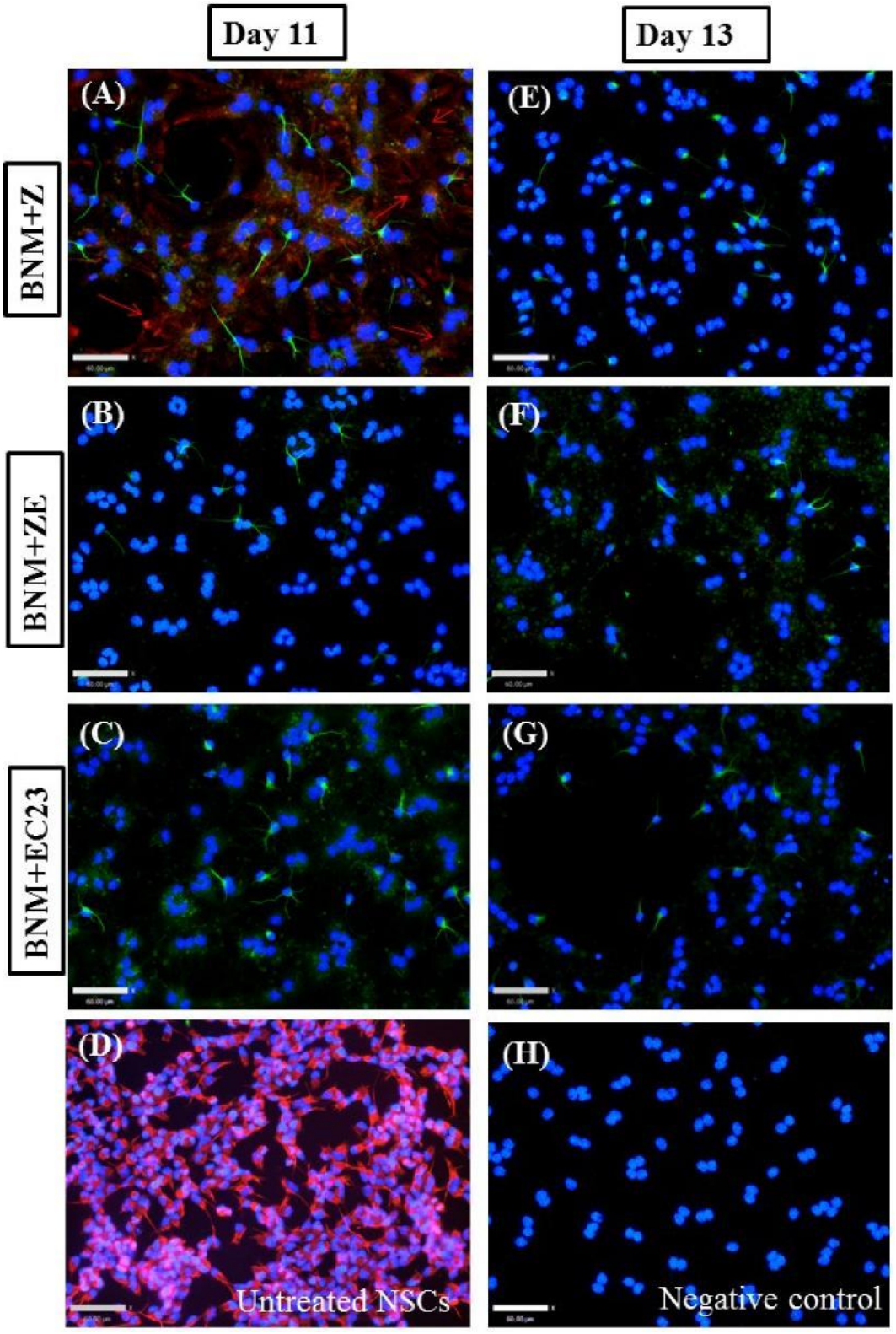


Figure 3-13 Neuron formation of the untreated adult mNSCs or after eleven- or thirteen-day treatment of BNM+Z, BNM+ZE, or BNM+EC23.

((A↓)~(G)) arethe expression of □-III tubulin (green), Nestin (red) conducted with double immunocytochemical staining and Dapi counterstain (blue). (H) illustrated staining without primary antibody in order to avoid possible auto-fluorescence from the cells. The scale bars were 60 μm. The results were constantly observed in two identical experiments.

After performing double immunocytochemical staining, the percentage of β-III tubulin-positive neurons was calculated by using the images. On day eleven, the BNM+Z-treated cells showed a higher percentage of 3-III tubulin-positive neurons than BNM+ZE and BNM+EC23 (Figure 3-14 purple). This trend was observed on day thirteen as well, especially compared BNM+Z with BNM+ZE (Figure 3-14 orange). It indicated that Rosiglitazone promoted neurogenesis more than EC23 or the combined treatment of Rosiglitazone with EC23 and the combination between Rosiglitazone and EC23 (BNM+ZE) did not promote EC23-induced neurogenesis.

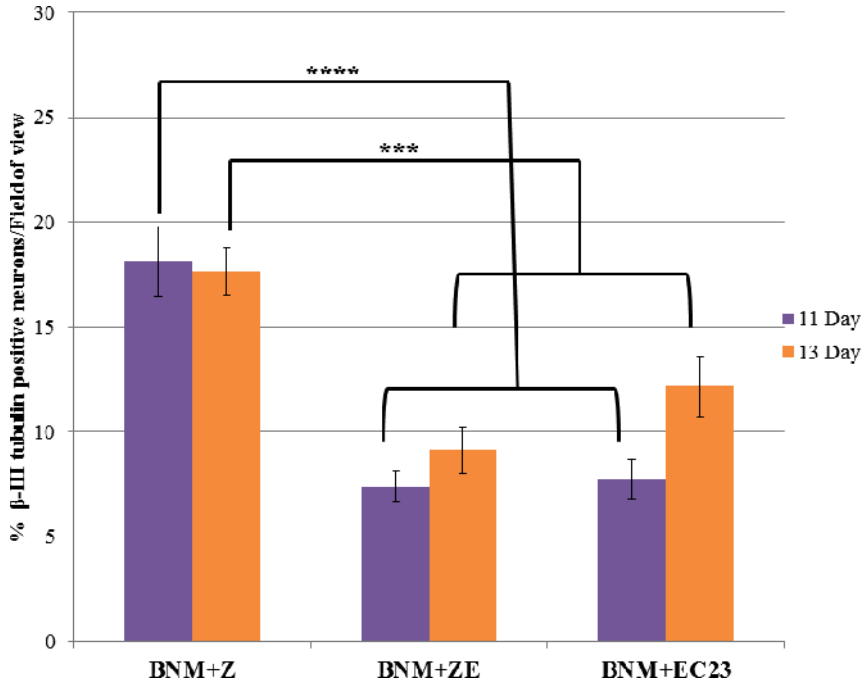


Figure 3-14 The percentage of 3-III tubulin positive neuron under BNM+Z, BNM+ZE or BNM+EC23 on day eleven and thirteen.
The numbers are presented by mean ± standard error of the mean from ten identical images. After analysing by one way ANOVA, P value *** was smaller than 0.001and **** smaller than 0.0001.

3.3.2 Glia formation from the adult mNSCs under Rosiglitazone treatment.

Results

In the previous section, it had been observed that some Nestin-positive undifferentiated adult mNSCs on day eleven and β-III tubulin-positive neurons on day eleven and thirteen when the cells were cultured in BNM+Z. This section is going to study if GFAP-positive astrocytes were detectable when the cells were cultured in BNM+Z for eleven and thirteen days. Some GFAP-expressing astrocytes were detectable cultured in BNM+Z at day eleven (Figure 3-15 (A) red) and day thirteen (Figure 3-15 (E) red). Interestingly, some Nestin-expressing neural stem cells were detectable on day eleven (↓ in Figure 3-15 (A)) which was also observed in the untreated adult mNSCs (Figure 3-15 (D) green). However, it was not observed in BNM+ZE treated- (Figure 3-15 (B) day eleven and (F) day thirteen) or BNM+EC23-treated (Figure 3-15 (C) day eleven and (G) day thirteen) group. Some GFAP-expressing astrocytes were also detectable under BNM+ZE (Figure 3-15 (B) day eleven and (F) day thirteen) and BNM+EC23 (Figure 3-15 (C) day eleven and (G) day thirteen). It showed that Rosiglitazone maintained the survival of the undifferentiated adult mNSCs on day eleven and promoted gliogenesis on day eleven and thirteen.

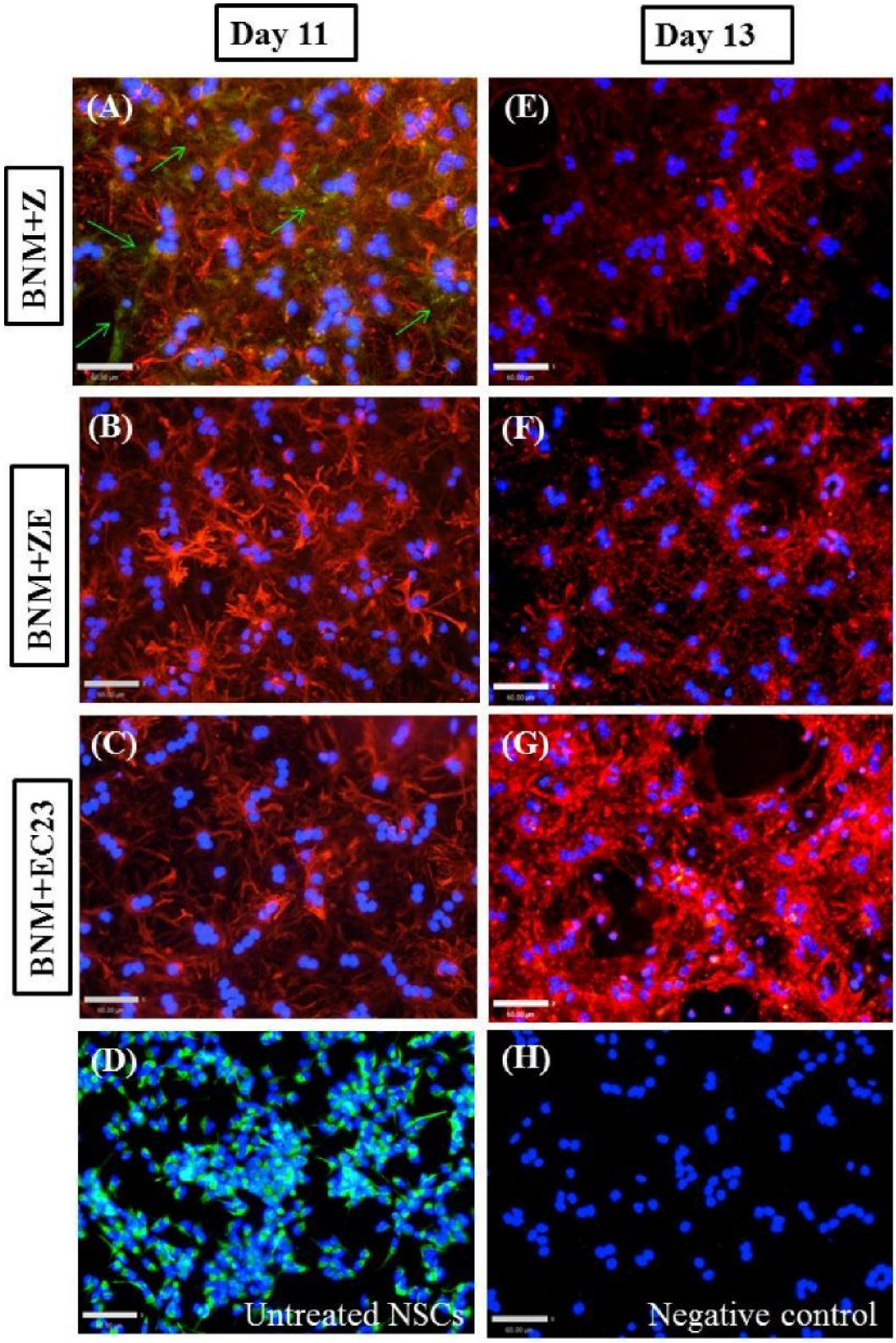


Figure 3-15 Glia formation of the untreated adult mNSCs or after eleven- or thirteen-day treatment of BNM+Z, BNM+ZE, or BNM+EC23.
((A↓)~(G)) illustrated images of double immunocytochemical staining GFAP (red), Nestin (green), and Dapi counterstain (blue). (H) illustrated staining conducted without primary antibody to eliminate possible auto-fluorescence from the cells. The scale bars were 60 μm. The images were selected from two identical experiments.

3.3.3 Effect of Rosiglitazone on the expression of NSC marker Nestin.

Nestin has been used as a marker for undifferentiated NSCs (Lendahl et al., 1990). Nestin is downregulated and is replaced by cell type-specific intermediate filaments, such as neurofilaments and GFAP during differentiation. In Figure 3-13 (A) and Figure 3-15 (A), some Nestin- expressing adult mNSCs were detectable under BNM+Z on day eleven. Detecting the trend of Nestin expression in an early time course would be able to determine the impact of Rosiglitazone on the expression of Nestin. This section aims to find out the dynamic of Nestin expression in Rosiglitazone containing conditions. In a day two time point, flow cytometry analysis revealed that a lesser portion located in Nestin-positive gate in BNM (Figure 3-16 (A) grey), BNM+Z (Figure 3-16 (A) blue), BNM+ZE (Figure 3-16 (A) green) and BNM+EC23 (Figure 3-16 (A) purple) than untreated NSCs (Figure 3-16 (A) red).The expression level of Nestin under BNM+Z decreased to 44.8% (Figure 3-16 (B) blue). In BNM+ZE, the expression level of Nestin decreased to 39.7% (Figure 3-16 (A) green) whilst it decreased to 49.3% in BNM+EC23 (Figure 3-16 (A) purple). This set of experiment was conducted with biological duplicates and showed no statistically significant difference from any comparison. It hinted that Rosiglitazone did not promote Nestin expression in an early time course as day two.

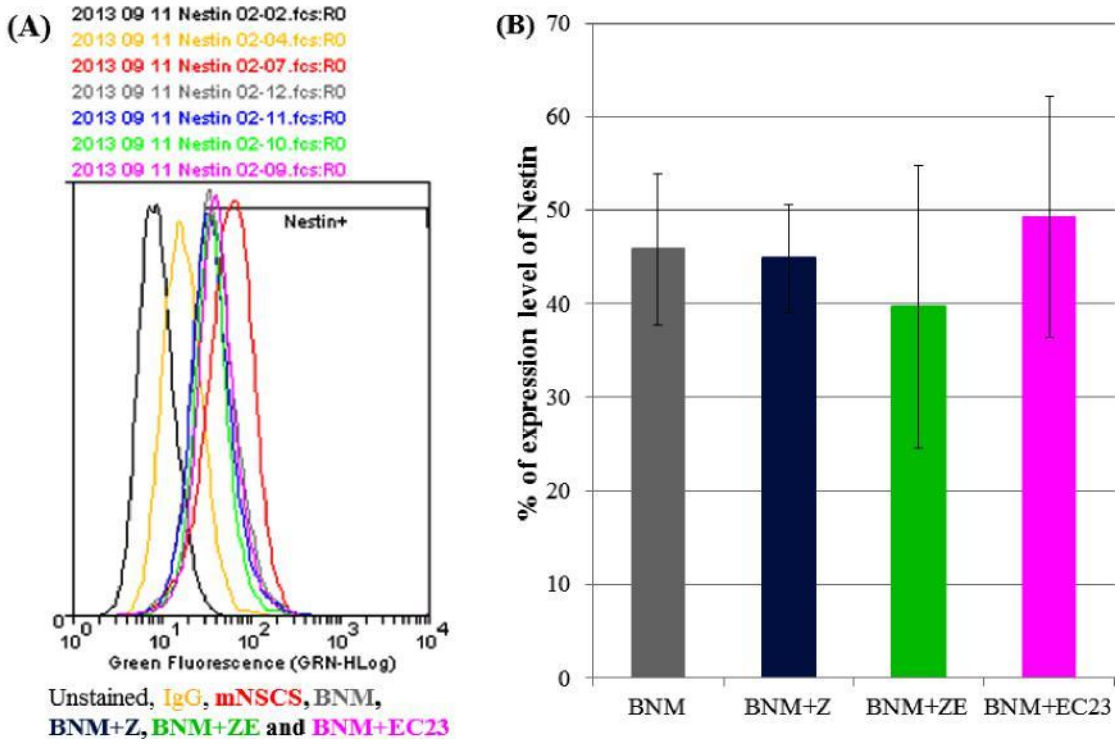


Figure 3-16 The expression level of Nestin after a two-day treatment of BNM+Z, BNM+ZE or BNM+EC23. (A) illustrated the Nestin expression histogram by flow cytometry. (B) illustrated statistics of duplicates and values were presented by mean □ standard error of the mean.

3.3.4 Impact of Rosiglitazone on the growth rate of adult mNSCs

The results of double immunocytochemical staining (Figure 3-13 and Figure 3-15) showed that the density of Dapi-positive nuclei in BNM+Z seems to be higher than the cells treated in BNM+ZE or BNM+EC23. Calculating the numbers of Dapi-positive nuclei will determine if higher numbers of cell survived in BNM+Z than in BNM+ZE or BNM+EC23 on day eleven and thirteen. After calculating the numbers of Dapi-positive nuclei, the BNM+Z (Figure 3-17 BNM+Z purple) group showed higher numbers of Dapi-positive nuclei than in BNM+EC23 (Figure 3-17 BNM+EC23 purple) or BNM+ZE (Figure 3-17 BNM+ZE purple) on day eleven. This trend was also observed on day thirteen (Figure 3-17 orange) but there was no statistically significant difference. It indicated that Rosiglitazone did support more cells to survive in a long term differentiation time course.

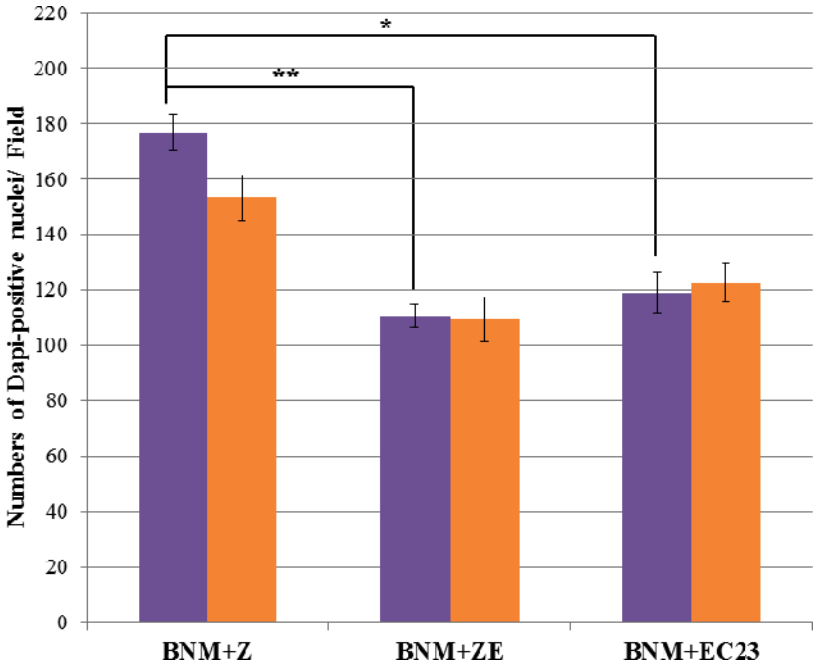


Figure 3-17 The numbers of Dapi-positive nuclei under BNM+Z, BNM+ZE and BNM+EC23 on day eleven and thirteen.
The number of Dapi-positive nuclei was calculated from ten images from each group. The values were presented by mean ± standard error of the mean. One way ANOVA analysis showed that * indicated the p value < 0.05 and ** < 0.01.

The data above suggested that Rosiglitazone had a tendency to support more cells survival in a longer differentiation time point. A cell counting experiment in a time course-dependent manner will determine if Rosiglitazone supports the survival of cells. In an early time course, the numbers of cell under BNM+Z slightly decreased from day tow to day four (Figure 3-18 red). This phenomenon was also observed in BNM (Figure 3-18 blue), BNM+ZE (Figure 3-18 green) and BNM+EC23 (Figure 3-18 purple) with no statistically significant difference. It suggested that Rosiglitazone did not promote proliferation of adult mNSCs in an early time course, from day two to four.

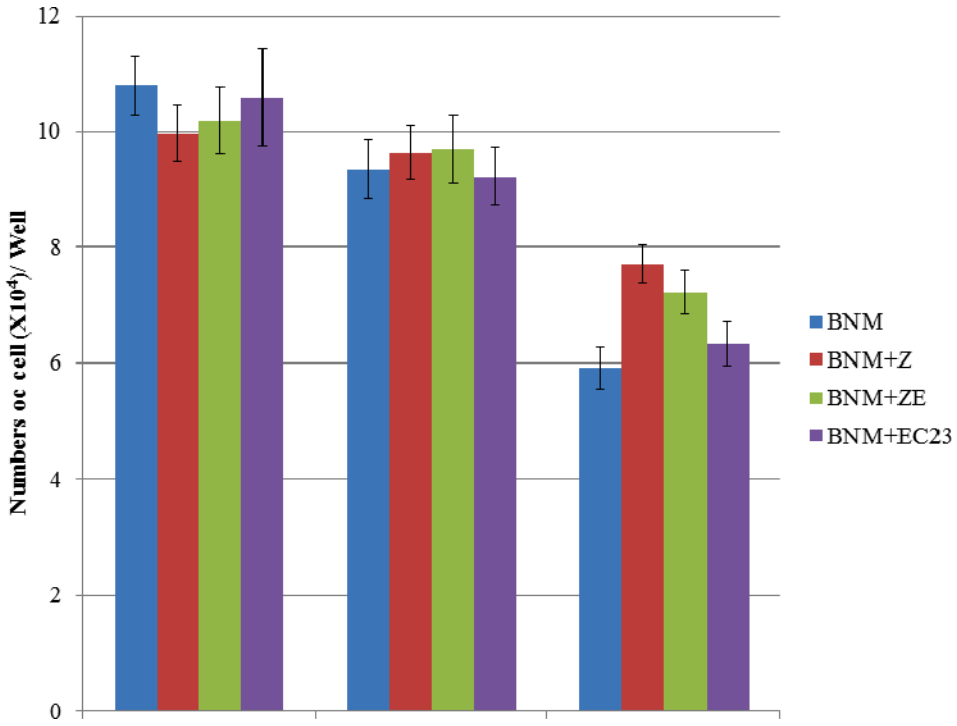


Figure 3-18 The growth rate of adult mNSCs under treatment of BNM+Z, BNM+ZE, or BNM+EC23 from day two to day four.
The results of quadruplicates are presented as mean □ standard error of the mean. A one way ANOVA analysis showed no statistically significant difference from any comparison. (P value on Day 4 BNM vs BNM+Z was 0.99 and BNM vs BNM+ZE was 0.99.)

3.3.5 The influence of serum in Rosiglitazone treatment

In the Retinoid-induced neurogenesis study above (Figure 3-12), there was higher percentage of 3-III tubulin-positive neurons and numbers of Dapi-positive nuclei being determined in serum free conditions than regular BNM. This section aims to study whether serum affects the survival of 13-III tubulin-positive neurons and Dapi-positive nuclei in Rosiglitazone containing condition. Calculating the percentage of 13-III tubulin-positive neurons and the numbers of Dapi-positive nuclei would explain whether serum had an impact on neurogenesis and the survival of cells on the activity of Rosiglitazone or combined Rosiglitazone and EC23 treatment.

Under BNM+Z, the percentage of 3-III tubulin-positive neurons in a serum free condition were slightly higher than in a serum containing condition on day eleven (Figure 3-19 (A) BNM+Z) whilst the percentage of 13-III tubulin-positive neurons in a serum free

condition remained to be similar withserum containing condition on day thirteen (Figure 3-19 (B) BNM+Z). However, lower percentage of β-III tubulin-positive neurons were observed in BNM+ZE and BNM+EC23 on day eleven (Figure 3-19 (A) BNM+ZE and BNM+EC23) than in serum free conditions; similar patterns were seen on day thirteen (Figure 3-19 (B) BNM+ZE and BNM+EC23). It suggested that foetal calf serum did not affect Rosiglitazone activity in the survival of 13-III tubulin-positive neurons and Dapi-positive nuclei but decreased the pre-neurogenic capability of EC23.

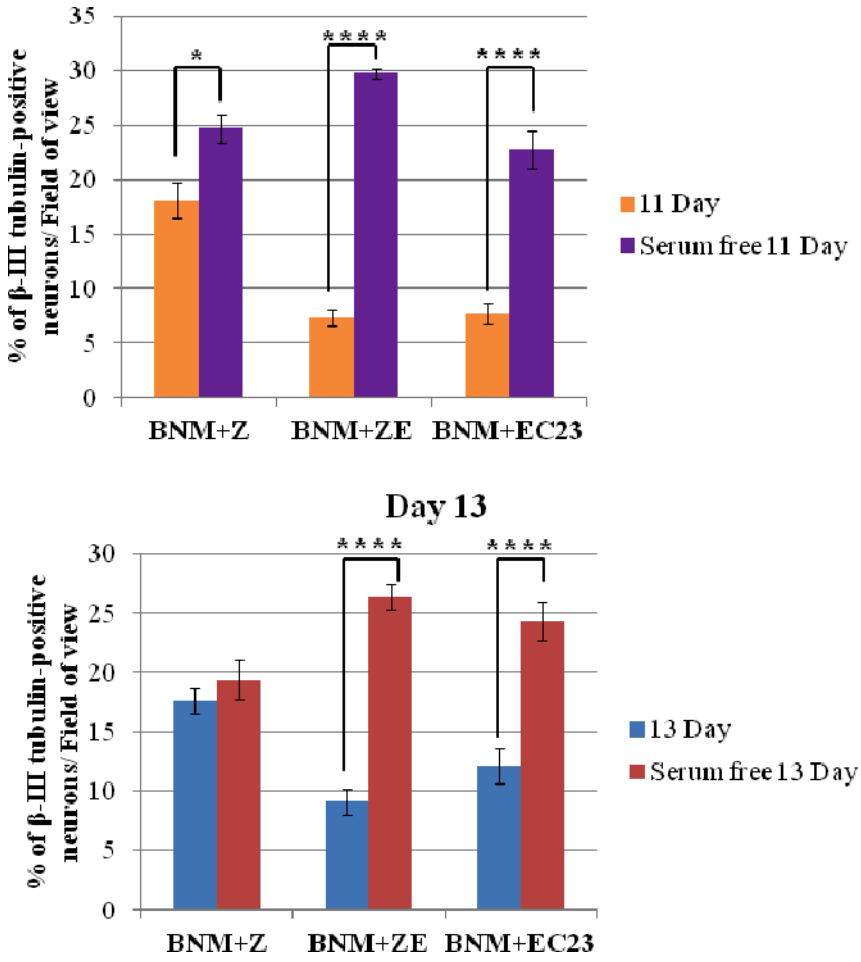


Figure 3-19 The percentage of 13-III tubulin-positive neurons under BNM+Z, BNM+ZE and BNM+EC23 with/without serum on day eleven and thirteen.
(A) illustrated the results on day eleven and (B) was day thirteen. The values were presented by mean ± standard error of the mean of ten images of different fields of view. After analysing by one-way ANOVA, P value * < 0.05 and **** < 0.0001.

The numbers of Dapi-positive nuclei in BNM+Z were similar with serum free condition on day eleven (Figure 3-20 (A) BNM+Z) and thirteen (Figure 3-20 (B) BNM+Z).

However, the numbers of Dapi-positive cell nuclei in BNM+ZE and BNM+EC23 were lower than serum free condition on day eleven (Figure 3-20 (A) BNM+ZE and BNM+EC23) and thirteen (Figure 3-20 (B) BNM+ZE and BNM+EC23). It suggested that foetal calf serum did not affect activity of Rosiglitazone in cell survival whereas it affected EC23 treatment even when combined with Rosiglitazone.

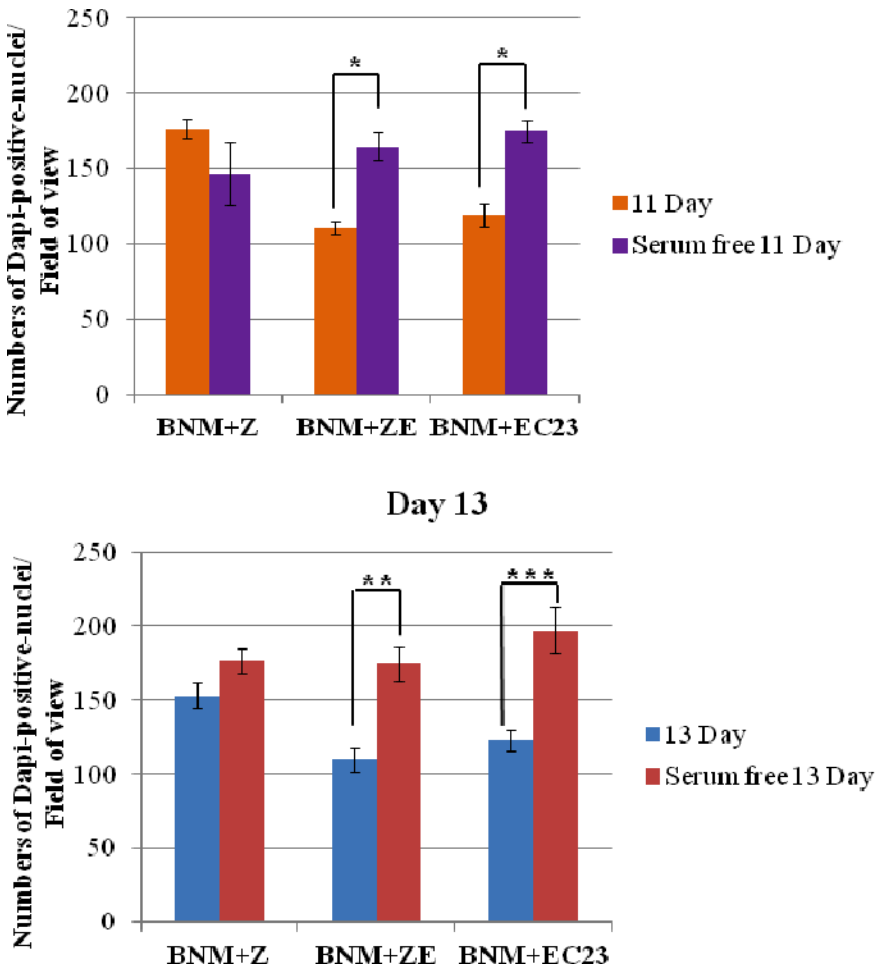


Figure 3-20 The numbers of Dapi-positive nuclei under BNM+Z, BNM+ZE and BNM+EC23 with/without serum on day eleven and thirteen.
(A) illustrated the results on day eleven and (B) was day thirteen. The values were presented by mean ± standard error of the mean of ten images of different fields of view. One way ANOVA analysed P value * < 0.05, < 0.01, and *** < 0.001.

Chapter 4. Discussion

This project studied the proliferation and differentiation capabilities *in vitro* of primary adult mNSCs generated from the lateral ventricle of the brain, using Retinoids, a PPAR agonist and an STAT 3 activator. The primary cultures of adult mNSCs were capable of doubling the population within 19.39 hours when the cells were cultured on laminin-coated environment and in an EGF- and FGF-containing medium. STAT3 activation did not promote the metabolic dynamic. It hinted that exogenous application of an STAT3 activator might not subsequently promote proliferation of the primary culture. After Retinoic acid treatment, GFAP-positive astrocyte were detectable from day four to thirteen and 13-III tubulin-positive neurons appear from day four to seven whilst it also appeared on day eleven and thirteen in serum free condition. After an EC23 treatment, both GFAP-positive astrocytes and 13-III tubulin-positive neurons were detectable from day four to thirteen whilst the percentage of 13-III tubulin-positive neuron was higher under serum free condition on day eleven and thirteen. The relative numbers of Dapi-positive cell nuclei and the percentage of 13-III tubulin-positive neuron were higher under EC23 than Retinoic acid suggesting EC23 would be a better chemical to study neurogenesis than Retinoic acid (refer to Appendix 2 Potential Publication). The percentage of EC23-induced 13-III tubulin-positive neuron was higher in serum free condition on day eleven and thirteen than regular BNM+EC23 suggesting that serum was not favourable in EC23-containing neural differentiation treatment. In the preliminary study of a PPARγ agonist Rosiglitazone, Nestin-positive NSCs were detectable on day eleven; this was not observed under either Retinoic acid or EC23. GFAP-positive astrocytes and -III tubulin-positive neurons were detectable on day eleven and thirteen in Rosiglitazone, the same under serum free conditions. Difference in number of Dapi-positive cell nuclei and 13-III tubulin-positive neurons in Rosiglitazone did not reach statistically significant difference from serum free condition on day eleven and thirteen suggesting that serum was not favourable in Rosiglitazone treatment. However, Rosiglitazone did not promote Nestin expression on day two, cell proliferation from day two to four, or the percentage of 13-III tubulin-positive neuron on day eleven or thirteen when the cells were treated with combined Rosiglitazone and EC23. It suggested that PPARγ activation was not involved in Retinoid-induced neurogenesis.

4.1. The proliferation of the primary culture generated from the lateral ventricle of adult mouse brain

It has been reported that the NSCs generated from the lateral ventricle of embryonic or postnatal rodent brains would be able to expand under EGF- and FGF-containing condition (Reynolds et al., 1992; Reynolds and Weiss, 1992; Morshead et al., 1994; Gritti et al., 1995; Weiss et al., 1996; Martens et al., 2000; Rietze et al., 2001). In this study, the primary culture of NSCs generated from the adult brain was also capable of expanding under EGF- and FGF-containing condition. When the adult mNSCs were cultured onto laminin-coated environment, the adult mNSCs doubled the amount of population within 19.39 hours. Alcock and Sottile published that the primary culture of the adult mNSCs generated from the lateral ventricle doubled the amount of population in 2.1 days in a monolayer culture condition (Alcock and Sottile, 2009). However, they did not specify how they generated the monolayer culture system. The initiating density of cell was $5X10^5$ cells/mL in their study. In order to determine the cell doubling time in linear phase of a growth curve, the initiating density of cell used in this project was $2.4X10^5$ cells/mL. It suggested that the primary culture of adult mNSC generated from lateral ventricle doubled the amount of population in a shorter time when the cells were cultured on a laminin-coated vessel.

From embryonic to mature brain, STAT3 is expressed constantly in CNS whilst its expression decrease in an aging brain (Bonni et al., 1997; De-Fraja et al., 1998; Niwa et al., 1998; De-Fraja et al., 2000). STAT3 acts to its binding sites in a cell-type-dependent manner so that STAT3 is predicted to be involved in a variety of cell responses. (Takeda et al., 1997; Matsuda et al., 1999; Cheng et al., 2011; Hutchins et al., 2013; Natarajan et al., 2014). For example, it maintains undifferentiated and proliferating status of mESCs. In a CNTF-induced STAT3 activation study, it triggered the embryonic rat NSCs to differentiate into GFAP-positive astrocytes (Bonni et al., 1997). A potent STAT3 activator Colivelin was used in this study to test the impact of STAT3 activation in the proliferation of the primary culture. The confluence of Colivelin-treated groups was not significantly different from the untreated adult mNSCs when observed under a bright field microscope (data not shown). In a PrestoBlue® test (Boncler et al., 2014), Colivelin did not promote cell metabolic dynamic from 1 fM to 100nM. It hinted that Colivelin might not subsequently facilitate the

proliferation of the adult mNSCs. Colivelin has been known to be potent at a fM range (Chiba et al., 2005) in activating STAT3 when it was used to study neural protection activity in neurodegenerative disease animal models *in vitro* (Chiba et al., 2005; Ciesler and Sari, 2013). Colivelin did not alter the metabolic dynamic in the primary culture of adult mNSCs, it might be due to the cells not responding to Colivelin. LIF is a commonly used STAT 3 activator (Matsuda et al., 1999) and it is capable of activating STAT3 in NSCs (Takizawa et al., 2001). Comparing LIF- induced STAT3 activation at protein level with Colivelin activity will be able to determine if the adult mNSCs respond to Colivelin.

In another aspect, De-Fraja et. al. mentioned that EGF induced high-level expression of p-STAT3Y705 (De-Fraja et al., 1998) in NSCs and p-STAT3Y705 is a major activated form. It indicated that the exogenous administration of STAT3 activator might not further increase the level of STAT3 activation when the adult mNSCs were cultured in EGF-containing medium. The protocol of using p-STAT3Y705 antibody is under optimisation so that the expression of p-STAT3Y705 at protein level under control or Colivelin treatments were unable to be analysed here.

4.2. Retinoid-induced astrocytogenesis and neurogenesis

Retinoic acid has been used for neurogenesis studied in NSCs *in vitro* (Weiss et al., 1996; Goffredo et al., 2008; Kim et al., 2009; Lu et al., 2009; Maltman et al., 2009; Christie et al., 2010; Lee et al., 2012b). In these Retinoid-induced neurogenesis studies, no much discussion was addressed on the loos of cell or cell death when Retinoic acid was used to induce neurogenesis. In my work, the numbers of adult mNSCs under Retinoic acid treatment decreased from day two to four compared with untreated control. On day four, Retinoic acid remarkably decreased the numbers of cell more than day two. Comparing with BNM-treated group, Retinoic acid decreased the numbers of cell more than BNM on day two and four. It suggested that Retinoic acid induced the loss of cell in an early time course. Christie et. al. (Christie et al., 2010) indicated that the density of cells in Retinoic acid- or EC23 treatment were around 16% lower than control group when they studied the neurogenesis capability of ReNcell 197VM human neural progenitors. Ninomiya et. al. (Ninomiya et al., 1997) described apoptotic cell death when they used Retinoic acid to study the neurogenesis

capability of P19 embryonal cancer Stem cells. Retinoic acid decreased the growth of cell and induced apoptosis were observed with 24 hours incubation. They observed DNA fragments and fragment nuclei in the Retinoic acid treated cells. The cell death happened after re-entry into S phase of cell cycle. They suggested that Retinoic acid prolonged the transition between S and G1 phase. In keratinocytes, Retinoic acid increased the mRNA and protein level of p53, caspase-3, -6, -7 and -9 which elements are associated with apoptosis (Mrass et al., 2004) indicating that cell loss may occur under Retinoic acid incubation. A TUNEL staining, propidium iodine (PI) cell cycle analysis, and BrdU incorporation analysis would be able to understand if Retinoic acid altered the pattern of cell cycle or induced apoptosis due to the loss of cell population. However, the protocol of PI cell cycle analysis is still under optimisation so no result of PI cell cycle analysis was available to be presented.

Nestin has been used as a marker to identify NSC (Lendahl et al., 1990). Nestin is downregulated and subsequently replaced by neural specific neurofiliments or glial specific intermediate filaments. In Retinoic acid incubation, the percentage of Nestin-positive population gradually decreased from day one to three compared to the untreated adult mNSCs. However, this set of experiments was conducted by using duplicates and no statistically significant difference was observed between treated and untreated groups. The protocols of J3-III tubulin and GFAP expression were under optimisation at this time of this study. Otherwise, the results of percentage of J3-III tubulin- and GFAP-positive population would explain if Nestin expression was replaced by J3-III tubulin or GFAP in differentiated cells.

As previously mentioned, Retinoic acid is commonly used in neurogenesis *in vitro* (Weiss et al., 1996; Goffredo et al., 2008; Kim et al., 2009; Lu et al., 2009; Maltman et al., 2009; Christie et al., 2010; Lee et al., 2012b). Because Retinoic acid is photo-sensitive, a novel synthetic analogue EC23 has been released recently (Maltman et al., 2009; Gluyas et al., 2012). In an early time course of neural differentiation study under BNM conditions, EC23 decreased the numbers of adult mNSCs since day two and maintained similar range of number of cells on day three and day four compared to cells being treated by BNM. That effect was milder than Retinoic acid without statistically significance. After a two day treatment in BNM+EC23, the expression level of Nestin was lower than the untreated adult mNSCs and similar with BNM-treated cells. However, the set of experiment was conducted by using duplicates and there was no statistically significance in any comparison. It indicated

that EC23 decreased the number of cells from day two to four and had tendency to attenuate the expression level of Nestin as early as day two.

In Retinoid-induced neurogenesis, Bain et. al. (Bain et al., 1995) published that the Retinoic acid-treated ESCs showed neural-like morphology and was 7.8 times higher than untreated control. Subsequently, Retinoic acid was used in a range of neurogenesis studies in ESCs, embryonic NSCs or embryonal cancer stem cells (Appendix 1) (Weiss et al., 1996; Goffredo et al., 2008; Kim et al., 2009; Lu et al., 2009; Maltman et al., 2009; Christie et al., 2010; Lee et al., 2012b). This study demonstrated Retinoic acid- or EC23-induced gliogenesis and neurogenesis in a time-dependent manner by using the primary culture of adult mNSCs. GFAP-positive astrocytes were detectable from day four to thirteen similar to what observed under serum condition on day eleven and thirteen. J3-III tubulin-positive neurons showed from day four to seven and disappeared on day eleven and thirteen; however under serum free condition J3-III tubulin-positive neurons appeared on day eleven and thirteen. It suggested that Retinoic acid supports the survival of neurons at a variant time course due to the contained serum. In observation of morphology of neurons, the J3-III tubulin-positive neurons showed a morphology with long neurites which was similar with morphology of an excitatory neuron when the cells were cultured in BNM+RA for five or seven days. After eleven day or thirteen day serum-free Retinoic acid treatment, the J3-III tubulin-positive neurons showed morphology with short neurites that indicated morphology of armature neuron or an interneuron. It suggested that J3-III tubulin-positive neurons survived in a serum free Retinoic acid treatment in a longer differentiation time frame but might support the survival of different phenotypes or maturity of neurons. However, there was no result of J3-III tubulin-positive neurons at an earlier differentiation time points as day five or seven. Comparing the expression of neuron-related markers, the morphology and electrophysiological activities of those differentiated neurons under serum containing Retinoic acid treatment with serum-free condition at earlier time-points as day five or seven would help clarify the role of serum in Retinoic acid-induced neurogenesis.

Under an EC23 treatment, some GFAP-positive astrocytes and J3-III tubulin-positive neurons were detectable from day four to thirteen. Calculating the numbers of J3-III tubulin-positive neurons, the percentage of J3-III tubulin-positive neurons decreased from day five to eleven but surprisingly increased on day thirteen. However, it had been observed different morphology among the J3-III tubulin-positive neurons. The J3-III tubulin-positive neurons

showed long neurites that were excitatory neuron-like morphology on day five and seven and short neurites that were a mature or interneuron-like morphology on day eleven and thirteen. It suggested that EC23 supported the survival of different neural morphology or maturity on differentiation time points. In serum-free condition, it was observed with more Dapi-positive cell nuclei and J3-III tubulin-positive neurons than in serum containing condition on day eleven and thirteen. The morphology of J3-III tubulin-positive neurons showed the same trend of neural morphology change as serum-containing condition. It suggested that serum was not required in EC23-induced neurogenesis (refer to Appendix 2 Potential Publication).

Since EC23 is a novel synthetic Retinoid, comparing its pro-neurogenic capability with Retinoic acid would establish if EC23 is a more portent replacement of Retinoic acid. In an early time course, EC23-induced loss of cells was milder than Retinoic acid. EC23 had potential to decrease the expression level of Nestin on day two, similar to what was observed with Retinoic acid. In a differentiation time course study, EC23 induced astrocytogenesis from day four to thirteen thus showing the similar gliogenesis capability with Retinoic acid. However, J3-III tubulin-positive neurons survived in EC23 from day four to thirteen whilst the J3-III tubulin-positive neurons only survived from day four to seven in Retinoic acid. In a serum free condition, J3-III tubulin-positive neurons were detectable in either Retinoic acid or in EC23 incubation on day eleven and thirteen. It suggested that EC23 was a good replacement of Retinoic acid in studying Retinoid-induced neurogenesis (refer to Appendix 2 Potential Publication).

4.3. The preliminary study of Rosiglitazone in Retinoic acid-induced neurogenesis.

In the molecular mechanism of Retinoid signalling, RXR requires another co-factor to form a homo/heterodimer, and PPAR1 is one of the co-factors (Rodriguez et al., 1994; Cullingford et al., 1998). PPAR1 is expressed in adult neural stem cells (Wada et al., 2006; Miglio et al., 2009) and PPARγ activation stimulated the growth rate of the embryonic mNSCs (Wada et al., 2006). In neurodegenerative disease animal models, PPARγ activation attenuate on-going neurodegeneration (Brodbeck et al., 2008; Escribano et al., 2010a; Lee et al., 2012a; Sadeghian et al., 2012). Therefore, a PPAR1 agonist Rosiglitazone was utilised to

study if PPAR□ activation could affect the response of adult mNSCs toward differentiation conditions containing Retinoids.

Nestin is a marker for undifferentiated NSCs (Lendahl et al., 1990). After conducting immunocytochemical staining, Nestin was expressed in the untreated adult mNSCs. Surprisingly, Nestin-positive adult mNSCs were also detectable on day eleven, which were not observed in neither EC23-treated nor combined Rosiglitazone and EC23-treated groups. Wada et al. published that PPARγ activation stimulated the expansion of mNSCs generated from embryonic (E13~14) mouse brains (Wada et al., 2006). In this study, the formula of Rosiglitazone treatment consisted Rosiglitazone and BNM. The BNM was for neuro- or gliogenesis, not for NSC expansion. It showed that Rosiglitazone had a tendency to maintain some adult mNSCs in BNM incubation but that effect was abolished by a combined treatment with EC23. Therefore, the expression level of Nestin was analysed by conducting flow cytometry in an earlier differentiation time point as day two. However, the expression level of Nestin decreased in Rosiglitazone, similarly to the trend seen with EC23 or combined Rosiglitazone and EC23-treated groups. It suggested that Rosiglitazone did not promote the expression of Nestin in an early differentiation time point.

In immunocytochemical staining results, the numbers of Dapi-positive cell nuclei in Rosiglitazone were higher than either combined Rosiglitazone and EC23 or EC23 incubation on day eleven and thirteen. Subsequently, the growth rate of the cells in untreated or differentiation conditions were conducted by containing the numbers of cell in an earlier differentiation time points from day tow to four. In Rosiglitazone incubation, the numbers of cell decreased from day two to four, similarly to the trend seen with BNM, EC23 and combined Rosiglitazone with EC23 treated-groups. It suggested that PPAR□ activation did not promote the proliferation of adult mNSCs in an early time course. Unfortunately, the data above did not explain the reason why Nestin-positive mNSCs was observed in immunocytochemical staining images after an eleven day treatment of Rosiglitazone. It might be because PPAR□ plays a different role in adult NSC than embryonic NSCs.

In glial and neural differentiation study, GFAP-positive astrocytes and β-III tubulin-positive neurons were observed in Rosiglitazone on day eleven and thirteen. It showed similarly trend to combined Rosiglitazone with EC23 or EC23 incubation on both days. The percentage of □-III tubulin-positive neurons in Rosiglitazone was higher than either combined Rosiglitazone and EC23 or EC23 on day eleven and thirteen. It indicated that

Discussion

Rosiglitazone itself had tendency to enhance the percentage of □-III tubulin-positive neurons and Rosiglitazone did not enhance the response of adult mNSCs toward differentiation conditions containing Retinoid.

Chapter 5. Conclusion

The primary cultures of adult mNSCs were generated from the lining of lateral ventricle. This project studied the proliferation and differentiation response of this primary culture by the treatment of Retinoids, a PPAR agonist and an STAT 3 activator. These primary cultures of these adult mNSCs doubled within 19.39 hours when they were culture onto laminin-coated environment and in an EGF- and FGF-containing medium. These cells also formed a true monolayer structure which was benefit to observe the following proliferation and differentiation tests. In the incubation of a potent STAT3 activator, Colivelin, the metabolic dynamic of these cells did not obviously increase as we hypothesised. It hinted that this exogenous application of an STAT3 activator might not subsequently promote proliferation of the primary culture in our system. In the neural differentiation study, the primary cultures were treated by Retinoic acid in a time-course dependent manner. Some GFAP-positive astrocyte were detectable from day four to thirteen and 13-III tubulin-positive neurons appear from day four to seven whilst it also appeared on day eleven and thirteen in serum free condition. When the primary cultures were incubated in EC23, both GFAP-positive astrocytes and 13-III tubulin-positive neurons were detectable from day four to thirteen whilst the percentage of 13-III tubulin-positive neuron was higher under serum free condition on day eleven and thirteen. In this condition, the relative numbers of Dapi-positive cell nuclei and the percentage of 13-III tubulin-positive neuron were higher than Retinoic acid. Thus, EC23 would be a better chemical to study neurogenesis than Retinoic acid (refer to Appendix 2 Potential Publication). In order to increase the percentage of neuron induced in Retinoids incubation, the presence and the absence of serum had been studied. The percentage of EC23-induced 13-III tubulin-positive neuron was higher in serum free condition on day eleven and thirteen than regular BNM+EC23. It indicated that serum was not favourable in EC23-containing neural differentiation treatment. In order to clarify if there is a synergic effect on neural differentiation when the primary cultures were treated by a combination of Retinoids with a PPARγ agonist, the preliminary study of a PPARγ agonist Rosiglitazone was conducted. After incubating in Rosiglitazone, Nestin-positive NSCs were detectable on day eleven; this was not observed under either Retinoic acid or EC23. GFAP-

positive astrocytes and □-III tubulin-positive neurons were detectable on day eleven and thirteen in Rosiglitazone that was also observed under serum free conditions. Compared the number of Dapi-positive cell nuclei and 13-III tubulin-positive neurons in Rosiglitazone to serum free conditions, the difference in number did not reach statistically significant difference on day eleven and thirteen. It suggested that serum was not favourable in Rosiglitazone treatment. However, Rosiglitazone did not promote Nestin expression on day two and cell proliferation from day two to four. The combined treatment of Rosiglitazone and EC23 did not promote the percentage of 13-III tubulin-positive neuron on day eleven or thirteen. It suggested that PPARγ activation was not involved in Retinoid-induced neurogenesis.

Chapter 6. Future Work

In this study, primary mNSCs generated from the lateral ventricle of adult brains proliferated in an EGF- and FGF-containing medium. The exogenous application of the STAT3 activator Colivelin did not promote the metabolic dynamic of the adult mNSC primary culture. There was not obvious different confluence of the cells cultured in an EGF- and FGF-containing medium or Colivelin treatments when observed under a bright field microscope. This result suggested that an extra STAT3 activator treatment might not subsequently facilitate the metabolic dynamic of the adult mNSCs in EGF-containing condition. Comparing the protein level of the activated STAT3 in EGF- and FGF-containing medium with Colivelin (100 nM and 100fM) treatments will clarify whether EGF has already activated the maximum amount of STAT3. It will contribute in understanding the role of EGF in maintaining adult mNSC proliferation. From another aspect, the adult mNSCs might not respond to Colivelin. Comparing the protein level of activated STAT3 of the adult mNSCs in LIF treatment with in Colivelin incubation will determine whether the adult mNSCs respond to Colivelin.

Loss of cells in Retinoic acid-containing differentiation treatment was found to be more severe than in EC23. This phenomenon in NSCs was not discussed in the published literature. A TUFNEL staining, and the expression level of *caspase*-3, -6 or -9 will clarify whether Retinoid-induced loss of cells was caused by apoptosis. Comparing the severity in Retinoic acid with that in EC23 will be an evidence to support that EC23 is a better chemical for studying Retinoid-inducing neurogenesis.

In the study of Nestin expression, it decreased in Retinoic acid treatment from day one to day three and day two in EC23 treatment. However, the dynamic change of neural or glial markers was not observed. Studying the dynamic expression of Nestin, β-III tubulin and GFAP of cells treated in Retinoic acid or EC23 will determine the time frame of Retinoid-induced neural differentiation. It will optimise the formula of Retinoid-containing neural differentiation treatment for increasing the numbers of neurons and determine whether Retinoids affect the expression of neural-associated markers.

In the neurogenesis study, the adult mNSCs were capable of differentiating into □-III tubulin-positive neurons in Retinoic acid- or EC23-containing neural differentiation treatment.

Future Work

Different morphologies of the 13-III tubulin-positive neurons were observed on different differentiation time points. Studying the expression of neural markers (Table 6-1) and electrophysiological activities of the differentiated neurons with variant morphologies will identify whether EC23-induced different phenotypes of neurons at different differentiation time points.

Table 6-1 Markers for identifying the different phenotypes of neurons

Markers	Targets
Tyrosine hydroxylase	Catecholaminergic, sensory and autonomic neurons
Glutamate transporter	Glutamatergic excitatory neurons
Choline acetyltranferase	Cholinergic neurons
Peripherin	Motor, ganglion neurons
Trk receptors	Sensory neurons
Neurogenin 2	Motor neurons
Neural nuclei antigen (NeuN)	Amateur neurons
Calretinin	Amateur neurons
Glutamate decarboxylase (GAD) 65/67	GABAergic interneurons
Parvalbumin	GABAergic interneurons
Glycine transpoter GLYT2	Glycinergic interneurons

In the neurogenesis study, the adult mNSCs differentiated into 13-III tubulin positive neurons in a differentiation time point-dependent manner when the cells were cultured in Retinoid-containing differentiation medium. It is necessary to determine the targeted genes of Retinoid receptors and clarify whether Retinoid promote the expression of genes-, RNA- or protein- related to neural differentiation, such as neurofilaments, neurotrophins, neurogenins etc. It will contribute to understand the mechanism of Retinoid-induced neurogenesis.

This project demonstrated that the adult mNSCs were capable of differentiating into □-III tubulin positive-neurons, and GFAP-positive astrocytes when the cells were cultured in a Retinoids-containing neural differentiation treatment. However, it is unknown if this primary culture of the adult mNSCs are able to differentiate into oligodendrocytes. An oligodendrocytosis study will contribute to the completion of the differentiation capability of the primary culture of adult mNSCs into neurons, astrocytes and oligodendrocytes. It will contribute to generate the cellular network among neurons, astrocytes and oligodendrocytes. It will help understanding the progression of neurodegenerative diseases and subsequently

develop treatments to stop the progression of neurodegeneration or to regenerate the damaged tissues.

Appendix

Appendix 1 The neural differentioation effects under Retinoid treatments in stem cell *in vitro*.

Retinoids	Cell type	Mechanism	Type of neuron	Reference
Retinoic acid (0.5 μM)	mESC[a]	↑□-III tubulin, neurofilament M and L, Brn-3, GAD[b] and GFAP.	1. Glutamate (Glu1-4 and 6) Kainate, NMDA, GABA (GAD[c]) and Glycin 2. Voltage- gated Na^+, K^+ and Ca^{2+} channels.	(Bain et al., 1995; Strübing et al., 1995)
Retinoic acid (10 ~ 100 nM)	mESC[a]	↑NCAM, synaptophysin, synaptobrevin, SV2[c], neurofilament M and L, GFAP	1. $GABA_A$, Glycine, AMPA, NMDA 2. Voltage- gated Na^+, K^+ and Ca^{2+} channels.	(Rohwedel et al., 1999)
Retinoic acid (non-specified)	mESC[a]	↑□-III tubulin	non-specified	(Zhao et al., 2004)
Retinoic acid (1 μM)	mNSC from telecephalons of E14.5 embryos	1. facilitate leukaemia inhibitory factor induced astrocyte differentiation by activating STAT3 2. activate promoter of *gfap* via histone H3 acetylation which allows STAT3 to gain access to promoter of *gfap* more efficiently which is enhanced by a histone deacetylase inhibitor, valproic acid	non-specified	(Asano et al., 2009)
Retinoic acid (0.5 ~ 1 μM)	mESC[a]	1. ↑ Wnt inhibitor Sfrp2[d], Sox6*, Brn2, neurogenin 2 and Tmem46 (Shisa 2) 2. ↓ cyclin E1	1. $GABA_A$, Kainate, AMPA, NMDA 2. Midbrain, hinbrain and spinal cord neurons	(Kim et al., 2009)
Retinoic acid (10 μM)	mESC[a]	↓Oct4, Sox 1* and ↑Nestin*, BLBP*, Olig2, Musashi, □-III tubulin, N-CAM, MAP 2[e], neural neclei and GFAP 2. ↑Erk[f] phosphorylation via MEK 1 3. ↓Wnt activity via ↑Sfrp2[c] 4. ↓ BMP_g/Smad 1	non-specified	(Lu et al., 2009)
Retinoic acid (5 nM)	mESC[a] (wild-type and Sox1-GFP+/+)	1. phosphorylate Rrk via fgf receptor 2. first ↑fgf8/Erk and then ↓ fgf4/Erk signalling via RAR/RXR complex 3. ↓pluripotency genes, such as, Nanog, Klf4 and Rex 1 4. ↑ Ngn 2, Mash 1 Sox 1* and Pax 6	neural stem cells	(Stavridis et al., 2010)
Retinoic acid (1 ~ 5 μM)	mESC[a], miPSC[h], mEC[i]	1. ↑□-III tubulin and Nestin*	neural stem cell	(Sartore et al., 2011)

Appendix

		2. ↓ survivin 3. ↑ Aneuploidy and micronuclei formation = mitotic checkpoint failure		
Retinoic acid (5 jtM)	mESC[a] (wild-type and LRRK2$_j$+/-)	1. ↓Naong, RNA binding protein Lin-28, oct4,Dppa2[k], Lefty2[l], secreted phosphoprotein, F-box protein-15 and zinc-finger protein 42 2. ↑Scn1a[m], Kcnq2[n], mGluR8[o] and iGluR1$_p$.	Gabaergic and glutamatergic neurons	(Schulz et al., 2011)
Retinoic acid (0.5 jtM)	mESC[a]	1. ↑□-III tubulin, N-CAM, Cytokeratin Endo A, Nestin, Pax 6 and Sox 1 2. ↓ E-cadherin, Oct4 and Nanog 3. ↓Akt and PI3K$_q$	neural stem cell	(Kotasova´ et al., 2012)
Retinoic acid (10 ~ 100 nM)	mESC[a]	1. ↑ GFAP, Gbx2, Map2 and Nestin* 2. ↓ Nanog	non-specified	(Akanuma et al., 2012)
Retinoic acid (1 nM ~ 1 jtM)	mESC[a], hNPC[r], Human neuroblastoma cell, rat hippocampal neural stem cell	1. FGF receptor 1 accumulation in cell nuclei 2. FGFR1-αRAR and FGFR1-αRXR complexes formation 3. FGFR1 interacts with orphan receptor Nur1 and Nur77 4. RAR/RXR complex formation 5. ↑ nuclear 100, □-III tubulin, fgfr1, fgf2 6. ↓ Oct 4	non-specified	(Lee et al., 2012c)
Retinoic acid	Sox 2 over-expressed mESC[a],	1. retain Sox 2 overexpression which downregulate expression of Oct3/4 2. ↑neuroD1 and synaptophysin expression	non-specified	(Drakulic et al., 2012)
Retinoic acid (1,2,4 or 8 jtM)	U87 glioma stem cell line	1. ↑GFAP, □-III tubulin and ↓CD133 after a seven day treatment at 1 or 2 jtM whilst the cells under 4 or 8 jtM suffered from shrinking or apoptosis 2. The differentiation capability of was the most efficient at 1 jtM and this capability decreased when the concentration increased. 3. The concentrations of Retinoic acid did not	non-specified	(Shi et al., 2013)

		affect the present of cells at G0/G1, S or G2/M phase. 4. RA induced differentiation of this cell type was not ideal in all concentration in the present of EGF and bFGF.		
Retinoic acid EC23 (1 □M)	Human neuro-progenitors and hESC[s]	1. ↑□-III tubulin, MAP 2, neurofilament and HB9[t]	motor neuron	(Christie et al., 2010)

a. mESC: mouse embryonic stem cell

b. GAD: Glutamic acid decarboxylase

c. SV2: synaptic vesicle protein 2

d. Sfrp2: secreted frizzled-related protein 2

e. MAP 2: microtubule associated protein 2

f. Erk: extracellular signal-regulated kinase

g. BMP: Bone morphogenic protein

h. miPSC: mouse induced pluripotent stem cell

i. mEC: mouse embryonal carcinoma

j. LRRK2: leucine-rich repeat receptor kinase 2

k. Dppa2: developmental pluripotency associated 2

l. Lefty 2: Left-right determination 2

m. Scn1a: voltage-gated Na^+ channel protein 1a

n. Kcnq 2: voltage-gated K^+ channel protein 2

o. mGluR8: metabotropic glutamate receptor 8

p. iGluR1: ionotropic glutamate receptor 1

q. PI3K: Phosphoinositide 3-Kinas

r.hPNC: human neural progenitor cells

s. hESC: human embryonic stem cell

t. HB9: motor neuron marker

*The cells transiently increase expression of Sox 1, Sox 6, Nestin and BLBP at neural stem cell stage.

Appendix

Appendix 2 Potential publication

Part of data were extracted and written this potential publication. This potential publication was written in the format for *the Journal of Neuroscience Methods*.

Appendix

The pro-neurogenic effect of EC23 is more potent than Retinoic acid

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Abstract

Recent studies have shown that the adult brain retains a population of stem cells in specific locations. Adult neural stem cells (NSCs) from the subventricular zone of the lateral ventricle have self-renewing and differentiation capability. These NSCs are capable of differentiating into astrocytes, oligodendrocytes and neurons *in vitro*. Study the proliferation and differentiation capability of the adult NSCs would provide a resource for developing new treatments for neurodegenerative diseases in the future. Retinoic acid has been described to promote neuron formation from the several different types of stem cell *in vitro*. Recently, a novel synthetic analogue EC23 has been released. This paper compares the pro-neurogenic capability of EC23 with Retinoic acid by using primary culture of adult mouse NSCs generated from the lining of lateral ventricle. We described a neural differentiation protocol by using EC23 in primary culture of adult ‘NSCs.

Key words: adult neural stem cell, Retinoic acid, EC23, neural differentiation

1. Introduction

The brain is not known to be self-repaired after injury or degeneration. It has been reported that adult brain contained a population of neural stem cell (NSC) in the lining of the subventricular zone(Smart, 1961; Blakemore, 1969; Privat and Leblond, 1972; Reynolds and Weiss, 1992; Lois and Alvarez-Bulla, 1993; Lois and Alvarez-BuyIIa, 1994; Morshead et al., 1994; Alvarez-Buylla et al., 2002; Episkopou, 2005; Goffredo et al., 2008). From embryogenesis to adult life, these cells differentiate into a range of cell types, such as interneurons, granular neurons, periglomerular neurons and neuroglia in the olfactory bulb (Smart, 1961; Altman, 1969; Luskin, 1993; Doetsch and Alvarez-Buylla, 1996; Weiss et al., 1996; Doetsch et al., 1997; Doetsch et al., 1999). The adult NSCs retain the capability to self-

renew, and undergo *de novo* neurogenesis and gliogenesis. *In vitro* evidence confirmed that these NSCs, which can be maintained using epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), are capable of differentiating into neurons and glia when treated with appropriate media (Reynolds et al., 1992; Reynolds and Weiss, 1992; Morshead et al., 1994; Gritti et al., 1995; Weiss et al., 1996; Martens et al., 2000; Rietze et al., 2001). Furthermore, it raises the possibility that subventricular zone in adult brain as a resource of precursor cells able to generate neurons for transplantation and neural replacement for neural injury or neural degeneration diseases. NSCs are also helpful to model and identify new treatments for neurodegenerative diseases in the future.

During embryogenesis, Retinoic acid acts as one of the morphogens guiding the development of the eye, forebrain and hindbrain (Shearer et al., 2012), especially in neuronal patterning, neural differentiation and axon outgrowth (Bain et al., 1995; Kastner et al., 1995; Maden, 2007; Lu et al., 2009; Elizalde et al., 2010). Experimental evidence showed that Retinoid treatments promoted neuron formation from NSCs *in vitro* (Weiss et al., 1996; Goffredo et al., 2008; Kim et al., 2009; Lu et al., 2009; Maltman et al., 2009; Christie et al., 2010; Lee et al., 2012b). The pro-neurogenic capability of Retinoic acid has been demonstrated by using several cell models (Bain et al., 1995; Strübing et al., 1995; Rohwedel et al., 1999; Kim et al., 2009; Christie et al., 2010; Schulz et al., 2011). Retinoic acid treatment promoted neurogenesis was 7.8 times higher than untreated embryonic stem cells (ESCs) (Bain et al., 1995).

Because Retinoic acid is photo-sensitive, stable analogues of Retinoic acid have been synthesised (Maltman et al., 2009; Gluyas et al., 2012). EC23, a stable synthetic analogue of Retinoic acid, has been used in few neural differentiation studies (Appendix 1) (Maltman et al., 2009; Christie et al., 2010; Gluyas et al., 2012). In a ligand-receptor assay, EC23 bonded intracellular Retinoic acid receptors RARα, RAR□ and RAR □ in a concentration dependent manner, from 0.1 nM to 1 □M (Gluyas et al., 2012) whilst Retinoic acid is from 3 to 100 nM (Allenby et al., 1993). However, the impact of EC23 on neuron formation and cell growth remain unclear.

We aimed to generate a neural differentiation protocol using EC23 in order to study the pro-neurogenic capability of the primary culture of adult mouse NSC isolated form the lining of lateral ventricle.

2. Methods and Materials

2.1 Adult mNSC culture

Wild type adult mNSCs were isolated from the lining of the lateral ventricle of adult mice (Clarke, 2003; Hutton and Pevny, 2008) and the method was adapted from Hutton and Pevny, 2008. The tissue lining the ventricle was harvested and incubated with 5mL Accumax (Innovate Cell Technologies®) at 37°C for 30 minutes with intermittent agitation. Subsequently, 5mL PBS were added and the suspension was centrifuged for 4 minutes at 200 G. After centrifugation, the cells were resuspended in 5mL PBS and allowed larger fragments to settle. The suspension was transferred to a fresh centrifuge tube and centrifuged for 4 minutes at 200 G. Finally, the adult mNSCs cell pellet was resuspended in a fresh serum-free medium . The medium contained 25 mL Neurobasal medium (no Glutamine and Phenol Red, Gibco®), 25 mL Dulbecco's Modified Eagle Medium (DMEM)-F12 Nutrient Mixture (no Glutamine, Gibco®), 1 mL B27 supplement (50x) (Gibco®), 0.5 mL N2 supplement (100x) (Gibco®), 0.5% Penicillin/Streptomycin (Sigma®), 0.2 % Heparin solution (5 μg/mL) (Stemcell Technologies®) as neural base medium (NB) and freshly added bFGF (AA10-155 Life Technologies®) (10 ng/mL) and EGF (Life Technologies®) (20 ng/mL) (Alcock and Sottile, 2009) right before applying to adult mNSCs.

2.2 Neural differentiation treatment

The basic neural differentiation medium (BNM) contained NB, 0.5% FCS and BDNF (human, Source Bioscience®) (10 μg/mL).The Retinoic acid (RA) treatment was BNM with 1 μM RA (Sigma®), and EC23 group was BNM with 1 μM EC23 (Stable Synthetic Retinoid EC23, Reinnervate®), The adult mNSCs were seeded on laminin-coated vessels and the density of cell is $2X10^4$/mL. Three days after seeding, the cells were treated with adult mNSC culture medium for an undifferentiated group or the five different neural differentiation treatments. The media were changed every two days and fixed with cold 4% parafomaldehyde (PFA) (VWR International®) in PBS at various time point. In a series of serum free experiments, fetal calf serum was removed from the formulas described above.

2.3 Immunocytochemical staining

Adult mNSCs were seeded on laminin- (10 □g/mL DMEM-F12) coated glass coverslips and the density of cells was $2X10^4$/mL. The cells were fixed with cold 4% PFA for 30 minutes at room temperature and then preserved in PBS. The cells were washed for 10 min in 0.1% Tween-20 (Sigma-Aldrich®) in PBS (PBT). And then, the cells were incubated

in blocking solution (0.1% Tween-20 in PBS, 0.2% FCS) for 30 min at room temperature. Afterwards, the cells were incubated in the primary antibody (Nestin, Rat-401®, Developmental Studies Hybridoma Bank 1:50, β-III tubulin D71G9 XP® mAb #5568, Cell Signaling 1:100 and GFAP Z0334®, Dako 1:200) diluted in 0.02% FCS blocking solution for 1.5 hour at room temperature. Subsequently, the cells were washed thrice with PBT for 15 min at room temperature and then incubated in the secondary antibody (Fluorescein Goat Anti-Rabbit IgG Antibody FI-1000®, Fluorescein Horse Anti-Mouse IgG Antibody FI-2000®, Texas Red Goat Anti-Rabbit IgG Antibody TI-1000®, and Texas Red Horse Anti-Mouse IgG Antibody TI-2000®, Vector Laboratories 1:200) diluted in 0.02% FCS blocking solution for 1 hour in the dark. After washing thrice with PBT for 15 min at room temperature, the glass coverslips were mounted on glass slides with Vectrashield® Mounting Medium containing DAPI (H-1200, Vectrolabs®). The slides were stored at room temperature in the dark at least four hours and fluorescent image were taken by a Nikon ECLIPE 90i Fluorescence Microscope, a Hamamatsu Digital Camera and Volocity® software (version 5.2.0, Improvision).

2.4 Cell counting

2.4.1 Dapi positive cell nuclei counting

The Dapi positive cell nucleus counting was calculated from ten identical images of different fields of view from each sample to determine the number of Dapi positive cell nuclei.

2.4.2 Percentage of β-III tubulin positive neurons

The β-III tubulin positive neuron counting was calculated from ten identical images of different fields of view from each sample to determine the number of Dapi positive cell nuclei. The percentage of β-III positive neuron was calculated by the numbers of β-III tubulin positive neuron divided by the numbers of Dapi positive cell nucleus from the same image. The values were presented by mean ± standard error of the mean.

2.4.3 Cell counting for cell growth rate study

mNSCs were seeded on laminin coated-24-well plates at a density of $2X10^4$/mL. After three days, the cells were treated with adult mNSC culture medium as undifferentiated control group or neural differentiation treatments. The cells were trypsinised and then incubated at 37°C up to 5 minutes. Subsequently, the cells were treated by 10% FCS in PBS

in order to stop trypsinisation. 10 □L supernatant of cells were counted using a Haemocytometer and cell numbers calculated in quadruplicates from the same batch at each condition and time point.

3. Result and discussion

3.1 Comparing the impact of EC23 on neurogenesis and cell growth with Retinoic acid

3.1.1 The growth rate of the Retinoids-treated adult mNSCs

During differentiation, NSCs divide into two different daughter cells: one is still NSC and the other is differentiated cell so that the number of cell will decrease during differentiation. It has been also reported that Retinoic acid increased the mRNA and protein level of p53, caspase-3, -6, -7 and -9 in keratinocytes which elements are associated with apoptosis (Mrass et al., 2004). A cell counting experiment was conducted to monitor the change of the numbers of cell under a series of neural differentiation treatments. The neural differentiation treatments started at day zero. After a two-day treatment of BNM, the number of the treated cells remained at the similar level with untreated adult mNSCs at day zero (Figure 3-9 red Day 2) which also observed in BNM+RA- (Figure 3-9 green Day 2) and BNM+EC23-treated (Figure 3-91 purple Day 2) groups. The numbers of treated-cells showed a decrease on day three and day four. The numbers of cell treated by BNM+RA decreased the most between day two and day four (Figure 3-9 green). It indicated that Retinoic acid attenuated the growth of adult NSCs the most.

Appendix

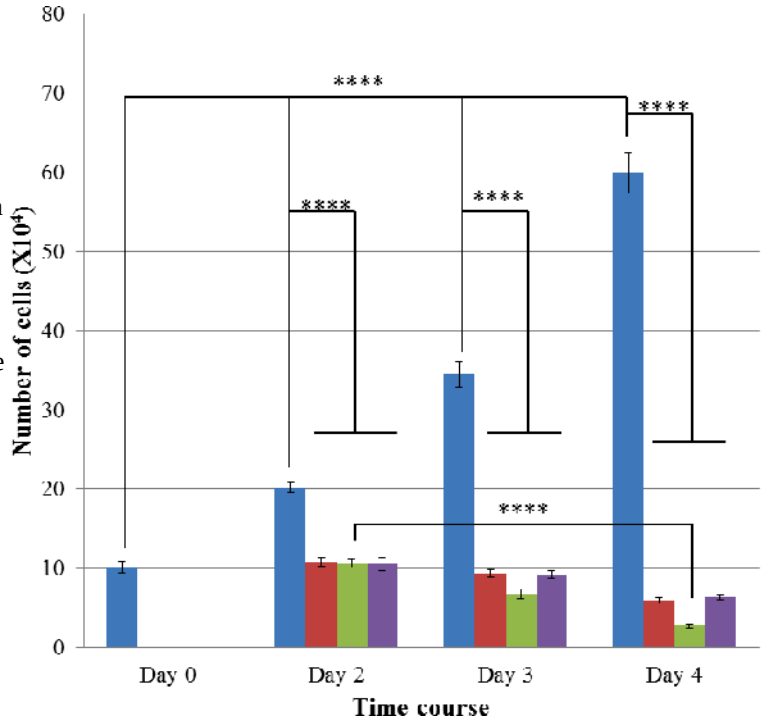


Figure 1 The numbers of the untreated adult mNSCs or cells by BNM, BNM+RA or BNM+EC23 from two to day four. The statistic is illustrated by standard error **** indicated the was smaller than by a one way analysis. treated day mean ± mean. p value 0.0001 ANOVA

3.1.2 Neurogenesis adult mNSCs Retinoic acid EC23 treatment.

of under or

Under a BNM+RA treatment, some J3-III tubulin-expressing neurons were detectable from day four (data not show), day five (Figure 3-4 (A) green) and day seven (Figure 3-4 (B) green). However, the J3-III tubulin-expressing neurons became undetectable on day eleven (Figure 3-4 (C) green) and day thirteen (Figure 3-4 (D) green). In the BNM +EC23- treated cells, some J3-III tubulin-expressing neurons were detectable from day four (data not show), day five (Figure 3-4 (F) green), day seven (Figure 3-4 (G) green), day eleven (Figure 3-4 (H) green) and day thirteen (Figure 3-4 (I) green). Comparing the morphology of J3-III tubulin-positive neurons under BNM+EC23 on day five and day seven with day eleven and thirteen, the J3-III tubulin-positive neurons showed long neurites on day five and seven but short neurites on day eleven and thirteen. The neurons with long neurites had excitatory-neuron-like morphology and the neurons with short neurites had inter-neuron-like morphology. In the untreated adult mNSC, only Nestin-expressing cells were detectable (Figure 3-4 (E) red) but not J3-III tubulin- positive cells. It concluded that BNM+RA or EC23 induced adult mNSCs to produce neurons from day four and the J3-III tubulin expressing neurons survived in BNM+RA until day seven but day thirteen in BNM+EC23.

Appendix

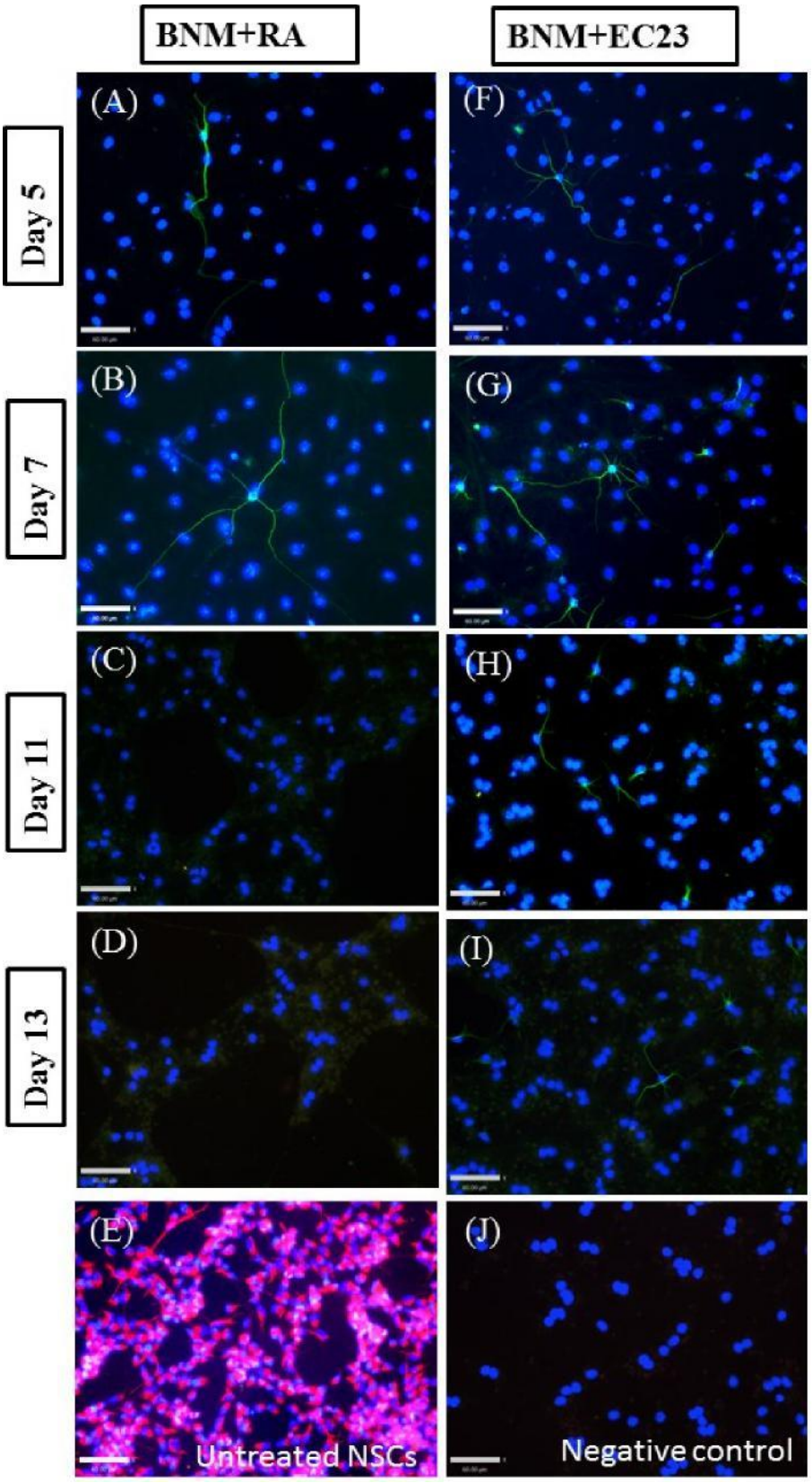


Figure 2 Retinoic acid and EC23 promoted neurogenesis in a differentiation time course.
Expression of □-III tubulin (green), Nestin (red) ((A)~(I)) and Dapi counterstain (blue) in a series of double immunocytochemical staining. (J) illustrated immunocytochemical staining without primary antibody, detecting possible auto-fluorescence from the cells. The scare bars were 60 μm.

Appendix

After counting the number of Dapi-stained nuclei and J3-III tubulin-positive neurons, it showed the percentage of J3-III tubulin-positive neurons under BNM+RA or BNM+EC23 on each differentiation time point (Figure 3-5). Under BNM+RA treatment, the percentage of J3-III tubulin-positive neurons slightly decreased from day five (Figure 3-5 BNM+RA blue) to day seven (Figure 3-5 BNM+RA red) without significant statistic different but disappeared on day eleven and thirteen (Figure 3-5 BNM+RA green and purple). In BNM+EC23, it is shown a trend that J3-III tubulin-positive neurons decreased from day five to day eleven (Figure 3-5 BNM+EC23 blue, red and green) but increased on day thirteen (Figure 3-5 BNM+EC23 purple). Comparing the percentage of J3-III tubulin-positive neurons under BNM+RA on day five (Figure 3-5 blue) and day seven (Figure 3-5 red) with BNM+EC23, the BNM+EC23 treated cells showed slightly higher percentage of J3-III tubulin-positive neurons then BNM+RA on both days but no statistic significant difference. However, the J3-III tubulin-positive neurons survived in a longer tern in BNM+EC23 then BNM+RA. It indicated that the J3-III tubulin-positive neurons survived in BNM+EC23 longer than BNM+RA and the inter-neuron-like cells develop slower than the excitatory-neuron-like cells.

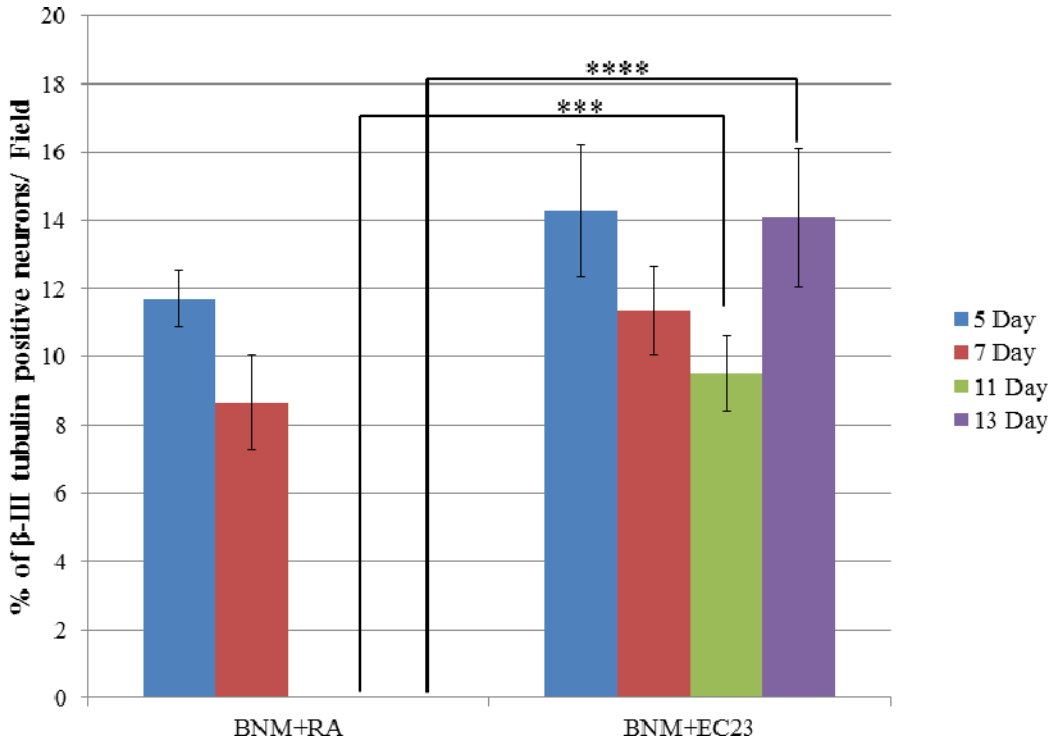


Figure 3 The numbers of the untreated adult mNSCs or treated by BNM, BNM+RA or BNM+EC23 from day two to day four.
The results are shown as mean ± standard error of the mean. **** indicated the p value < 0.0001 by a one-way ANOVA analysis. (P values: Day 3 BNM vs BNM+RA was 0.81and BNM+RA vs BNM+EC23 was 0.86. Day 4 BNM vs BNM+RA was 0.34 and BNM+RA vs BNM+EC23 was 0.54.)

3.1.3 Gliogenesis of adult mNSCs under Retinoic acid or EC23 treatment.

Some GFAP-positive astrocytes were detectable under BNM+RA treatment from day four (data not show) to day thirteen (Figure 3-6 red (A) day five, (B) seven, (C) eleven and (D) thirteen). It also observed under BNM+EC23 from day four (data not show) to thirteen (Figure 3-6 red (F) day five, (G) seven, (H) eleven and (I) thirteen). However, only Nestin was detectable in untreated adult mNSCs (Figure 3-6 green (E)) but not GFAP. Thus, some adult mNSCs went on glial genesis under Retinoic acid or EC23 treatment.

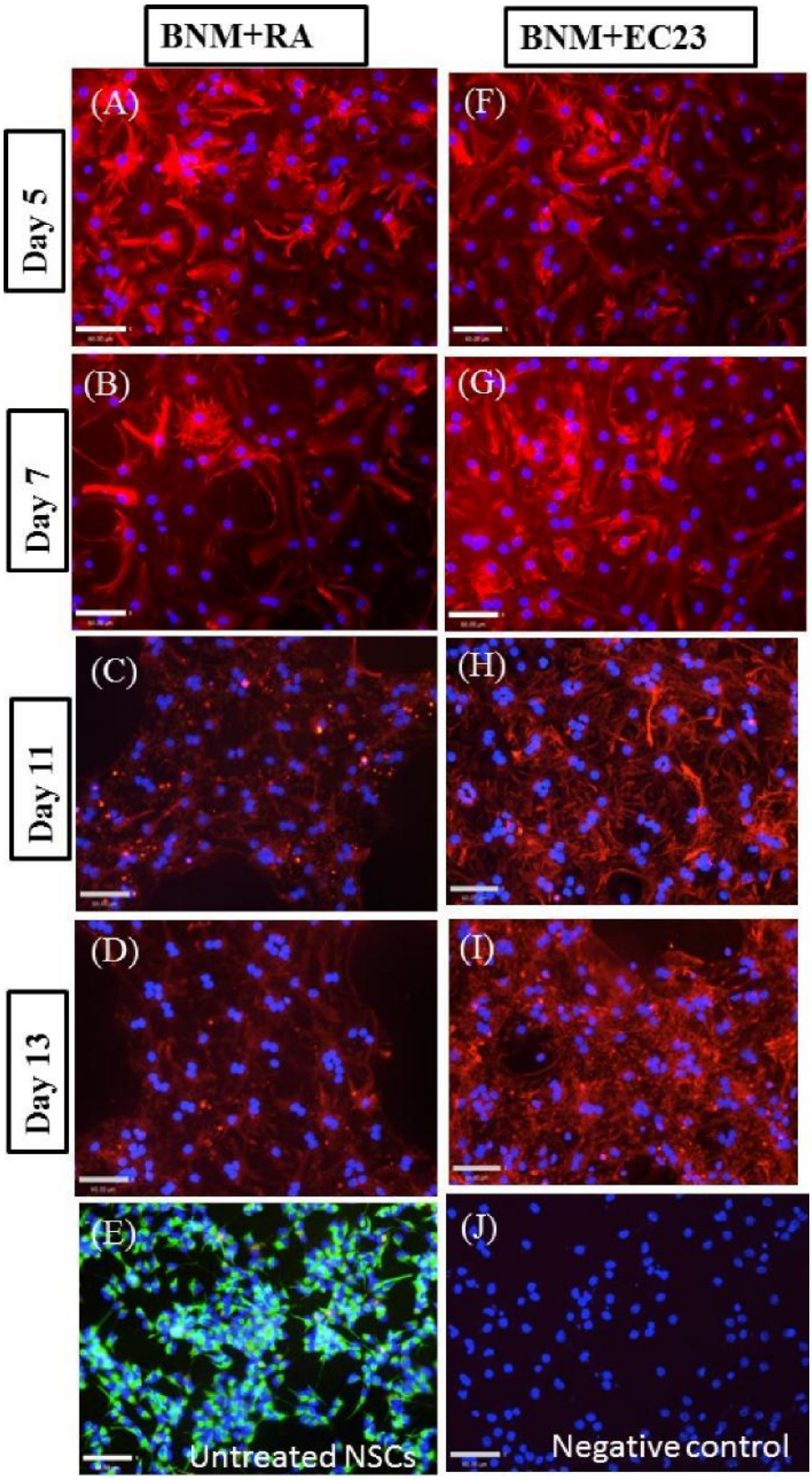


Figure 4 Retinoic acid and EC23 promoted glia formation in a time course differentiation.
The double immunocytochemical staining of GFAP, Nestin (green) ((A)~(I)) and Dapi counterstain (blue). (J) illustrated staining without primary antibody, eliminating possible false positive signals from auto-fluorescence of cells. The scare bars were 60 μm.

3.2 The influence of serum in Retinoic acid-induced neurogenesis of adult mNSCs

Serum has been reported to be used as a main element in Retinoic acid-induced neurogenesis (Weiss et al., 1996; Goffredo et al., 2008; Kim et al., 2009; Lu et al., 2009; Maltman et al., 2009; Christie et al., 2010; Lee et al., 2012b). In the results of neural differentiation study, 3-III tubulin-positive neurons were undetectable in BNM+RA conditions on day eleven (Figure 3-10 (A)) and thirteen (Figure 3-10 (B)).It suggested that the formula of BNM+RA might not support the survival of neurons on day eleven and thirteen. Therefore, a foetal calf serum free neural differentiation formula was tested. Surprisingly, some 3-III tubulin-positive neurons were detectable under serum free BNM+RA on day eleven (Figure 3-10 (C) green) and thirteen (Figure 3-10 (D) green) which was not seen in the untreated adult mNSCs (Figure 3-10 (I)). In BNM+EC23, there were more Dapi-positive nuclei and β-III tubulin-positive neurons being observed in serum free conditions than regular BNM+EC23 on day eleven and thirteen (Figure 3-10 compared (E) to (G) and (F) to (IT)). It indicated that serum was not required in Retinoid-induced neurogenesis.

Appendix

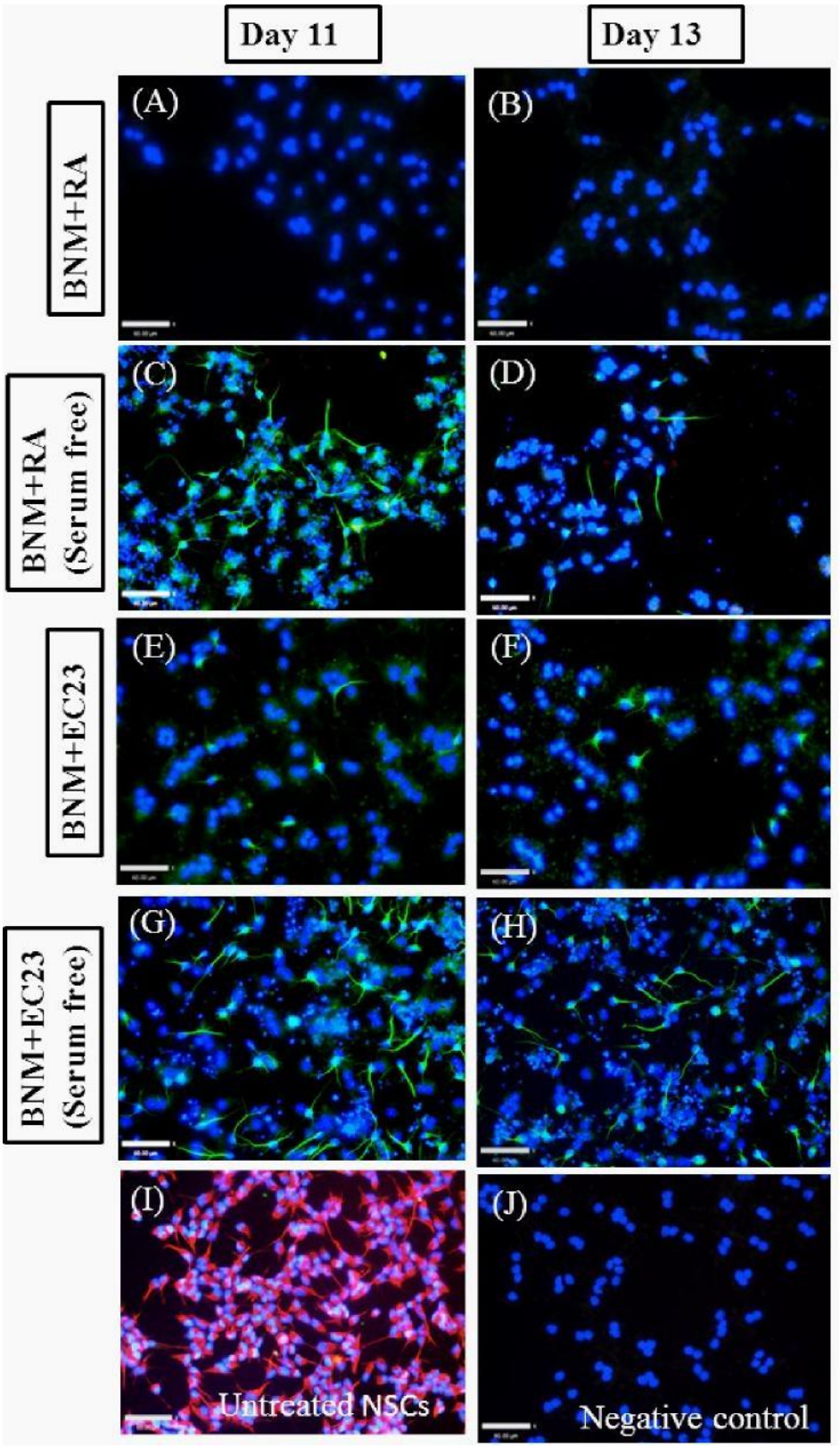


Figure 5 The expression of 3-III-tubulin under Retinoic acid or EC23 treatments with/without serum. (A)~(I) were double immunocytochemical staining of 3-III tubulin (green), Nestin (red) and Dapi counterstain (blue). (J) illustrated staining without primary antibody, eliminating possible false positive signals from auto-fluorescence of cells. The images were taken by a fluorescent microscope with Nikon i90 camera and analysed by Volocity® software. The scare bars were 60 μm.

Appendix

After performing double immunocytochemical stainings (Figure 3-10), it seems that there are more Dapi-positive nuclei in serum-free condition than regular BNM in both RA and EC23 treatments on day eleven and thirteen. Calculating the numbers of Dapi-positive nuclei would represent the numbers of cells under an observed field. In BNM+RA-treated cells, the number of Dapi-positive nuclei slightly decreased comparing day eleven (Figure 3-11 (A) 11 day) with thirteen (Figure 3-11 (A) 13 day) without statistically significance. This trend was observed in serum free BNM+RA on day eleven (Figure 3-11 (A) serum free 11 day) and thirteen (Figure 3-11 (A) serum free 13 day). The numbers of Dapi-positive nuclei in BNM+EC23 remained stable on day eleven (Figure 3-11 (B) 11 day) and thirteen (Figure 3-11 (B) 13 day). However, the numbers of Dapi-positive nuclei under serum free BNM+EC23 were higher than under serum containing condition (Figure 3-11 (B) compared serum free 11 day with 11 day and serum free 13 day with 13 day). It indicated that higher numbers of Dapi-positive cell nuclei remained under serum free BNM+EC23 on day eleven and thirteen and serum was not required in EC23 treatment.

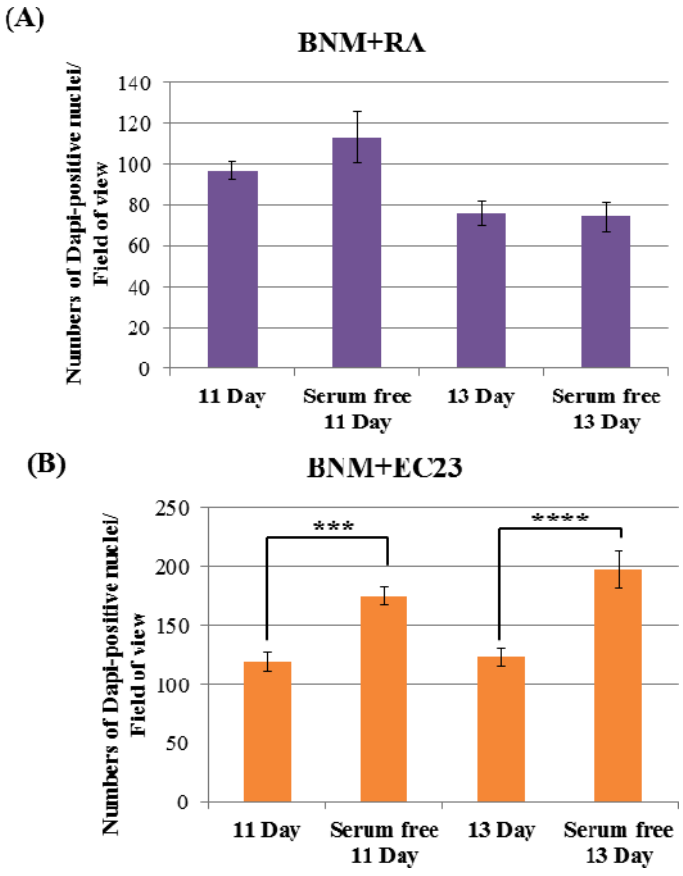


Figure 6 The numbers of Dapi-positive cell nuclei under Retinoids with or without foetal calf serum.
(A) illustreated the numbers of Dapi-positive nuclei in Retinoic acid treatment and (B) illustrreated EC23. The resuts are presented by mean ± standard error of the mean. After one way ANOVA analysis, ** indicated the p value was smaller than 0.01, *** was smaller than 0.001 and **** was smaller than 0.0001.

Appendix

In the images of double immunocytochemical staining (Figure 3-10), some β-III tubulin-positive neurons was detectable under serum free BNM+RA on day eleven (Figure 3-10 (C) green) and thirteen (Figure 3-10 (D) green). It seems that more 13-III tubulin-positive neurons were observed in EC23 groups (Figure 3-10 compared (E) to (G) and (F) to (IT)). After calculating the percentage of 13-III tubulin-positive neurons, there was around 17% 13-III tubulin-positive neurons in serum free BNM+RA on day eleven (Figure 3-12 (A) serum free 11 day) and 15 % on day thirteen (Figure 3-12 (A) serum free 13 day). In BNM+EC23, the cells treated under serum-free formula also showed the percentage of β-III tubulin-positive neurons was around 10% higher than regular BNM+EC23 on both days (Figure 3-12 (B) compared serum free 11 day with 11 day and serum free 13 day with 13 day). Under serum free condition, BNM+EC23 showed slightly higher percentage of β-III tubulin-positive neurons then BNM+RA on day eleven (Figure 3-12 compared (B) serum free 11 day with (A) serum free 11 day) and significant higher on day thirteen (Figure 3-12 compare (B) serum free 13 day with (A) serum free 13 day ****). It indicated that foetal calf serum free BNM+EC23 would provide β-III tubulin-positive neurons to survive in a long term differentiation time course and EC23 would be a better chemical to use for neural differentiation study than Retinoic acid.

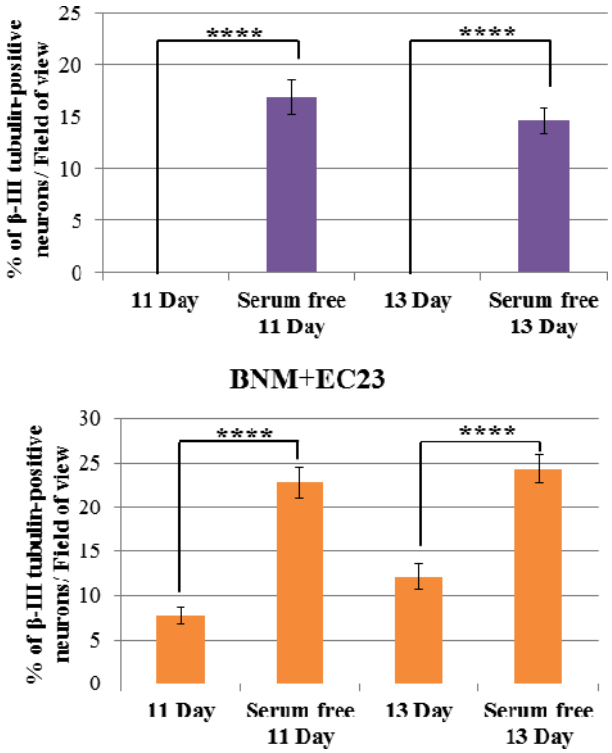


Figure 7 The percentages of 3-III tubulin-positive neurons under eleven- or thirteen-day treatment of Retinoids with or without fetal calf serum.
(A) inllustrated the result in Retinoic acid and (B) illustrated EC23 groups. The resuts are presented by mean ± standard error of the mean. One way ANOVA analysis showed that p value was smaller than 0.0001 (****).

4. Conclusion

EC23 promoted neurogenesis from day four to day thirteen whilst the 3-III tubulin-positive neuron in Retinoic acid was not detectable under on day eleven and thirteen in serum containing media. The numbers of Dapi-positive nuclei and the percentage of 3-III tubulin-positive neurons under EC23 were higher than Retinoic acid. However, removing foetal calf serum from the basic neural differentiation medium, the 3-III tubulin- positive neurons were observed in Retinoic acid treatment on day eleven and thirteen. The number of Dapi-positive nuclei and the percentage of 3-III tubulin- positive neurons under EC23 were lower than serum free EC23 condition. It suggested that serum free EC23 neural differentiation formula would be a favourable condition in studying Retinoid-induced neurogenesis.

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