

For my family

ABSTRACT

Eun Hyuk, C. The role of polycomb repressive complex 2 in postnatal subventricular zone neural stem/progenitor cell self-renewal and multipotency University College.

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The murine subventricular zone (SVZ) in a brain contains a population of stem cells and daily produces tens of thousands of neurons throughout lifetime. However, the mechanisms of SVZ neural stem/progenitor cell (NSPC) maintenance, differentiation and cell-fate specification are still not clear. To understand these parameters via histone methylations with bivalent mechanism, the SVZ NSPCs were first isolated by using a culture technique called neurosphere assay (NSA). It has been a challenge to culture pure cell populations of SVZ subtypes, so the NSA was initially validated.

The H3K27me3 mark, which has a dominant role in the bivalent mechanism, has not been studied in postnatal and adult SVZ *in vivo*, yet their role has been implicated to control the shift of embryonic cortical neurogenesis to gliogenesis. Therefore, we have first investigated whether H3K27me3 marks are present in the postnatal and adult SVZ NSPC population and whether their marks have been changed after stroke or demyelination in central nervous system (CNS) by immunohistochemistry. With the confirmation of H3K27me3 mark present in SVZ NSPCs, the presence of H3K27me3 catalyzer, called polycomb repressive complex 2 (PRC2) core components (Eed, Ezh2, Suz12) including Jarid2, was investigated and confirmed in postnatal SVZ *in vitro* by qRT-PCR and Western blot. To understand the role of PRC2 enzymatic activity in postnatal SVZ neurosphere self-renewal and multipotency, Eed was down-regulated by using lentiviral mediated delivery of shRNA. Also, PRC2 dependent or independent function of Jarid2 was examined via knockdown approach. The lack of Eed in the neurospheres resulted the attenuation of self-renewal and oligodendrogenesis, whereas the Jarid2 knockdown neurospheres showed the decreased proliferation with no SVZ NSPC differentiation. Based on these knockdown studies, it suggests Eed and Jarid2 might not share their function in the postnatal SVZ NSPCs to govern postnatal SVZ NSPC self-renewal and multipotency.

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This thesis have been conceptualized and experimentally designed by myself with inputs from my supervisor, Dr. Francis Szele and ad hoc supervisor, Prof. Neil Brockdorff.

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All other experiments including quantification and analysis have been performed by myself with the great helpful guidance from my colleagues in the Department of Physiology, Anatomy and Genetics.

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Abbreviations

Ara-C	Cytosine- β -D-arabinofuranoside
BCA	Bicinchoninic Acid
bHLH	basic helix-loop-helix
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
ChiP	Chromatin immunoprecipitation
ChiP-seq	Chromatin immunoprecipitation-sequencing
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CT-1	Cardiotrophin-1
DCX	Doublecortin
DIV	Day in vitro
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
dpi	day post infection
Eed	Embryonic ectoderm development
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EM	Electronic microscopy
ESC	Embryonic stem cell
Ezh2	Enhancer of zeste homolog 2
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FGF2	Fibroblast growth factor 2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCV	Ganciclovir
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate aspartate transporter
H3K27me3	Histone 3 lysine 27 trimethylation
JAK-STAT	Janus kinase-signal transducers and activators of transcription
LIF	Leukemia inhibitory factor
MCAO	Middle cerebral artery occlusion

mRNA	messenger ribonucleic acid
NEPC	Neural epithelial cell
Ngn	Neurogenin
NPC	Neural progenitor cell
NSA	Neurosphere assay
NSC	Neural stem cell
NSPC	Neural stem/progenitor cell
O4	Oligodendrocyte 4
Olig	Oligodendrocyte lineage gene
PB	Phosphate Buffer
PBS	Phosphate buffered saline
PcG	polycomb group
PDGFR- α	Platelet-derived growth factor receptor-alpha
PFA	Paraformaldehyde
Ph3	phosphohistone 3
PRC2	Polycomb repressive complex 2
PSA-NCAM	Polysialylated neuronal cell adhesion molecule
qRT-PCR	quantitative real time polymerase chain reaction
RGC	Radial glial cell
RMS	Rostral migratory stream
RNAi	ribonucleic acid interference
RT-PCR	reverse transcription polymerase chain reaction
Shh	Sonic hedgehog
shRNA	short hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
Smo	Smoothened
SSEA1	Stage-specific embryonic antigen 1
Suz12	Suppressor of zeste homolog 12
TBS	Tris-buffered saline
TE	Tris-EDTA
TrxG	Trithorax group
TU	Transducing Unit
β III tubulin	Class III beta tubulin

Chapter 1

Introduction

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Chapter 1

Introduction

1.1. The SVZ Neurogenesis: Life-Long Maintenance Of Endogenous Neural Stem/Progenitor Cells Via Spontaneous Transformation

Adult neurogenesis is an exceptional phenomenon for neuronal development in the adult brain. It mostly occurs in the murine hippocampus and the subventricular zone (SVZ) throughout the entire lifetime (Gage et al., 1995a; Gould et al., 1999; Palmer et al., 1997; Richards et al., 1992). The exact function of adult neurogenesis in normal behavior is not clearly understood, yet ongoing neurogenesis contributes to short term olfactory and hippocampal memory formation (Kriegstein and Alvarez-Buylla, 2009; Shors et al., 2002; Zhao et al., 2008a). Particularly, SVZ adult neurogenesis has been highlighted as a source for producing new neurons for repairing injured or diseased lesion sites in the central nervous system (CNS), although complete functional recovery has not been shown (Arvidsson et al., 2002). However, the adult SVZ neurogenesis in humans becomes attenuated during childhood and is no longer found in the adult SVZ (Sanai et al., 2011). In order to maintain or reinvigorate human SVZ neurogenesis for therapeutic applications in adult brains, it is important to understand how neural stem cell (NSC) populations are maintained from embryogenesis through to the adult SVZ. Thus, in this thesis I specifically looked for mechanisms regulating self-renewal and multipotency in the postnatal SVZ neural stem/progenitor cells (NSPCs).

1.1.1. Neuroepithelial Cells Transform Into Radial Glial Cells In Early Embryonic Brains

In brain development, adult SVZ NSCs are originally derived from neuroepithelial cells (NEPCs), which are found in a pseudostratified epithelial layer called the ventricular zone (VZ) in the neural plate and neural tube (Gotz and Huttner, 2005) (Figure 1-1). In the VZ, NEPCs are anchored on the apical (ventricular) surface of the neural tube via adherens junctions and these cells send long processes to the basal (pial) lamina, which lies on the opposite side of the epithelial layer (Huttner and Brand, 1997; Wodarz and Huttner, 2003). Like typical epithelial cells, they also contact neighboring cells via tight junctions (Aaku-Saraste et al., 1996; Campbell and Gotz, 2002; Zhadanov et al., 1999).

To expand the VZ, NEPCs undergo mitosis for self-renewal, and they also differentiate into multiple lineages in the developing cerebral cortex (McCarthy et al., 2001). An NEPC can give rise to either two NEPCs which expand the VZ through symmetrical division, or one NEPC and a neuron or a basal progenitor expanding the cortex through asymmetrical division (Huttner and Brand, 1997; Wodarz and Huttner, 2003). Based on these previous studies, the NEPC population was proven to truly be the neural stem cells, which are able to continuously self-renew and to differentiate into multiple lineages. Much work in the past two decades has elucidated extrinsic and intrinsic molecular regulatory mechanisms that govern NEPC development.

At approximately embryonic day 9–10 (E9-10) NEPCs, expressing Nestin as an intermediate filament (Hartfuss et al., 2001) and Prominin (CD133) in the apical plasma membrane (Corbeil et al., 2001; Weigmann et al., 1997), acquire astroglial cell features. Throughout this thesis, these cells are henceforth called radial glial cells (RGCs) (Figure 1-1b,c, 1-2). Most RGC properties are similar to NEPCs such as

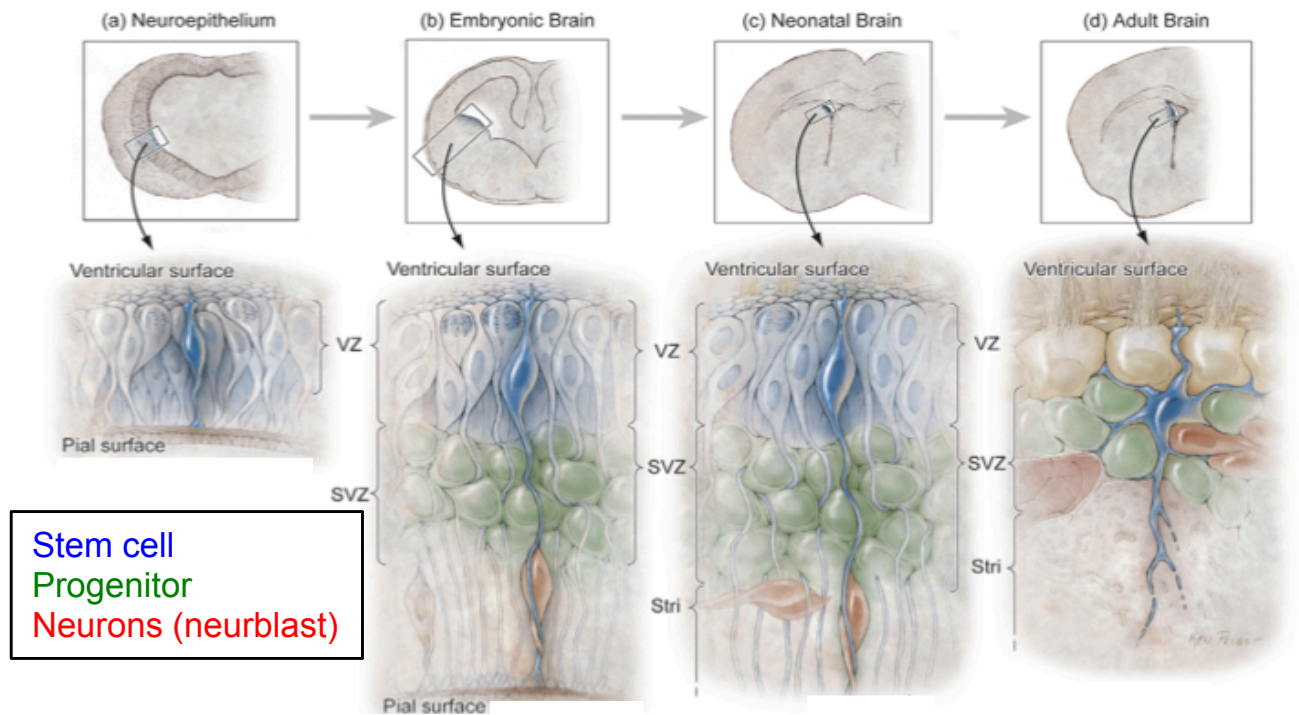


Figure 1-1. Cellular morphology of neural stem cells during embryonic to adult transformation.

From the embryonic neuroepithelium to the adult brain, the apical side of neural stem cells always contacts the ventricular surface. Although some physiological features have changed, they always give rise to intermediate progenitor cell populations as well as neurons (neuroblasts). Interestingly, a ventricular zone (VZ) can be still found in neonatal (P0-2) brain, but it disappears by P4. *The image is modified from Merkle, F.T. and Alvares-Buylla, A. 2006 Curr. Opin. Cell. Biol.*

adherens junctions, apical surface, basal laminin contact, Nestin and Prominin expression and down-regulated apical-basal polarity. However cell-cell tight junctions are converted to adherens junctions in RGC (Aaku-Saraste et al., 1996; Gotz and Huttner, 2005; Stoykova et al., 1997). Also, RGCs additionally express brain lipid-binding protein (BLBP), astrocyte-specific glutamate transporter (GLAST), vimentin, Tenascin C (TN-C), s100 calcium binding protein β (s100 β) and Glutamine Synthetase (GS) (Kriegstein and Alvarez-Buylla, 2009; Mori et al., 2005). The RGC begins to directly establish contact with endothelial cells, facilitating cerebral vasculature development (Mission et al., 1991; Takahashi et al., 1990). Taken together, this strongly suggests that NEPCs are directly transformed into the RGC.

1.1.2. Spatiotemporal Cell Fate Restriction Of Radial Glial Cells

As the transformation from NEPCs to RGCs occurs with the few physiological changes as described above, the stem cell properties of RGCs become attenuated compared to NEPCs. RGCs can still expand the VZ and give rise directly to neurons or the cell-fate restricted intermediate progenitors such as oligodendrogenic (oIPCs) or neurogenic (nIPCs) progenitor cells (Figure 1-2). However, it is controversial whether one RGC can give rise to multiple cell lineages, as examined by *in vivo* viral lineage tracing experiments (Grove et al., 1993; He et al., 2001; Parnavelas et al., 1991; Reid et al., 1995; Walsh and Cepko, 1988, 1992; Williams et al., 1991; Yung et al., 2002) and *in vitro* experiments (Luskin et al., 1988; Qian et al., 2000).

The attenuation of RGC fate commitment is temporally regulated. This was demonstrated using Cre/loxP cell fate-mapping. After cre recombinase, driven by a promoter of early RGC cell marker (Blbp) expressed at E10, was crossed with transgenic mice, all the CNS cells were labeled (Anthony et al., 2004). This evidence

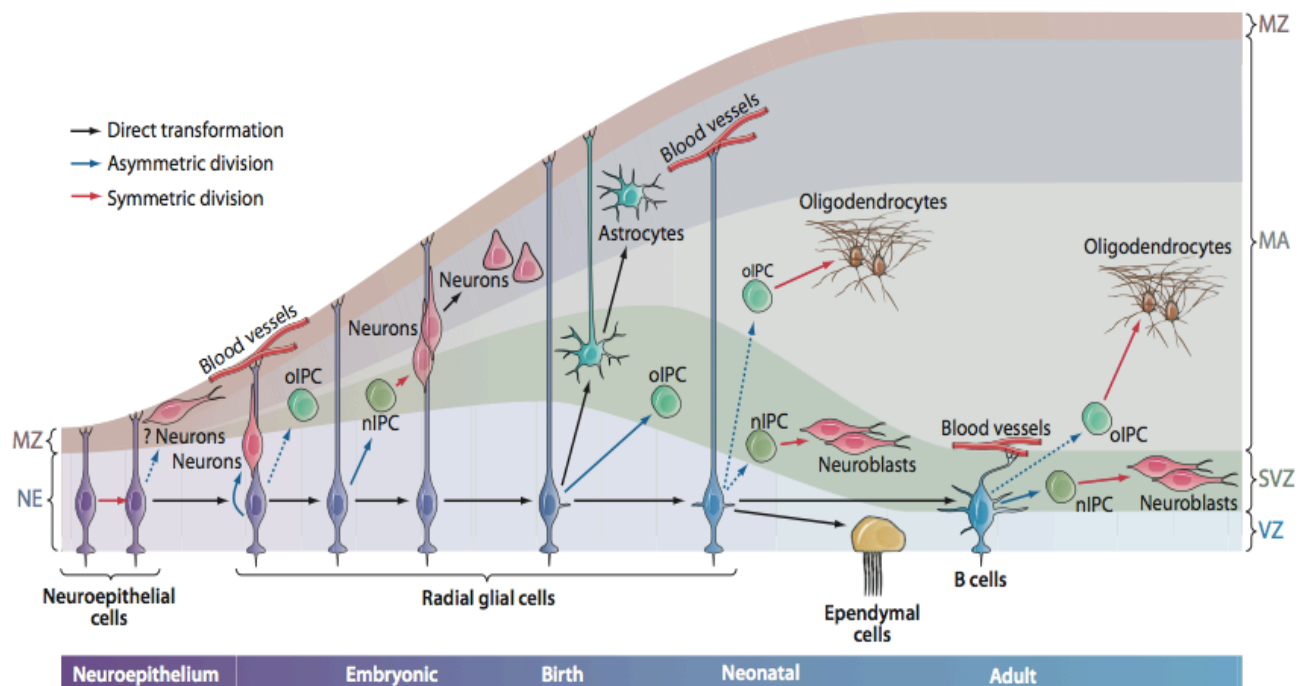


Figure 1-2. The spontaneous transformation of neural stem cells during cortical development.

The neuroepithelial cells are found in early neural tube formation. In order to expand cortical layers from the pseudostratified layer, they transform into radial glial cells during E9-10. The more cell fate committed radial glial cells give rise to differentiated cells with intermediate progenitor cells (IPC) including neurogenic IPC (nIPC) and oligodendrogenic IPC (oIPC). With the nIPC cells, radial glial cells produce neurons in the cortex until approximately E17 (neurogenesis). Following this neurogenic period, glia are produced from radial glial cells until neo- or postnatal periods (gliogenesis). During postnatal stages, a subset of radial glial cells transform into specialized astrocytes with primary cilia contacting the lateral ventricle; these are considered to be adult SVZ neural stem cell. Another subset of radial glia turn into ependymal cells. *The image is adapted from Kriegstein, A. and Alvarez-Buylla, A. 2009 Annu. Rev. Neurosci.*

indicates all the RGCs in E10 are multipotential. In contrast, the cre driven by the human glial fibrillary acidic protein (hGFAP) promoter for RGC population, expressed at E14, labels a large number of neurons in dorsal telencephalon, whereas in ventral telencephalon almost all the oligodendrocytes and only a few neurons are marked (Malatesta et al., 2003). In the cortical RGCs, the RGC fate commitment was first demonstrated during the neurogenic period around E12-E13 (Noctor et al., 2004). For instance, the specific cell types like deeper layer of cortical projection neurons are only born from cortical RGCs in earlier period of the embryonic cortical neurogenesis (Desai and McConnell, 2000).

Besides the temporal control for RGC fate determination, there is regional control of RGC fate that can be observed in 4 main regions: the septum, the cortex, the lateral ganglionic eminence (LGE) and the medial ganglionic eminence (MGE) based on the different types of neuronal production with different combinations of transcriptional factors expression (Kriegstein and Alvarez-Buylla, 2009). For example, the cortical pyramidal neurons only come from Pax6 expressing RGCs in cortical regions. This region-specific differentiation is also found in astrocyte (Hochstim et al., 2008) and oligodendrocyte (Kessaris et al., 2006) generation. Taken together, this evidence suggests that the RGC fate is spatiotemporally determined with the RGC population becoming more heterogeneous as the brain develops.

1.1.2.1. The cortical radial glial cell fate determination by extracellular factors

Due to the early RGC fate restriction, which can be retained until the postnatal stage *in vivo* (Merkle et al., 2007), it is important to understand the mechanism of RGC fate determination during embryonic brain development. Although different regions of RGCs are governed by different transcription factors, the cortical RGC fate

determination has been best studied. Thus, I will discuss about how cortical RGCs are regulated for their fate determination.

As shown in the cell fate-mapping studies by Malatesta et al. in 2003, the cortical RGCs primarily generate neurons between E13 and E16 in dorsal VZ/SVZ. Later on, the RGCs become more biased towards gliogenesis starting from E18 till the postnatal stage (Alvarez-Buylla and Lim, 2004) (Figure 1-2). In order to examine whether this phenomenon is due to extrinsic stimulations, the E15 neuronal progenitor cells (NPC, referring as both RGC and intermediate progenitor cells) are differentiated on neurogenic and gliogenic brain slice cultures, which give rise to neurons and glia respectively (Morrow et al., 2001). Based on this *in vitro* experiment, the extrinsic cues have been shown to play important roles determining NPC fate, as either neurogenic or gliogenic.

During RGC neurogenesis, several extracellular factors were found to be important for maintaining this neurogenic period (Anderson, 2001). The platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and Wnt signaling are potential candidates (Erlandsson et al., 2001; Hirabayashi and Gotoh, 2005; Hirabayashi et al., 2004; Israsena et al., 2004; Jin et al., 2002; Johe et al., 1996; Li et al., 1998; Muroyama et al., 2004). Both PDGF and VEGF increase the proliferation of NPCs during the neurogenic period, whereas Wnt signaling promotes differentiation (Figure 1-3A). The latter is mediated by the β -catenin and TCF complex, which enhance the expression level of neurogenin (Ngn) 1 and Ngn 2 at E13.5 (Hirabayashi et al., 2004; Israsena et al., 2004). This has been validated by the conditional knockout of the β -catenin gene, which results in the lack of both Ngn 1/2 expression in the embryonic cortex (Machon et al., 2003). As Ngn 1 and 2 can negatively influence astrocytic gene activation at the transcriptional level

(Sun et al., 2003), they are necessary for the neurogenesis in embryonic cortex (Fode et al., 2000). Therefore, the role of Wnt signaling has been shown to be very important for cortical neurogenesis.

In addition to these three extracellular factors, Notch signaling is an important mediator for NPC neurogenesis. This has been demonstrated by conditional inactivation of mindbomb-1 (Mib-1), a ligase for Notch ligands (Deltalike and Jagged), in nestin and hGFAP positive cells (Yoon et al., 2008). The loss of Mib-1 disrupts Notch signaling resulting in premature differentiation of nIPCs. In addition, the lack of Notch signaling between RGCs and either nIPCs or neurons results in the depletion of RGC populations in the VZ (Dong et al., 2012; Yoon et al., 2008); this provides evidence that Notch signaling is essential for RGC survival and maintenance of neurogenesis.

In contrast to the aforementioned function, Notch signaling, stimulated in newborn neurons, has another important role in regulating the appropriate proportion of neurons and initiating gliogenesis through multiple different downstream cell signaling cascades (Bertrand et al., 2002; Namiyama et al., 2009). For example, persistent expression of Hes1, a gene downstream of Notch signaling, negatively influences neuronal differentiation by suppressing the function of proneural basic helix-loop-helices (bHLHs) such as Mash1 and Ngn 1 until postnatal stages (Hirabayashi and Gotoh, 2005; Louvi and Artavanis-Tsakonas, 2006; Muroyama et al., 2004). At the same time, Hes1 and Hes5 directly induce Janus kinase (JAK) 2 to activate the signal transducers and activators of transcription (STAT) 3, which promotes gliogenesis (Kamakura et al., 2004) (Figure 1-3B). This shift from neurogenesis to gliogenesis was observed around E16-18, and during the gliogenesis period this JAK-STAT pathway is also induced by the ciliary neurotrophic factor

(CNTF), leukemia inhibitory factor (LIF) and cardiotrophin-1 (CT-1) (Hirabayashi and Gotoh, 2005).

Besides Notch and JAK-STAT signaling, epidermal growth factor (EGF) and bone morphogenic protein (BMP) can also promote the gliogenesis in RGCs (Burrows et al., 1997; Panchision and McKay, 2002), and these signaling pathways are interrelated. For example, both CNTF- and CT-1 induced JAK-STAT signaling activates STAT3 function as a transcriptional factor (Johe et al., 1996; Miller and Gauthier, 2007) (Figure 1-2B). This active form of STAT3 directly interacts with co-activator CBP/p300 and the active form of Smad1, which is stimulated by BMP2 (Nakashima et al., 1999; Sauvageot and Stiles, 2002; Sun et al., 2003). This complex can induce glial cell markers such as α 100 β and GFAP. In addition, the GFAP promoter region has a STAT-responsive element, which can be directly recognized by the complex (Bonni et al., 1997). Based on these combinations of extracellular signaling, it is possible to shift cortical RGCs from a neurogenic to gliogenic fate.

Oligodendrogenesis begins after neurons and astrocytes are generated, migrate and localize in the cortex. During neurogenesis, the oligodendrocyte specific bHLH factor, Olig2, is co-expressed with Ngn2, so NPCs produce motor neurons rather than oligodendrocytes. However, after Ngn expression is suppressed, Nkx2.2 is expressed along with Olig2 to give rise to oligodendrocytes (Ross et al., 2003). Also, induction of Sonic hedgehog (Shh) signaling enhances promotion of oligodendrogenesis by stimulating Olig 1/2 expression, which can inhibit astrogenesis (He and Sun, 2007).

In summary, the cortical RGCs have separate time periods for neurogenesis and gliogenesis. Although this fate shift paradigm may not be shared with other RGCs in other regions, the several extracellular signaling described above are certainly important to govern the cortical RGC fate determination.

1.1.3. Radial Glial Cells Transform Into Astrocyte Like Stem Cells In The Postnatal SVZ Development

After the VZ persists until P2- P3, the periventricular region becomes the SVZ (Merkle et al., 2004) (Figure 1-1). The postnatal SVZ contains much more cells when compared to the adult SVZ although it becomes thinner during the first two weeks of the postnatal stage (Merkle et al., 2004; Peretto et al., 1999; Tramontin et al., 2003). Also, the olfactory ventricle becomes closed and forms the rostral migratory stream (RMS) (Peretto et al., 1999). Although the postnatal RGCs do not produce the same types of neurons that were generated by embryonic RGCs in the same region, the boundary of embryonic RGC fate restriction is still retained in the periventricular region with the region-specific transcription factor expression (Kohwi et al., 2007; Merkle et al., 2007; Young et al., 2007). For example, the ventrolateral SVZ that expresses *Gsh2* from the LGE and MGE produces dopaminergic and calbindin positive olfactory bulb (OB) periglomerular cells (PGCs), whereas dorsal SVZ expressing *Emx1* from cortical VZ gives rise to calretinin positive PGCs. Interestingly, this cell fate determination is due to a cell-autonomous mechanism rather than extracellular stimulation. It was demonstrated by engrafting different regions of RGCs into different locations in the SVZ, and the transplanted RGC fate did not alter (Kohwi et al., 2007; Merkle et al., 2007; Young et al., 2007).

Alongside the production of these OB interneurons, the postnatal RGCs can still produce parenchymal astrocytes and oligodendrocytes (Kriegstein and Alvarez-Buylla, 2009; Merkle et al., 2004). Also, the ventricular surface attached RGCs transform into astrocyte-like stem cells, as validated by overlapping RC1 expression between RGCs and astrocyte-like stem cells (Mission et al., 1991), fluorescent dye (Dil) labeling of RGCs (Alves et al., 2002) and viral labeling/time-lapse imaging of

RGCs (Merkle et al., 2004; Noctor et al., 2008). In support of this notion, the apical attachments of the RGC derived astrocyte-like stem cells are seen carrying primary cilia in the center of pinwheel structures found in the wall of the lateral ventricle, as visualized in whole mount staining (Mirzadeh et al., 2008). Moreover, the RGCs also give rise to a mature ependymal cell population during 1-4 postnatal weeks (Spassky et al., 2005) (Figure 1-1A,B). It is not clear whether one RGC can give rise to all these different cell types in postnatal SVZ, but it is well established that RGCs can spontaneously transform into astrocyte-like stem cells and ependymal cells and still maintain both neurogenesis and gliogenesis in postnatal SVZ.

1.1.4. Adult SVZ Neurogenesis And Gliogenesis

1.1.4.1. Astrocyte-like cells are the stem cells in adult SVZ

The astrocyte-like stem cells, transformed from cell fate restricted RGCs, have further attenuated their plasticity compared to the early embryonic NEPCs or RGCs, but they can still produce neurons, astrocytes and oligodendrocytes in adult brains. Previous studies have demonstrated that astrocyte-like SVZ stem cells can regenerate and multiply *in vivo* (Doetsch et al., 1997; Menn et al., 2006). First of all, it was demonstrated by infusing brains with cytosine- β -D-arabinofuranoside (Ara-C), to eliminate actively mitotic cells, namely, transit amplifying cells and neuroblasts in the SVZ. After 6 days, these cells were replenished by the astrocyte-like stem cells which are generally quiescent and therefore resistant to Ara-C depletion (Doetsch et al., 1999). To validate that the quiescent stem cell populations were astrocyte-like cells, the authors used a transgenic mouse expressing the receptor for avian leucosis virus under a GFAP promoter (GFAP-Tva mice) (Holland and Varmus, 1998). This allowed specific labeling of astrocytes with the replication-competent avian leucosis

virus encoding alkaline phosphatase (RCAS-AP). Interestingly, SVZ astrocytes, transit amplifying progenitor cells, migrating neuroblasts and then OB interneurons had the AP label (Doetsch et al., 1999), confirming that the astrocyte-like stem cells in the SVZ give rise to OB interneurons. In order to further validate this finding, all the GFAP positive cells were killed by infusing Ganciclovir (GCV) into the lateral ventricle of transgenic mice that expressed herpes simplex virus thymidine kinase (HSV-TK) in GFAP positive cells. This prevented the replenishment of new cells *in vivo* and *in vitro* (Imura et al., 2003; Morshead et al., 2003).

Furthermore, the regionally pre-determined cell fate in postnatal SVZ is still observed in the adult OB interneuron production. For instance, the cortical derived stem cell population becomes diminished in the adult SVZ, but it still produces calretinin positive OB interneurons. In contrast, the LGE derived stem cells give rise to all the calbindin positive OB interneurons (Kriegstein and Alvarez-Buylla, 2009). Also, other studies indicate that the astrocyte-like stem cell populations give rise to parenchymal NG2 positive oligodendrocyte progenitor cells to produce oligodendrocytes in adult brains (Aguirre et al., 2004; Menn et al., 2006) as well as astrocytes in adult SVZ (Doetsch et al., 1999). The multipotency of adult SVZ NSPC is found infrequently *in vivo*, but it can easily be demonstrated in adult SVZ neurospheres (Doetsch et al., 1999).

In order to maintain the quiescent adult SVZ astrocyte-like stem cell population, the primary cilia on the surface of the lateral ventricle have important functions as cellular antennae. This function has been implicated to respond extracellular signaling such as Shh since postnatal SVZ (Han et al., 2008; Huangfu and Anderson, 2005; Singla and Reiter, 2006). Conditionally knocking out Smoothed (Smo), a downstream effector of Shh signaling, in Nestin positive cells

results in the depletion of both astrocyte-like stem cell and transient amplifying progenitor cell populations (Machold et al., 2003). This evidence suggests that the maintenance of postnatal and adult SVZ stem cell populations is still influenced by extracellular signaling. In addition to Shh signaling, recent studies suggest that both lateral ventricle and vasculature niches provide a microenvironment for stem cells to be maintained and to promote neurogenesis (Kokovay et al., 2010).

In sum, the previous experiments provided compelling *in vivo* evidence to convince the field that neurogenic and gliogenic astrocyte-like cells are the stem cells in the adult SVZ. Although there is some evidence to suggest that ependymal cells in the adult SVZ may act like neural stem cells after brain injury (Carlen et al., 2009; Johansson et al., 1999), the data have so far not been reproduced by other laboratories.

1.1.4.2. Heterogeneity in adult SVZ

The adult SVZ is composed of various cell types. Unipolar or bipolar astrocyte like stem cells (also known as B cells) (Mirzadeh et al., 2008) give rise to transient amplifying progenitor cells (also known as C cells), which can rapidly divide to generate neuroblasts (also known as A cells), that migrate through RMS to differentiate into OB interneurons (Doetsch et al., 1997) (Figure 1-4). Additionally, ependymal cells (directly transformed from RGCs), microglia (derived from peripheral macrophages), niche astrocytes, capillary blood vessels and pericytes are also found in the adult SVZ (Doetsch, 2003; He and Sun, 2007; Kriegstein and Alvarez-Buylla, 2009) (Figure 1-4).

Subpopulations of cells in the SVZ express different markers. For example, SVZ astrocyte like-stem cells were shown to express Sox2, Musashi, SSEA1, GFAP, Nestin, GLAST, Prolaminin (CD133), and platelet-derived growth factor receptor- α

(PDGFR- α) (Jackson et al., 2006). Niche astrocytes also expressed GFAP, but they could be separated from the stem cell population by epidermal growth factor receptor (EGFR) expressed only on the stem cells and other stem cell markers (Beckervordersandforth et al., 2010; Pastrana et al., 2009). Transient amplifying progenitor cells express EGFR (Doetsch et al., 2002; Pastrana et al., 2009) as well as Mash1 (Pastrana et al., 2009), whereas neuroblasts expressed polysialylated neuronal cell adhesion molecule (PSA-NCAM), doublecortin (Dcx) and class III beta tubulin (β III-tubulin) (Zhao et al., 2008a) (Figure 1-4). Finally, ependymal cells express s100 β and vimentin (Mirzadeh et al., 2008) (Figure 1-4). These markers are not restricted to individual cell-types, so recent studies have utilized more than one cell type marker to identify the individual cell types by using perspective fluorescence-activated cell sorting (FACSorting) approaches (Beckervordersandforth et al., 2010; Pastrana et al., 2009). It has been challenging in the last two decades to identify and isolate individual cell types from the heterogeneous cell populations in the adult SVZ. An alternative way to identify individual cell types is with electron microscopy (EM). Because individual cell type morphologies are significantly different from one another, it is possible to dissect out the specific subtypes in SVZ cells. For instance, a light cytoplasm with an irregular cell outline is typical of stem cells, whereas a large spherical shape with frequent nuclear invaginations is characteristic of transient amplifying progenitor cells. In contrast, elongated cells with dark cytoplasms are most likely neuroblasts (Doetsch et al., 1997). Although this imaging technique allows identifying the individual SVZ cell types *in vivo*, it is still difficult to isolate the cell populations from SVZ.

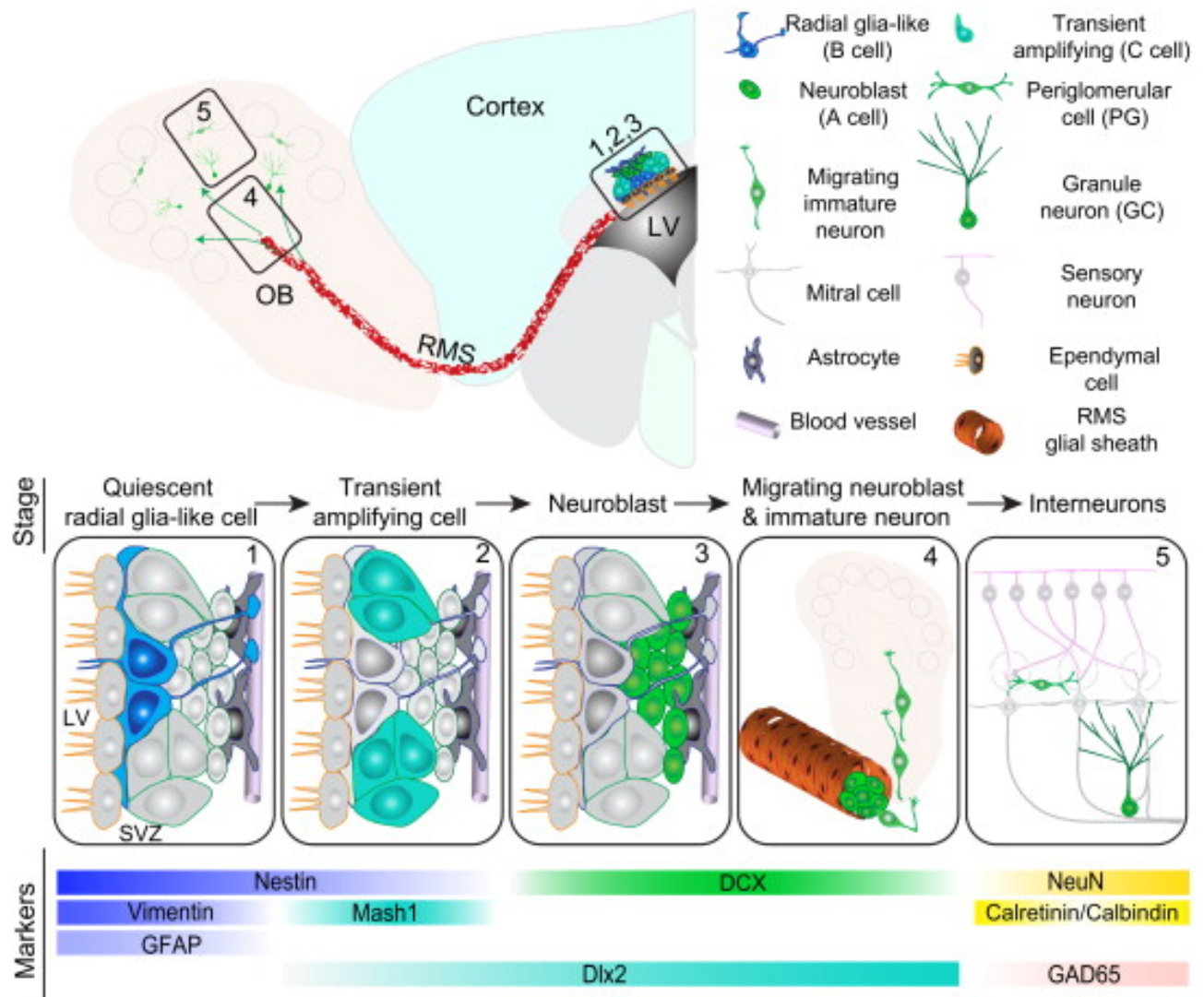


Figure 1-4. The adult SVZ cell types and their markers.

The schematic indicates the quiescent astrocyte-like stem cells (B cells) give rise the rapidly dividing transient amplifying progenitor cells (C cells), which derive to migrating neuroblasts (A cells). The neuroblasts migrate along RMS to become olfactory bulb interneurons. The common cell type markers are largely overlapping from one type to the other. *The image is adapted from Ming, G. and Song, H. 2011 Neuron.*

1.1.5. SVZ Neural Stem/Progenitor Cell Culture: Neurosphere Assay

The NSA was used to provide *in vitro* evidence at the single cell level that the adult brain contains cells that can behave as both self-renewing and multipotential stem cells. It has become the most common method for culturing CNS NSPCs since 1992, when it was initially established by Reynolds and Weiss (Reynolds and Weiss, 1992). This primary cell culture system allows NSPCs to be maintained and expanded as they float freely in culture medium, containing two mitogens: epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2). Over the last two decades, this technique has been applied to examine the stemness of the NSPCs isolated from various brain regions in different developmental stages. Particularly NSA studies with SVZ NSPCs have demonstrated that the transient amplifying progenitor cells (Dlx2 positive cells) can act as the multipotent stem cells after EGF exposure (Doetsch et al., 2002). As NSA allows measurement of two aspects of stem cell characteristics: self-renewal and multipotency, NSA has been implicated as a useful tool to examine the responses of NSPCs after intrinsic or extrinsic modifications. In chapter 3, I will discuss the evolution of this technique and my refinement of its use for this thesis.

1.1.5.1. Self-renewing SVZ Neurospheres

The self-renewal capacity of purported stem cells in the NSA can be inferred from the ability to serially form neurospheres from dissociated single cells. Since *bona fide* stem cells in the CNS are known to be quiescent (Gritti et al., 1999; Jandial et al., 2008; Reynolds and Weiss, 1996; Rietze et al., 2001; Vescovi et al., 1999; Zhang et al., 1997), only 1-10% of the cells in culture can form new spheres and thus act like NSCs (Reynolds and Rietze, 2005). Only EGF and FGF2 responsive NSPCs can form neurospheres, consequently, non-stem/progenitor cells will die off. Therefore, the

number of neurospheres per starting cells is an indicator of the self-renewal capacity, whereas measuring the size of the individual neurospheres is a frequent measure of NSC proliferation, although neurosphere size is also influenced by cell death. Thus in addition to these parameters, cell death rates and viability should be monitored in the NSA.

1.1.5.2. Multipotential SVZ Neurospheres

As previously mentioned, the NSA can be used to assess multipotency of spheres. If one NSC becomes a neurosphere at clonal dilution, single neurospheres can give rise to one, two or all three different neural lineages, and are then referred as mono-, bi- or tri-potential neurospheres, respectively. This differentiation can be initiated by withdrawing the EGF and FGF2 from the culture medium following attachment of the neurospheres on particular substrates. After 7 days of growth in differentiation medium, markers of differentiation can be detected; O4 (oligodendrocytes), β III-tubulin (neurons) and GFAP (astrocytes) (Reynolds and Weiss, 1992). For example, SVZ derived neurospheres commonly give rise to approximately 10-15% oligodendrocytes, 20-30% neurons and 60-70% astrocytes (Doetsch et al., 1999; Singec et al., 2006). Although the SVZ *in vivo* is primarily neurogenic rather than gliogenic, the ratio among three different lineages is not recapitulated in the NSA. This suggests the stem cell niche - the microenvironment in which NSPCs reside – can influence SVZ NSPC fate determination (Conti et al., 2005). Also, Noggin expressed by ependymal cells *in vivo* acts as antagonist of BMP to induce neurogenesis in the adult SVZ (Lim et al., 2000), but ependymal cells are rare to find in the neurospheres.

In sum, the NSA technique has provided a tremendous advantage that allowed the field of CNS NSPC to evolve over the last two decades. However, the assay has its limitations. The exact physiological features of NSPCs *in vivo* have not been recapitulated *in vitro* due to the absence of the stem cell niche environment, but it can be utilized to measure the capacity of intrinsic NSPC characteristics.

1.2. Histone 3 Lysine 27 Trimethylation To Regulate SVZ NSPC Self-renewal And Multipotency

1.2.1. The Role Of Intrinsic Regulation During Cortical Development

Various extracellular signaling pathways are involved in the neurogenic to gliogenic cell fate shift during embryonic cortical development (details mentioned in 1.1.2.2. section). The role of these signaling pathways has been oversimplified as Wnt signaling promoting neurogenesis, and JAK-STAT and Notch signaling only promoting gliogenesis. The expression of their ligands and receptors is not temporally restricted (Hirabayashi and Gotoh, 2005); such that each signaling cascade has also been implicated in different functions at different time points. More and more evidence is suggesting that epigenetic mechanisms may regulate these functional switches.

Similar to Notch signaling described in 1.1.2.2., most of these extracellular signaling ligands and receptors are expressed throughout embryogenesis (Hirabayashi and Gotoh, 2005). For example, Wnt signal ligands and receptors are expressed from E8 till approximately E17 (Hirabayashi et al., 2004). At E10, the Wnt signaling up-regulates cyclinD 1/2 and cMyc to induce NPC proliferation, whereas at E13.5 it up-regulates Ngn 1/2 (Hirabayashi and Gotoh, 2005). Although it had been not clear how this change is regulated during this 3 day period, a recent study has indicated that

histone methylation regulates the suppression of Ngn 1/2, which promotes neurogenesis but inhibits gliogenesis, toward the end of neurogenic period (Hirabayashi et al., 2009) (Figure 1-3A).

The duality of Wnt signaling effects during cortical development has also been found with the JAK-STAT pathway. The CNTF-induced JAK-STAT signaling causes STAT3 to become phosphorylated in both the neurogenic and gliogenic period (Kamakura et al., 2004). The activated STAT3 promotes the GFAP expression only during gliogenesis (Figure 1-4B) because the GFAP promoter is inactivated by DNA methylation during the neurogenic period (Takizawa et al., 2001). Rather than promoting GFAP transcription during the neurogenic period, the LIF- and CNTF-induced JAK-STAT play a role in NPC self-renewal, similar to Notch signaling (Chambers et al., 2001; Kamakura et al., 2004).

Whereas extracellular signaling is critical for fate shifts during cortical development, these pathways can function differently at different time points. What do other factors need to be considered in order to fully understand the radial glial cell fate shift? As bHLH genes (Ngn 1/2 and Olig 1/2) and GFAP expression is regulated by histone and DNA methylations, epigenetic regulation has been recognized as an important factor to govern cell fate determination.

1.2.2. An Important Epigenetic Mark, H3K27me3, For The Developmental Gene Regulation

1.2.2.1. Mechanism of histone methylation for bivalent gene regulation

As described in section 1.2.1., the key developmental genes including Ngn 1/2, Olig 1/2 and GFAP in NPC are regulated by histone and DNA methylation during neural development (Mohn et al., 2008). DNA methylation of CpG nucleotides permanently

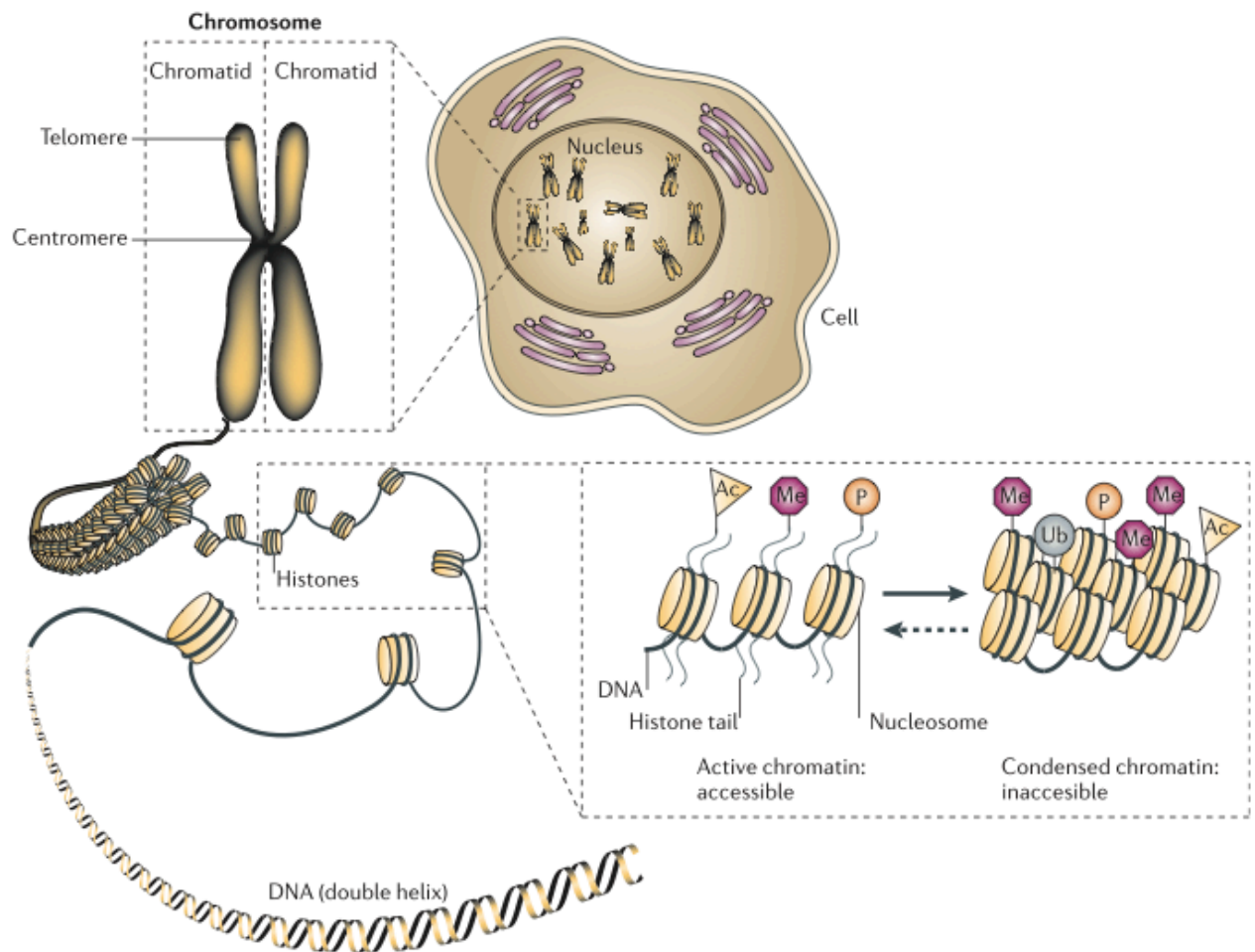


Figure 1-5. Chromatin remodeling via histone modification.

In a nucleus, genomic DNA is compactly organized to form a chromatin. To packing a long piece of DNA in stable condition, it has been wrapped around a histone, which stacks to one another and forms a chromatin. To regulate chromatin configuration as relaxed or condensed, the N-terminal histone tail is covalently modified by acetylation, methylation, phosphorylation, or ubiquitination. This dynamic chromatin structure change allows for gene expression to be either activated or inhibited. *The image is adapted from Sparmann, A. and van Lohuizen, M. 2006 Nat. Rev. Cancer.*

suppresses gene expression (Li, 2002) unless DNA demethylase subsequently removes the methyl-group (Reik, 2007). Histone methylation is a less permanent mechanism when compared to DNA methylation. During histone methylation, the N-terminal tail of histone, wrapped by DNA, is recognized by a site-specific methyltransferase and post-translationally modified by adding a methyl group (Bernstein et al., 2007) (Figure 1-5). Similar to DNA methylation, this can be also enzymatically reversed by histone demethylase. The histone methylation sites are histone 3 lysine 4 trimethylation (H3K4me3), H3K9me3, H3K27me3, H3K36me3 and H4K20me3 (Mikkelsen et al., 2007). Similar to DNA methylation, most histone methylations suppress their target gene expression, except H3K4me3 and H3K36me3, which promote target gene expression. Previous studies in embryonic stem cells (ESCs) have identified some promoters with both H3K4me3 and H3K27me3 marks, and this unique combination is referred to as '**bivalent**' chromatin mark (Azuara et al., 2006; Bernstein et al., 2006; Mikkelsen et al., 2007) (Figure 1-6A). Due to their opposite effects on gene expression regulation this provides the '**poise**' status of gene expression and allows changes in either direction. These bivalent marks are found in approximately 22% of CpG-rich promoters, and those genes are either housekeeping genes or key developmental genes during stem cell differentiation (Mikkelsen et al., 2007). Interestingly, these bivalent marks have also been found in neural genes, including Ngn1/2, Pax6, Sox1, Sox3, Nkx2.2, Mash1, Olig1/2, etc. (Mikkelsen et al., 2007).

Unlike H3K27me3, H3K4me3 alone shows a strong positive correlation between its intensity of trimethylation and target gene expression, but this is not the case for the bivalent site in the CpG-rich promoter (Mikkelsen et al., 2007). This

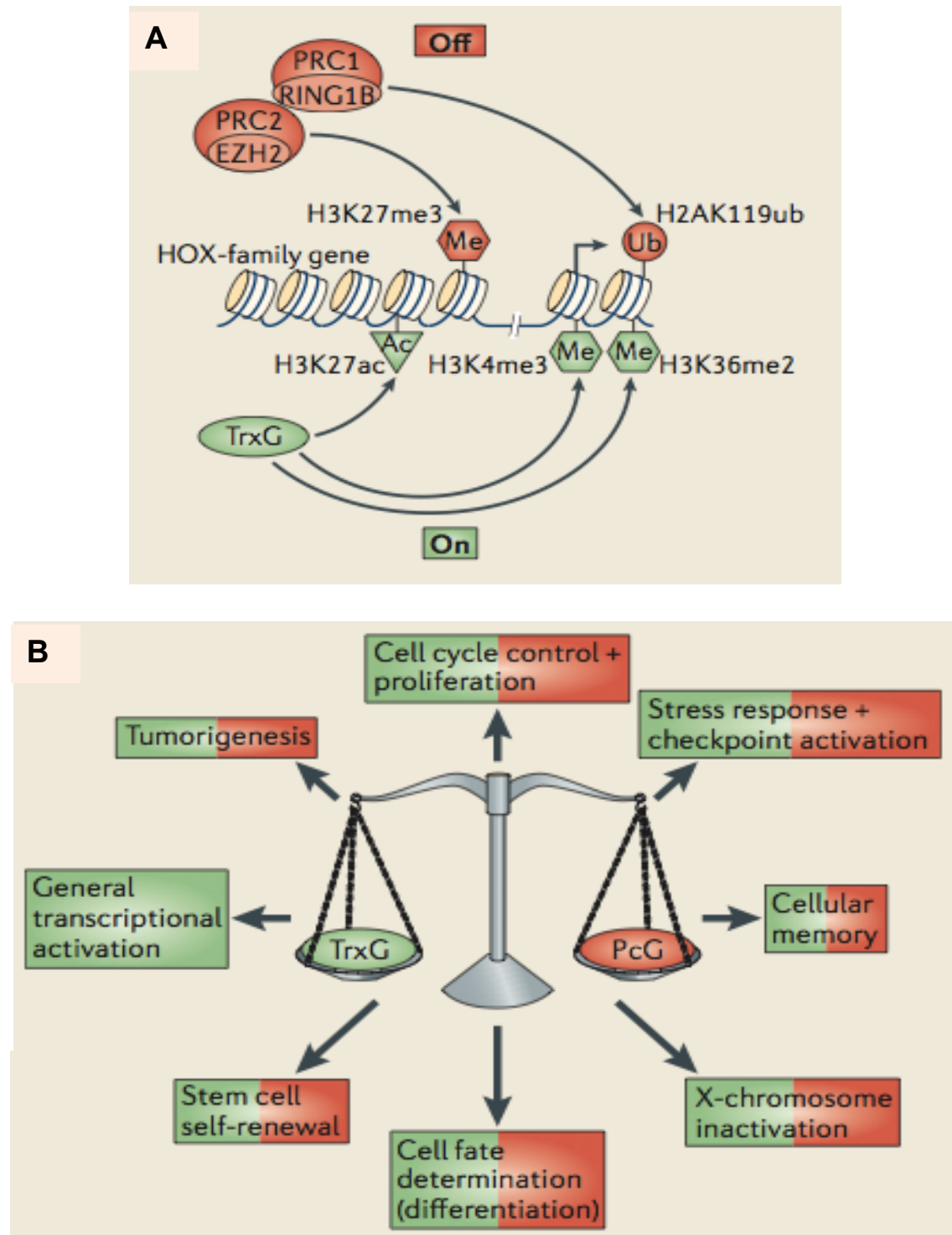


Figure 1-6. Trithorax and Polycomb group antagonist functions.

(A) A schematic for TrxG and PcG mechanisms of methylation, ubiquitylation and acetylation. Note that PRC2 and PRC1 work in concert. (B) Trithorax group (TrxG) and polycomb group (PcG) functions are required to be balanced in order to maintain multiple biological features. *The image is modified from Schuettengruber, B. et al. 2011 Nat. Rev. Mol. Cell. Biol.*

suggests that H3K27me3 mark has a dominant effect on gene suppression compared to H3K4me3 activity. Since these embryonic cortical developmental genes [bHLH genes (i.e. Ngn 1/2, Oligs 1/2) and GFAP] also have bivalent sites on their promoters this suggests that epigenetic regulation may be more important to control these key developmental genes than extracellular signaling.

1.2.2.2. The interaction between polycomb complex group and trithorax group

The H3K4me3 and H3K27me3 marks are catalyzed by trithorax group (TrxG) and polycomb complex group (PcG), respectively. The functions for PcG and TrxG were first identified in silencing and maintaining the *Drosophila* homeobox genes (HOX genes) for proper developmental segmentation (Akam, 1987). For the H3K4 trimethylation process, histone-modifying complex of TrxG is composed of MLL, WDR5, ASH2L, RbBP5 and CFP1 (Schuettengruber et al., 2007). MLL has the SET domain for histone methyltransferase activity (Gu et al., 1992; Tkachuk et al., 1992). In contrast, the PcG has two groups: polycomb repressive complex (PRC) 1 and PRC 2. PRC1 is composed of Ring1A, Ring1B, Bmi1, M33 (Cbx family) and Rae28 (Phc family), whereas PRC2 is composed of Eed, Ezh2, Suz12, Jarid2 and Pcl2/Mtf2 (Surface et al., 2010) (Figure 1-7A). For the H3K27me3, PRC2 is directly associated with the catalytic activity, whereas PRC1 monoubiquitines on H2A (H2A_{ub1}) to maintain the repressive state of transcriptions after PRC2 catalytic activity (Buchwald et al., 2006). For the catalytic activities, Ring1B in PRC1 has the ubiquitinase functional domains while Ezh2 in PRC2 has the SET domain for methyltransferase activity.

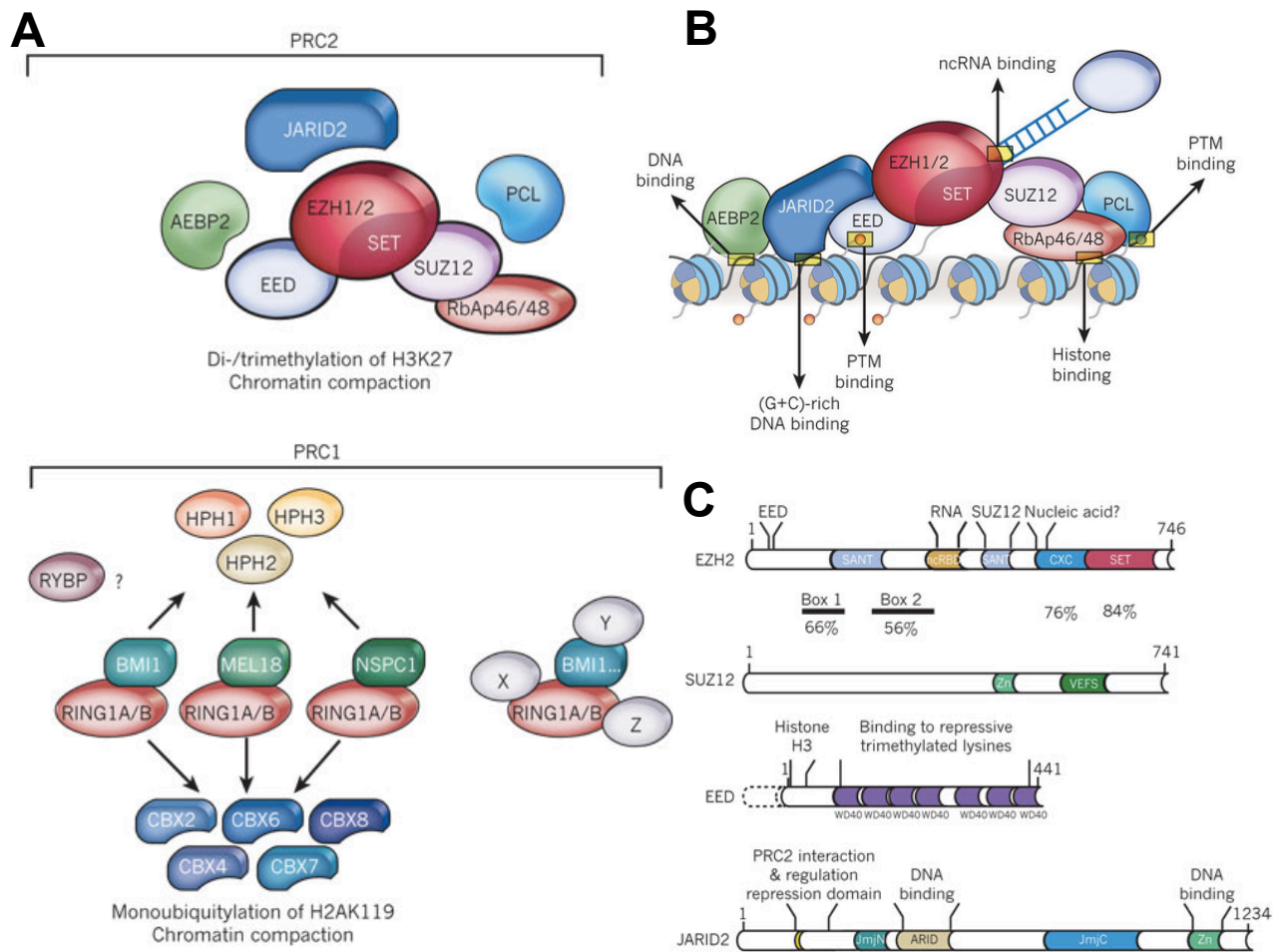


Figure 1-7. Schematics of polycomb group (PRC1/2).

(A) Shows all purported PRC1/2 components and indicates how they interact with one another.

(B) In mammalian systems, each component of PRC2 is physically associated with one another and support H3K27me3 catalytic activity.

(C) Schematics of Ezh2, Eed, Suz12 and Jarid2 genes.

The image is adapted from Margueron, R. and Reinberg, D. 2011 Nature.

The H3K4me3 has been found in both CpG-rich and CpG-poor promoter regions to regulate housekeeping genes, cellular replication and metabolism associated genes (Mikkelsen et al., 2007). In contrast, the H3K27me3 was only found in CpG-rich promoter regions, where it regulates genes associated with complex expression pattern, developmental, morphogen and cell surface molecules (Mikkelsen et al., 2007). Although bivalent marks, observed in approximately 22% of CpG-rich promoters, may not seem a large proportion of the genome, the effects of this bivalent mechanism has been implicated in cell cycle control (Liu et al., 2007), X-chromosome inactivation (Pullirsch et al., 2010), stem cell self-renewal (Ang et al., 2011) and cell fate determination during stem cell differentiation (Bagchi et al., 2007) (Figure 1-6B).

1.2.3. Important Epigenetic Factors To Regulate SVZ Neural Stem/Progenitor Cell Stemness

As development proceeds, the cell fate potential of SVZ stem cells generally becomes more restricted and specified. However, it has been previously shown that alteration of the methylation state of cells can change the self-renewal and cell fate choice of RGCs. When Ezh2 was floxed out by Emx1-Cre at E9.5 decreased neurogenesis in embryonic cortex was observed (Pereira et al., 2010). However, when Ezh2 from PRC2 (or Ring1B from PRC1) was floxed out in nestin positive cells at E13.5, the neurogenic phase in the embryonic cortex was extended (Hirabayashi et al., 2009). Hirabayashi et al. in 2009 suggested this phenotype could be due to loss of target gene suppression such as Ngn 1/2. In addition, Ezh2 knockdown in cultured NSCs from E14 telencephalon showed the reduction of oligodendrogenesis but induction of astrogenesis, whereas Ezh2 overexpression resulted in the opposite effects (Sher et al.,

2008). As PRC2 is important to initiate the PcG function, H3K27me3 is very critical for cell fate shift during cortical development. These data suggest that similar mechanisms may still be at play in the postnatal SVZ.

Several other pieces of evidence indicate that epigenetic factors regulate SVZ NSPC proliferation and differentiation. First of all, the role of Bmi-1, a subunit of PRC1 required H3K27me3 mark, in VZ (embryonic) and SVZ (postnatal and adult) has been investigated by knockout, knockdown and overexpression approaches (Fasano et al., 2007; Fasano et al., 2009; He et al., 2009; Molofsky et al., 2003). Bmi-1 knockout mice showed signs of postnatal growth retardation and neurological defects although they survived until adulthood (Molofsky et al., 2003). In the culture system, postnatal SVZ NSC from Bmi-1 knockout mice had reduced self-renewal with up-regulation of p16 and p19, although it did not affect cell survival and differentiation processes. By depleting p16 in the Bmi-1 knockout NSC, the self-renewal defect could be partially rescued. This Bmi-1 knockout study had been extended to embryonic cortical VZ and adult SVZ *in vivo* and *in vitro* by using Bmi-1 shRNA (Fasano et al., 2007). Bmi-1 knockdown in embryonic and adult NSCs also showed a lack of NSC proliferation and self-renewal although this activity was mediated by p21 instead of p16/p19. Based on these two studies, Bmi-1 is necessary for NSC self-renewal and proliferation.

Two years later, this statement was validated by Bmi-1 overexpression experiments, which demonstrated increased NSC proliferation and self-renewal *in vitro* not *in vivo* (Fasano et al., 2009; He et al., 2009), while p16/p19 were attenuated *in vitro* (He et al., 2009). In addition to self-renewal and proliferation of NSCs, Bmi-1 overexpressed adult SVZ neurospheres produced neurons after multiple passages, while the control group showed a lack of neurogenesis (Fasano et al., 2009). Based on

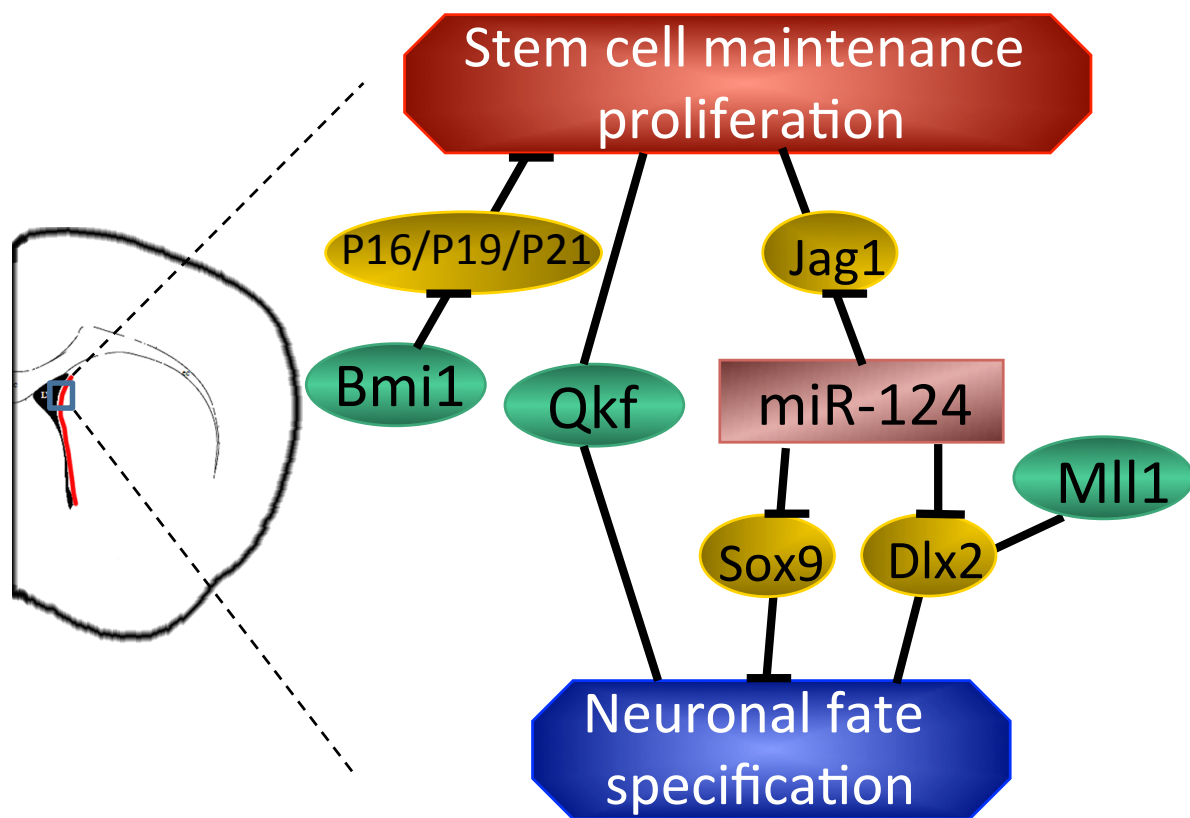


Figure 1-8. Known epigenetic regulation of SVZ NSPCs.

Recent evidence has shown that epigenetic regulators (Qkf, Mll1 and Bmi1) and a non-coding RNA (miR-124) can modulate SVZ NSPC proliferation and fate specification (Cheng et al., 2009; Fasano et al., 2007; Fasano et al., 2009; He et al., 2009; Lim et al., 2009; Merson et al., 2006; Molofsky et al., 2003). These regulators and ncRNA functions could be linked by H3K27methylation.

these studies, SVZ NSC self-renewal and proliferation are regulated by Bmi-1 expression levels, but it is unknown whether this phenomenon is mediated by PRC1 activity (Figure 1-8).

Secondly, Mll1, which is known as a H3K4 trimethyltransferase for bivalent activity of H3K27me3 in ESCs, guides postnatal SVZ NSCs towards neuronal differentiation rather than oligodendrogenic differentiation mediated by *Dlx2* genes (Lim et al., 2009) (Figure 1-8). After Mll1 depletion in postnatal SVZ NSCs *in vivo*, abnormally increased NSC proliferation was shown. This phenotype is the opposite effect of Bmi-1 deficient NSCs, but it has not been demonstrated whether NSC proliferation is regulated by a bivalent mechanism.

Thirdly, Querkopf (Qkf), a MYST family histone acetyltransferase, has an important role for NSC stemness in embryonic VZ and adult SVZ (Merson et al., 2006; Thomas et al., 2000). The Qkf mutant mice, generated by hypomorphic mutation to suppress 90% of the genes, showed a reduction in embryonically VZ NSC proliferation, numbers of cortical plate neurons and numbers of cortical interneurons (Thomas et al., 2000). The mutant mice survived until adulthood and showed a reduction in lateral ventricle SVZ NSC proliferation, smaller size of OB, reduction of PSA-NCAM positive neuroblast chains in whole mount section *in vivo* and reduction of neuronal differentiation *in vitro* (Merson et al., 2006) (Figure 1-8). However, the specific downstream mechanism of Qkf is still unknown.

Lastly, the role of microRNA-124, highly present in adult SVZ neuroblasts, is implicated in regulating a state of balance between proliferation and neuronal production (Cheng et al., 2009). miR-124 knockdown in adult SVZ show that the proliferation of neuroblast *in vivo* and transient amplifying cell *in vitro* was significantly increased. In contrast, the overexpression of miR-124 *in vivo* and *in vitro*

resulted in increased neuronal formation. *Dlx2*, *Sox9* and *Jag1*, which were directly targeted by miR-124 in adult SVZ neuroblasts, are involved in the balance between proliferation and neuronal production (Figure 1-8).

Despite all the evidence, it is still unclear how these modifiers mechanistically regulate NSPC properties, yet the functional significance of Bmi-1 and Mll1 (a H3K4 trimethyltransferase for bivalent activity of H3K27me3 in ESCs) support the notion that H3K27me3 mark and PRC2 function are important in governing postnatal and adult SVZ NSPC proliferation and differentiation.

1.2.4. Polycomb Repressive Complex 2 Function In Mammalian Cells

The function of PRC2 as H3K27 di- and tri-methyltransferase has been conserved from plants to vertebrates during evolution (Bracken et al., 2006; Kirmizis et al., 2004; Kwong et al., 2008; Lee et al., 2006; Negre et al., 2006; Oktaba et al., 2008; Schuettengruber et al., 2009; Schwartz et al., 2006; Squazzo et al., 2006; Tolhuis et al., 2006) although the composition of PRC2 can be changed from one species to another (Cao and Zhang, 2004a; Schwartz and Pirrotta, 2007). In order to identify the role of individual PRC2 components, most genes have been mutated, and loss of all the core components is embryonically lethal (Surface et al., 2010). Therefore, several research groups have genetically modified and biochemically examined the role of individual PRC2 components in ESCs (Ketel et al., 2005; Kuzmichev et al., 2005; Kuzmichev et al., 2002; Margueron et al., 2009; Margueron et al., 2008; Muller et al., 2002; Pasini et al., 2004; Shen et al., 2008; Su et al., 2003). After these extensive biochemical studies, the catalytically essential core components in mammalian cells were defined as Ezh2 [catalytic SET domain contain histone methyltransferase (Margueron and Reinberg, 2011)], Eed [WD40 domain repeat containing protein for

protein-protein interaction (Xu and Min, 2011), maintenance and propagation of H3K27me3 (Margueron et al., 2009; Tie et al., 2007; Xu et al., 2010)] and Suz12 [C-terminal region containing a zinc finger domain (Yamamoto et al., 2004)] (Figure 1-7B,C).

When one of these components is lost, PRC2 does not form and PRC2 activity disappears. Thus, it has been possible to examine the PRC2 function in developmental, stem cell and cancer biology by genetically removing or suppressing one or two of the core components rather than all of them (Jaenisch and Young, 2008; Jones and Baylin, 2007; Ng et al., 2008; Pien and Grossniklaus, 2007; Pietersen and van Lohuizen, 2008; Rajasekhar and Begemann, 2007; Schwartz and Pirrotta, 2007; Simon and Lange, 2008; Sparmann and van Lohuizen, 2006; Ting et al., 2006; Whitcomb et al., 2007).

Previous studies showed that PRC2 function is consistently preserved in different systems including cortex, epidermis, spinal cord, thymocyte, limb, cardiac muscle, hepatocytes, preadipocytes, skeletal muscle, central foregut and small intestine (Akizu et al., 2010; Aoki et al., 2010; Benoit et al., 2012; Caretti et al., 2004; Delgado-Olguin et al., 2012; Ezhkova et al., 2011; Ezhkova et al., 2009; He et al., 2012; Hirabayashi et al., 2009; Juan et al., 2011; Pereira et al., 2010; Richie et al., 2002; Stojic et al., 2011; Wang et al., 2010; Wyngaarden et al., 2011; Xu et al., 2011). However, PRC2 recruitment toward their target sites in mammalian cells has not been clearly defined yet. In recent studies on PRC2 function in ESCs, PRC2 recruitment is regulated by a novel gene called Jarid2 (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009) (Figure 1-7B), but the role of Jarid2 has not been studied in postnatal and adult brains yet.

1.2.4.1. Role of Jarid2 as PRC2 recruitment

The Jarid2 gene contains both a nuclear localization signal and an AT-rich interaction domain called ARID which allows the protein to directly bind to DNA and act as a transcription factor during heart development (Lee et al., 2000; Motoyama et al., 1997; Takeuchi et al., 1999; Takeuchi et al., 1995) (Figure 1-7C). Early Jarid2 mutant mice studies have shown that the mutant mice can only survive until E13-15.5, due to lethal defects in the neural tube and heart (Lee et al., 2000; Takeuchi et al., 1999; Takeuchi et al., 1995; Toyoda et al., 2000). Because the role of Jarid2 is important for embryonic development, Jarid2 functional studies have been mainly carried out in only ESCs (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009).

How PRC2 is recruited to chromosomes in mammalian cells is an open question. Recent PRC2 studies in ESCs demonstrated that Jarid2, which has a DNA binding domain, was a strong potential candidate for PRC2 recruitment due to the following biochemical studies: (1) Jarid2 binding sites on DNA in ESCs are largely common with PRC2 target sites, as confirmed by chromatin-immunoprecipitation assay (ChIP on ChIP and ChIP-sequencing) and (2) Jarid2 and PRC2 core components directly interact at the protein level, as confirmed by co-immunoprecipitation (Co-IP) (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009). By knockdown and knockout of Jarid2, the H3K27me3 intensity on pluripotency genes such as Oct4 and Nanog was significantly reduced (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010), suggesting that Jarid2 is directly related to the PRC2 activity in ESCs to regulate ESC stemness.

However, Jarid2 function in PRC2 has been controversial due to inconsistent H3K27me3 intensity after Jarid2 knockdown or knockout. As a recruiter of PRC2, the

absence of Jarid2 resulted in decreased amounts of H3K27me3 as would be expected (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010). However, in contrast, the Jarid2 knockout and knockdown can also result in increased amounts of H3K27me3 (Shen et al., 2009) or produce no changes in H3K27me3 intensity (Peng et al., 2009). For the increment of H3K27me3, Shen et al. in 2009 have reported that Jarid2 in PRC2 acts as an enzymatic inhibitor (Shen et al., 2009). Therefore, it has been challenging to elucidate the exact function of Jarid2 in stem cells and how it acts to finely tune stem cell self-renewal and differentiation via H3K27me3.

During neuronal differentiation of ESCs, Jarid2 expression levels were significantly decreased (Li et al., 2010) similar to PRC2 core components (Sher et al., 2008). However, PRC2 plays a crucial role in regulating the neurogenesis and gliogenesis in cortical NSCs until perineonatal stages; therefore the next question to ask is whether PRC2 function in postnatal SVZ is dependent on Jarid2 in order to govern SVZ NSPC fate choices and differentiation.

1.2.4.2. Jarid2 has a PRC2 independent function

Jarid2 has been recognized as a regulator in early development of neuronal, cardiac, liver, and hematopoietic tissues in certain genetic backgrounds (Jung et al., 2005; Lee et al., 2000; Takeuchi et al., 1995), but its function in postnatal and adult stages is not clear. Jarid2 mutant mice showed a significant increase in NPC proliferation, which was correlated with increased expression of NPC markers: Musashi1, Mash1, Sox2, β III-tubulin, and PECAM (Takahashi et al., 2007). However, cyclin D1 knockout mice carrying the mutant Jarid2 do not show this abnormal NPC proliferation, suggesting cyclin D1 is necessary for Jarid2 proliferation mechanism (Takahashi et al., 2007). In another report, overexpression of flag-Jarid2 in NIH 3T3 cells revealed

that Jarid2 first interacts with the H3K9 methyltransferases G9a and GLP to methylate the cyclin D1 promoter region and suppress its gene expression (Shirato et al., 2009). In addition, Jarid2 can directly regulate Notch gene expression in endothelial cells by being associated with H3K9 methyltransferase (Mysliwiec et al., 2011; Mysliwiec et al., 2012). However, this result was not replicated in ES cells; instead, Jarid2 interacts with PRC2 (Pasini et al., 2010; Shen et al., 2009). Therefore, Jarid2 can be found as a PRC2 independent gene, and it could possibly function as more than a DNA binding protein in different tissue and developmental stage.

1.3. Overall Aims Of This Thesis

The objective of this thesis has been primarily to understand the stemness of postnatal SVZ NSPC populations *in vitro* and how epigenetic regulators can regulate the NSPC self-renewal and differentiation properties. Due to the technical limitation of isolating pure cell populations from postnatal SVZ, a validated primary neural stem cell culture technique called the neurosphere assay has been utilized to isolate NSPC cell populations *in vitro* to examine the self-renewal and differentiation capacities. However, there was no standardized protocol for the primary NSPC culture, so the first aim of this thesis was to characterize and optimize the neurosphere assay by using a specific protocol that had been previously reported (Chapter 3). As the level of H3K27me3 mark has not been previously shown in postnatal and adult brain, it was necessary to confirm whether there is H3K27me3 level within the SVZ; which cell type has more or less of H3K27me3 marks; and whether this mark would be altered after stroke or demyelination in CNS (Chapter 4). With the confirmation of H3K27me3 mark within the postnatal SVZ, the presence of PRC2 core components (Eed, Ezh2 and Suz12) including Jarid2 were examined at both transcriptional and

translational levels (Chapter 4). The loss of PRC2 function in postnatal SVZ NSPC population was conducted by lentiviral mediated delivery of the Eed knockdown in neurospheres, and the PRC2 dependent or independent function of Jarid2 was also tested by the same knockdown approach (Chapter 5). Overall, this thesis argues that *in vivo* postnatal SVZ NSPCs can be modeled by an *in vitro* system while still maintaining the NSPC self-renewal and differentiation properties, and these NSPC properties can be altered by the suppression of endogenous Eed and Jarid2 expression levels. Unlike other stem cells, these two molecules do not show the similar phenotypic change for the postnatal SVZ NSPC; they might not share their roles in the NSPC population.

Chapter 2

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Chapter 2

Material And Methods

2.1. Tissue Culture

2.1.1. Generation Of Neurospheres

2.1.1.1. Generation of Neurospheres

Postnatal day 4-5 (P4-5) mice were anaesthetized by intraperitoneal injection of pentobarbital (50 mg/kg) and decapitated. Brains were rapidly excised and placed in ice-cold Neurobasal+ [Neurobasal (Gibco 21103), B-27 (2%; Invitrogen 17504-044), N-2 supplement (1%; Invitrogen 17502-048), L-glutamine (2 mM; Invitrogen 25030), heparin (20 mg/ml; J.T. Baker M916-00), penicillin/streptomycin (1X; Gibco 15070)]. Brains were then sliced into 500 µm thick coronal sections using a McIlwain tissue chopper. Sections were then transferred back to ice-cold Neurobasal+. The entire striatal SVZ flanking both ventricles was microdissected by using 27x³/₄ gauge syringe needles. Microdissected SVZ tissues were pooled and centrifuged for 15 seconds. The pellets were minced with fine iris scissors. Three hundred µl of accutase (Sigma A6964) pre-warmed at 37°C was added and tissue gently triturated before it was incubated at 37°C for 10 minutes (min). Enzymatically dissociated tissues were further triturated until they became single cells. Completely dissociated cells were mixed with 3 mL of Neurobasal+ and centrifuged at 2000 xg for 10 min. The supernatant was discarded, and the pellet was resuspended in 1 ml Neurobasal+GF [Neurobasal+ with 20 ng/ml EGF (Sigma E9644) and 20 ng/ml bFGF (R&D Systems 233-FB)]. Viable cell concentration was determined by Trypan Blue exclusion (J.T. Baker X242-03). After quantifying the viable cells under hemocytometer, the cell suspensions were diluted at a concentration of 100 cells/µl with Neurobasal+GF and cultured in T12.5 flask, pre-coated with Poly-HEMA to avoid adherence. Eighty

percent of Neurobasal+GF medium was changed every 2 days. For secondary and tertiary neurosphere generation, the primary or secondary neurospheres on days *in vitro* (DIV) 4 were pooled and centrifuged at 2000 xg for 10 min. The pellet was dissociated with accutase as described above. The washing, cell counting, and seeding steps were performed as described above.

2.1.1.2. Poly-HEMA Coating

Poly-HEMA (Poly 2-hydroxyethyl methacrylate; Sigma P-3932) 600 mg was added to 50 mL of 95% ethanol to make 12 mg/mL (10X concentrated) stock solution. Due to the lack of solubility, the mixture was vigorously vortexed several times. Undissolved Poly-HEMA was incubated at 37°C overnight with frequent vortexing. For the working concentration (1X), the stock solution was diluted with 95% ethanol to a 1:10 ratio and let it sit for at least 10 min for complete homogenization. To coat the tissue culture flasks, 2 mL of 1X Poly-HEMA solution was applied to 12.5 mL flasks. The cap for the flask was left opened for air-drying in a sterile hood at least 1 day before using it. To make 120 mg/mL for higher concentration of Poly-HEMA coating, 3 g of Poly-HEMA was added into 25 mL of 95% ethanol. The mixture was stirred on 37°C hot plate.

2.1.1.3. Neurosphere Quantification

Each neurosphere on day *in vitro* (DIV) 4 were imaged using a Leica DM IRB inverted microscope. Spheres greater than 25 µm in diameter were considered as a neurosphere and counted. Representative neurospheres from the flask were captured and analyzed for diameter by using Openlab 3.1.3 and Volocity software (Improvision).

2.1.1.4. Neurosphere Differentiation

For neurosphere differentiation, 8-well chamber slides (VWR 734-2050) were coated with substrates like poly-D-lysine/laminin. Poly-D-lysine (200 μ L of 0.1 mg/mL; Sigma P6403) was applied to each well. The slides were incubated for 1-2 hours (hrs) at room temperature or wrapped with parafilm to incubate overnight at 4°C. Poly-D-lysine was removed and stored at 4°C for reuse, and the slides were washed 3 times with autoclaved distilled water for 5 min at room temperature. After the final wash, the slides were air-dried. The stock concentration of natural mouse laminin (1 mg/mL; Gibco 23017-015) was diluted down to 10 μ g/mL in 1X phosphate buffered saline (PBS). One hundred μ L of this working concentration of laminin was applied to cover each well and incubated at 37°C for 1 hr. To apply neurospheres in DIV 4 on these substrates, the laminin was aspirated, and Neurobasal+ (200 μ L) was added into each well. A single or multiple spheres was selected by using P-20 pipette and added onto the coated chamber. For single cell differentiation, all the neurospheres were centrifuged at 172 xg for 10 min. The cell pellets were resuspended with Accutase and incubated at 37°C for 10 min. Dissociated cells were placed into each chamber at a density of 2×10^5 cells/mL and cultured for 12 hrs in Neurobasal+GF medium followed by 7 days with Neurobasal+. When changing Neurobasal+ every 2-3 days, the differentiated cells were imaged using a Leica DMIRB inverted microscope. By 7 days after plating, the cells were fixed with 4% paraformaldehyde (PFA) for 15 min followed by 3 times washes with 1X PBS for 15 min.

For neurosphere differentiation on Matrigel (BD Biosciences 354234) and Geltrex (Gibco A1413302), Matrigel and Geltrex had to be thawed in 4°C for overnight. The cold 50 μ L of Matrigel (0.25 mg/mL) and 100 μ L of Geltrex (12-18 mg/mL) solutions were applied to each well of 8-well chamber slides. The coated

slides were incubated at 37°C for 1 hr until a neurosphere was applied onto the substrates.

2.1.2. Embryonic Stem Cell Culture

The embryonic stem cell (ESC) line ESF116, cultured on a feeder cell layer, was obtained from Professor Paul Fairchild's laboratory in University of Oxford and used within 30 passages of the original vial. To isolate ESCs, the ESCs were passaged 2-3 times in gelatin-coated flasks to remove feeder cells. When 80% ESC confluency was reached, medium was removed, and cells washed with 1X PBS. One mL of TrypLE express (1X; Invitrogen 12605-010) was applied to cells and incubated at 37°C for 6 min. After tapping the flask, the cells were incubated at 37°C for 6 min. Detached cells were triturated by adding 1 mL of feeder medium [Dulbecco's Modified Eagle Medium (D-MEM, Lonza BE12-604F), Fetal Bovine Serum (FBS, 10%; Gibco 16000-036), Glutamine (1%; PAA M11-004), Penicillin/Streptomycin (1%; PAA P11-010), Na Pyruvate (1%; PAA S11-003) Non-Ess AAs (1%; PAA M11-003), β -Mercaptoethanol (0.1%; Sigma M7522)] into the flask. The dissociated cells were washed with 3 mL of feeder medium and spun down at 1300 xg for 5 min. After discarding the supernatant, the cell pellet was resuspended by 1 mL of ES medium [feeder medium + ESGRO (LIF, 0.1%; Millipore ESG1107)]. After triturating the cells with P-1000 tips, the cells were first diluted in 1/10. Subsequently they were diluted in 1/5 and 1/3 to minimize the feeder cell contamination. To seed the cells, the flask were coated with gelatin; six milliliters of autoclaved gelatin (0.1%; Sigma, G1890) dissolved in 1X PBS was added into each T75 flask. The flask was incubated at 37°C for 20-30 min. By aspirating the gelatin solution, the flask was rinsed with 1X PBS and ready to be used.

2.1.3. Immortalized Cell Line Cultures

Human embryonic kidney (HEK) 293 FT (Invitrogen R700-07) and HEPA 1-6 (ATCC CRL-1830) were used within 30 passages of the original vial. Cells were grown in D-MEM (1X, Invitrogen 32430-100) supplemented with FBS (10%; Invitrogen 16000-036) and penicillin-streptomycin (1%; Invitrogen 15140-122) at 37°C in 5% CO₂. Within 3-5 days, the cells were washed with 1X PBS and fed with fresh medium. To be passaged, the cells were treated with 1 mL of 0.05% Trypsin-EDTA (1X; Gibco 25300-054) and incubated at 37°C for 2 min. The detached cells were completely dissociated by trituration. For maintaining cell lines, 1/10 diluted cells were seeded into new flasks. When exact cell numbers were required for seeding, viable cell numbers were determined with trypan blue exclusion.

2.1.4. Freezing And Thawing Stock Cells

To freeze down the cells, cells need to be 80% confluent so that they are in optimum growth conditions prior to freezing. After washing with 1X PBS once, the cells were treated with 1 mL of 0.05% Trypsin-EDTA and incubated at 37°C for 2 min. The detached cells were spun down, and supernatant was removed. The cell pellet was resuspended with 1mL of freezing medium [D-MEM:FBS:Dimethyl sulfoxide (DMSO; Sigma C6164) mixed in 6:3:1 ratio respectively]. Resuspended cells were placed in cryovials, and frozen at -80°C for overnight. The completely frozen cells were transferred into liquid nitrogen.

To take cells out from the frozen stock, the cells taken out from -80°C storage were briefly placed in 37°C water bath. The thawed cells were mixed with 5 mL of 1X PBS and spun down at 180 xg for 2 min. The supernatant was discarded, and the cells

were resuspended in 1 mL of appropriate medium. One third and two thirds of cells were seeded into separate flasks to monitor the condition of cell growth.

2.2. Gene Expression Determination

2.2.1. Transcriptional Level Detection

2.2.1.1. Isolation of RNA and DNase treatment

For total RNA extraction from both tissues and cells, the Qiagen mini RNeasy Kit (Qiagen 74104) was utilized. For total RNA extraction from neurospheres, they were first dissociated by accutase as above, and single cells were counted on a hemocytometer as described in section 2.1.1.1. With maximum number of 1×10^7 single cells per reaction, cells were centrifuged for 5 min at 300 xg, and the supernatant discarded. The cell pellet was resuspended by adding 600 μ L of Buffer RLT with 1% β -Mercaptoethanol. Resuspended cells were vigorously vortexed and triturated with pipette. The cell lysate was directly put into a QIAshredder spin column (Qiagen 79654) and centrifuged for 2 min at 13,200 rpm on a bench top centrifuge. The homogenized lysate, eluted solution, was mixed with 70% RNase free ethanol by titrating several times and transferred to RNeasy spin column. Seven hundred μ L of samples in the column was centrifuged for 15 seconds at 10,000 rpm. After discarding flow-through, 700 μ L of Buffer RW1 was added to the column and centrifuged for 15 seconds at 10,000 rpm. Again, flow-through was discarded, and 500 μ L of Buffer RPE was added to the column to be centrifuged for 15 seconds at 10,000 rpm. Finally 500 μ L of Buffer RPE was added to the column to be centrifuged for 2 min at 10,000 rpm. The column was placed into a new collection tube and centrifuged for 1 min at 10,000 rpm. To elute total RNA, 50 μ L of RNase/DNase free water was added to the column and centrifuged for 1 min at 10,000 rpm. The eluted

total RNA was quantified by using Nanodrop (Thermo scientific). The quality of RNA was determined by 260/280 and 260/230 ratios; the generally accepted ratios are 1.8 - 2.0 and 2.0 – 2.2, respectively.

For micro-dissected SVZ and striatum tissues, the maximum weight as 30 mg of the dissected tissues isolated from 6-10 P4 pups were instantly frozen in dry ice followed by crushing them with a pestle. From here, tissues were treated with Buffers to isolate total RNA as described above.

For DNase treatment, the total RNA (up to 10 µg) was used in a 50 µL reaction. In the reaction, 5 µL of 10X TURBO DNase buffer and 1 µL of TURBO DNAase (Ambion AM1907) were added with RNase/DNase free water to make 50 µL in total. The sample was incubated at 37°C for 20-30 min followed by adding 5 µL DNase Inactivation Reagent. Thoroughly mixed sample was incubated for 5 min at room temperature and centrifuged for 1.5 min at 10,000 xg. The supernatant was saved for DNA free RNA in -80°C.

2.2.1.2. Reverse transcriptase Polymerase Chain Reaction

For first-strand cDNA synthesis, the DNase treated RNA (up to 5 µg) was mixed with 125 ng of random primer (Invitrogen 48190-011) and 10mM of dNTP Mix (Invitrogen 18427-013) in 20 µL reaction. The mixture was heated at 65°C for 5 min followed by incubation on ice for 1 min. By adding 4 µL of 5X First-Strand Buffer, 1 µL of 0.1M DTT and 1 µL of SuperScript III Reverse Transcriptase (Invitrogen 18080-093) into the mixture with gentle titration, the sample was incubated in following orders of temperature and time: 25°C for 5 min, 55°C for 1 hr, 70°C for 15 min. The final product of cDNA concentration was considered as equivalent to the initial quantity of the DNased RNA.

2.2.1.3. Polymerase Chain Reaction and Quantitative Real Time PCR

2.2.1.3.1. Polymerase Chain Reaction

The following components were added together: 0.75 μL 50mM MgCl_2 , 0.5 μL 10mM dNTP, 2.5 μL 10X polymerase chain reaction (PCR) buffer (Invitrogen 10966-018), 0.1 μL Platinum Taq polymerase (Invitrogen 10966-018), 1 μL 10 μM gene specific Forward primer, 1 μL 10 μM gene specific Reverse primer, 10 ng of DNA and 13 μL of DNA free water. This PCR mixture was thermocycled as follows: first step 94°C for 2 min; 40 cycles of 94°C for 30 seconds, 54°C for 30 seconds, 72°C for 45 seconds; last step 72°C for 5 min. The final PCR products were electrophoresed on 2%(W/V) agarose gels. (Please see the table 2.1. for gene specific primer sequences)

2.2.1.3.2. Quantitative Real Time PCR

The difference between qRT-PCR and PCR was qRT-PCR used cDNA instead of DNA plasmid and SYBR Green master mix (Applied Biosystems 4309155). Thus, the qRT-PCR reaction components: 12.5 μL SYBR Green Master Mix, 1.75 μL 1 μM gene specific Forward primer, 1.75 μL 1 μM gene specific Reverse primer, 25 ng/ μL of cDNA and 6.5 μL of DNA free water. The qRT-PCR reaction thermocycle was set on StepOnePlus Real-Time PCR system (Applied biosystems 4376600): first step 94°C for 1 min; 40 cycles of 94°C for 30 seconds, 54°C for 30 seconds, 72°C for 1 min; last step 72°C for 4 min. The Ct values were automatically calculated by setting the threshold from the first five-cycle noise. All the Ct values were first normalized to the internal control as 18s and GAPDH. The normalized values (delta Ct) was normalized against to experimental controls such as scramble shRNA to achieve delta delta Ct. The fold difference was yielded followed by the equation:

Table 2.1. Gene specific primers pairs for PCR/RT-PCR/qRT-PCR

Gene name	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Reference
18s	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG	Schmittgen et al JBBM 2000
βIII tubulin	GAGGAGGAGGGGAGATGA	GAGCTGGTGAGAAGCAAAGC	Pastrana et al PNAS 2009
c-Myc	CAGCAGCGACTCTGAAGAAG	GACCTCTTGGCAGGGGTTT	Takahashi et al Cell 2006
Eed	TCCTGTCAGTAAGTAAAGATCATGC	ATCTCTGTGCCCTTCCACAC	
EGFR	GCTTGCAACGGTTCTCTCTC	CCGTTATCCATCCCTGACTC	Pastrana et al PNAS 2009
Ezh2	CTTCCATGCAACACCCAAC	TCCCTCCAGATGCTGGTAAC	
GAPDH	GTCTCCTGCGACTTCAGC	TCATTGTCATACCAGGAAATGAGC	
GFAP	CAGGCAATCTGTTACACTTGG	TGTCTGCTCAATGTCTTCCCTA	Pastrana et al PNAS 2009
Jarid2	AGGCGGTAAATGGGCTTCTTGTA	AGCCTGGGCCTTTTCCTCGAC	Shen et al Cell 2009
Klf4	CACCATGGACCCGGGCGTGGCTGCCAGAAA	TTAGGCTGTTCTTTTCCGGGGCCACGA	Takahashi et al Cell 2006
MASH1	ATGCAGCTACTGTCCAAACG	AACAGTAAGGGGTGGGTGTG	Pastrana et al PNAS 2009
Oct4	CTGAGGGCCAGGCAGGAGCACGAG	CTGTAGGGAGGGCTTCGGGCACTT	Takahashi et al Cell 2006
Sox2	GGTTACCTCTTCCTCCCACTCCAG	TCACATGTGCGACAGGGGCAG	Takahashi et al Cell 2006
Suz12	TTTCTTCCACAAAAATGATAAGC	TGGCAAACCTTTCACAAGCAG	

Fold difference = $2^{-(\Delta\Delta Ct)}$. The maximum and minimum of errors were first calculated as $\Delta\Delta Ct + \text{standard deviation (STD)}$ and $\Delta\Delta Ct - \text{STD}$ to obtain RQ maximum and RQ minimum respectively. The RQ values were converted to fold difference by using the fold difference equation: RQ fold difference = $2^{-(RQ_{\text{max or min}})}$. To get the error bar values for maximum and minimum, the error bar maximum = RQ maximum fold difference - fold difference, and the error bar minimum = fold difference - RQ minimum fold difference. (Please see the 2.1. for gene specific primer sequences)

2.2.2. Translational Level Detection

2.2.2.1. Protein extraction

Cells in suspension/tissues were washed once with 1X PBS and pelleted by centrifugation at 1,000 xg for 10 min at 4°C. Supernatant was discarded, and the pellet of cells/tissues was resuspended with 1 mL of ice cold 1X PBS. With extensive pipette trituration, the dissociated cells/tissues were spun down at 1,000 xg for 10 min at 4°C. Adherent cell culture media was removed and cells washed once with 1X PBS, then 1 mL ice cold 1X PBS added, cells mechanically scraped from the plate and spun down at 1,000 xg for 10 min at 4°C. To lyse the cells, equivalent volume (25 µL for 48-well plate; 50 µL for 6-well plate; 75 µL for 6 cm dish) of lysis buffer [50 mM Tris, pH 8.0, 150 mM NaCl, 1% Igepal, 0.5% NaDeoxycholate, 0.1% SDS, 1X protein inhibitor cocktail (Roche 11836170001)] was applied to the pellet followed by extensive trituration. After incubation for 10 min on ice, the lysate was cleared by centrifugation at 1,200 xg for 10 min at 4°C. The supernatant was saved for the quantification and stored at -20°C for overnight storage otherwise -80°C.

2.2.2.2. Bicinchoninic Acid (BCA) Protein Assay for Quantification

Protein content was quantified using the BCA assay. The protein standard [bovine albumin (BSA), Sigma P0914-10AMP] was serially diluted from 0 to 1 mg/mL. Each sample was diluted 1 in 10 with lysis buffer. All the samples were made as duplicates in a 96-well plate and mixed with 100 μ L of a mixture of copper (II) sulfate solution (Sigma C2284-25ML) and bicinchoninic acid solution (Sigma B9643-1L) in 1 in 50 ratio. The plate was sealed and incubated at 37°C for 30 min. The absorbance of BCA reaction was detected by using a plate reader (Thermo/LabSystems 352 Multiskan). Quantification of protein samples was calculated by their absorbance intensity reflected to the absorbance of known protein standard result.

2.2.2.3. Western blot

Protein (10 μ g) was boiled in 5X Laemmli buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β -mercaptoethanol, 0.01% bromophenol blue) for 10 min. Protein was then separated by SDS-polyacrylamide gel electrophoresis and transferred to a 0.45- μ m PVDF membrane (Millipore, Billerica, MA). Blots were incubated in blocking buffer [Tris buffered saline (TBS), 1% Tween-20, 3% BSA (Sigma A9418-50G)] and probed by using the primary antibodies suspended in blocking buffer (See Table 2.2. in Appendix for specific dilution and information of primary antibodies). Membranes were washed three times with TBS + 1% Tween-20. Goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA), suspended in blocking buffer were applied. After three times of wash with TBS + 1% Tween-20, chemiluminescence was produced by using an ECL+ kit (Amersham, RPN2132). Images were photographed using a charge-coupled device CCD camera (UVP, Cambridge, UK) and analyzed using LABWORKS software 4.6 (UVP).

2.3. Basic Molecular Cloning

2.3.1. Miniprep

A single bacterial colony from a LB agarose plate containing 0.1 mg/mL ampicillin or a stab from bacterial glycerol frozen stock was added into a 1.5 mL of LB culture to grow overnight in 37°C with shaking at 225 rpm. High turbid 1.5 mL culture was transferred into a 1.5 mL eppendorf tube and cells pelleted in a bench top centrifuge at 5000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was thoroughly resuspended with 70 µL STET buffer (8% sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris-Cl pH 8) by trituration. The bacterial cell lysate was mixed with 200 µL alkaline SDS solution (1% SDS, 0.2 M NaOH) by inverting the eppendorf tube several times. 150 µL Neutralisation buffer (7.5 M ammonium acetate) was added to the sample mixed, and the tube incubated on ice for 5 min. The sample was centrifuged at 13,000 rpm in 4°C for 20 min to pellet DNA. Supernatant was removed and the pellet mixed with 250 µL of isopropanol by inversion. Followed by centrifugation at 10,000 rpm for 8 min, the DNA pellet was washed by adding 200 µL of 70% ethanol. After repeating the centrifugation, the supernatant was discarded and the pellet was air dried for 10 min. The DNA was resuspended with 50 µL of TE (10mM Tris-Cl, pH 7.5 1mM EDTA) containing 5 µg/mL RNaseA and stored at -20°C.

2.3.2. Maxiprep

Bacterial miniculture (1.5 mL) was set up by following the protocol mentioned in section 2.3.1. The small culture was tipped into a 250 mL LB + 0.1 mg/mL ampicillin overnight (15-16h) culture in 37°C with shaking at 225 rpm. The turbid culture was centrifuged for 15 min in 4°C at 6000 rpm. The supernatant was discarded. The rest of the protocol was followed by Qiagen maxiprep kit (12963). The bacterial pellet was

resuspended with 15 mL of P1 buffer (with RNase A). The pellet was incubated in 37°C shaker for 10 min. The 15 mL of P2 buffer was added to resuspended solution, The mixture was occasionally swirled and kept for 5 min at room temperature. The 15 mL of chilled P3 buffer was added to the mixture and mixed by repeated inversion. The mixture was incubated on ice for 20 min and the bacterial lysate spun down at 9000 rpm in a J 10.5 rotor (Beckmann J2 centrifuge) for 35 min at 4°C. Fifteen milliliter of QBT solution was used to equilibrate the column and the supernatant was filtered through a double layer of Kimwipe tissues. After the filtration, the column was washed with 30 mL of QC buffer twice followed by adding 15 mL of pre-warmed 55°C QF buffer to elute the DNA into Oakridge tubes. Ten and a half milliliters of isopropanol was added and centrifuged at 4°C for 30 min at 14,000 rpm in J017 rotor in Beckman J2 centrifuge. After discarding the supernatant, 3.5 mL of 70% Ethanol was added without mixing or swirling. The mixture was spun down at 4°C for 30 min at 14,000 rpm in J-17 rotor. After discarding supernatant, the pellet was air dried for 15 min and resuspended with 250 uL of TE buffer overnight. To collect the DNA, the resuspended solution was spun down for 5 min and supernatant was collected.

2.3.3. Restriction Enzyme Digest

For the 100 µL restriction enzyme digest reaction, 5 µg of DNA plasmid with 10 µL of enzyme preferred Buffer (supported New England Biolabs), 2-3 µL of enzyme and rest of volume for DNA free water. If enzyme required BSA (20X), then 5 µL of BSA (20X) was added. The mixture was incubated at the appropriate temperature overnight, and the digested mixture was run on a TAE gel for electrophoresis.

2.3.4. Transformation

For transformation of DNA plasmid to DH5a (competent *E. coli* strain; gifted from Neil Brockdorff's laboratory), tubes of cells were removed from the -80°C freezer and slowly thawed on wet ice with gently tube tapping to resuspend cells. The DNA plasmid (1 ng to 50 ng) was directly added to cells. To mix the plasmid, the cells were gently tapped rather than trituration or vortexing. The cells were incubated on wet ice for 30 min and then incubated in a 37°C water bath for exactly 45 seconds for the heat shock. The cells were placed on ice immediately for at least 2 min. After adding 450 mL of SOC medium at room temperature, the cells were incubated at 37°C with shaking at 225 rpm for 1 hr. The transformed cells (10-100 mL) were spread on LB + 0.1 mg/mL ampicillin plate and incubated at 37°C overnight. The DNA plasmid containing colonies appeared on the plate the next day.

2.4. Lentivirus Production And Titration

2.4.1. Production Of Lentivirus

HEK 293 FT (Invitrogen R700-07) cells were used for calcium phosphate transfection. In a 10 cm dish, 1×10^5 HEK 293 FT cells were seeded and cultured for 12 hrs to reach 80% confluency. The culture medium was described in 2.1.3. section. Four hrs prior to transfection, the culture medium was replaced with 9 ml of fresh DMEM+GlutaMAX + 10% FBS (No Pen/Strept). Four hrs later, the DNA cocktails [4.5 µg of psPAX2, 1.5 µg of pMD2.G, 6 µg of shRNA in pLKO.1, 80 µl fresh 2.5M CaCl_2 (C-3306 Sigma) and volume adjusted to 500 µl with autoclaved distilled water] were slowly mixed with 500 µl of 2X HEPES buffered saline (HEBS), pH 7.05 [8 g of 280 mM NaCl, 0.37 g of 10 mM KCl, 0.13 g of 1.5 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1 g of 12 mM Dextrose/glucose, 5 g of 50 mM HEPES, free acid and volume adjusted to 500

mL with autoclaved distilled water]. The precipitate mixture (1 mL) was slowly added dropwise to the 9 ml of media covering the cells while the plate was constantly swirled. After 12 hrs, the culture medium was changed with 8 ml of regular medium containing pen/strep. After 48 hrs, the supernatant was collected and replaced with 8 mL of fresh medium. The collected supernatant was spun down for 7 min at 2000 rpm at 4°C. The supernatant was filtered through 0.45 µm membrane to exclude 293 cell contamination. Filtered virus containing solution was spun down in a 32Twi rotor for 1hr 40 min at 25000 rpm in 4°C. One hundred µl of autoclaved PBS was added on the side of the ultracentrifuge tube to avoid direct contact with pellet and incubated overnight at 4 °C. The resuspended pellet solutions were pooled together and aliquoted into 20 µl per tube and stored at -80 °C.

2.4.2. Lentiviral Titration

The 30,000 of HEK 293 FT cells were seeded in a 24 well plate the day before the infection. The viral stock stored at -80°C was serially diluted with PBS and added onto the cells directly. To maximize the cell and virus contact, 500 uL of virus mixed medium was added per well. After 72 hrs incubation, no viral infection control cells were trypsinized and heamocytometer counted as in section 2.1.1.1. The GFP positive cells were counted in random fields of the wells and the lentiviral titration was calculated by following equation:

$$\text{Transducing Units (TU) / mL} = (P * N / 100 * V) * 1/DF$$

(P=% of GFP+ cells; N= number of cells at time of transduction; V= volume of dilution added to each well; DF= dilution factor)

The 5x objective lens provided a view of 0.0238 cm² (actual size). The surface area of each well in 24 well plates was 1.9 cm². Thus, each view in 5x objective lens represented 1.25% of total surface area.

2.4.3. Lentiviral Infection Of Neurospheres

With known titers of each viral stock, the viral stock volume to be used was calculated (MOI 1 or 2) according to the following equation:

$$\text{Volume from viral stock (mL)} = (N / T) * \text{the number of MOI}$$

(N= number of cells to be infected; T= titer of the viral stock)

In order to allow cells to recover from the neurosphere passage, lentiviral infection was carried out after 12 hrs after single cell dissociation. The lentivirus was added to Neurobasal+GF with 6 µg/mL of polybrene, and this mixture was applied to cells. After 4 hrs of lentiviral exposure to cells, the medium was immediately changed with fresh medium.

2.5. Immunohistochemistry And Confocal Microscopy

2.5.1. Immunohistochemistry

2.5.1.1. Immunocytochemistry of neurospheres

The cells were washed with 1X PBS 3 X 5 min (3 x 5 wash). Cells were then incubated with PBS+ [donkey serum (10%; Sigma D9663); Triton X-100 (0.1%; VWR 28817.295) in 1X PBS] for 1 hr in room temperature to increase permeability. After this blocking step, the cells were incubated with primary antibodies, diluted in PBS+ to their own specific dilution factors, overnight at 4°C (See table 2.3. in Appendix for all antibodies used). After 3 x 5 wash with 1X PBS, secondary fluorescent Alexa antibodies, corresponding to the primary antibody species, were

used in 1:500 diluted concentration for 1 hr incubation in room temperature. After another 3 x 5 wash, DAPI (1:1000 dilution for working concentration; Sigma D9542) was applied to cells for 15 min followed by 3 x 5 wash with phosphate buffer (PB). The cells were coverslipped with flourosave (Calbiochem 345789).

For the O4/GFAP/ β III tubulin triple imunohistochemistry, PBS+ (10% donkey serum; no Triton-X) was used for 1 hr room temperature incubation followed by O4 antibody incubation at 4°C for overnight. The cells were washed with 1X PBS three times 5 min and secondary fluorescent Alexa fluorophore-conjugated antibodies applied as described above. After secondary antibody incubation, 1X PBS 3 x 5 wash was performed, and the cells were incubated with PBS+ (10% donkey serum; 1% Triton-X) for 1 hr in room temperature. The GFAP and Tuj1 (for β III tubulin staining) antibodies were applied to the cells for overnight incubation at 4°C. The following day, cells were washed 3 x 5 min with 1X PBS , incubated with secondary fluorescent Alexa antibody for 1 hr at room temperature and washed with 1X PBS again as described above. After 1X PBS wash, 1:1000 diluted DAPI was applied to cells for 15 min followed by 3 x 5 wash with PB. The cells were mounted with flourosave.

2.5.1.2. Perfusion of P4 and adult brains and microtome sectioning

Postnatal mice were killed with over-dose of pentobarbitone (>200 mg/kg body weight) via intraperitoneal (IP) injection, and brains surgically removed. Entire brains were immersed in 4% paraformaldehyde (PFA) in 0.1M phosphate buffer saline (PBS) overnight at 4°C. Fixed brains were then incubated in 30% sucrose in 0.1M phosphate buffer (PB) until they sunk and then were frozen at -80°C.

For the adult brains, mice were administrated an over-dose of pentobarbitone

(1 g/kg body weight) via IP injection and transcardially perfused with 25 mL of ice-cold saline to flush out blood, followed by 25 mL of 4% PFA. As described for P4 mice brains, the adult brains were dissected from the skull and incubated with 4% PFA and 30% sucrose, sequentially. When brains sunk, they were frozen down at -80°C.

The frozen brains were coronally sectioned with a microtome (Leica SM2000R; Leica, Milton Keynes, UK). The thickness of each section was set as 30 µm, and sections were obtained across a large anterior posterior range: from the olfactory bulb until the hippocampus. The sections were put in cryoprotectant (300 g sucrose, 300 mL ethylene glycol, 900 mL 0.1 M PB) and stored at -20°C.

2.5.1.3. Immunohistochemistry of sections

The general immunohistochemistry protocol was mentioned in 2.5.1.1. section. The brain sections were washed with 1X PBS for 5 min three times (3 x 5 wash). The sections were incubated in 50 mM glycine in PBS (0.2 g glycine, 50 mL PBS) for 15 min. After 3 x 5 wash, the sections were blocked with PBS+ for 1 hr in room temperature. After this blocking step, the primary antibodies was diluted in the PBS+, and sections were incubated in the antibody for overnight at 4°C (See table 2.3. in Appendix for all the antibodies that have been used). After 3 x 5 wash, secondary fluorescent Alexa antibodies, corresponding to the primary antibody species, were used in 1:500 diluted concentration for 1 hr incubation in room temperature. Once again with 3 x 5 wash with 1X PBS, DAPI was applied to cells for 15 min followed by 3 x 5 wash with PB. The sections were mounted with flourosave (Calbiochem 345789).

2.5.2. Confocal microscopy and image analysis

All the immunohistochemically stained coronal sections and neurospheres were imaged with a Zeiss LSM 710 laser scanning confocal microscope with 40X oil immersion (Zeiss Plan-NeoFluar 40X/1.3 Oil DIC) and 20X (Zeiss Plan-Neofluar 20X/0.50) objective lenses. Using the Zeiss Zen software, the four laser excitations (405, 488, 561 and 633 nm) were manually set and used to excite DAPI, Alexa Fluor 488, Alexa Fluor 567 and Alexa Fluor 647 fluorophores (Invitrogen) respectively. With the setting of pinhole as 1 airy unit (AU) on the 561 channel, the other channels shared the same pinhole size setting, and images were acquired with identical settings across experimental groups. .lsm image file were analyzed with LSM Image Browser 4.2 (Zeiss), Volocity (Improvision) and ImagJ (NIH) software.

To measure the intensity of H3K27me3 and DAPI images, an individual nucleus was marked and measured the averaged fluorescence intensity per pixels. The averaged intensity was normalized by the non-specific signals in the same image. This normalized H3K27me3 fluorescence intensity values from individual cells were divided by their normalized DAPI intensities followed by the same calculation.

2.6. Statistics

The numerical data were presented as mean $\pm$ standard deviation. Differences between two groups were assessed using Student's t-test and three or more groups with analysis of variance (ANOVA) with Bonferroni's post-hoc test. Groups were considered significantly different when at least a 95% confidence level ($p < 0.05$) was obtained. Analysis and graphical representation were performed with Microsoft Excel 2012 and GraphPad Prism 5 (GraphPad, La Jolla, CA, USA).

Chapter 3

SVZ Neurosphere Assay

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Chapter 3

SVZ Neurosphere Assay

3.1. Introduction

Bona fide stem cells in the adult CNS (Gritti et al.; Jandial et al.; Reynolds and Weiss; Rietze et al.; Vescovi et al.; Zhang et al.) and other organs are known to be rare and quiescent. To understand the biological features of these cell populations, there has been a major international program to isolate and expand the limited cell numbers *in vitro*. In 1992, Brent Reynolds and Sam Weiss first introduced the neural stem cell culture called ‘neurosphere assay’ by growing dissociated cells from the adult mouse striatum in mitogens like EGF. (Reynolds and Weiss, 1992). Not like the traditional neuronal cell line culture, artificially derived from tumors or immortalized neuronal cells by introducing oncogenes (Frederiksen et al., 1988; Gage et al., 1995b; Snyder et al., 1992), this primary cell culture method demonstrated a single stem cell from brain tissues could self-renew and differentiate into multiple lineages in complete medium with supplements. By using this approach, various research laboratories successfully cultured brain neurospheres derived from multiple species, ages and regions; now it has been cited in more than 1000 publications.

3.1.1. Isolation And Expansion Of Neural Stem/Progenitor Cell Population From Heterogeneity Of SVZ Cell Composition

As previously described in Chapter 1, the postnatal and adult SVZ consists of heterogeneous cell populations (Figure 1-1). Isolating only stem cells from these intermixed SVZ cells has been a challenge. Several laboratories have attempted to use prospective FACsorting to separate each subtype of SVZ cell population, but as of yet there is no universally accepted method because there are few specific cell surface

markers for individual SVZ cell types (Beckervordersandforth et al., 2010; Pastrana et al., 2011; Rietze et al., 2001). After the adult brain was found to have neural stem cells with the neurosphere culture (Reynolds and Weiss, 1992), Lois et al. in 1993 demonstrated the SVZ is neurogenic and Morshead et al. in 1994 demonstrated the SVZ contains the stem cell population by forming neurospheres from micro-dissected SVZ tissues, while other brain tissues, including cortex and striatum, could not give rise to substantial numbers of neurospheres (Lois and Alvarez-Buylla, 1993; Morshead et al., 1994). Subsequently, it was demonstrated that SVZ neurospheres could self-renew and spontaneously differentiate into multiple neural cell lineages, but a neurosphere was composed of a mixture of stem and progenitor cells (Doetsch et al., 1999; Suslov et al., 2002).

For the purposes of culturing pure stem cell population from SVZ, the heterogeneity of cells within a neurosphere was a problem. To try to resolve this issue, Singec et al. in 2006 first attempted to record single cell growth by using video time-lapse microscopy imaging (Singec et al., 2006). Initially it was thought that a single neurosphere was generated from a single cell, but single cells or single spheres could easily adhere to one another in the free-floating culture condition (Singec et al., 2006). This made it impossible to know if multiple lineages came from individual cells or were due to clumping of separate lineage restricted progenitors. Since it was difficult to control the movement of every single cell or neurosphere in this culture condition, the monolayer culture method (culturing a single cell layer on the bottom of a culture flask) was introduced for the SVZ NSPC culture (Scheffler et al., 2005). Since individual cells would attach to the bottom of flask, it was thought cell movement would be more controllable. However, these cells were found to be vibrantly motile on the surface of the culture flask, and it seemed cell-cell contact still

spontaneously occurred (Coles-Takabe et al., 2008; Mori et al., 2006; Singec et al., 2006). Therefore, culturing single cells per well (clonal analysis) was considered to be the best method to avoid cell clumping, but it rarely yielded neurospheres, was difficult to perform on a routine basis and was not practical for every single experiment. Due to these reasons, low concentrations of cells (between 0.2 to 20 cells per μL) have been commonly utilized (Fischer et al., 2011; Ortega et al., 2011) although in these conditions cell-cell or sphere-sphere adhesion could still occur during the culture (Coles-Takabe et al., 2008; Mori et al., 2006; Singec et al., 2006).

Due to these limitations of the neurosphere cell culture technique when using the whole SVZ, recent studies demonstrated it was possible to separate *in vivo* SVZ stem and progenitor cells with a combination of two SVZ stem cell markers (hGFAP/Prominin and GFAP/EGFR) and culture them separately (Beckervordersandforth et al., 2010; Pastrana et al., 2009). The selectively isolated SVZ stem cells from both approaches gave rise to significantly higher numbers of neurospheres per starting cells than the ones from the non-stem cell populations. By using immunohistochemistry and qRT-PCR, the SVZ cell type markers were detected, and interestingly the neurospheres expressed not only SVZ stem/progenitor cell expressing markers like GFAP, GLAST and Mash1 but also differentiation markers like $\beta\text{III-tubulin}$ (Beckervordersandforth et al., 2010; Pastrana et al., 2009). This suggested that neurospheres could spontaneously differentiate into the three different neural cell lineages during self-renewal stage of NSA.

The SVZ neurosphere assay has been readily used by several research groups and recapitulates *in vivo* SVZ heterogeneity in the absence of endogenous stem cell microenvironment. However, heterogeneity within a neurosphere is still an issue for culturing pure populations of stem cells, but this biological feature may be necessary

for forming neurospheres. Therefore, the neurosphere assay experiment should not be interpreted as NSCs or NPCs only effects; it is from the SVZ heterogeneous cell population effects.

3.1.2. Various Protocols For SVZ Neurosphere Culture

Because of the optimizations mentioned above, the neurosphere assay protocol has been slightly modified from one laboratory to another over the past two decades (Table 3-1). Among these mouse SVZ neurosphere assay protocols, the following three conditions in the SVZ cell culture assay have been most commonly modified:

1. Different complete media
2. Different combination of growth factors and supplements to make defined media
3. Defined media with or without serum

Each parameter has an important role for SVZ cell self-renewal, differentiation and cell fate choice; the biological read-outs of neurospheres from different protocols are not the same or can be conflicting even though the neurospheres are generated from the same tissue (Pastrana et al., 2011). Since there is no standardized protocol for the neurosphere assay, the characterization of neurospheres produced following a particular protocol would help to understand the neurosphere phenotype changes after biological and chemical treatments.

Table 3-1. Mice SVZ Neurosphere protocols since 1992

Paper	Senior authors	Year/publication	Pubmed search: "SVZ" "neurosphere" "mice"	Neurosphere media					flask/plate	Dissociation	Cell density	Differentiation
				Cell types	Media	Growth factors	Hormones	Others				
Reynolds BA.	Weiss S.	1992	3-8 months old adult striatal tissue	Adult mouse striatum including and excluding subependyma	serum free media	EGF 20 ng/mL	N/A	N/A	non adhesive	Enzymatically dissociate	104 cells/cm ²	Poly-L-Ornithine
Morshead CM, et al	van der Kooy D.	1994	Neuron	subependyma	serum free media	EGF 20 ng/mL	N/A	N/A	non adhesive	Enzymatically dissociate	104 cells/cm ²	N/A
Reynolds BA.	Weiss S.	1996	Dev Biol	3-8 months old adult striatal tissue	serum free media	EGF 20 ng/mL	N/A	N/A	non adhesive	Enzymatically dissociate	104 cells/cm ²	Poly-L-Ornithine
Weiss S. et al	Reynolds BA.	1996	Neurosci	Adult CD1 mice SVZ	DMEM/F-12	EGF 20 ng/mL, bFGF 20 ng/mL or both	5 mM HEPES, 0.6% glucose, 3 mM sodium bicarbonate, 2 mM glutamine, 20 nM progesterone, 60 uM putrescine and 30 nM sodium selenite	1.33 mg/mL of trypsin, 0.67 mg/mL of hyaluronidase, and 0.2 mg/mL of kynurenic acid	non-coated	1.33 mg/mL of trypsin, 0.67 mg/mL of hyaluronidase, and 0.2 mg/mL of kynurenic acid in aCSF and 0.7 mg/mL of progesterone	5-10 viable cells/ul	15 ug/mL poly-L-ornithine
Gritti A. et al	Vescovi AL.	1996	Neurosci	3-8 months old CD1 mice striatal tissue including subependyma	DMEM/F-12	EGF 20 ng/mL, FGF2 20ng/mL or both	2 mM L-glutamine, 0.6% glucose, 9.6 gmg/mL putrescine, 5.2 ng/mL sodium selenite, 0.1 mg/mL transferrin and 2 ug/mL heparin	1.3 mg/mL trypsin, 0.67 mg/mL hyaluronidase and 0.2 mg/mL kynurenic acid in aCSF and 0.7 mg/mL of progesterone	60mm dish	1.3 mg/mL trypsin, 0.67 mg/mL hyaluronidase and 0.2 mg/mL kynurenic acid in aCSF and 0.7 mg/mL of progesterone	3,500 cells/cm ²	polyornithine, No GFs and FBS
Johansson CB, et al	Frisen J	1999	Cell	Adult SVZ and spinal cord	DMEM/F-12	EGF 20 ng/mL and bFGF 20 ng/mL	B27, 2 mM glutamine, 100 U/mL penicillin and 100 ug/mL streptomycin	0.7 mg/mL hyaluronic acid, 0.2 mg/mL kynurenic acid, and 1.33 mg/mL trypsin in HBSS with 2 mM glucose	unknown	0.7 mg/mL hyaluronic acid, 0.2 mg/mL kynurenic acid, and 1.33 mg/mL trypsin in HBSS with 2 mM glucose	single cell/96-well	N/A
Doetsch F. et al	Alvarez-Buylla A.	1999	Cell	adult CD1 mice lateral walls	DMEM-F12 (GIBCO)	EGF 20 ng/mL	N2 (GIBCO), 2 mM glutamine, 0.6% (w/v) glucose, and 15 mM HEPES	0.25% trypsin/1 mM EDTA, 0.7 mg of ovomucoid inhibitor and 1 mg of DNase	uncoated 35 mm dishes	Papain, 0.7 mg of ovomucoid inhibitor and 1 mg of DNase	5,556 cells/cm ²	10ug/mL laminin, No EGF
Lim D.	Alvarez-Buylla A.	1999	PNAS	P6-10 or adult microdissected SVZ	DMEM-F12 (GIBCO)	EGF 20 ng/mL	N2, B27 (Both: GIBCO), 2 mM glutamine, 0.6% (w/v) glucose, 10% FBS and 15 mM HEPES	0.25% trypsin/1 mM EDTA, 0.7 mg of ovomucoid inhibitor and 1 mg of DNase	poly-D-lysine	0.25% trypsin/1 mM EDTA, 0.7 mg of ovomucoid inhibitor and 1 mg of DNase	5,556 cells/cm ²	10ug/mL laminin, No EGF
Gritti A. et al	Vescovi AL.	1999	Neurosci	4-8 months old CD1 mice striatal tissue excluding subependyma or subependyma alone	DMEM/F-12	EGF 20 ng/mL, FGF2 20ng/mL or both	2 mM L-glutamine, 0.6% glucose, 9.6 gmg/mL putrescine, 5.2 ng/mL sodium selenite, 0.1 mg/mL transferrin and 2 ug/mL heparin	1.3 mg/mL trypsin, 0.67 mg/mL hyaluronidase and 0.2 mg/mL kynurenic acid in aCSF and 0.7 mg/mL of progesterone	35 mm Petri dishes	1.3 mg/mL trypsin, 0.67 mg/mL hyaluronidase and 0.2 mg/mL kynurenic acid in aCSF and 0.7 mg/mL of progesterone	3,500 cells/cm ²	polyornithine, No GFs and FBS
Rietze RL. et al	Bartlett PF.	2001	Nature	8-10 weeks old adult CBA mice periventricular tissue	NS-A basal serum free media	EGF 20 ng/mL and bFGF 10ng/mL	2 mM L-glutamine, 0.6% glucose, 60 mM putrescine, 20 nM progesterone, 30 nM sodium selenite and 100 mg/mL apo-transferrin	10 mM HEPES, 200 g/mL EDTA, 0.5 mM trypsin and 0.001% DNase	N/A	10 mM HEPES, 200 g/mL EDTA, 0.5 mM trypsin and 0.001% DNase	10 cells/cm ²	poly-L-ornithine, No GFs and 1% FBS
Laywell ED, et al	Steindler DA.	2002	Methods in mol biol	Not mentioned	DMEM/F-12	EGF 20 ng/mL and bFGF 10ng/mL	10% FBS	6 well plates coated with poly-HEMA	trypsin/EDTA	trypsin/EDTA	Not mentioned	poly-L-ornithine and laminin
Haughey NJ, et al	Mattson MP.	2002	Neuromol Med	8-10 weeks old human fetuses cortical tissue	Neurobasal culture media (Gibco BRL)	EGF 20 ng/mL and bFGF 20 ng/mL	N/A	uncoated plastic flask	mechanical dissociate	mechanical dissociate	1000 cells/ul	No GFs; 1% FBS

Table 3-1. cont.

Paper	Senior authors	Year/publication	Cell types	Neurosphere media				flask/plate	Dissociation	Cell density	Differentiation
				Media	Growth factors	Hormones	Others				
Li L. et al	Ross AH.	2002 Mol Cell Neurosci	postnatal mouse striatal tissue	serum free media	EGF 20 ng/mL	N/A	N/A	non adhesive	mechanical dissociate	104 cells/cm ²	Poly-L-Ornithine
Doetsch F. Alvarez-Buylla A.	Doetsch F. Alvarez-Buylla A.	2002 Neuron	adult CD1 mice lateral walls	DMEM-F12 (GIBCO)	EGF 20 ng/mL	0.02 mg/ml insulin	N2 (GIBCO), 2 mM glutamine, 0.6% (w/v) glucose, and 15 mM HEPES	uncoated 35 mm dishes	Papain, 0.7 mg of ovomucoid inhibitor and 1 mg of DNase	5,556 cells/cm ²	10ug/mL laminin, No EGF
Parmar M. et al	Kokaia Z.	2003 Mol Cell Neurosci	4-6 weeks old female NMRI mice	DMEM-F12	EGF 20 ng/mL, bFGF 10 ng/mL or both	N/A	B27, 2 mM L-glutamine, 15 mM HEPES, 0.6% glucose, 0.1% NaHCO ₂ , penicillin/streptomycin	cell culture plates	HBSS without Ca ²⁺ and Mg ²⁺ , 0.015 M HEPES, 5.4 mg/mL D-glucose, 1.33 mg/mL trypsin, 80 U/mL DNase, 0.7 mg/mL hyaluronidase and 2 mg/mL kynereinic acid	10 cells/ul or 50 cells/ul	poly-L-lysine, no GFs, 1% serum
Eriksson C. et al	Wictorin K.	2003 Exp Neurol	E13.5-14.5 LGE tissues	DMEM-F12 (GIBCO)	EGF 20 ng/mL and FGF2 10 ng/mL (all: TEBU)	defined hormone mix, 0.6% glucose, 100 µg/ml transferrin, 0.6 mM putrescine, 30 nM sodium selenite, 2 mM insulin and 20 glutamine, 0.1% sodium bicarbonate, 5 mM Hepes (all from Sigma) and penicillin (100 U)/streptomycin (100 µg/ml; GIBCO)		uncoated T75 plastic flask	0.1% trypsin, 0.05% DNase	100,000 cells/mL	Poly-L-lysine, DMEM/F12, a defined hormone and salt mixture, No GFs, 1% fetal bovine serum
Soares S.	Sotelo C.	2004 Neurosci	Adult or neonatal mice SVZ	NSA media (Cliniscience, Montrouge, France)	EGF 20 ng/mL and FGF2 10 ng/mL (all: TEBU)	Insulin and progesterone	glucose, NaHCO ₃ , HEPES, apo-transferrin, putrescine, selenium (all: Sigma), L-glutamine (Invitrogen) and 10% FBS	poly-D-lysine	Papain	5,000 cells/cm ²	N/A
Palma V. et al	Altarba AL	2005 Developm ent	postnatal or adult mice SVZ	Neurobasal media	EGF 10 ng/mL	0.02 mg/ml insulin	N2, 2 mM glutamine, 0.6% glucose, antibiotics and 15 mM HEPES	uncoated flask	0.3% papain, 0.7 mg of ovomucoid inhibitor and 1 mg of DNase	1x10 ⁶ cells per bottle	10ug/mL laminin, No EGF
Chen J. et al	Chopp M.	2005 J Neurosci	normal adult male C57BL/6J SVZ	DMEM/F-12	EGF 20 ng/mL and bFGF 20 ng/mL	0.025 mg/mL insulin, 6.3 ng/mL progesterone	2 mM L-glutamine, 0.6% glucose, 9.6 µg/mL putrescine, 0.1 mg/mL apotransferrin and 5.2 ng/mL sodium selenite	N/A	0.25% trypsin-EDTA	30 cells/ul or 20 cells/ul	Lamin, DMEM/F-12, 2% FBS and EGF (For neurite outgrowth) OR matrigel and 2% B27 (For migration)
Wang TW. Parent et al	JM.	2005 Developm ent	P15 CD1 mice SVZ	DMEM/F-12	EGF 20 ng/mL and FGF2 10 ng/mL	a defined hormone and 2.5µg/mL insulin	0.6% glucose, 5 mM HEPES, 3 mM sodium bicarbonate, 2 mM glutamine, 100 µg/mL transferrin, 60 µM putrescine, 30 nM sodium selenite and 20 nM progesterone	N/A	1.3 mg/mL trypsin, 0.67 mg/mL hyaluronidase and 0.2 mg/mL kynereinic acid in aCSF and 0.7 mg/mL ovomucoid	1,062-2,831 cells/cm ²	1x10 ⁵ cells per well in 24-well plates coated with poly-ornithine, no GFs
Katakows ki M. et al	Chopp M.	2005 Neurosci Latt	2-3 months old C57 mice SVZ	DMEM/F-12	EGF 20 ng/mL and bFGF 20 ng/mL	0.025 mg/mL insulin, 6.3 ng/mL progesterone	2 mM L-glutamine, 0.6% glucose, 9.6 µg/mL putrescine, 0.1 mg/mL apotransferrin and 5.2 ng/mL sodium selenite	N/A	0.25% trypsin-EDTA	20 cells/ul	Lamin, DMEM/F-12, 2% FBS and EGF (For neurite outgrowth) OR matrigel and 2% B27 (For migration)
Meissner KK. et al	Doering LC.	2005 Brain Res	1 year old C57BL/6J SVZ	DMEM/F-12	EGF 20 ng/mL and bFGF 20 ng/mL	N/A	1.33 mg/mL of trypsin, 0.67 mg/mL of hyaluronidase, and 5-10 viable 0.2 mg/mL of kynereinic acid	non-coated plate		poly-L-lysine, No GFs	

Table 3-1. cont.

Paper	Senior authors	Year/publication	Cell types	Neurosphere media				flask/plate	Dissociation	Cell density	Differentiation
				Media	Growth factors	Hormones	Others				
Scheffler B. et al	Steindler DA	2005 PNAS	P8 and adult C57/B6 SVZ	DMEM/F-12	EGF 20 ng/mL and bFGF 20 ng/mL	N/A	100 units/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B (collectively, abx) N2 supplements, 35 µg/ml bovine pituitary extract, abx, 5% FCS (HyClone)	uncoated T75 plastic tissue-culture dishes	0.005% trypsin	1,000 cells/cm ²	10 µg/mL polyornithine and 1 µg/mL laminin at densities of $\approx 2 \times 10^5$ cells per cm ²
Mishra SK. et al	Zimmermann H.	2006 Development	8-9 weeks old C57BL/6J SVZ	DMEM/F-12	EGF 20 ng/mL and FGF2 10 ng/mL	N/A	B27, 10 mM HEPES, 100 U/ml penicillin, 10 µg/ml streptomycin	uncoated culture dish	0.5 mg/ml papain (14 U/mg), 0.1 mM EDTA, 0.7 mg/ml trypsin inhibitor, 1000 U/ml Dnase I, Accutase II	single cell/well (96-well)	Dissociated with Accutase (5000 cells/overslip) on 0.25 mg/ml poly-L-ornithine and 2% FBS
Councill JH. et al	LaMantia A-S.	2006 Neuroscience	>7 weeks old male CF1 SVZ	Neurobasal-A	EGF 20 ng/mL and bFGF 20 ng/mL	N/A	B27, N2 and 1% penicillin/streptomycin	24 well dish	Hyaluronidase/Trypsin with DNase and trypsin inhibitor	5,000 cells/well (24-well)	poly-D-lysine and laminin
Kosaka N. et al	Ochiya T.	2006 FASEB J.	E14.5 ganglionic eminence SVZ	DMEM/F-12	EGF 20 ng/mL and bFGF 20 ng/mL	N/A	B27, 1% antibiotic Antimycotic solution	various size plate	mechanically dissociate	2.5 cells/ul	15 µg/mL poly-L-ornithine, 1 µg/mL Fibronectin and 1% FBS with or without cytokine (EGF-4 or FGF-2)
Ocbina PJ. et al	Szele FG.	2006 Mol Cell Neurosci	13-14 weeks old male CD1 mice SVZ	Neurobasal media	EGF 20 ng/mL and FGF2 20 ng/mL	N/A	N2, B27, 2 mM L-glutamine, 0.1 µg/ml heparin, 100 U/ml penicillin and 100 µg/ml streptomycin	poly-Hema coated flask	100 ng/ml papain	10 cells/ul in 25cm ²	matrigel or poly-D-lysine (20 µg/mL)/laminin (2.5µg/mL), No GFs
Kirkham DL. et al	Doering LC.	2006 BMC Neuroscience	1-4 weeks old mice SVZ	DMEM/F-12	EGF 20 ng/mL and bFGF 10 ng/mL	20 nM progesterone and 0.1 mL insulin-transferrin-selenium	0.03M glucose, 0.005 M HEPES, 60µM putrescine, 2.0 mL B27 and 7.32 µl heparin	24 well dish	0.13 mg kynurenic acid, 0.66 mg type 1-S hyaluronidase, 1.3 mg trypsin and 1.6 µl of ovomucoid	10 cells/ul	0.5 mg insulin, 1.61 mg putrescine, 5.0 mg transferrin, 0.46 gm D(+) galactose and 200 µL insulin-transferrin-sodium selenite
Busch C. et al	Drews U.	2006 J Cell Sci	<2 months old SVZ	DMEM/F-12	FGF2 20 ng/mL	N/A	N2	15 µg/ml poly-L-ornithine and 2 µg/ml fibronectin	1.5 mg/ml trypsin, 0.35 mg/mL Dnase and 0.7 mg/ml trypsin inhibitor	500 cells/ul	N/A
Dizon MLV. et al	Szele FG.	2006 Neuroscience	Adult male CD1 mice SVZ	Neurobasal media	EGF 20 ng/mL and FGF2 20 ng/mL	N/A	N2, B27, 2 mM L-glutamine, 0.1 µg/ml heparin, 100 U/ml penicillin and 100 µg/ml streptomycin	poly-Hema coated flask	100 ng/ml papain	50 cells/ul in 25cm ²	matrigel and No GFs
Torroglosa A. et al	Estrada C.	2007 Stem cells	P7 CD1 SVZ	DMEM/F-12	EGF 20 ng/mL	20 nM progesterone and 25 µg/mL insulin	100 µg/ml transferrin, 30 nM sodium selenite, 60 µM putrescine, 2 mM glutamine, 33 mM glucose, 5 µg/ml gentamicin, 5 mM HEPES and 0.125 µg/ml fungizone	For the monolayer, polyornithine with 1% FCS	1 mg/mL trypsin, 0.2 mg/mL kynurenic acid and 0.7 mg/ml ovomucoid	100 cells/ul in 96-well	N/A

Table 3-1. cont.

Paper	Senior authors	Year/publication	Cell types	Neurosphere media				flask/plate	Dissociation	Cell density	Differentiation
				Media	Growth factors	Hormones	Others				
Maslov AY et al	Pruitt SC.	2007	Stem cells Adult 129/Sv SVZ	DMEM/F-12	EGF 20 ng/mL and bFGF 20 ng/mL	N/A	B27	96-well microtiter plate	0.25% trypsin	30 cells/ μ L	N/A
Merkle FT. Alvarez- et al	Buylla A.	2007	Neonatal CD1 anterior, dorsal and ventral of SVZ	DMEM/F-12	EGF 20 ng/mL and bFGF 20 ng/mL	N/A	100 units/ml penicillin, 100 μ g/ml streptomycin, and 250 ng/ml amphotericin B (collectively, abx) N2 supplements, 35 μ g/ml bovine pituitary extract, abx, 5% FCS (HyClone)	uncoated T75 plastic tissue-culture dishes	0.005% trypsin	1,000 cells/ cm^2	10 μ g/ml polyornithine and 1 μ g/ml laminin at densities of $\approx 2 \times 10^5$ cells per cm^2
Raponi E. Delouime et al	JC.	2007	Glia P2 C57/B16 mice SVZ	DMEM/F-12	EGF 10 ng/mL and bFGF 10 ng/mL	none	B27 and N2	noncoated Petri dishes	2.5 mg/mL trypsin and 1 μ g/mL DNase	5 cells/ μ L	poly-L-lysine, No GFs, higher conc N2 and B27 and 3%FBS
Ferron SR. et al	Farinas I.	2007	Neot Adult CD1 mice SVZ	DMEM/F-12	EGF 20 ng/mL and bFGF 10 ng/mL	0.02 mg/mL insulin and 160 nM progesterone	0.6% glucose, 0.1% NaHCO ₃ , 5 mM HEPES, 2 mM L-Gln and 50 U/mL penicillin/streptomycin	24 well culture plate	0.9 mg/mL papain, 0.2 mg/mL L-Cys and 0.2 mg/mL EDTA	5 cells/ μ L or 2,600 cells/ cm^2	N/A
Young Bartlett KM. et al	PF.	2007	J P2 and P100 C57BL/6 mice SVZ	INS-A basal serum free media	EGF 20 ng/mL and bFGF 10 ng/mL	25 mg/mL insulin	2 mM L-glutamine, 0.6% glucose, 60 mM putrescine, 20 nM progesterone, 30 nM sodium selenite and 100 mg/mL apo-transferrin	tissue culture plates	10 mM HEPES, 200 g/mL EDTA, 0.5 mM trypsin and 0.001% DNase	3,500 cells/ cm^2 (for primary neurosphere), 1 cell per well (for secondary neurosphere) and 1% FBS	poly-L-ornithine, No GFs
Hitoshi S. Ikenaka et al	K.	2007	J Neurosci Res.	serum free media	EGF 20 ng/mL and bFGF 10 ng/mL	25 mg/mL insulin, 100 mg/mL transferrin, 20nM progesterone, 60mM putrescine, and 30nM selenium	0.6% glucose (Sigma), 2 mM glutamine (GIBCO), 3 mM sodium bicarbonate (Sigma), and 5 mM HEPES buffer (Sigma)	uncoated 24-well plate	1.33 mg/mL trypsin, 0.67 mg/mL hyaluronidase, 0.2 mg/mL kynurenic acid, 70 μ m cell strainer and trypsin inhibitor	5 cells/ μ L	N/A
Ishii Y. et al	Funa K.	2008	Mol Cell Neurosci	NeuroCult Stem Cell Basal Medium and DMEM/F-12	EGF 20 ng/mL and bFGF 10 ng/mL	N/A	NeuroCult proliferation supplement and N2	polyHema	mechanical dissociate	10 cells/ μ L	poly-L-lysine and laminin, No GFs
Yasuda T. Adams et al	DI.	2008	Mol Cell Neurosci	DMEM/F-12	EGF 20 ng/mL and bFGF 10 ng/mL	N/A	5.6 mM HEPES, 0.7% glucose, 15 mM NaHCO ₃ , 200 U/mL penicillin/streptomycin and 10% mouse NeuroCult NSC proliferation supplements, 2% BSA and 2 μ g/mL heparin	T12.5 or T25 flasks/96well plates	0.05% trypsin-EDTA and 40 μ m cell strainer	2-10 $\times 10^3$ cells/ cm^2 or 10 ³ cells/well in 96-well plates	N/A

Table 3-1. cont.

Paper	Senior authors	Year/publication	Cell types	Neurosphere media				flask/plate	Dissociation	Cell density	Differentiation
				Media	Growth factors	Hormones	Others				
Barraud P. Franklin et al	Neurosci Cell	2008 Mol	P0-P10 CD1 mice SVZ	DMEM/F-12	EGF 20 ng/mL and bFGF 20ng/mL	N/A	1:3 mixture of DMEM/F12, 2% B27, 1% penicillin/streptomycin and 4 ng/mL heparin	uncoated flask	Collagenase IA, 0.25% DNase I and 40 µm cell strainer	100 cells/µL	laminin, collagen
Das S.	Basu A.	2008 J Neurosci	P7 BALB/c mice SVZ	DMEM/F-12	EGF 20 ng/mL and FGF 6.29 ng/mL and 1.0ng/mL progesterone	25 µg/mL insulin and 10 ng/mL D-biotin, 16.11 µg/mL putrescine, 5 ng/mL selenium, 50 µg/mL apo-transferrin and 50 µg/mL gentamycin		6 well plate	10 mg/mL Trypsin and 50 µg/mL DNaseI; Accutase	3 x 10 ⁴ cells/cm ²	poly D-lysine
Nicoleau C. et al	Coronas V.	2009 Stem cells	P1-3 C57Bl6 SVZ	serum free media	EGF 20 ng/mL and HGF 20 ng/mL	N/A	B27, 15 mM HEPES (Gibco), 1 mM sodium pyruvate, 25 mM D-glucose, 100 U/mL penicillin and 100 µg/mL streptomycin	24 well plate	0.025% trypsin and 0.265 mM EDTA	5,000 cells/well in a 24 wells plate	N/A
Armesilla-Diaz A. et al	Neurosci	2009	E13.5 mouse olfactory bulb	DMEM/F-12	EGF 20 ng/mL and bFGF 20ng/mL	25 µg/ml insulin (Sigma), 10% 10x hormone mix (0.6% glucose, 0.1% NaHCO ₃ , 5 mM Hepes, 1 mg/mL apo-transferrin, 96 mg/mL putrescine, 0.2 µM progesterone and 0.3 µM sodium selenite)	0.6% glucose, 0.1% NaHCO ₃ , 5 mM Hepes, 2 mM L-glutamine, 50 U/ml penicillin/streptomycin, 7 U/ml heparin and 0.4% BSA	GFR-Matrigel coated plates	Mechanically dissociate and Accutase	25,000 cells/cm ²	GFR-Matrigel, No GFs and 2% FCS
Pastrana E. et al	Doetsch F. PNAS	2009	adult CD1 mice lateral walls	DMEM/F-12 (GIBCO)	bFGF 10 ng/mL, PDGF-AA 20 ng/mL or EGF 20 ng/mL	insulin 0.02 mg/ml	N2 (GIBCO), 2 mM glutamine, 0.6% (w/v) glucose and 15 mM HEPES	uncoated 35 mm dishes	Papain, 0.7 mg of ovomucoid inhibitor and 1 mg of DNase	0.3 cell/µL	10µg/mL laminin, No EGF
Kinoshita MO. et al	Furuwa S. Lett.	2009 Neurosci	8 weeks old mice SVZ	DMEM/F-12 and NeuroCult proliferation medium	EGF 20 ng/mL	N/A	10% FBS	poly-Hema coated plate	trypsin/EDTA	Not mentioned	poly-L-lysine, NeuroCult murine basal medium with murine neural stem cell differentiation supplements

Table 3-1. cont.

Paper	Senior authors	Year/publication	Cell types	Neurosphere media				flask/plate	Dissociation	Cell density	Differentiation
				Media	Growth factors	Hormones	Others				
Raedt R. et al	Boon P.	2009 Epilepsy Res.	Adult male C57BL/6xDBA2/J mice SVZ	NS-A basal serum free media	EGF 20 ng/mL and bFGF 20ng/mL	N/A	2 mM L-glutamine, 3 mM D-glucose, 1% N2, 100 U/ml penicillin and 100 U/ml streptomycin	96-well flat-bottomed plate	0.5 mM Na2EDTA, 1.0 mM cysteine, 0.9 mg/ml papain, 1 mg/ml Dnase and 0.5 mg/ml ovomucoid	6,9930 cells/cm ²	50,000 cells/cm ² on matrigel, No GFs and 3% FCS
Nakatani Y. et al	Yu RK.	2010 Glycobiology	P2, P10, P30 and adult SVZ	Neurobasal-A medium	EGF 20 ng/mL and FGF2 20ng/mL	N/A	B27, 2 mM L-glutamine	uncoated 96-well plates	0.15% trypsin, 0.1% DNase I and 0.07% trypsin inhibitor	100 cells/ μ L	poly-l-ornithine, fibronectin, No GFs and 1% FBS
Persson A. et al	Kuhn HG.	2010 Neuroscience	P9 C57BL/6 mouse SVZ	Neurobasal-A medium	EGF 20 ng/mL and bFGF 10ng/mL	N/A	B27, 100 U penicillin, 100 μ g streptomycin, 2 mM Glutamax and 2 ug/ml heparin	Not mentioned	TrypLE express	60 cells/ μ L	Matrigel, No GFs and No heparin
Akiba Y. et al	Baker H.	2010 Biochem Biophys Res Commun	P2 C57BL/6J mice SVZ	DMEM/F-12	EGF 20 ng/mL and bFGF 20 ng/mL	N/A	100 units/ml penicillin, 100 μ g/ml streptomycin, and 250 ng/ml amphotericin B (collectively, abx) N2 supplements, 35 μ g/ml bovine pituitary extract, abx, 5% FCS (HyClone)	uncoated T75 plastic tissue-culture dishes	0.005% trypsin	1,000 cells/cm ²	10 μ g/ml polyornithine and 1 μ g/ml laminin at densities of $\approx 2 \times 10^4$ cells per cm ²
Obermair Fl. et al	Thallmair M.	2010 Stem Cell Res.	6-8 weeks old C57BL/6 mice SVZ	DMEM/F-12	EGF 20 ng/mL and FGF2 20 ng/mL	N/A	N2, 2 mM glutamine and 5 ug/ml heparin	plastic tissue culture dishes	2.5 U/ml papain, 250 U/ml DNaseI, 1 U/ml dispase and 70 μ m cell strainer	none; Percoll density gradient	polyornithine/laminin, No GFs, N2, 0.5% FBS and 1 μ M forskolin
Del Valle I. et al	Silva A.	2010 Neuroscience	E13.5 FVB mouse embryo OB and 6-8 weeks old FVB SVZ	DMEM/F-12	EGF 20 ng/mL and bFGF 20 ng/mL	N/A	0.6% glucose, 0.1% NaHCO3, 5 mM Hepes, 25 μ g/ml insulin, 1 mg/ml apotransferrin, 96 mg/ml putrescine, 0.2 μ M progesterone and 0.3 μ M sodium selenite	6 well plate	1 mg/ml papain	10,000 cells/cm ²	5 $\times 10^4$ cells/cm ² , 0.5% FCS, No GFs
Li L. et al	Bartlett PF.	2010 J Neurosci	E12, P2 and 3-4 months old C57BL/6 mice SVZ	NeuroCult Neural Stem Cell Basal Medium	EGF 20 ng/mL and bFGF 10ng/mL	N/A	16 mM HEPES (Sigma-Aldrich), 100 U/ml penicillin/streptomycin, NSC proliferation supplements, 2% BSA and 2 μ g/ml heparin	96-well plates	0.05% trypsin-EDTA and 0.014% trypsin inhibitor type S, and 1 cell I-S from soybean	640, 320, 160, 80, 40, 20, 10, per well	poly-l-ornithine, laminin and 1% FCS
Cheng A. et al	Shih J.C.	2010 J Neurosci	E14.5, E17.5 and P2 mouse dorsal telencephalon	DMEM/F-12	EGF 30 ng/mL and bFGF 30 ng/mL	N/A	B27	uncoated plastic culture flask	0.05% trypsin-EDTA	10 cells/ μ L for embryonic and 50 cells/ μ L for neonatal	N/A
Capilla-Gonzalez V. et al	Garcia-Verdugo JM.	2010 Eur. J Neurosci	E2, E5 and E8 C57BL/6 male mice SVZ	DMEM/F-12	EGF 20 ng/mL and bFGF 6.3 ng/mL	N/A	2 mM L-glutamine, 0.6% glucose, 9.6 mg/ml insulin and putrescine, 5.2 ng/ml sodium selenite, 0.1 mg/ml transferrin, 2 mg/ml heparin and antibiotic/antimycotic	35 mm Petri dishes	1.3 mg/ml trypsin, 0.67 mg/ml hyaluronidase and 0.2 mg/ml kynurenic acid in aCSF and 0.7 mg/ml ovomucoid	3500 cells/cm ²	polyornithine, No GFs and FBS

Table 3-1. cont.

Paper	Senior authors	Year/publication	Cell types	Neurosphere media				flask/plate	Dissociation	Cell density	Differentiation
				Media	Growth factors	Hormones	Others				
Popa et al	N. et Boucraut J	2011	Glia P5 C57BL/6J mice SVZ	DMEM/F-12 (3:1)	EGF 10 ng/mL and bFGF 20 ng/mL	Insulin and progesterone 20 nM	transferring, selenium ITS additive (insulin, transferrin, selenium), 100 U/mL penicillin/streptomycin and B27	Poly-L-lysine coated culture flask	trypsin-EDTA	Not mentioned	0.01 mg/mL Poly-L-lysine, No GFs and 1% FCS
Bouab et al	M. Fernandes KJ	2011	Neuroscience 2 and 12 months old C57BL/6 mice SVZ	DMEM/F-12 (3:1)	EGF 20 ng/mL and FGF2 25 ng/mL	N/A	1% Penicillin/Streptomycin (Wisent), 1 µg/mL Fungizone (Invitrogen), 2% B27	6 well Falcon plate	Papain	5 cells/µL	40 cells/µL (15–20,000 cells/cm ²) onto 0.02 mg/mL poly-L-lysine, No GFs, and 1% FBS
Beatus et al	P. Bartlett PF	2011	Dev Neurobiol 6-10 weeks old C57BL/6 mice SVZ	NeuroCult NSC basal medium	EGF 20 ng/mL and FGF2 10 ng/mL	N/A	mouse NeuroCult NSC proliferation supplements, 2% BSA and 2 µg/mL heparin	96 well plate	0.1% trypsin-EDTA, 0.014% w/v trypsin inhibitor type I-S 1,000 from soybean and 40 µm cell sieve	1,000 cells/well in 96 well plate	N/A
Kitada et al	M. Dezawa M	2011	Anat Rec 8 weeks old male C57BL/6 mice SVZ	neurobasal medium	EGF 20 ng/mL and bFGF 20 ng/mL	N/A	B27	Not mentioned	0.25% trypsin, 1 mM EDTA, 10% FCS and 100-µm Cell Strainer	Not mentioned	N/A
Merlo et al	S. Sortino MA	2011	Neuropharmacology 2 months old CD1 male mice SVZS	DMEM/F-12	EGF 20 ng/mL	N/A	B27, 2 mM glutamine	uncoated 35 mm dishes	trypsin-EDTA	5,556 cells/cm ²	10 µg/mL laminin and No EGF
Prüss et al	H. Priller J	2011	Neuroscience ~8 weeks old C57BL/6 mice SVZ	serum free NBMA medium	EGF 20 ng/mL and bFGF 10 ng/mL	N/A	B27 and L-glutamine	low-attachment culture flasks	0.05% trypsin-EDTA	3.5 × 10 ³ cells/cm ²	No GFs
Roitbak et al	T. Cunningham LA	2011	Exp Neurol 4 weeks old C57BL/6 mice SVZ	DMEM/F-12	EGF 10 ng/mL and bFGF 10 ng/mL	25 µg/mL insulin and 20 µM progesterone	EGF 10 ng/mL, 2.5 mM L-glutamine, 3 mM sodium bicarbonate, 16 µg/mL putrescine, 30 nM sodium selenite and 100 µg/mL apo-transferrin	uncoated tissue culture	MACS Neural tissue dissociation kit	8-10 cells/µL	poly-L-lysine
Khodosevich et al	K. et al Monver H	2011	J Cell Sci. P4 C57BL/6 mice SVZ	Neurobasal media	EGF 20 ng/mL and bFGF 20 ng/mL	N/A	B27, 200 mM L-glutamate 100 U/mL penicillin/streptomycin	Not mentioned	30 U of papain, 0.0005% DNase and trypsin inhibitor	100 cells/µL	No GFs
Zhou et al	Q. Doughty ML	2011	BMC Neuroscience 8-10 weeks old C57BL/6 mice SVZ	mouse NeuroCult NSC Basal Medium	rh EGF 20 ng/mL and rh FGF-b 10 ng/mL	N/A	mouse NeuroCult NSC proliferation Supplement and 2 µg/mL Heparin	Not mentioned	Neural Tissue Dissociation kit and 0.025% Trypsin-EDTA	100 cells/µL	2,000 cells/well in poly-D-lysine, No GFs

Table 3-1. cont.

Paper	Senior authors	Year/publication	Cell types	Neurosphere media				flask/plate	Dissociation	Cell density	Differentiation
				Media	Growth factors	Hormones	Others				
Fotovati A. et al	Dunn SE.	2011 Cancer Res.	E14.5-E17.5 C57BL/6 mice SVZ	DMEM/F-12	EGF 20 ng/mL and bFGF 10 ng/mL	25 µg/mL of insulin and 20 nM progesterone	2 mg/mL heparin, 100 µg/mL of apo-transferrin, 60 µM putrescine and 30 nM sodium selenite	ultralow attachment plate	mechanical dissociate	50 cells/µL	poly-DL-ornithine/gelatin, no GFs, 1% FBS, BNP-4,
					rh EGF 20 ng/mL and rh FGF-b						
Zhou Q. et al	Doughty ML.	2011 Neurosci Lett	8-10 weeks old C57BL/6J mice SVZ	mouse NeuroCult NSC Basal Medium	mouse NeuroCult NSC FGF-b	mouse NeuroCult NSC Proliferation Supplement and 2 µg/mL Heparin		Not mentioned	Neural Tissue Dissociation kit and 0.025% Trypsin-EDTA	100 cells/µL	2,000 cells/well in poly-D-lysine, No GFs
Song HW. et al	Lee JE.	2011 Life Sci	p0-p2 ICR mice SVZ	DMEM/F-12	EGF 20 ng/mL and bFGF 20 ng/mL	6.3 ng/mL progesterone and 0.025 mg/mL insulin	B27, 2 mM L-glutamine, 0.6% glucose, 9.6 µg/mL putrescine, 5.2 ng/mL sodium selenite, 0.1 mg/mL transferrin and 2 µg/mL heparin	24 well plate	1.3 mg/mL trypsin, 0.67 mg/mL hyaluronidase and 0.2 mg/mL kynurenic acid in aCSF and 0.7 mg/mL ovomucoid	30 cells/µL	polyornithine, No GFs and FBS

The Szele laboratory previously utilized the neurosphere assay to study stem cell characteristics and migration of SVZ cells after brain injury and molecular mechanisms (Dizon et al., 2006; Ocbina et al., 2006). As my thesis primarily focused on understanding the stemness of postnatal SVZ NSPCs, I have extensively characterized the SVZ neurosphere assay condition before this was used in functional studies.

3.2. Results

3.2.1. SVZ Neurosphere Optimization And Control Of Self-renewal

According to the protocol published by Szele laboratory (Dizon et al., 2006; Ocbina et al., 2006), I carried out fifteen trials of the SVZ neurosphere assay (Table 3-2). From these trials, successfully reproduced experiments were less than 15% due to lack of cell survival. Therefore, I characterized and optimized the protocol with regards to the previous other SVZ neurosphere publications.

3.2.1.1. Starting cell densities

Without changing the contents of the medium and the supplements, I first tried to culture the neurospheres at three different cell concentrations per volume (25, 50 and 100 cells per μL in T12.5 flask). Among these conditions, I found neurospheres from only the 100 cells/ μL culture condition 4 days later (Figure 3-1A); the other two conditions were not sufficient to generate neurospheres from single cells during 4 days (data not shown). The total number of primary neurospheres was 459 ± 60.5 per starting 200,000 cells, and individual neurosphere diameter was $50.7 \pm 3.13 \mu\text{m}$. To expand the SVZ NSPC population, the neurospheres were dissociated, and 100 cells/ μL were seeded to make secondary neurospheres over the next 4 days. The number (2016 ± 58.6 per starting 200,000 cells) and diameter ($88.9 \pm 7.1 \mu\text{m}$) of secondary neurospheres were significantly increased compared to the primary neurospheres (Figure 3-1I, J).

As the secondary neurospheres should have a higher concentration of NSPCs, these neurospheres were stained with several SVZ NSPC markers to indicate their identities. Postnatal SVZ NSPC population *in vivo* has been defined by staining for Sox2 and EGFR (markers for SVZ NSPC population), vimentin (a marker for radial

Table 3-2. Trials of Neurosphere Assay Followed by Previously Published Protocol

Number of trials	Neurosphere	total # of live cells (1×10^5)	total # of death cells (1×10^5)	Cell viability (%)	Enough live cells to passage?
1	Primary	9.00	N/C	N/A	Yes
	Secondary	12.40	N/C	N/A	Yes
	Tertiary	6.55	N/C	N/A	Yes
2	Primary	5.00	N/C	N/A	No (too few)
	Secondary	0.00	N/C	N/A	No (all death)
3	Primary	11.00	N/C	N/A	No
	Secondary	0.00	N/C	N/A	No (all death)
4	Primary	9.10	8.90	50.56	Yes
	Secondary	0.00	0.00	0.00	No (all death)
5	Primary	6.60	N/C	N/A	Yes
	Secondary	0.00	0.00	0.00	No (all death)
6	Primary	2.50	0.30	89.29	Yes (worked)
	Secondary	2.00	2.00	50.00	No (all death)
7	Primary	9.10	0.90	91.00	No (unhealthy)
	Secondary	0.50	0.00	100.00	Yes
	Tertiary	0.30	0.00	100.00	No
8	Primary	3.20	1.40	69.57	Yes
	Secondary	10.30	0.70	93.64	No (unhealthy)
	Tertiary	N/A	N/A	N/A	No (all death)
9	Primary	13.60	0.90	93.79	No (too few)
	Secondary	0.80	0.90	47.06	No
10	Primary	7.70	0.20	97.47	No (too few)
	Secondary	0.40	1.70	19.05	No (too few)
	Tertiary	0.10	0.17	37.50	No (too few)
11	Primary	8.70	1.10	88.78	No (too few)
	Secondary	0.50	0.33	60.02	No (too few)
		0.50	0.78	39.12	No (too few)
		0.00	N/A	N/A	No (all death)
12	Primary	10.50	0.00	100.00	Yes
	Secondary	3.80	1.40	73.08	No (too few)
		1.30	3.50	27.08	No (all death)
		0.80	0.39	67.28	No
13	Primary	5.00	1.72	74.40	Yes
	Secondary	0.36	0.06	85.71	No (too few)
		0.08	0.02	80.00	No (too few)
		0.04	0.08	32.20	No (too few)
		0.08	0.00	100.00	No (too few)
		0.02	0.00	100.00	No (too few)
14	Primary	5.50	0.04	99.31	No (too few)
	Secondary	0.71	0.71	49.86	No (too few)
		0.04	2.00	1.96	No (all death)
15	Primary	8.00	2.05	79.60	Yes
	Secondary	3.27	2.80	53.87	Yes (small size)
	Tertiary	1.27	0.06	95.81	Yes

N/C: Not Countable; N/A: Not Applicable.

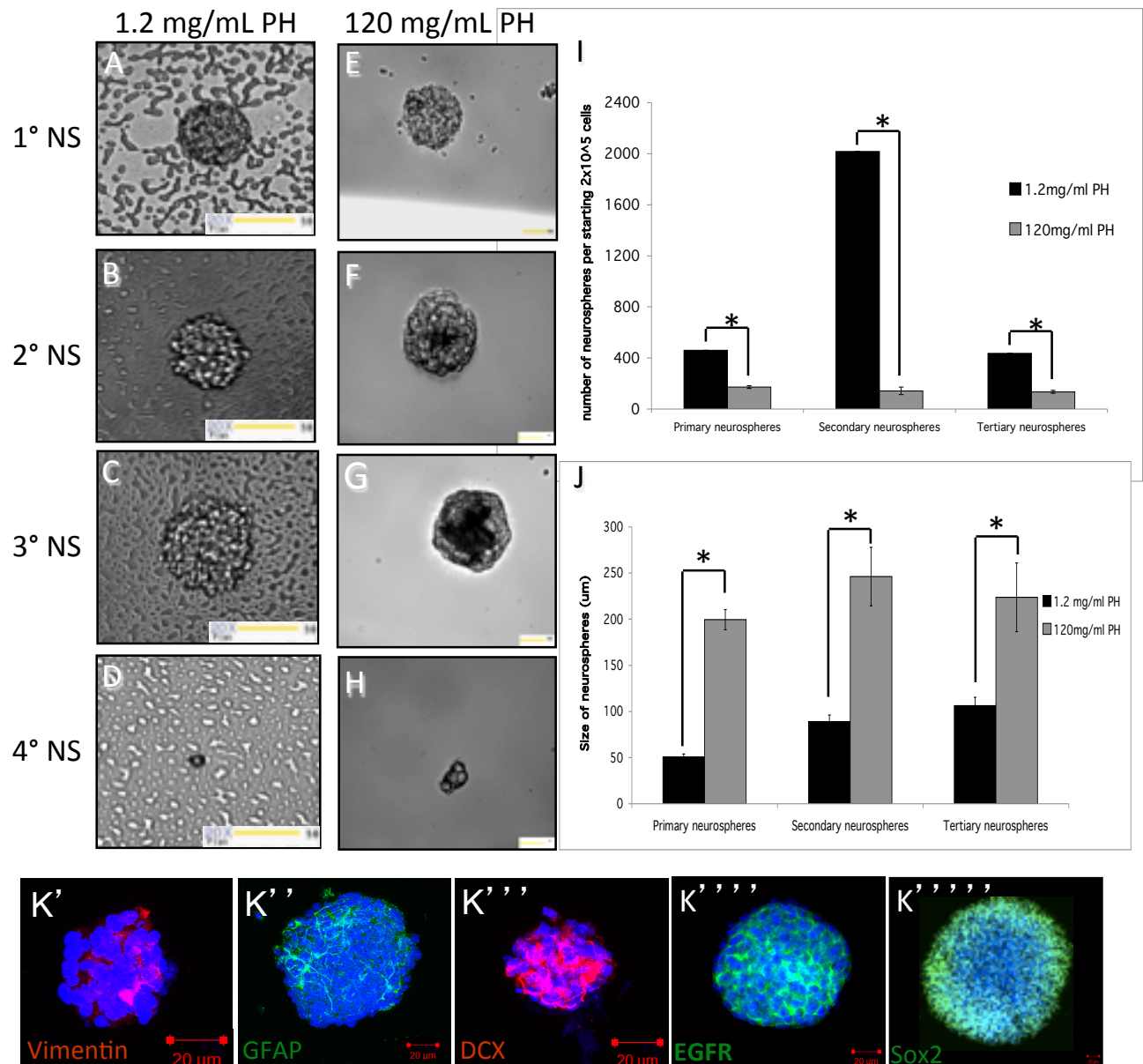


Figure 3-1. P4 SVZ neurosphere self-renewal.

The neurosphere assay was performed to first assess SVZ NSPC proliferation.

(A-D;E-H) One hundred single cells per microliter were seeded in 1.2 mg/mL and 120 mg/mL concentrated poly-HEMA coated flasks, respectively. The neurospheres were imaged and monitored every 24 hr.

(I,J) The number and diameter of individual neurospheres was measured 4 days after the passage. * $P < 0.05$.

(K) Immunocytochemistry was carried out on secondary neurospheres for vimentin, GFAP, DCX, EGFR and Sox2 to indicate the heterogeneity of individual neurospheres. Scale bar=20 μm (all the data were from n=3 experiments).

glial cells), GFAP (a marker for astrocytes and radial glial cells) and Dcx (a marker for neuroblasts), which were all found in neurospheres (Figure 3-1K series). Thus, these stainings assured that the neurospheres were heterogeneous.

To measure the capacity of SVZ neurosphere self-renewal under this culture condition, secondary neurospheres were passaged until the cells no longer formed neurospheres. After 4 days, tertiary neurospheres were formed and their diameters ($106.3 \pm 9.0 \mu\text{m}$) were larger than secondary neurospheres although the number of neurospheres (435 ± 36 per starting 200,000 cells) was approximately 78 % decrease (Figure 3-1I, J). After passaging the tertiary neurospheres, it was rare to observe any spheres during the next 4 days (Figure 3-1D). By monitoring the cells with a microscope, most cells remained as live and healthy single cells (Figure 3-1D). Due to this growth pattern, secondary neurospheres were utilized for all subsequent biological studies.

3.2.1.2. Adherence of neurospheres

Floating (self-renewing) neurospheres had a tendency to settle on the bottom of flasks and differentiate spontaneously (Figure 3-2A,B). To examine how non-adhesive culture conditions would affect maintenance of self-renewing neurospheres, I cultured them in two different concentration of poly-HEMA coated flasks and non-treated plates.

The Szele laboratory previously used 1.2 mg/mL poly-HEMA pre-coated culture flasks and found most neurospheres floated in the medium (Dizon et al., 2006; Ocbina et al., 2006). The settled neurosphere sizes varied and neurospheres of similar diameters could either float or adhere (Figure 3-2B). These results suggested neurosphere settlement was not due to the weight of individual neurosphere. The

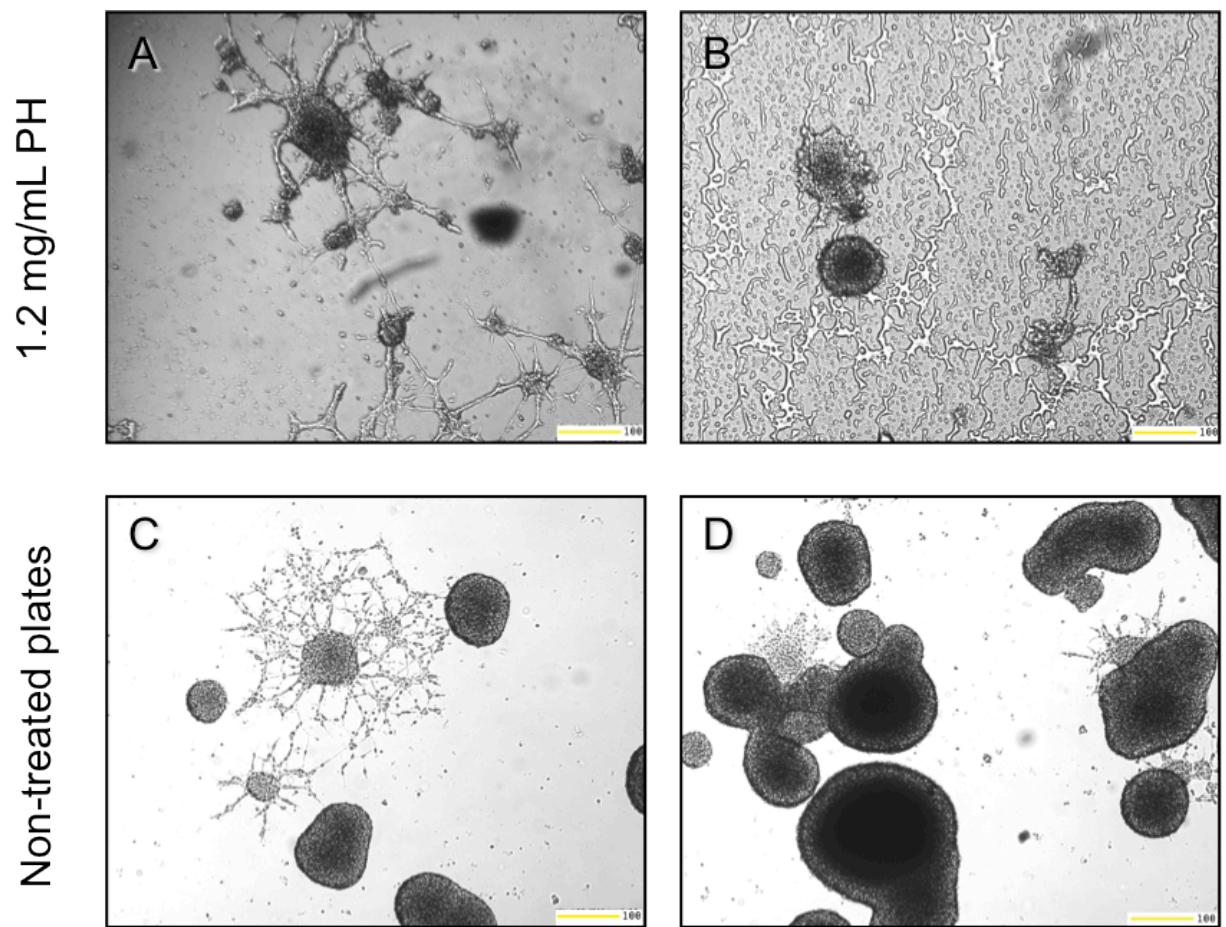


Figure 3-2. Neurosphere settlement to the bottom of pre-coated flasks or non-treated plates.

Four days after passaging, some neurospheres were adhere to the bottom of poly-HEMA coated flasks (A,B) and also to non-treated plates (C,D). Adherence to the bottom of the plate was not correlated with neurosphere size.

(A,C) Cells migrated from attached neurospheres and appeared to differentiate.

(C,D) Neurospheres in non-treated plates also clustered together and occasionally fused to make large spheres.

All the images were taken with 20X objective lens. Scale bar for A-D=100 μ m (all the data were from n=3 experiments)

initially attached neurospheres usually came off by gently tapping the flask. However, firmly attached neurospheres on the flask started to generate multiple processes and seemed to differentiate.

Because all the tissue culture flasks and plates were treated to be adhesive on the bottom of surface, I next used non-treated plates to generate neurospheres though settled-down and adherent neurospheres were still found on the bottom of the plates (Figure 3-2C). Also I found that neurospheres in the plates were more adhesive to one another (Figure 3-2D), so they were difficult to dissociate into single cells during secondary and tertiary neurosphere passages, which was not found in poly-HEMA flask culture condition.

I then tried alternative non-adhesive culture conditions by increasing the poly-HEMA concentration up to 120 mg/mL. This method completely resolved the issues of neurosphere adhesion to the flask, but the number of primary neurospheres was significantly decreased (172.00 ± 18.33 per starting 200,000 cells) (Figure 3-1I), whereas the primary neurosphere diameter ($199.41 \pm 18.92 \mu\text{m}$) was significantly increased (Figure 3-1J). In addition, these parameters were not significantly changed from primary to secondary (141.67 ± 51.47 per starting 200,000 cells with $246.14 \pm 54.96 \mu\text{m}$) and tertiary (134.17 ± 20.83 per starting 200,000 cells with $223.57 \pm 64.42 \mu\text{m}$) neurospheres (Figure 3-1I,J). At the same time, the quaternary neurospheres did not form, similar to what was seen in the lower poly-HEMA culture condition (Figure 3-1H). These data suggested non-adhesive culture conditions provide no change of neurosphere self-renewal and proliferation after few passages, even though neurospheres were cultured with the same condition of media. As it has previously been validated that the cells substantially self-renew and proliferate after each passage

(Reynolds and Rietze, 2005), I have decided to culture the neurospheres in 1.2 mg/mL poly-HEMA coated flasks.

3.2.1.3. A role of supplements in survival

The commercially available N2 and B27 serum-free supplements have been utilized either alone or separately by many laboratories in the neurosphere culture (Table 3-1). Otherwise, few laboratories individually added the each component for N2 and/or B27 into complete media (Table 3-1). To examine the role of both N2 and B27 serum-free supplements in the neurosphere culture, the supplements were withdrawn while various amounts of EGF and FGF2 were added in the culture medium to test any synergistic effects between growth factors and supplements.

During the 4 days of the primary neurosphere culture, the neurospheres cultured in different conditions were monitored every 24 hrs; 0, 20 or 40 ng/mL of both EGF and FGF2 were added to culture medium without supplements. Dissociated single cells were able to form primary neurospheres quickly, and their diameter was proportional to growth factor concentration (Figure 3-3). After passaging these neurospheres, secondary neurospheres were not formed (Figure 3-3) showing the N2 and B27 supplements were necessary for successful self-renewal. All the cells remained as single cells and eventually became debris (data not shown). This feature of neurospheres was also observed when I used the culture medium, including supplements, which were stored at 4°C for more than a week (Table 3-2). Thus fresh supplements were vital for SVZ neurosphere survival, and EGF and FGF2 could not compensate the role of supplements in passaging primary neurospheres.

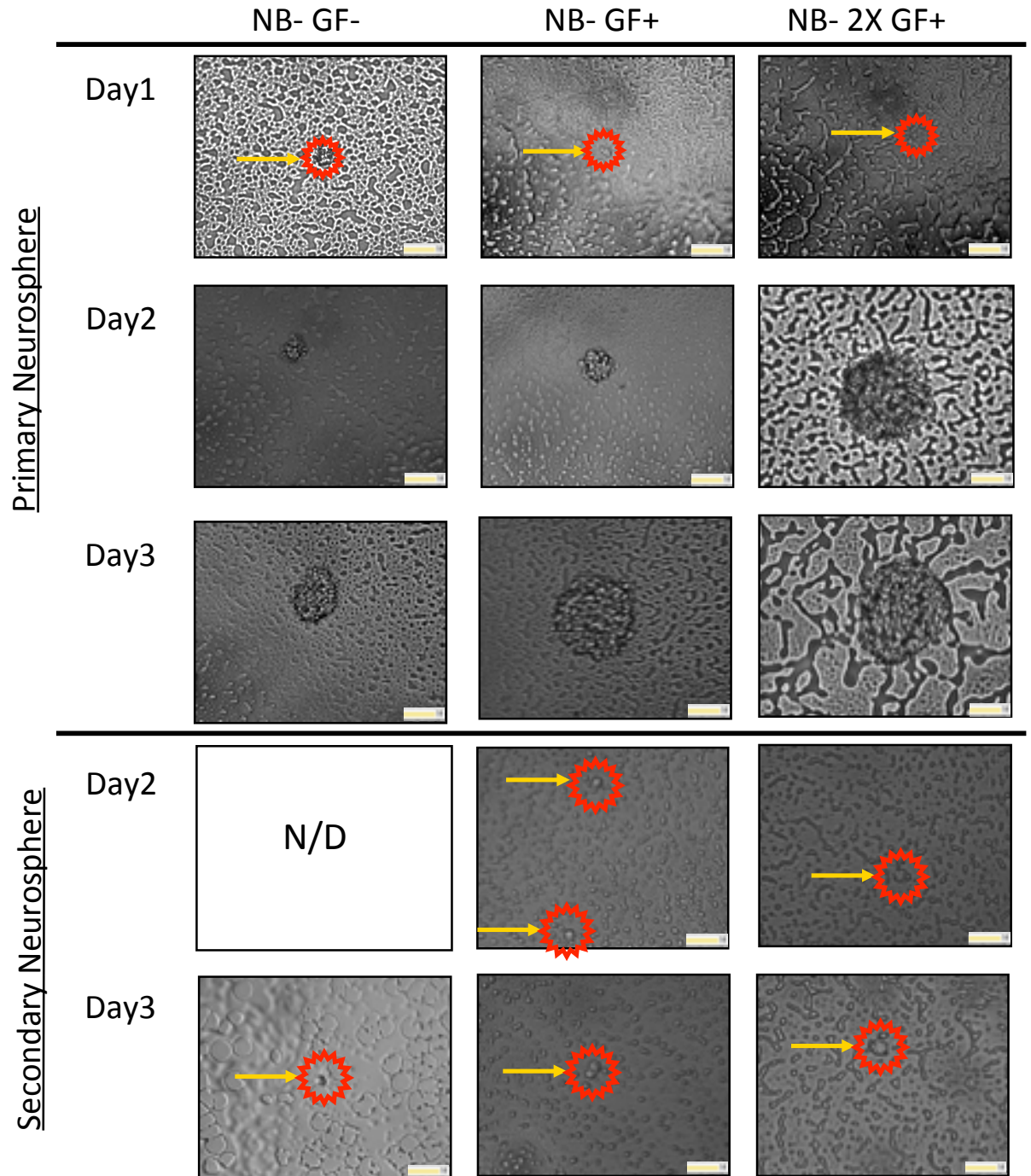


Figure 3-3. Neurosphere self-renewal without N2 and B-27 supplements and various growth factor concentrations.

The neurosphere assay was performed without N2 and B27 supplements (NB-), with no growth factors (GF-), with 20 ng/mL of GFs (GF+), or with 40 ng/mL of GFs (2X GF+). During primary and secondary neurosphere culture, the cells and neurospheres were monitored and photomicrographed at 20x magnification. The background pattern seen in most panels was due to the 1.2 mg/mL poly-HEMA coat. The 1st day after passage, secondary neurospheres could not be detected. Also, secondary neurospheres in the NB-, GF- condition were not detectable (N/D). Scale bar=50 μ m (All the data are from n=2 experiments.)

3.2.1.4. Modulating neurosphere self-renewal with EGF and FGF2

The role of EGF and FGF2 in the neurosphere assay was previously examined by withdrawing one or the other (Gritti et al., 2002; Gritti et al., 1999). Based on these studies, adding both growth factors in the culture medium would increase neurosphere self-renewal and proliferation. Therefore, most laboratories including ours have utilized both 20 ng/mL of EGF and 10 or 20 ng/mL of FGF2 (Table 3-1). However, it is not clear why these particular combinations have been used and how neurospheres would differently respond to the subtle changes in the concentrations of these growth factors.

With the same neurosphere culture condition, various concentrations of EGF and FGF2 were applied: 20 ng/mL of EGF and 10 ng/mL of FGF2; 20 ng/mL of EGF and 40 ng/mL of FGF2; 40 ng/mL of EGF and 20 ng/mL of FGF2 and 10 ng/mL of EGF and 20 ng/mL of FGF2. The primary neurospheres under these conditions did not show any statistically significant difference in number of neurospheres generated, number of live single cells, cell viability and the neurosphere diameter all at 4 DIV (Figure 3-4 A, C, E, G). However, secondary neurospheres grown in 20 ng/mL of EGF and 10 ng/mL of FGF2 had significantly lower cell viability than in 10 ng/mL of EGF and 20 ng/mL of FGF2 (ANOVA with Bonferroni's post-hoc test, p value < 0.005) although the rest of measurements did not show any significant differences (Figure 3-4 B, D, F, H). Also, there was not any particular abnormal phenotype of neurospheres from the four different conditions (data not shown). In sum, neurosphere self-renewal and proliferation were not affected by these various EGF and FGF2 concentrations in the SVZ neurosphere culture condition that I have used.

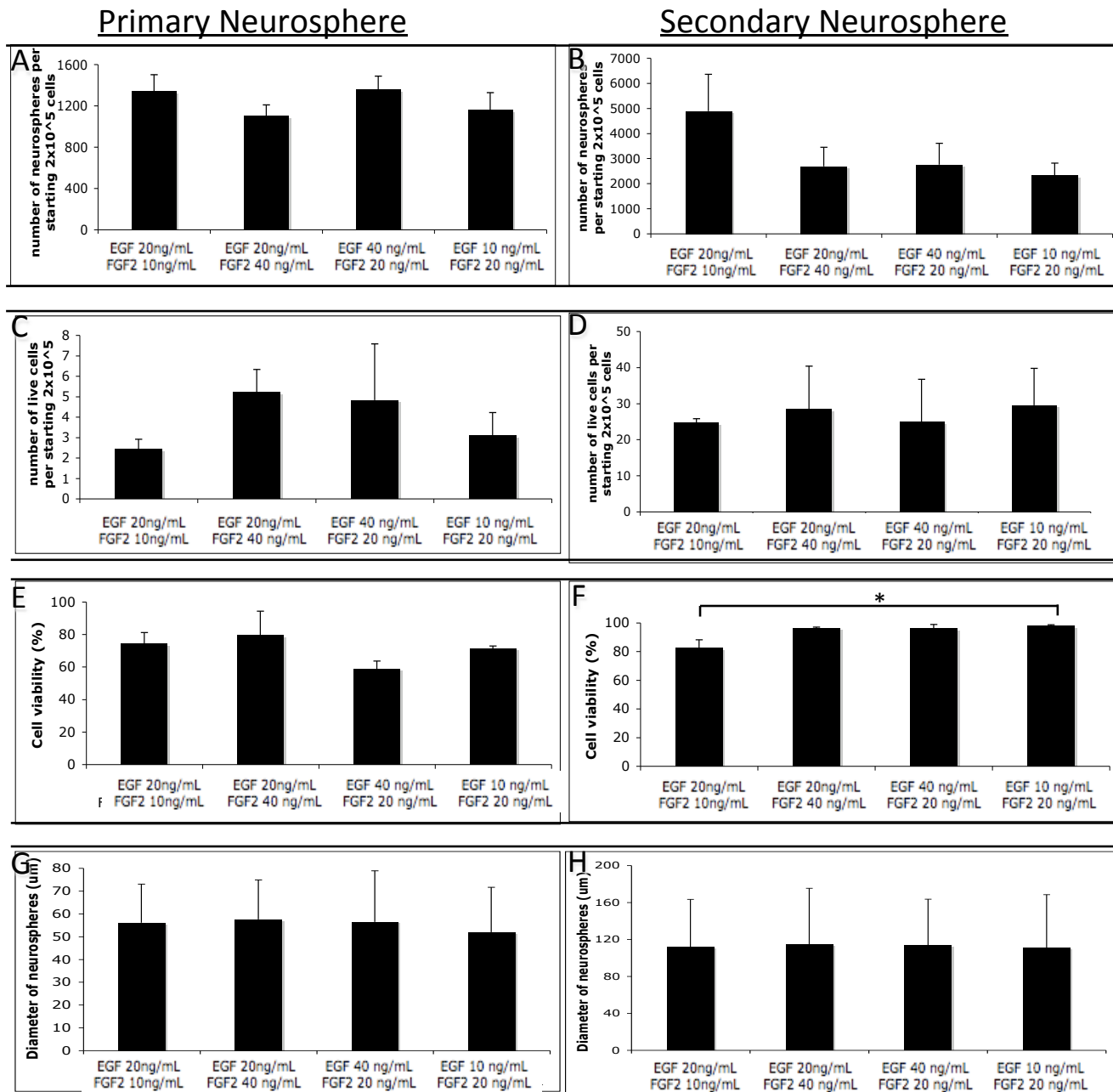


Figure 3-4. Neurosphere self-renewal in different concentrations of EGF and FGF2.

Two different concentrations (10 and 40 ng/mL) of EGF and FGF2 were used in 4 different combinations.

(A-H) After 4 days of culture in each condition neurospheres numbers, diameters, number of live single cells and cell viability were measured. Except cell viability from secondary neurospheres, there was no significant difference among four different conditions.

(F) Among all the measurements, only cell viability on secondary neurospheres cultured in EGF 20 ng/mL and FGF2 10 ng/mL was significantly lower than cell viability cultured in EGF 10 ng/mL and FGF2 20 ng/mL. (* $P < 0.05$) (All the data are from $n = 3$ experiments.)

3.2.2. SVZ Neurosphere Spontaneous Differentiation

For neurosphere differentiation, it was common for researchers to withdraw the growth factors (EGF and FGF2) from the culture medium and to attach neurospheres onto various substrates. In these conditions, neurospheres can differentiate into astrocytes, oligodendrocytes and/or neurons, whose markers were immunohistochemically distinguishable after 7 days of spontaneous differentiation. However, the percentage of cell types and multipotency of neurospheres varies from one publication to the other. To understand the possible reasons for the inconsistency between these results, I examined differentiation (1) on different substrates (poly-D-lysine/laminin, Matrigel or Geltrex), (2) of either secondary or tertiary neurospheres and (3) neurospheres exposed to different amount of growth factors during self-renewal.

3.2.2.1. Specific substrate for neurosphere differentiation

To recreate the most physiologically relevant *in vitro* condition for neurosphere differentiation, the natural extracellular matrix (Matrigel, laminin and Geltrex) extracted from the Engelbreth-Holm-Swarm tumor is commonly used (Table 3-1). Because this extracellular mix is made by a transformed cell line, the exact concentrations and ratios of its constituents can vary from batch to batch. As such it is more comparable to sera containing media than to defined media.

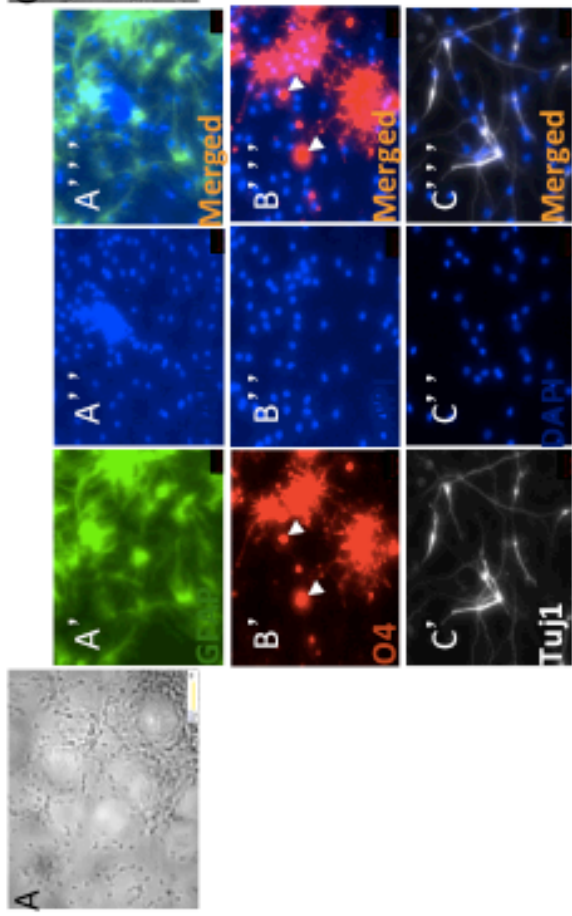
First, differentiated secondary neurospheres on natural mouse laminin coated on Poly-D-lysine could generate cells of all three neural lineages after 7 days (Figure 3-5A). The astrocytes were detected with an anti-GFAP antibody and could be categorized into two types: (1) one looked like a star or bush - like protoplasmic astrocytes *in vivo* and (2) the other looked like a radial glia which had long processes

without the bushy morphology around the cell body (Figure 3-5A'-A'''). The neurons stained with the Tuj1 antibody had short or long processes or both, and were often found connected with neighboring neurons (Figure 3-5C'-C'''). Unlike the astrocytes and neurons, oligodendrocytes stained with the O4 antibody had several branched curly processes (Figure 3-5B'-B'''), and all the oligodendrocytes looked very similar to one another. In the O4 staining, the red dots were found (Figure 3-5B' arrowhead). They did not overlap with any nucleus (DAPI) staining and spread out the entire neurosphere, so I considered them as non-specific signals. Also, the different cell types were consistently found from every single neurosphere; no uni- or bi-potential neurospheres were found.

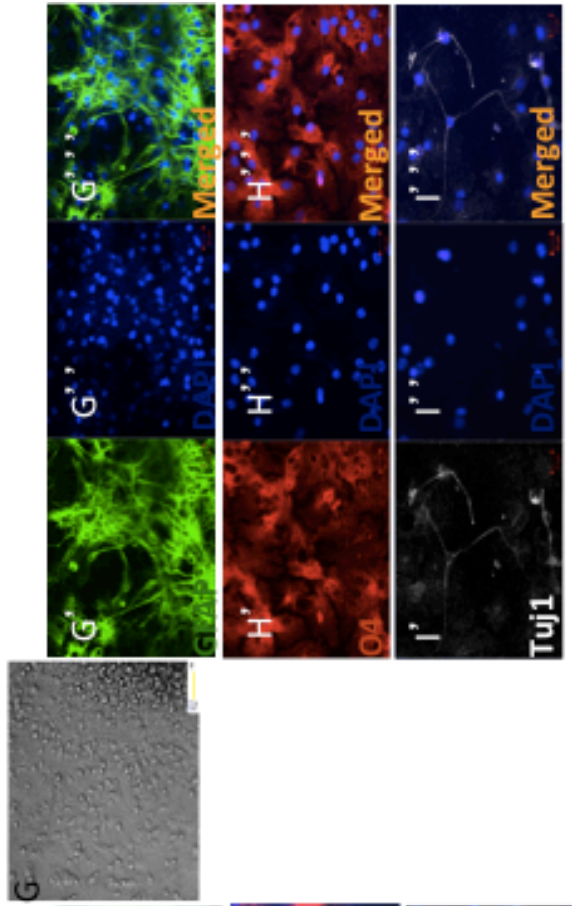
Matrigel is commonly used for providing a 3D scaffold for neurospheres unlike the 2D structure of laminin. Using secondary neurospheres to differentiate on Matrigel for 7 days, all three neural cell lineages were detected by immunocytochemistry (Figure 3-5D'-F'''). Unlike laminin, the GFAP positive cells all appeared to have morphology of protoplasmic astrocytes. The neurons were similar to the ones on laminin, but the oligodendrocytes did not have the curly looking branches (Figure both E' and N'). I carried out negative controls by omitting primary antibodies and also tested if secondary antibodies cross-reacted with the wrong species from primary antibodies; neither scenario occurred (Figure 3-5J-M). Thus, O4 immunoreactivity seemed to be a true signal. In addition to this, when the neurosphere size was less than 100 μm , neurospheres would give rise cells of mixed lineages which have been termed “asterons” (Laywell et al., 2005), which expressed both astrocytic and neuronal markers within a single cell (Figure O series).

Lastly, Geltrex, another extracellular matrix commonly used for differentiating human ESC towards the neuronal lineage, was used to examine SVZ neurosphere

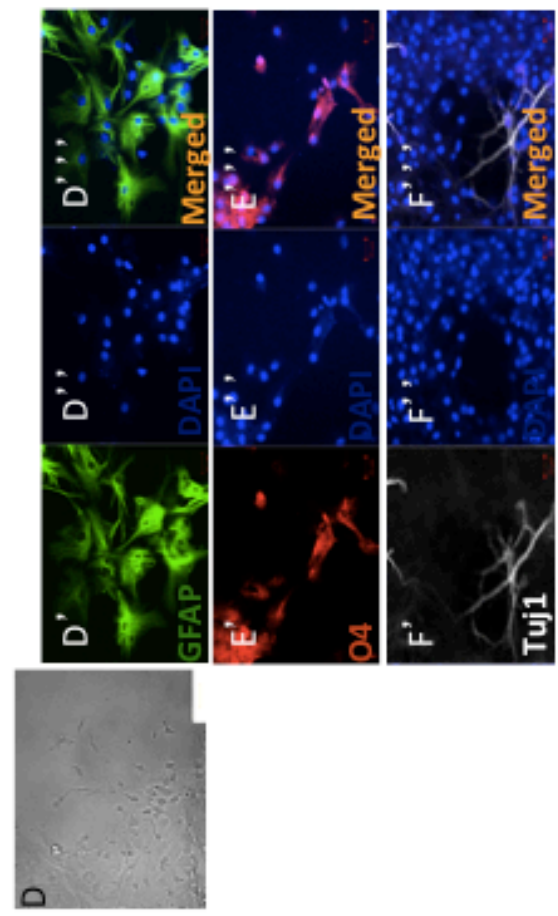
Poly-D-Lysin/Laminin



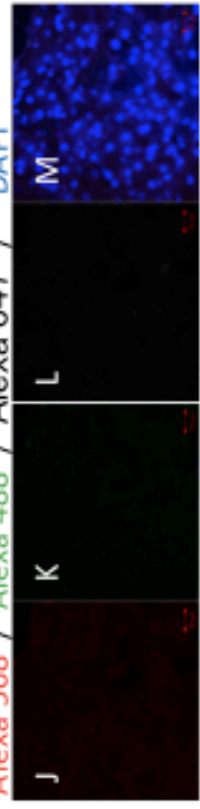
Geltrex



Matrigel



Alexa 568 / Alexa 488 / Alexa 647 / DAPI



Matrigel

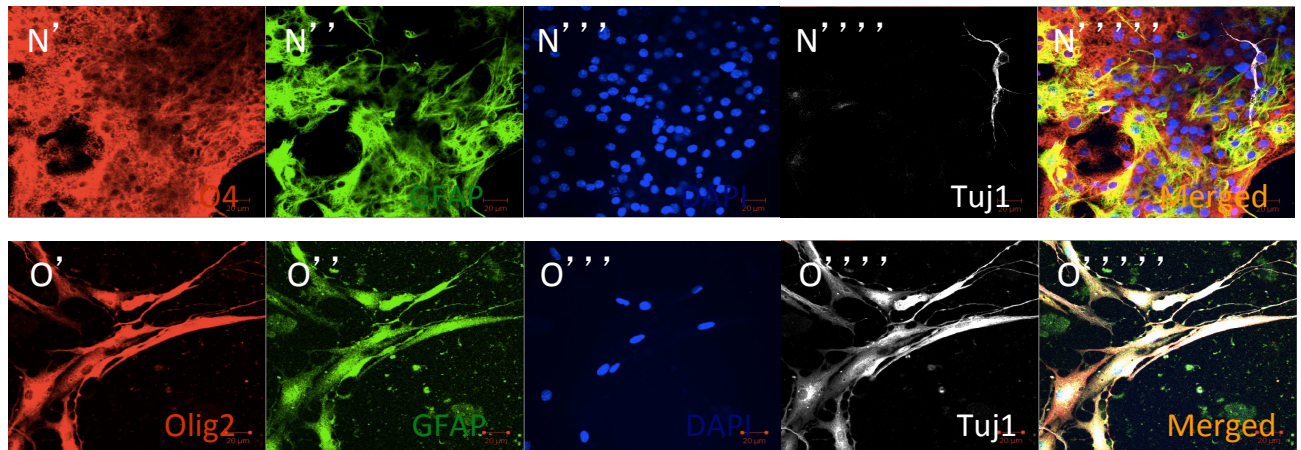


Figure 3-5. Different substrates for secondary neurosphere differentiation.

(A-I) To test spontaneous differentiation of secondary neurospheres, they were plated onto Laminin, Matrigel or Geltrex respectively. After 7 days, bright field images were taken of differentiated neurospheres (A,D,G) and then immunocytochemistry for the three neural cell lineage markers was carried out: GFAP for astrocytes, β III-tubulin for neurons and O4 for oligodendrocytes (A'-I'''). Scale bar for A,D,G = 50 μ m, for A'-I'''=20 μ m. (n=3)

(J-M) No primary antibody control for all the secondary antibodies to rule out background staining. (n=3)

(N) Triple immunohistochemistry on single neurosphere differentiation on matrigel. O4 staining overlapped with GFAP staining without detecting any specific oligodendrocytes. (n=3)

(O) A neurosphere whose diameter was less than 100 μ m differentiated for 7 days on matrigel and stained with specific antibodies. Every single cell showed all three lineages' markers. (n=3)

differentiation. By differentiating the same secondary neurospheres on Geltrex, the GFAP positive astrocytes resembled the ones from laminin. Meanwhile, the neurons' processes were much longer and thinner than the ones from laminin and Matrigel (Figure 3-5I'-I'''), and unclearly defined O4 positive cells were similar to the ones from Matrigel (Figure 3-5H'-H'''). Although three different extracellular matrixes were extracted from the same cell line, differentiated SVZ neurospheres were not the same. Only the oligodendrocytes from laminin substrate had the proper morphology of oligodendrocytes while Matrigel and Geltrex did not. Therefore, laminin was chosen to be used for all the neurosphere differentiation experiments.

3.2.2.2. Difference between secondary and tertiary neurospheres differentiation

Because passaged neurospheres contain a higher proportion of multipotent stem cells, I compared the capacity of multipotency from secondary and tertiary neurospheres.

As described above, secondary neurospheres gave rise to all three different neural lineages. Examination of entire differentiated neurosphere revealed that radial glial like astrocytes extended their long processes outwards from the center of the sphere (Figure 3-6A). Protoplasmic astrocytes were spread out on entire surface of the differentiated neurosphere. Meanwhile, the neurons were clustered together as bundles, and most of them were localized at both the edge and center of neurospheres. It was rare to see evenly distributed individual neurons from a differentiated neurosphere. Very few oligodendrocytes were found and they were randomly spread from the core of the sphere. The number of individual lineage cells per neurosphere was quantified (Figure 3-6B). The ratio between three different lineages was consistent from one secondary neurosphere to the other. The majority of cells were astrocytes (48.80 ± 4.99 %) with relatively fewer neurons (7.57 ± 0.35 %) and quite a small percentage of oligodendrocytes (1.84 ± 0.35 %).

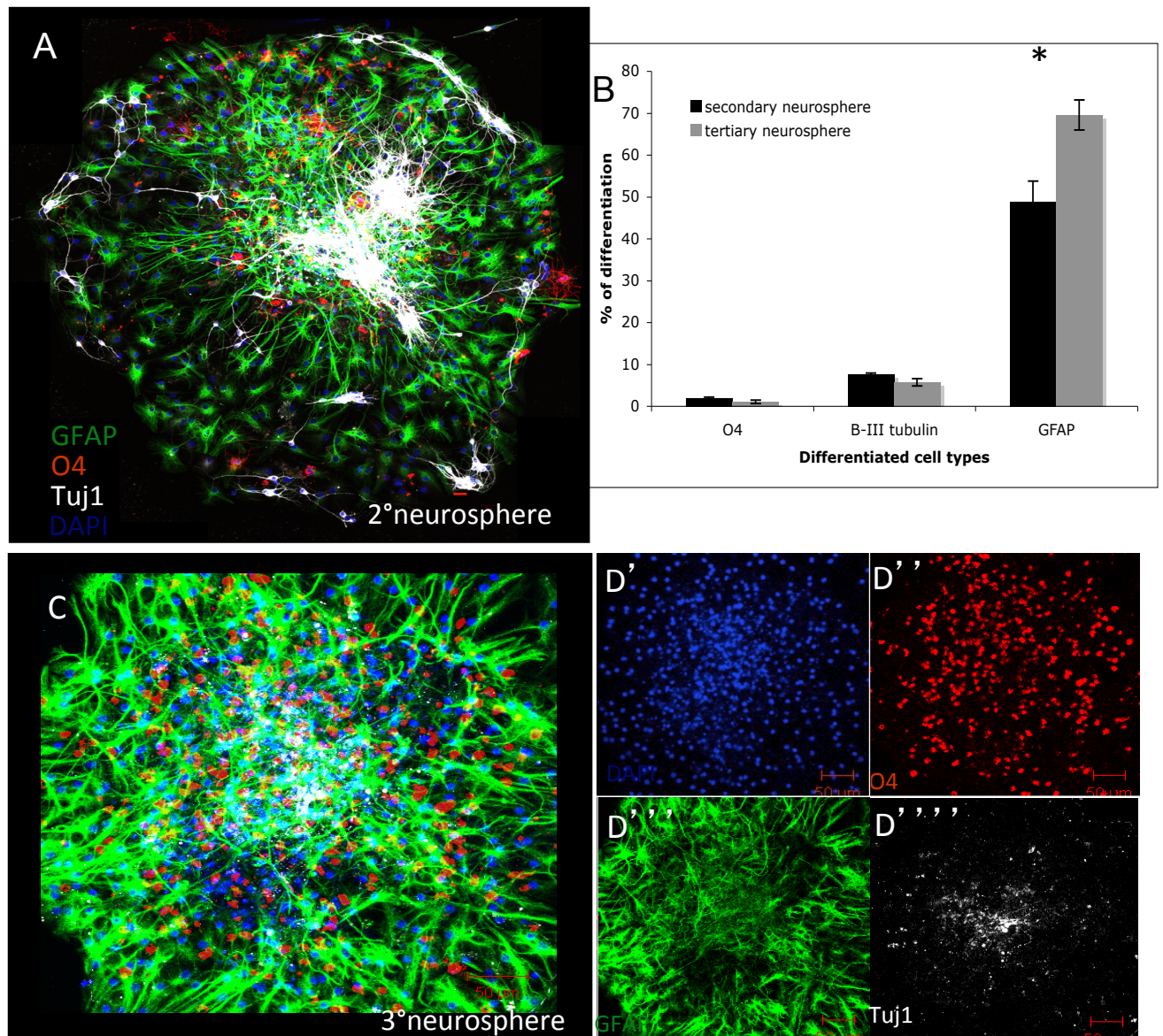


Figure 3-6. Secondary and tertiary neurosphere differentiation on poly-D-lysine/laminin.

(A-D) Secondary and tertiary neurospheres were spontaneously differentiated on poly-D-lysine/laminin for 7 days in the absence of growth factors. Differentiated neurospheres were stained with specific antibodies for astrocytes (GFAP), neurons (β III-tubulin) and oligodendrocytes (O4). Scale bar = 50 μ m.

(B) Quantitative analysis shows that the majority of differentiated cells were astrocytes. There was significant difference between the number of astrocytes generated from secondary and tertiary neurospheres. $*P < 0.05$

(C-D) Differentiated tertiary neurospheres were spontaneously differentiated on poly-D-lysine/laminin for 7 days in the absence of growth factors. The cells gave rise to neurons and oligodendrocytes with short processes while astrocytes did not seem to be altered in terms of their phenotypic changes. (All the data are from n=3 experiments.)

Similar to secondary neurosphere differentiation, tertiary neurospheres gave rise to three different lineages after 7 days of differentiation. The majority population from tertiary neurosphere differentiation was GFAP positive astrocytes although radial glial like astrocytes were not found (Figure 3-6D'''). The quantity of GFAP positive astrocytes (69.55 ± 6.20 %) from differentiated tertiary neurospheres was significantly higher than from differentiated secondary neurospheres (Figure 3-6B). Meanwhile, the neurons had shorter processes and did not migrate out from the core of neurospheres, while the O4 and DAPI double positive cells did not have curly processes, which appeared during maturation of oligodendrocytes (Figure 3-6C,D). However, the proportion of neurons (5.70 ± 1.54 %) and oligodendrocytes (1.00 ± 0.75 %) from single tertiary neurospheres was not significantly different from the secondary neurosphere differentiation (Figure 3-6B).

By comparing phenotypes and quantification of the differentiated secondary and tertiary neurospheres, tertiary neurospheres generated shorter processes of neurons and no curly phenotypic oligodendrocytes. Considering the phenotypes of the three different lineages, secondary neurosphere differentiation showed the most relevant postnatal SVZ *in vivo* cell types, so I chose secondary neurospheres for the rest of the neurosphere differentiation assays.

3.2.2.3. Neurosphere differentiation as spheres versus dissociated cells

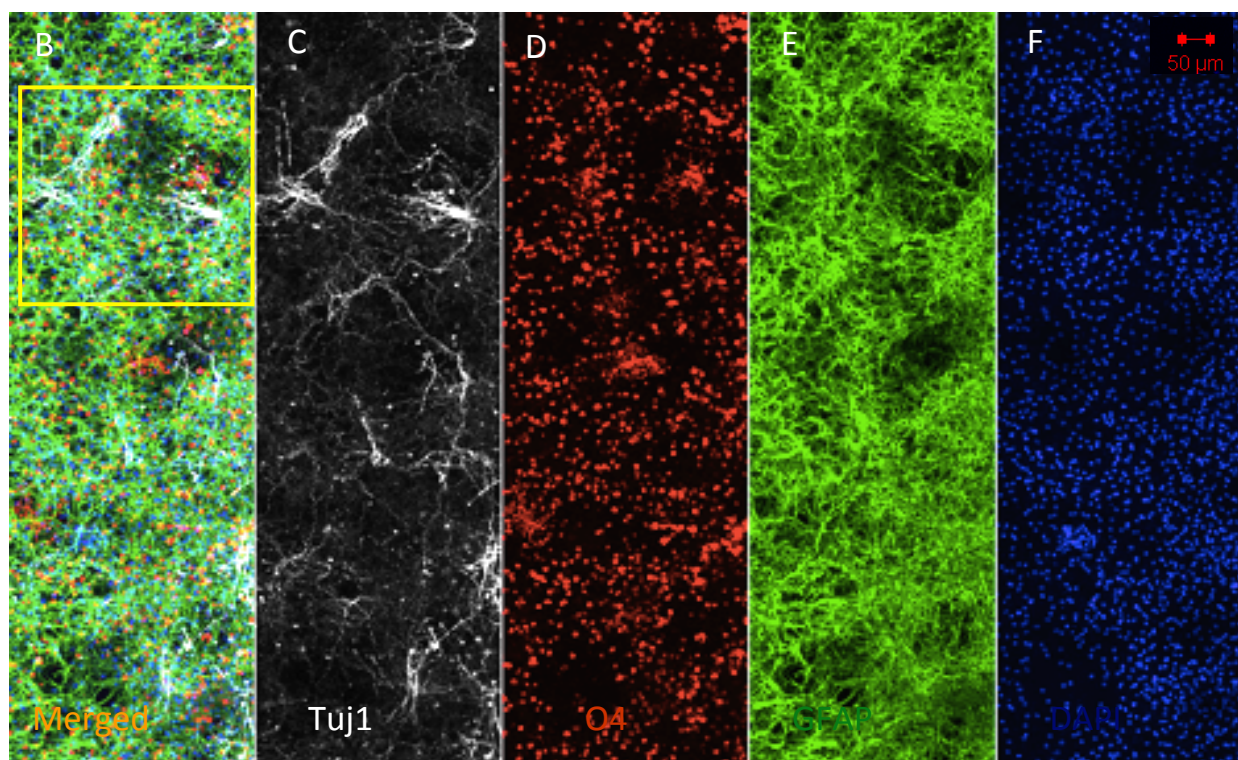
Besides differentiating single neurospheres per well, there were two other methods to differentiate neurospheres: (1) as multiple neurospheres or (2) as dissociated single cells. The density of single cells or neurospheres was a critical factor for cell viability and growth rate during the neurosphere self-renewal, but it was unclear if

differentiating one or multiple neurospheres or single cells affects the process of spontaneous differentiation or the cell fate choice.

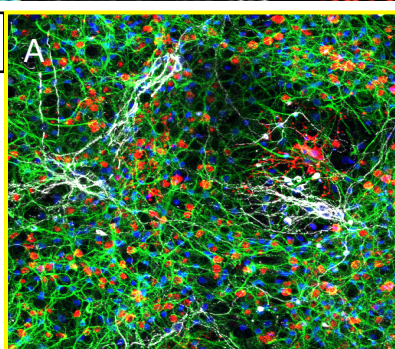
Four to eight various size (100 - 150 μm) secondary neurospheres were differentiated in the same size chambers, which were used for single neurosphere differentiation. After 7 days, all the neurospheres had differentiated into the three neural lineages (oligodendrocytes 0.71 ± 0.27 %; neurons 4.80 ± 1.48 %; astrocytes 51.41 ± 16.41 %) (Figure 3-7G), and the percentage of lineages was similar to single neurosphere differentiation (Figure 3-7M). Cell morphological differences was not observed, and the localization of astrocytes, neurons and oligodendrocytes within a neurosphere was not altered either.

Because neurospheres were adjacent to one another, each cell type from two different neurospheres were in contact. First, astrocyte populations on the border between two neurospheres were all intermixed without creating any specific boundaries that distinguished the individual differentiated neurosphere territories (Figure 3-7G,H). The neurons from two different neurospheres were linked to one another via long processes (Figure 3-7G,I). And, fewer oligodendrocytes with curly processes randomly spread from the core of the spheres (Figure 3-7J), and the red dots were also found but did not overlap with any DAPI positive nuclei.

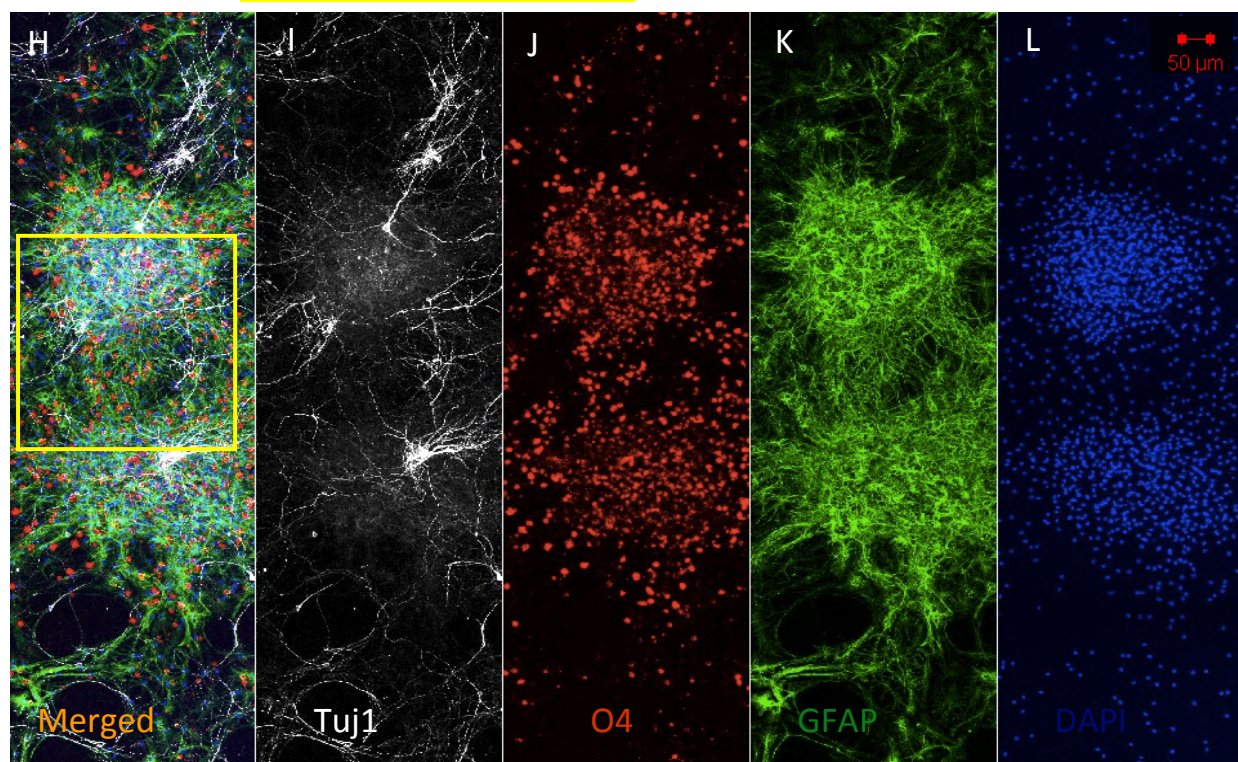
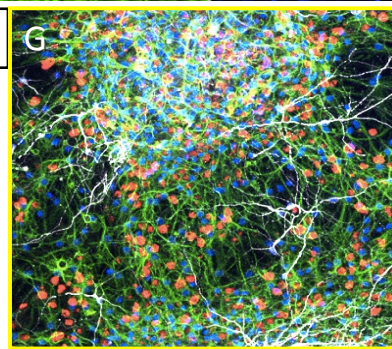
Differentiation of single cells, enzymatically dissociated from secondary neurospheres, resulted in no significant differences in cell fate choice compared to multiple sphere differentiation (oligodendrocytes 0.70 ± 0.07 %; neurons 6.36 ± 2.78 %; astrocytes 59.20 ± 7.92 %) (Figure 3-7M). Although the secondary neurospheres were completely dissociated into single cells before they were settled on laminin, nuclear staining indicated that the single cells were not evenly distributed on the laminin surface (Figure 3-7F). In the cell clustered regions, GFAP and Tuj1 positive



Single cell



Sphere



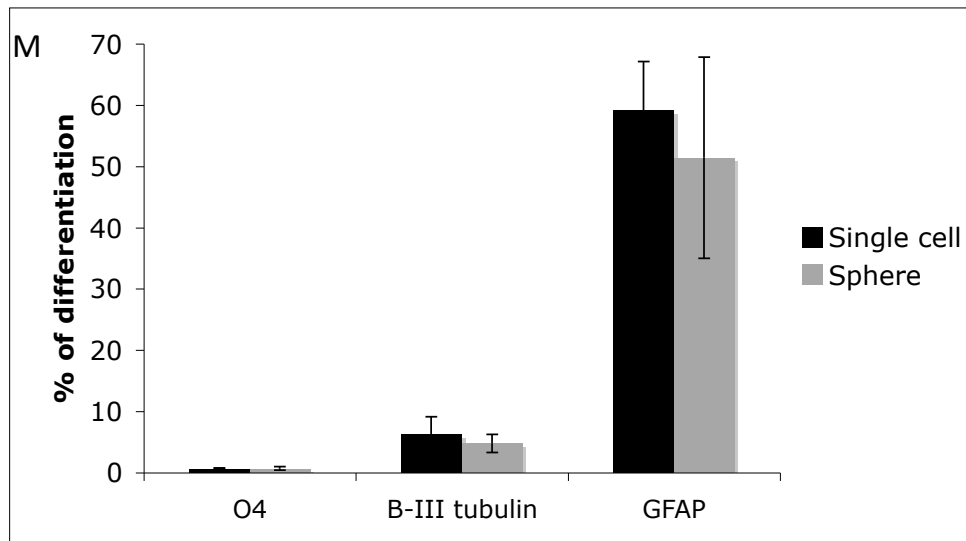


Figure 3-7. Differentiation of neurospheres plated as spheres versus single cells.

(A-L) Secondary neurospheres were differentiated on poly-D-lysine/laminin from single cell dissociates (A-F) versus from spheres. (G-L) After 7 days, differentiated neurospheres three neural cell lineage markers were examined. The magnified images (A,G) correspond to yellow boxes (B,H) respectively. Scale bar = 50 μ m

(M) Quantification of triple immunohistochemistry staining on differentiated neurosphere cells. All differentiated cells were divided by DAPI positive cells. There was no significant difference between the two different methods for neurosphere differentiation. (All the data are from n=3 experiments.)

cells were predominant, whereas oligodendrocytes were found in less cell dense areas (Figure 3-7A,C,E). Most GFAP positive cells had long processes, resembling radial glia-like astrocytes. Also, neurons were bundled together and connected to one another by short and long processes. In sum, neurosphere fate choice was not altered by two different methods for neurosphere differentiation, but the phenotype for the individual cells was different.

3.2.2.4. Differentiation of NSPC cultured with various EGF And FGF2

concentrations

Overexpressing EGF receptor caused neurosphere differentiation to be biased in cell fate determination towards a glial lineage upon EGF exposure (Burrows et al., 1997). However, it is still unclear whether different combinations of EGF and FGF2 concentrations in culture could affect not only cell differentiation but also cell fate determination.

To examine this, I have set four different conditions: 20 ng/mL of EGF and 10 ng/mL of FGF2; 20 ng/mL of EGF and 40 ng/mL of FGF2; 40 ng/mL of EGF and 20 ng/mL of FGF2 and 10 ng/mL of EGF and 20 ng/mL of FGF2. After differentiation of neurospheres cultured in these four different conditions, differentiated cells were immunohistochemically stained and quantified (Figure 3-8A-E). There were no significant morphological differences amongst the four conditions, but the extent of differentiation varied. Undifferentiated cell numbers from 20 ng/mL of EGF and 40 ng/mL of FGF2 condition was $32.28 \pm 10.28 \%$, which was significantly higher than from 10 ng/mL of EGF and 20 ng/mL of FGF2 condition ($7.66 \pm 0.36 \%$; ANOVA with Bonferroni's post-hoc test, p value < 0.005) (Figure 3-8E).

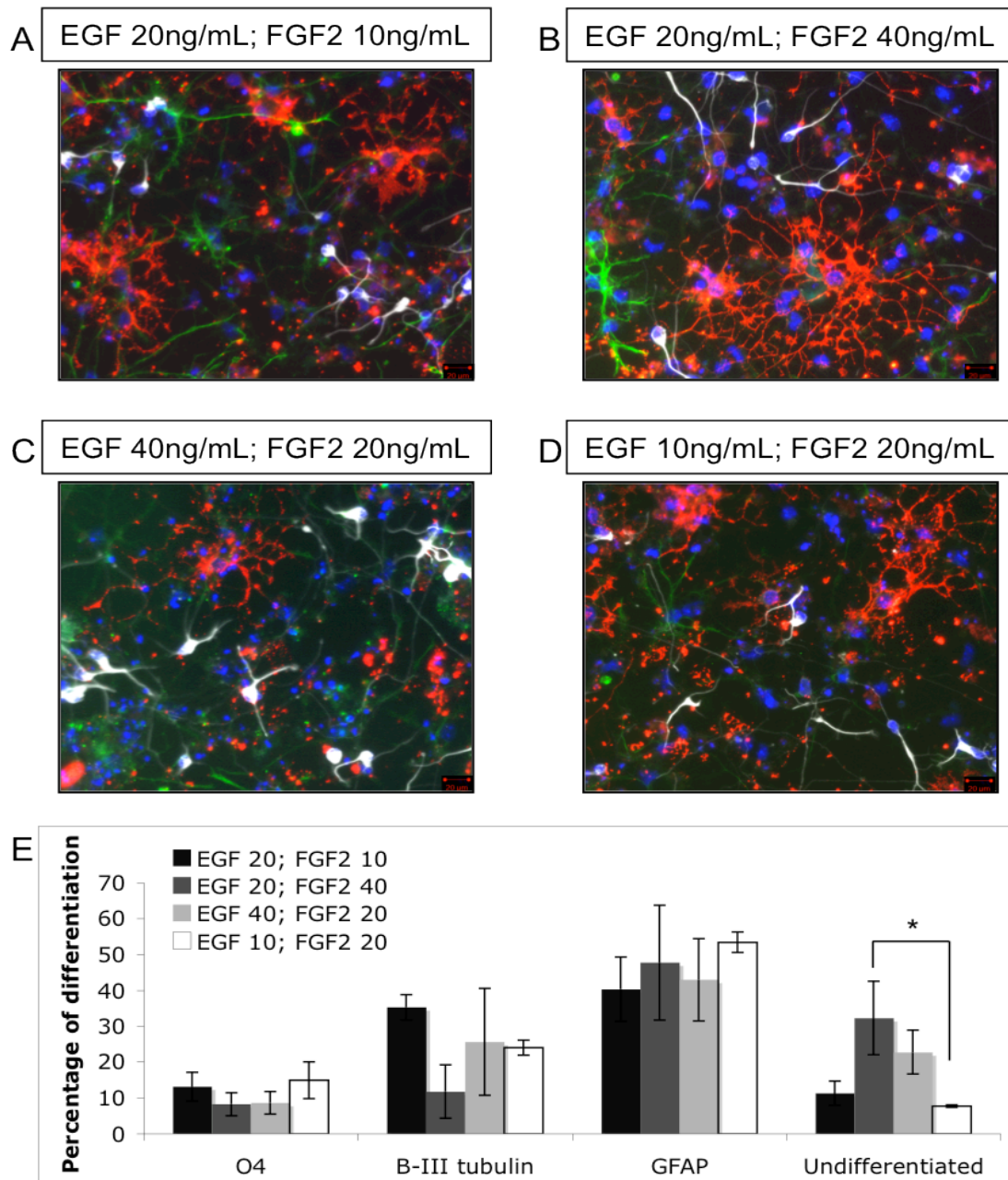


Figure 3-8. Differentiation of secondary neurospheres cultured in various concentrations of EGF and FGF2.

(A-D), Neurospheres were cultured in four different combinations of EGF and FGF2 during self-renewal and then differentiated for seven days. No growth factors were added during differentiation period. Differentiated neurospheres were stained with specific antibodies: astrocytes (GFAP: green), neurons (B-III tubulin: white) and oligodendrocytes (O4: red) and nucleus (DAPI: blue). Scale bar=20 μ m.

(E) Quantitative analysis shows that EGF 20 ng/mL and FGF2 40 ng/mL condition had significantly more undifferentiated cells than EGF 10ng/mL and FGF2 20 ng/mL. * $P<0.005$ (All the data are from n=3 experiments)

3.3. Discussion

From the characterization of neurosphere assay and refinement of the protocol, I have defined the role of neurosphere culture components for both self-renewal and differentiation. Also, I modified the pre-existing protocol to make the cell culture to be more reliable and reproducible. With 1.2 mg/mL of poly-HEMA coated flasks, 100 cells/ μ L were cultured for 4 days in the presence of fresh supplements with 20 ng/mL of each EGF and FGF2. After passaging the primary neurosphere to remove non-stem/progenitor cell populations, a secondary neurosphere was differentiated on poly-D-lysine/laminin. Seven days later, approximately 70% of a secondary neurosphere was fully differentiated into three different neural cell lineages with a specific ratio (<10% oligodendrocytes: ~10% neurons: ~60% astrocytes). This ratio was consistent if the neurospheres were differentiated as either multiple spheres or dissociated single cells although the morphology of astrocytes was distinctively different. Lastly, various EGF and FGF2 concentrations did not affect the neurosphere self-renewal except that reduction of FGF2 resulted in lower cell viability during neurosphere self-renewal. Meanwhile, twice more EGF and FGF2 in this culture condition attenuated the neurosphere differentiation process although this did not change the self-renewal process.

3.3.1. Reliable And Reproducible Neurosphere Culture

In the field of stem cell research, the free-floating sphere assay has been recognized as a simple primary cell culture to easily expand stem or progenitor cell populations. However, if the cell culture condition is not properly characterized and optimized for the particular cell types, it is difficult to reproduce any reliable data. With regard to my first 15 trials of the neurosphere assay (Table 3-2), the major issue was cell

viability. Without the ability to maintain adequate culture conditions for cells to live and grow, it is difficult to reproduce consistent cell behaviors. Therefore, it is important to establish a reliable cell culture condition to perform any of stem cell characterizations such as examining biological effects after genetic modification or drug treatments.

3.3.1.1. How to increase cell viability

In order to improve cell viability of the neurosphere culture, I found several factors that need to be considered: (1) fine SVZ micro-dissection, (2) sufficient cell density and (3) usage of proper cell culture medium (i.e. serum, supplements and growth factors).

From a practical point of view, precisely micro-dissecting SVZ tissue is technically demanding. In the early 90's, micro-dissected SVZ explants and the neurosphere assay were required to prove that the SVZ is a neurogenic compartment by producing far more neurospheres and neurogenic explants than the cortex or striatum (Lois and Alvarez-Buylla, 1993; Morshead et al., 1994). Since then it has become clear that fine micro-dissection of SVZ is required for not only limiting the source of brain tissue but also increasing cell viability in the culture. Either dissecting SVZ from coronally or sagittally sectioned brains, it is important not to include striatal tissue since it contains myelin as well as post-mitotic striatal cell bodies, which become debris in the neurosphere culture. The more dead and dying non-stem/progenitor cells or tissues can lower cell culture pH to prevent neurosphere growth (Fischer et al., 2011; Heinrich et al., 2011; Ortega et al., 2011; Pacey, 2006). Although the data were not shown in this chapter, one of the main problems in my

first 15 trials of neurospheres were likely due to unskillful SVZ dissection. Therefore, proper micro dissection of SVZ is necessary for reproducible neurosphere cultures.

As previously mentioned, cell density is an essential factor to determine multipotency of individual stem cells (clonality) of the culture as well as the degree of cell or sphere aggregation. Due to this, single cell culture or clonal density is preferred (Table 3-1). I tested three different cell concentrations initially, but fewer than 100 cells per microliter did not give rise to neurospheres, as mentioned in section 3.2.1.1. Because autocrine and paracrine signals from cells are required for healthy growth, cell density is directly related to sphere forming efficiency (Pastrana et al., 2011). Whereas the identity of these signals is unknown, their necessity could be the reason why single cell cultures were not reliably reproducible. Also, clonal densities of 0.2 - 20 cells/ μ L require 7-10 days of culture for each passage in order to obtain 40-150 μ m size neurospheres (Chojnacki and Weiss, 2008; Coles-Takabe et al., 2008; Ferron et al., 2007; Fischer et al., 2011; Heinrich et al., 2011; Ortega et al., 2011). This may be why I could not obtain neurospheres with 25 and 50 cells per microliter during 4 days. In sum, the cell density does not seem to directly affect cell viability in the culture, but it is important for the sphere forming process. Without consistent cell density from one experiment to another, it is difficult to compare the number and size of neurospheres.

For any type of cell culture work, it is crucial to understand the major components in the culture medium and use it properly at optimum conditions. According to the previous SVZ neurosphere studies, Dulbecco's modified eagle's medium (DMEM) with F-12 and neurobasal medium are commonly used (Table 3-1). Although the components of both media are not equivalent to one another, various extra amino acids, vitamins, inorganic salts or other components are added separately

to make both media to be equivalent. Besides the complete medium, several laboratories have used serum in their culture condition (Akiba et al., 2010; Kinoshita et al., 2009; Laywell et al., 2002; Merkle et al., 2007; Scheffler et al., 2005; Soares and Sotelo, 2004). Even though the serum was heat inactivated, it could still promote cell viability and initiate differentiation of floating neurospheres (Louis S.A., 2003). Most of all, I have also found that the commercially available N2 and B27 serum-free supplements are important for cell viability in neurosphere assay. As previously mentioned in 3.2.1.3., cells without both supplements could not self-renew or proliferate and gradually died, regardless of the absence or presence of growth factors. At least the composition of N2 serum-free supplement is known as a mixture of human transferrin, recombinant insulin, progesterone, putrescine and selenite, but the exact composition of B27 serum-free supplement is proprietary and not published. Due to variability between batch to batch for these supplements (Ortega et al., 2011), several research groups have replaced N2 and B27 with known concentrations of individual components in the medium (Table 3-1).

Lastly, the concentration of growth factors, especially FGF2, significantly affected only cell viability. The role of EGF and FGF2 has been known to have great effects on neurosphere proliferation (Gritti et al., 1999), but there was no significant change from four different combinations of growth factors. In contrast, I have shown that neurospheres cultured in 10 ng/mL FGF2 with 20 ng/mL EGF had a statistically significant reduction of cell viability in secondary neurospheres compared to the 20 ng/mL FGF2 with 10 ng/mL EGF condition. There has not been a direct comparison between these two different combinations of growth factors previously, but Vescovi et al. in 1993 have demonstrated that EGF alone could promote cell survival, whereas FGF2 alone did not enhance cell survival and the presence of no growth factors

resulted in significant decreases in cell survival (Vescovi et al., 1993). It seems the exact synergistic function between the two growth factors has implication on cell survival and it is important to choose a particular combination of EGF and FGF2 throughout the experiment. Otherwise, the result would be inconsistent and difficult to reproduce neurosphere data.

3.3.1.2. Differentiation of neurospheres depends on both intrinsic and extrinsic factors

The differentiation of NSPCs *in vivo* requires both intrinsic and extrinsic factors to determine when and what they become. For the process of spontaneous neurosphere differentiation *in vitro*, these factors are still significantly important for cell fate choice and differentiation.

As previously mentioned in 3.2.2.1., different substrates could give rise to different phenotypes of astrocytes, neurons or oligodendrocytes. In the previous studies on SVZ neurosphere differentiation (Table 3-1), laminin had been used as the most common substrate for generating uni-, bi- or multipotential neurospheres. Fewer studies have used Matrigel for SVZ neurosphere differentiation (Armesilla-Diaz et al., 2009; Dizon et al., 2006; Ocbina et al., 2006), and some utilized this substrate for studying cell migration in the neurospheres (Dizon et al., 2006; Katakowski et al., 2005; Ocbina et al., 2006). In contrast, Geltrex has never been used previously for SVZ neurosphere studies, but it has been used for neuronal differentiation from human embryonic stem cell (Fridman et al., 1990; Kubota et al., 1988; Salcedo et al., 2001).

By examining all three substrates, I found differences in differentiated cells. While cells had differentiated, they did not simply disperse outward from the core of

spheres in an even manner. The cells on laminin particularly circled around and form several small ring configurations with no cells in the middle. In contrast, on the Matrigel and Geltrex, this circular cellular arrangement was not found, but oligodendrocyte detection was technically difficult. In addition, phenotypes of GFAP positive cells on laminin had both protoplasmic astrocytes and radial glial cell bodies, whereas on Matrigel and Geltrex had only protoplasmic astrocytes. Because the exact concentrations of every constitution of the substrates were not published, it is difficult to speculate the cause of this phenomenon. However, similar to the differences in the differentiation of the neurospheres due to various substrates differences have also found in cell migration; Matrigel can allow neuroblasts to chain migrate (Dizon et al., 2006; Ocbina et al., 2006) while this feature was not found on laminin (Gurok et al., 2004). Since specific types of laminins *in vivo* in embryonic VZ and adult SVZ could regulate the astrocyte morphologies (Kazanis and French-Constant, 2011; Kazanis et al., 2010), it is important to choose specific substrates and be aware that different substrates and batch variability of the same substrate could produce different results in neurosphere cell differentiation.

Even though extrinsic cues can support neurosphere differentiation, if the cells are already intrinsically programmed to prohibit differentiation or conversely to a predetermined cell fate, it can be difficult to change these parameters with only external stimulation during differentiation. As I have shown in 3.2.2.2., when secondary and tertiary neurospheres were differentiated on the same substrate and same culture conditions, they did not give rise to the same phenotypic astrocytes, neurons and oligodendrocytes and percentage of differentiation. Based on the comparison between secondary and tertiary neurospheres during self-renewal, the number and size of these two neurospheres were distinctively different. This indicates

that the one extra passage of neurospheres could affect both self-renewal and differentiation. Previous studies have also mentioned that the levels of SVZ cell marker expression (i.e. Nestin, Sox2, Musashi1, Gsh2, Er81, Dlx2 and Mash1) were different between primary and serially passaged neurospheres (Ahlenius et al., 2009). Also, the authors have shown that further passaged neurospheres still highly expressed differentiated cell markers similar to primary neurospheres. Similarly, my data does not support the notion that further passaged neurospheres contain a higher concentration of self-renewing stem cells. In addition, it has been described that there would be more astrocytes and smaller neurons with serial passaging (Singec and Quinones-Hinojosa, 2008). Therefore, different neurospheres from different numbers of passages have different gene expression profiles, which make their differentiation properties variable and is not necessary for more stemness.

Since the neurosphere self-renewal process itself allows changes within the neurosphere cell-autonomous properties, I have examined whether the self-renewing neurospheres exposed to different concentrations of growth factors may influence their differentiation process. As I have demonstrated, the different combination of both EGF and FGF2 concentration did not produce any alteration in cell fate. However, the neurospheres cultured in 20 ng/mL EGF and 40 ng/mL FGF2 had attenuated differentiation capability compared to the ones in the 10 ng/mL EGF and 20 ng/mL FGF2 culture condition. Although a previous study has shown that overexpressing EGFR in neurospheres could make cells biased towards a more glial rather than neuronal fate (Burrows et al., 1997), this study utilized only EGF rather than a different combination of both EGF and FGF2 concentration. Therefore my data suggests that different combinations of both growth factors may not alter the cell fate determination but could change the differentiation capability of neurospheres.

3.4. Conclusion

Based on the previous neurosphere assay protocols, I have characterized and optimized the culture conditions: cell density, degree of adhesiveness for the culture flasks, proper culture medium and supplement conditions, amount of growth factors, substrates for differentiation and secondary and tertiary neurospheres differentiation. By comparing and contrasting different conditions and reagents, I have realized the alteration within each parameter can change the neurosphere behavior and differentiated cell morphologies. Therefore it is necessary to properly characterize all other neurosphere culture protocols and to standardize SVZ neurosphere protocols in order to compare individual works generated by different laboratories.

Chapter 4

H3K27 Trimethylation Signature In Postnatal SVZ With The Expression Of PRC2 Components

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Chapter 4

H3K27 Trimethylation Signature In Postnatal SVZ With The Expression Of PRC2 Components

4.1. Introduction

4.1.1. Presence Of H3K27me3 Marks And Their Possible Implication In The SVZ

During brain development, the SVZ NSPC has been dynamically transformed but maintains the capability for neurogenesis throughout life. The postnatal SVZ is a little studied area compared to the embryonic proliferative zones and the adult SVZ although its vibrant process of biological changes is very important to be considered in order to understand how to maintain neurogenesis from embryonic cortex to adulthood.

To understand SVZ NSPC characteristics, the field of stem cell biology identified a list of genes expressed in SVZ NSPCs and attempted to elucidate their roles via gain and loss of function experiments. However, microarray studies have suggested the process of transformation or maintaining the stemness of SVZ NSPCs probably requires dozens to hundreds of genes that are regulated simultaneously (Geschwind et al., 2001; Ivanova et al., 2002; Ramalho-Santos et al., 2002; Terskikh et al., 2001). Thus, it may be an unreasonable challenge to have an overarching comprehension of these processes by manipulating one or few genes at a time.

Recent studies have demonstrated that epigenetic modifications could provide global gene regulation. This is especially demonstrated when ESCs are differentiated into NPCs, and epigenetic modifications such as histone and DNA methylation govern transcriptional changes of neural lineage genes: neural, neuronal and astrocytic genes (Hirabayashi and Gotoh, 2010; Mohn et al., 2008). Although the data

were not directly measured from SVZ NSPCs, several studies on selective epigenetic regulators in SVZ NSPCs and their target gene regulations showed similar results (details describe in Chapter 1) (Hirabayashi et al., 2009; Lim et al., 2009; Pereira et al., 2010)

Specifically, neural genes including Sox1, Pax6, Ngn1, etc., are regulated via H3K27me3 (Mohn et al., 2008). According to the chromatin-immunoprecipitation (ChIP)-sequence (ChIP-seq) data published by Mikkelsen et al. in 2007, SVZ NSPC related genes, such as stem cell markers (GLAST, Musashi, GFAP and Mash1) and differentiation markers (GFAP, Olig2, Dlx1, Dlx2, Neurog1 and Neurog2), had a lack of H3K27me3 in NPCs compared to ESCs and embryonic fibroblasts (MEF) (Figure 4-1B,C) (Mikkelsen et al., 2007). Meanwhile, the H3K27me3 signature did not change for the pluripotency genes (Oct4, cMyc, Klf4 and Nanog) since these genes were regulated by DNA methylation instead (Figure 4-1A) (Deb-Rinker et al., 2005; Freberg et al., 2007). Based on the pattern of H3K27me3 signature among ESCs, NPCs and MEFs, the level of H3K27me3 for the neural genes could be used as an indicator for cell identity. Furthermore, this mark could possibly help to dissect out the subset of SVZ specific cell types such as stem cells, transient amplifying progenitor cells and neuroblasts.

Based on early embryogenesis studies, di- and tri-methylation of lysine 27 on histone 3 is catalyzed by PRC2 (Ezh2, Eed and Suz12) (Cao et al., 2002; Cao and Zhang, 2004b; Czermin et al., 2002; Kirmizis et al., 2004; Kuzmichev et al., 2002). To examine the function of PRC2 in embryonic cortex NSPCs, Ezh2 function has been removed in Nestin positive cells at E13 (Hirabayashi et al., 2009) and in Emx1 positive cells at E12 (Pereira et al., 2010). After confirming H3K27me3 abolishment,

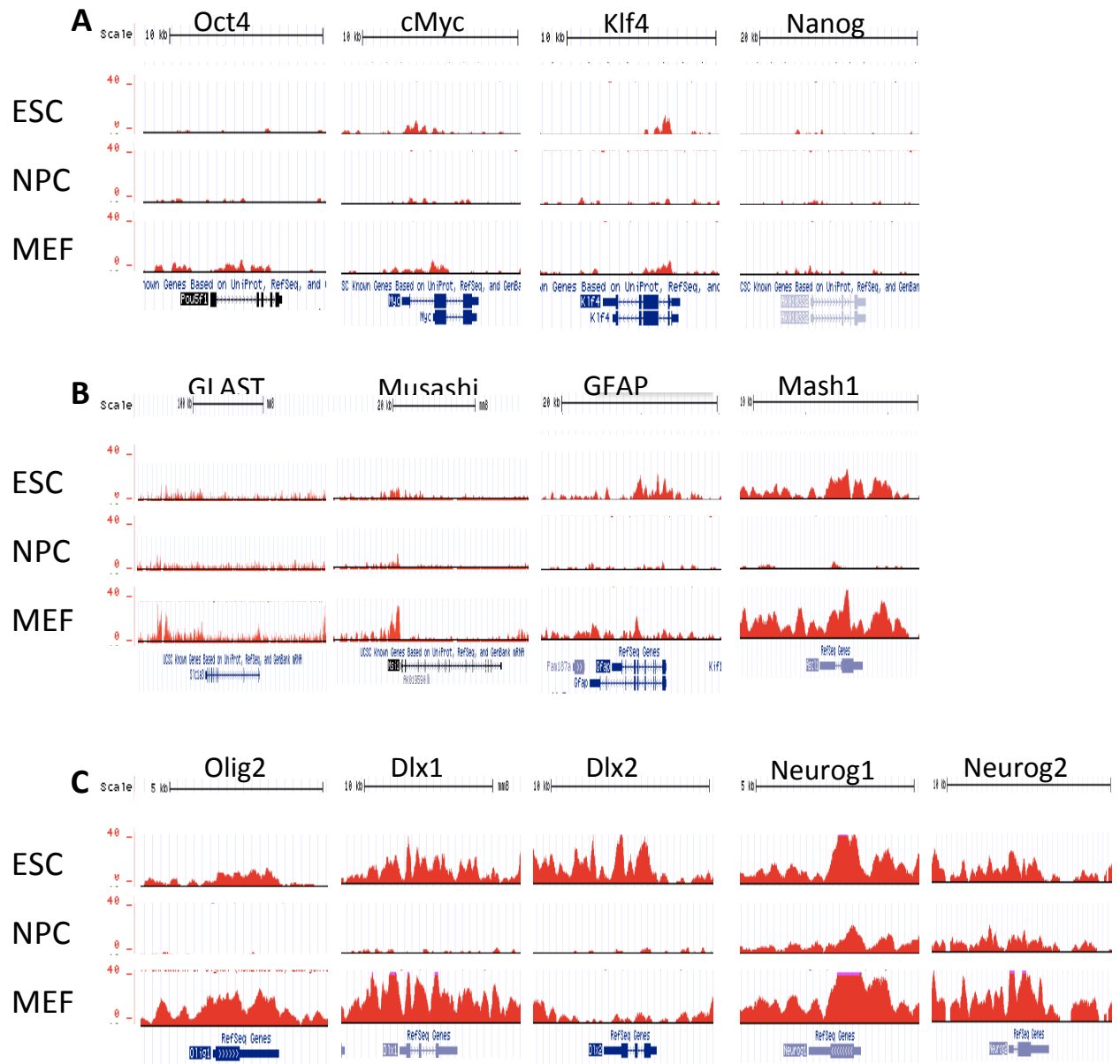


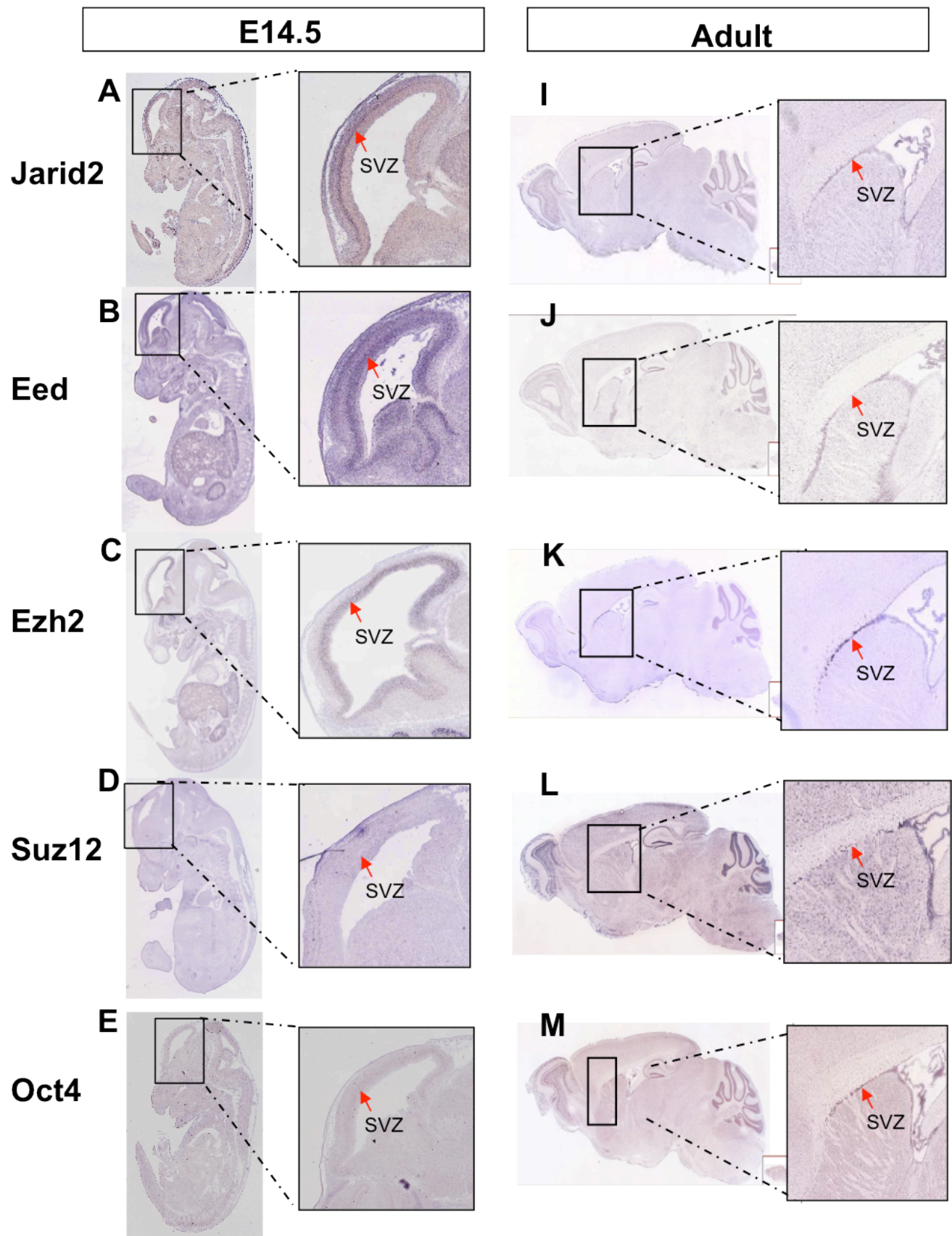
Figure 4-1. Comparison of pluripotent genes, SVZ NSPC markers and neural differentiation markers by ChIP assay in ESC, NPC and MEF for H3K27 trimethylation.

H3K27 trimethylation levels of (A) pluripotency genes (Oct4, cMyc, Klf4 and Nanog), (B) SVZ NSPC markers (GLAST, Musashi, GFAP and Mash1) and (C) differentiated neural cell markers (Olig2, Dlx1, Dlx2, Neurog1 and Neurog2) were compared with ESC, NPC derived from ESC and MEF. *Figures were compiled and modified from the UCSC Genome Browser.*

loss of Ezh2 function from nestin positive cells was shown to result in abnormal extension of neurogenesis with a delay in gliogenesis. Also, the Ezh2 deficient Emx1 positive cells increased cell proliferation during the neurogenic period but not in gliogenic period and showed an abnormal amount of neuronal production of cortical layers 2/3 and 5/6. These two studies prove that (1) Ezh2 is a necessary component of PRC2 for H3K27 trimethylation, (2) H3K27me3 is present from E12 till P0 and (3) loss of Ezh2 and H3K27me3 resulted in dysregulated NSPC proliferation and differentiation processes *in vivo* and *in vitro*. Based on these results and knowing that SVZ has life-long neurogenesis, I asked if PRC2 genes are continuously expressed in postnatal SVZ, and if they are necessary for global gene regulation? If so, is PRC2 function involved in the SVZ NSPC self-renewal and multipotency?

Kim et al. in 2007 demonstrated that Eed expression was maintained from E14.5 till adult brains. In embryonic and postnatal brains, Eed interacted with Ezh2 at the protein level until P28, but it began to interact with Mll1 in adult brains instead (Kim et al., 2007). However, it is not sure whether this feature applies to the SVZ and whether PRC2 genes are even still expressed in the postnatal SVZ. Due to the current technical limitations with specificity of PRC2 antibodies, it has been difficult to detect PRC2 protein using immunohistochemistry. Alternatively, *in situ* hybridization showed that Jarid2, Eed, Ezh2 and Suz12 are all expressed in the E14.5 proliferative zones and in the adult SVZ (Figure 4-2A-D; I-L respectively) except that Suz12 expression in E14.5 SVZ was very weak (Figure 4-2D). However, it is not clear whether the PRC2 components are still expressed in the postnatal SVZ.

In summary, current epigenetic studies have indicated that the role of PRC2 and H3K27me3 signature could be important for SVZ NSPC identity and plasticity, but their role in postnatal SVZ has not been investigated yet. Thus far, I would like to



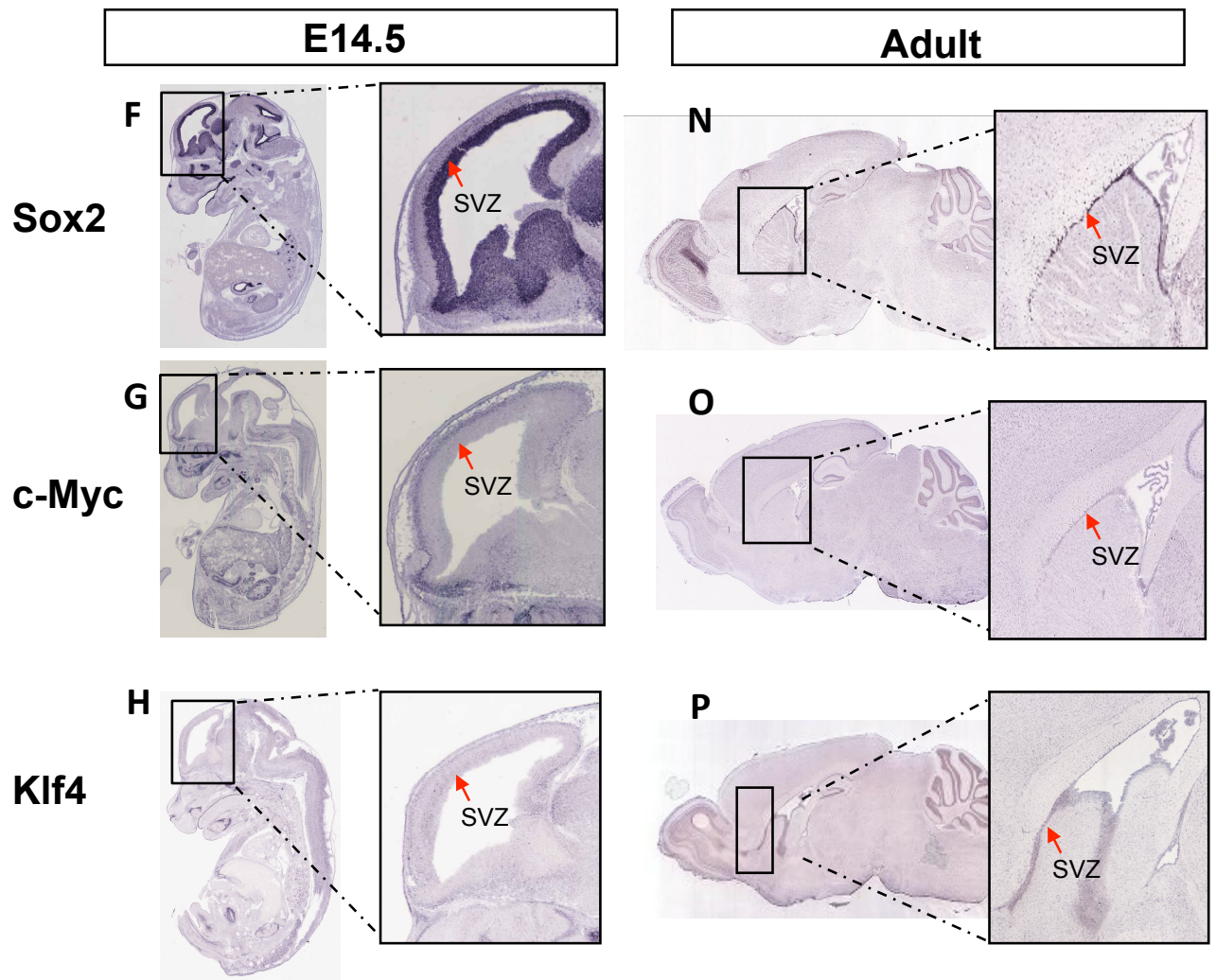


Figure 4-2. *In situ* hybridization of pluripotent genes in embryonic and adult SVZ.

The transcription level of *Jarid2*, *Eed*, *Ezh2*, *Suz12*, *Oct4*, *Sox2*, *cMyc* and *Klf4* detected in embryonic cortical SVZ (red arrows) (A-H) and adult SVZ (I-P). In the embryonic cortical SVZ, all genes were present but is different intensities. *Adapted from Genepaint.org*. The adult SVZ expressed all eight genes. *cMyc* appeared to be expressed just above the level of detection. *Adapted from the Allen Brain Atlas*.

investigate (1) the level of H3K27me3 intensity in the postnatal and adult SVZ, (2) the PRC2 expression level with PRC2 regulatory transcription factors (Oct4, cMyc, Klf4 and Sox2) to better understand the postnatal SVZ NSPC identity, (3) whether PRC2 gene expression is only restricted to the NSPC population or not and lastly (4) the possible H3K27me3 intensity changes in brain injury and disease models such as middle cerebral artery occlusion (MCAO) and cuprizone models, which alter the SVZ NSPC properties (Goings et al., 2008; Young et al., 2012).

4.2. Results

4.2.1. Histone 3 Lysine 27 Trimethylation In Postnatal And Adult SVZ *In Vivo*

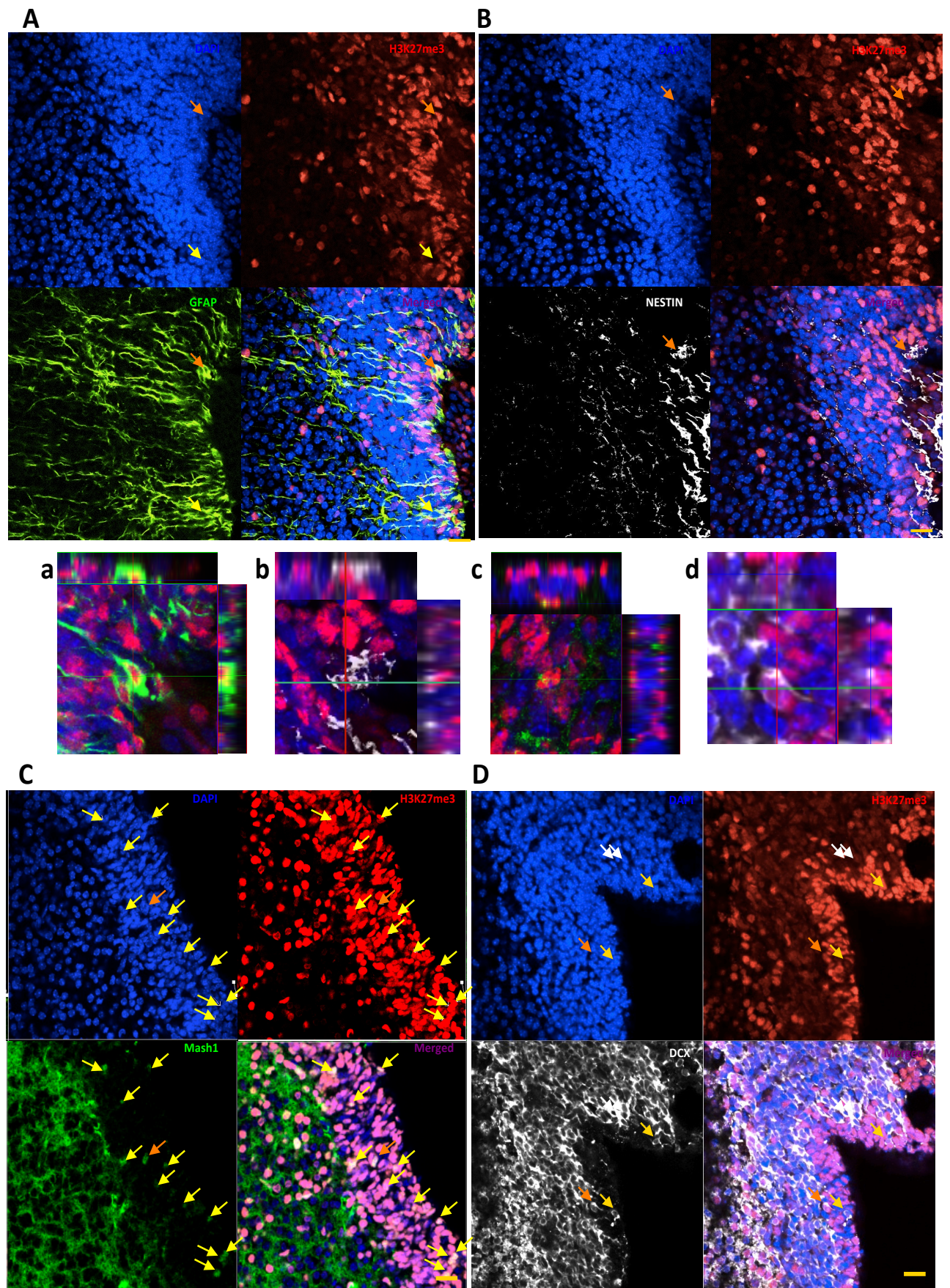
As it had not been demonstrated whether H3K27me3 remained in neurogenic niches, I determined H3K27me3 presence in both postnatal and adult SVZ. To understand the amount and distribution of H3K27me3 -marks in postnatal and adult brain, I used a H3K27me3 specific antibody on coronally sectioned postnatal (P4) and adult (6 weeks old) brains and examined immunofluorescence with confocal microscopy (Figure 4-3A,B). GFAP double immunofluorescence was carried out on the same sections in order to determine the extent of astrocyte lineage H3K27me3. P4 brains retained some radial glial cells carrying long processes, whereas adult brains had GFAP positive protoplasmic (grey matter) and fibrous (white matter) astrocytes instead (Figure 4-3A-f). In P4 brains, the majority of DAPI positive cells were labeled with the anti-H3K27me3 antibodies, except for in the striatum where immunofluorescence was significantly lower and only a minority of cells was labeled (Figure 4-3a). Although most GFAP positive cells, which have smaller nuclei, did not have H3K27me3 marks, most cells with relatively larger nuclei showed intense H3K27me3 immunofluorescence. These features were also found in the adult cortex although GFAP positive cells had changed their morphology (Figure 4-3B). In the postnatal dorsolateral SVZ, a subset of cells were H3K27me3 positive, and these cells, which had different intensities of labeling, were generally adjacent to the lateral ventricle rather than the striatal side of the SVZ (Figure 4-3c). However, in the adult brain, H3K27me3 was found throughout the dorsolateral SVZ except for one or two cells per field that were negative (Figure 4-3d). Lastly, the H3K27me3 labels in striatal cells were significantly different in postnatal compared to adult brains (Figure

Figure 4-3. H3K27me3 expression in postnatal and adult brains.

(A) Immunohistochemistry in postnatal (P4) and (B) adult (6 wks) brains coronal sections was carried out with anti-H3K27me3 antibodies to compare the difference of H3K27me3 signatures in two different brain developmental stages. (a-f) Higher magnification images of yellow boxes in Figure 4-3 A,B. The number of H3K27me3 positive cells per total cell numbers in striatum from both brains were significantly different although cortex and SVZ was not distinctively different. Scale bar=500 μ m (A,B) 20 μ m (a-f) (All the data are from n=3 experiments.)

4-3A,B). The adult striatal cells were significantly more H3K27 trimethylated when compared to the P4 striatal cells. Striatal images taken at higher magnification indicated that $84.13\% \pm 5.51$ of adult striatal cells were H3K27me3 positive, and these had larger nuclei than the rest of striatal cells (Figure 4-3f). In contrast, most striatal cells in P4 brains had the smaller nuclei compared to the adults, and the reduction of H3K27me3 positive striatal cell numbers ($36.84\% \pm 8.48$) were statistically significant (p value: 8.49×10^{-5} ; Figure 4-3e).

In order to further investigate the difference between postnatal and adult SVZ with regards to the H3K27me3 signature, I immunolabeled SVZ with cell type markers; Nestin (radial glia cells, transient amplifying cells and neuroblasts), GFAP (radial glia cells in postnatal SVZ or astrocyte-like stem cells in adult brain and niche astrocytes), s100 β (ependymal cells), Mash1 (transient amplifying cells) and Dcx (neuroblasts). This would help to dissect out which subset of SVZ cells has the H3K27me3 marks and how much qualitative H3K27me3 intensity is present in them. In the postnatal SVZ, GFAP and H3K27me3 co-localization was observed in the lateral ventricle side of SVZ (Figure 4-4A, a *orange arrow*). The morphology of the GFAP and H3K27me3 double positive cells were that of radial glial cells, carrying a long process. At the same time, there were GFAP positive but H3K27me3 negative



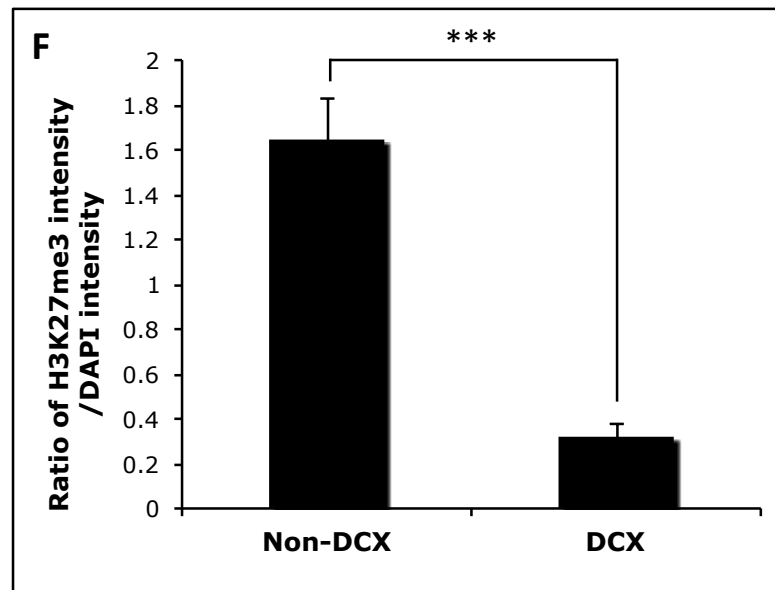


Figure 4-4. H3K27me3 expression in postnatal SVZ cell types.

(A-D) Co-immunohistochemistry for H3K27me3 and SVZ cell type markers was carried out: GFAP (astrocytes and radial glial), Nestin (radial glial, transient amplifying and neuroblasts), Mash1 (transient amplifying cells) and Dcx (neuroblasts). (a-d) Higher magnification cells pointed to with of the orange arrows from A-D, respectively. All the different SVZ cell type markers were co-labeled with H3K27me3 staining. Scale bar = 20 μ m

(F) H3K27me3 intensities from non-DCX and DCX positive cells in SVZ were measured and normalized by DAPI intensity. The ratio of normalized H3K27me3 intensity between two groups was significantly different (** $p < 0.001$). (All the data are from n=3 experiments.)

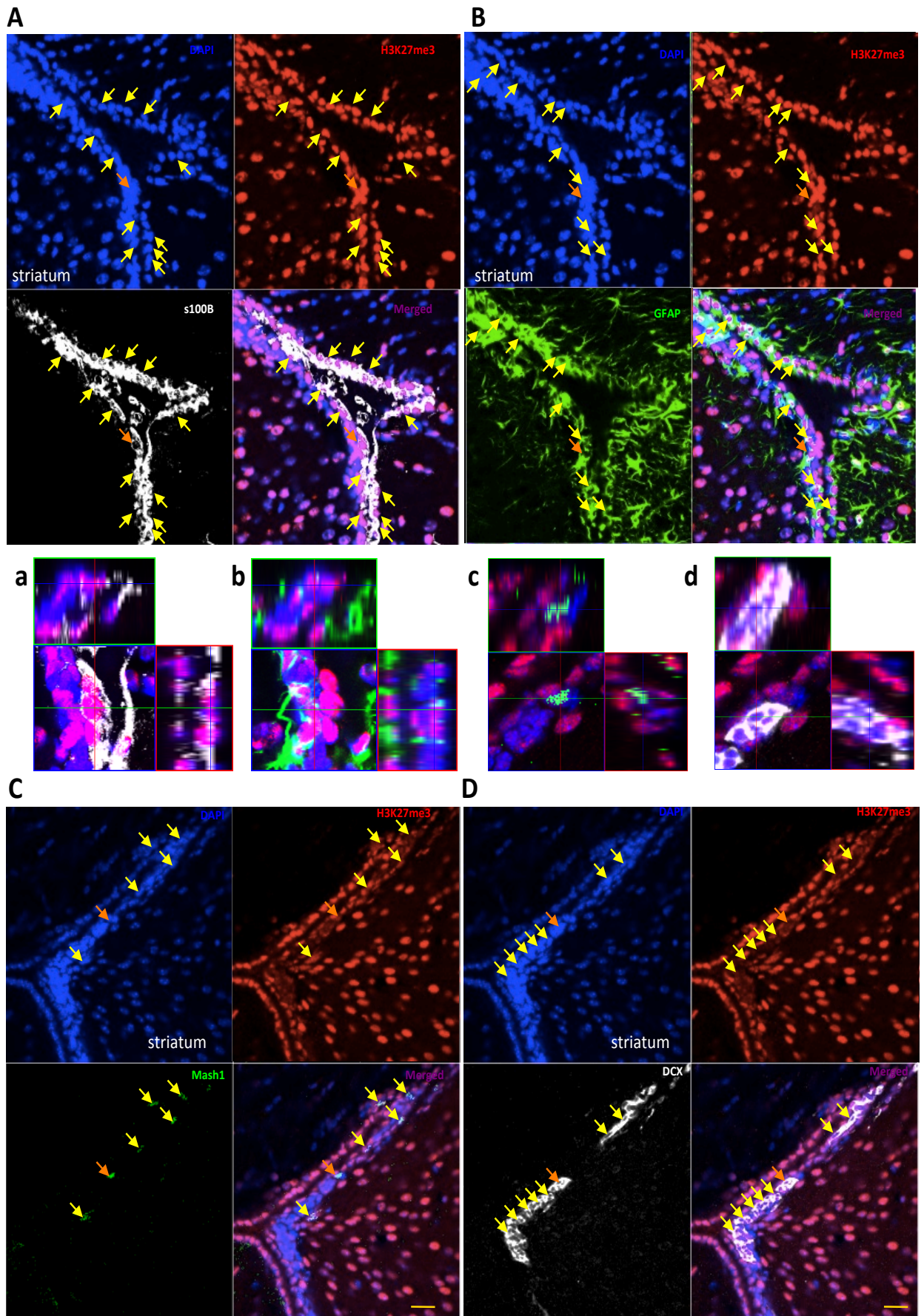
cells (Figure 4-4A *yellow arrow*). For Nestin and H3K27me3 co-staining, selective Nestin positive cells were co-stained with H3K27me3 (Figure 4-4B.b *orange arrow*). As a large number of H3K27me3 cells expressed neither GFAP nor Nestin, other SVZ cell type markers such as Mash1 (for transient amplifying cells) and Dcx (for neuroblast cells) were co-immunolabeled with anti-H3K27me3. Figure 4-4C shows that all Mash1 positive nuclei in the SVZ were co-localized with H3K27me3, whereas many but not all Dcx positive cells were co-immunolabeled with H3K27me3 (Figure 4-4D *white arrows*). Dcx positive cells adjacent to the lateral ventricle had

significantly lower intensity of H3K27me3 immunolabeling compared to non-Dcx positive cells in SVZ ($p < 0.001$, Figure 4-4D,F *all the yellow and orange arrows*).

In the adult SVZ, H3K27me3 positive cells were co-labeled with s100 β , GFAP, Mash1 and Dcx (Figure 4-5A-D *orange and yellow arrows*). The intensity of H3K27me3 marks appeared to be consistently higher in most ependymal cells, whereas in SVZ GFAP positive cells and Mash1 positive cells, it was variable (Figure 4-5A-C). Compared to non-DCX positive cells in SVZ such as s100 β , GFAP or Mash1 positive cells, all Dcx positive cells within the SVZ expressed significantly lower levels of H3K27me3, which at times was just above the level of detection ($p < 0.05$, Figure 4-5D,F).

4.2.2. PRC2 And Its Regulatory Transcription Factors Expression Level To Possibly Define Postnatal SVZ NSPC Identity *In Vivo* And *In Vitro*

Previously we hypothesized the level of H3K27me3 mark intensity may help dissect out SVZ cell type differences, but this was not possible since all the postnatal SVZ cell types were partially co-labeled with H3K27me3 mark, whereas all the cells in adult SVZ were co-localized with H3K27me3 mark. However, both postnatal and adult SVZ NSPCs had consistently higher intensity of H3K27me3 signals compared to neuroblasts as mentioned in 4.2.1. Thus, it became important to determine the expression of PRC2 components to investigate their role to govern H3K27me3 marks in the NSPC population. While PRC2 gene expressions were screened, transcriptional regulatory factors such as Oct4, Sox2, cMyc and Klf4 for regulating PRC2 gene expression were also examined in the postnatal SVZ (Peng et al., 2009; Ura et al., 2008).



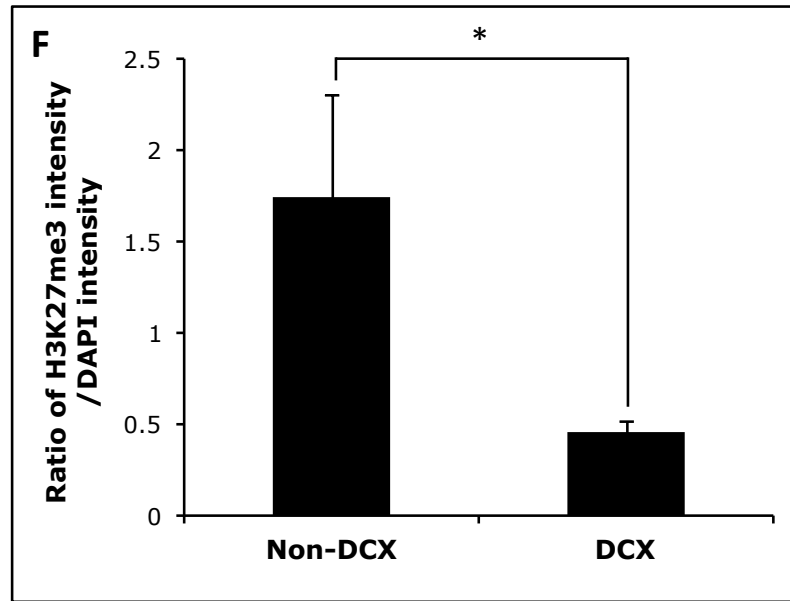


Figure 4-5. H3K27me3 expression in adult dorsolateral SVZ cell types.

(A-D) Co-labeling H3K27me3 and SVZ cell type markers: GFAP (astrocytes and radial glial), s100 β (ependymal cells), Mash1 (transient amplifying cells) and Dcx (neuroblasts). (a-d) Higher magnification of the orange arrow pointed cells from A-D respectively. All the different SVZ cell type markers were co-labeled with H3K27me3 staining. Scale bar = 20 μ m

(F) H3K27me3 intensities from non-DCX and DCX positive cells in SVZ were measured and normalized by DAPI intensity. The ratio of normalized H3K27me3 intensity between two groups was significantly different (* $p < 0.05$). (All the data are n=3 experiments.)

Immunohistochemistry was attempted to identify the expression and localization of PRC2 core components (Eed, Ezh2, Suz12) and Jarid2 in postnatal SVZ brain sections. However, multiple attempts revealed non-specific and uninterpretable labeling of cells and extracellular matrix with all four antibodies suggesting the antibodies were not suitable for immunohistochemistry (data not shown). As an alternative method, postnatal SVZ and striatal tissues were micro-dissected and total RNA extracted to synthesize cDNA. Since the expression and

function of both PRC2 and its regulatory transcription factors were previously described and demonstrated in ESCs, all the gene expression measurements were compared to ESCs as a positive control. Semi-quantitative reverse transcriptase (RT)-PCR indicated that the transcriptional levels for PRC2 components and the transcription factors were clearly detectable in ESC (Figure 4-6A). SVZ secondary neurospheres had all the messenger RNAs (mRNAs) for PRC2 genes and the transcription factors except Oct4. Similarly, all genes were detected from SVZ and striatum tissues except Oct4 and Klf4 (Figure 4-6A).

Because these semi-quantitative RT-PCR results did not indicate quantitative differences in transcription levels, cDNAs from four different samples were used for quantitative real time (qRT)-PCR to identify how much PRC2 and transcription factors were expressed relative to ESCs. To normalize expression levels to internal controls, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 18s were initially examined. From the preliminary studies, the same quantity of cDNAs from ESCs and SVZ neurospheres were used to detect GAPDH transcription level by using qRT-PCR, but their Ct values were consistently different by two. In contrast, 18s as an internal control did not show lesser than 1 Ct value difference. Thus, I used 18s as the qRT-PCR internal control.

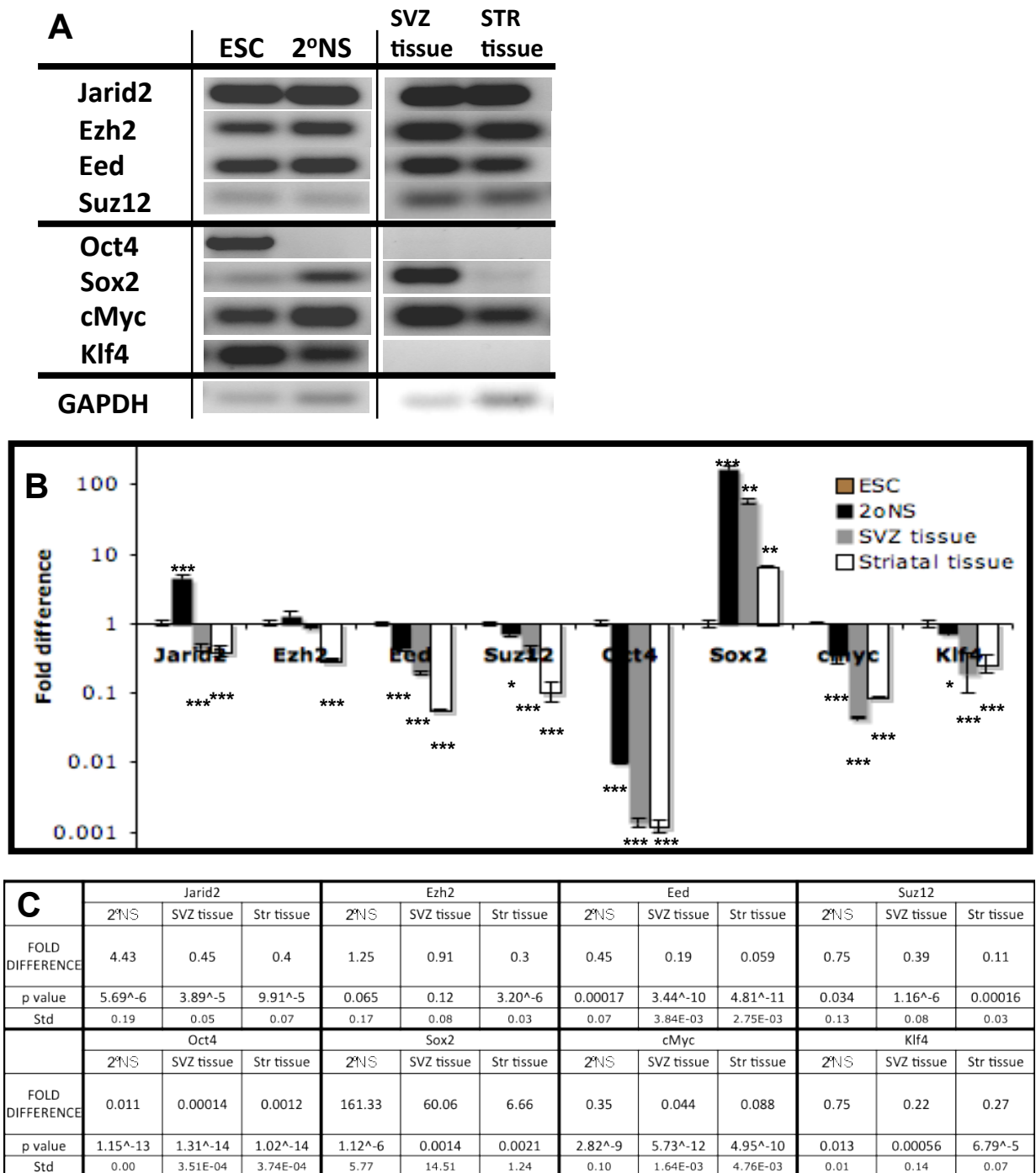


Figure 4-6. Transcriptional levels of PRC2 and its regulatory transcription factors from ESC, SVZ neurospheres, SVZ and striatum.

PRC2 and its regulatory transcription factors were semi-quantitatively (A) and quantitatively (B) measured from neurospheres, ESCs, and P4 SVZ and striatal tissues. GAPDH was used as a loading control for semi-quantitative PCR analysis, and 18s was used as an internal control for quantitative-Real time PCR analysis. ESCs have been used as an experimental control for the gene expression level (C) The fold difference compared to ESC, whose unit was set as 1; Student t-test p values; and standard deviation (Std) were listed as a table. (All the data are n=3 experiments.)

After normalizing raw Ct values to 18s as an internal control, all genes were normalized to ESCs as an experimental control once again (Figure 4-6B,C). Jarid2 transcriptional level in SVZ secondary neurospheres was about 4.43 ± 0.19 fold increased compared to ESCs, but its levels in the SVZ and striatal tissues were significantly decreased as 0.45 ± 0.07 and 0.40 ± 0.05 fold difference respectively (Figure 4-6B,C). Ezh2 mRNA expression in neurosphere was greater than in ESCs (1.25 ± 0.17) but less in the SVZ (0.91 ± 0.08) however neither change was significantly different compared to ESCs. In contrast, striatal tissue expressed significantly less Ezh2 mRNA compared to ESCs (0.3 ± 0.08) (Figure 4-6B,C). Also, Eed and Suz12 mRNA expression levels in neurospheres, SVZ and striatal tissues were all significantly decreased compared to ESCs (Figure 4-6B,C). Taken together, all the transcriptional levels of PRC2 core components in the P4 striatal tissue showed more reduction than the SVZ tissue and neurospheres when compared to ESCs.

In contrast, the transcription factor expression in SVZ and striatum tissue and SVZ neurospheres, was significantly decreased except for Sox2 (Figure 4-6B,C). Sox2 was known to be expressed in the SVZ and to govern multipotency of SVZ NSPCs (Collignon et al., 1996; Miyagi et al., 2006; Wood and Episkopou, 1999; Zappone et al., 2000). I found its transcription level in neurospheres was 161.33 ± 5.77 fold greater than ESCs. Also, SVZ and striatum had 60.06 ± 14.51 fold and 6.66 ± 1.24 fold greater than ESCs respectively (Figure 4-6B).

In summary, quantitative analysis of PRC2 gene expression levels indicated that the SVZ, the striatum and neurospheres had significantly lower mRNA expression levels compared to ESCs although these differences were not detected by semi-quantitative analysis. In addition, Oct4, cMyc and Klf4 transcriptional levels in the SVZ, striatum and neurospheres were significantly lower than in control ESCs,

but Sox2 mRNA was not. Interestingly, all the transcription factors' mRNA levels in neurospheres were significantly different from those in SVZ tissue (Oct4 *p* value: 2.594×10^{-5} ; Sox2 *p* value: 2.549×10^{-4} ; cMyc *p* value: 2.380×10^{-3} ; Klf4 *p* value: 2.665×10^{-4}). Therefore, these transcriptional changes could be due to the neurosphere culture environment.

These qRT-PCR data from SVZ tissue and its neurospheres indicated the transcription levels of PRC2 and its regulatory transcription factors. However, it was still unclear which subset of SVZ cells expressed these genes, so I utilized *in vivo* SVZ FACsorted cell cDNA, obtained from the Doetsch laboratory (Columbia University) (Pastrana et al., 2009), to measure endogenous transcriptional levels of transcription factors from individual adult SVZ cell types including striatal astrocytes (Figure 4-7A). By using GAPDH as a loading control, the transcription factors and Jarid2 transcriptional level were semi-quantified from striatal astrocytes, niche astrocytes, SVZ stem cells, transient amplifying progenitor cells and neuroblasts (Figure 4-7B). Jarid2 and cMyc expression levels were consistent throughout all five samples. Also, Sox2 was highly present in all three SVZ cells but had lower expression levels in striatal and niche astrocytes. Interestingly, Klf4 was only expressed in the neuroblast population while it was not present in any other cell type in both SVZ and striatum. Also, Oct4 was highly expressed in niche astrocytes while its lower expression was detected in SVZ stem/progenitor cell populations (Figure 4-7B).

Collectively the mRNA levels of all the PRC2 components as well as PRC2 regulatory transcription factors were detected from postnatal SVZ cells except Oct4 and Klf4. Although Klf4 expression level was detectable from postnatal SVZ

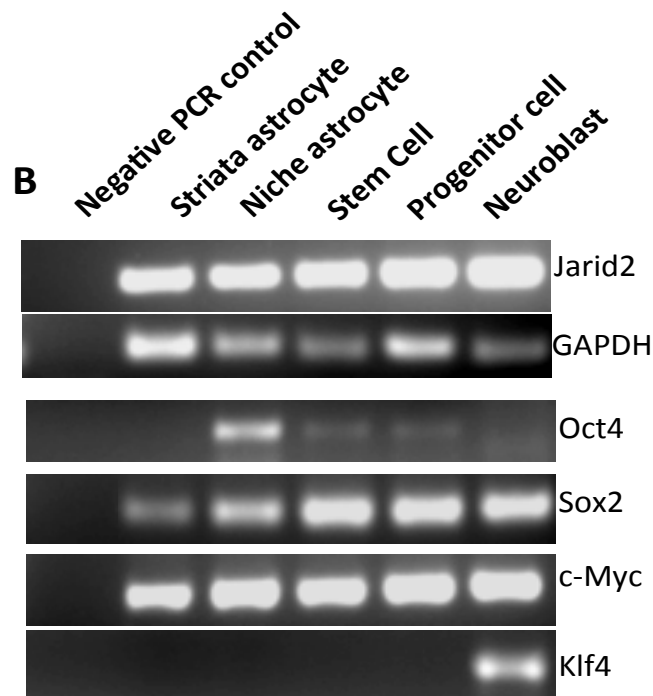
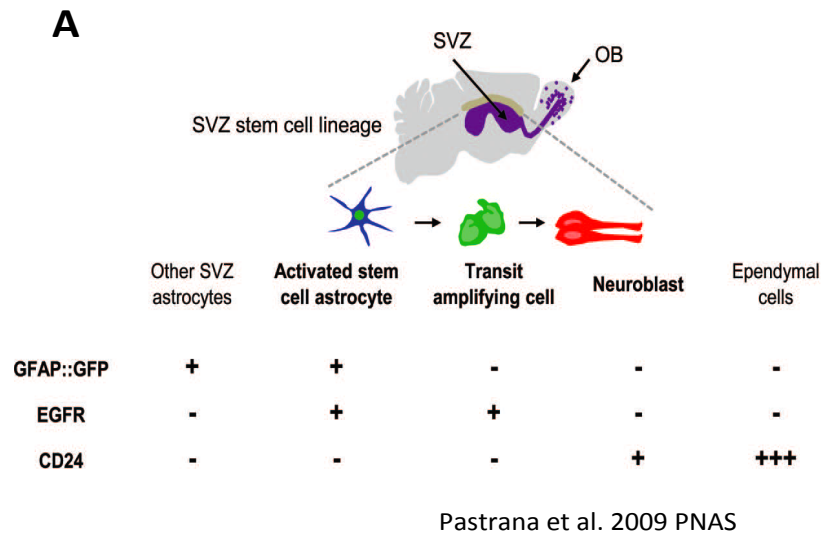


Figure 4-7. Transcriptional levels of pluripotent genes and Jarid2 from SVZ and striatal FACsorted cell types.

(A,B) cDNA extracted from FAC sorted SVZ/striatal cells were used to semi-quantitatively examine the transcriptional level of Jarid2, Oct4, Sox2, c-Myc and Klf4. GAPDH was a positive loading control (n=1) Except Oct4 and Klf4, the rest of genes were all expressed from the subset of SVZ and striatal cells. *Schematic (A) was adapted from Pastrana et al. 2009 PNAS*

neurospheres and adult SVZ neuroblasts, it was not in the postnatal SVZ tissue. Also the transcriptional level of Oct4 was not detectable from postnatal SVZ tissues and neurospheres, but its level was found at the FACsorted cDNA of SVZ niche astrocytes, stem cell and progenitor cell populations. In sum, this study suggested that (1) PRC2 expression was not restricted to only stem cell regions, (2) SVZ secondary neurospheres had different transcriptional levels than SVZ tissue and (3) the transcriptional profiles from the neurospheres seemed to represent a mixture of all the SVZ stem/progenitor cell types.

4.2.3. PRC2 and Its Regulatory Translational Level In SVZ Neurospheres

As described in 4.2.2., the transcriptional profiles of PRC2 and its regulatory transcription factors in SVZ secondary neurospheres resembled those from SVZ tissues. I next determined the translational levels of PRC2 and transcription factors in ESCs and SVZ secondary neurospheres.

Western blotting was performed with the gene specific antibodies that were previously validated in ESCs (Figure 4-8A), and the protein levels were compared to the levels of mRNA measured by qRT-PCR. The western blot results indicated that Jarid2 protein levels in secondary neurospheres were lower than ESCs, which did not correlate with the qRT-PCR result mentioned in 4.2.2. (Figure 4-8A). Another exception was Klf4, whose transcriptional level in neurospheres was lower than in ESCs, but its translational level in neurospheres was higher (Figure 4-8A). Lastly cMyc protein level in neurospheres was not proportional to its transcriptional level (Figure 4-8A). Otherwise, the protein levels of Oct4, Sox2 and all PRC2 core components were correlated to their mRNA expression levels (Figure 4-6B).

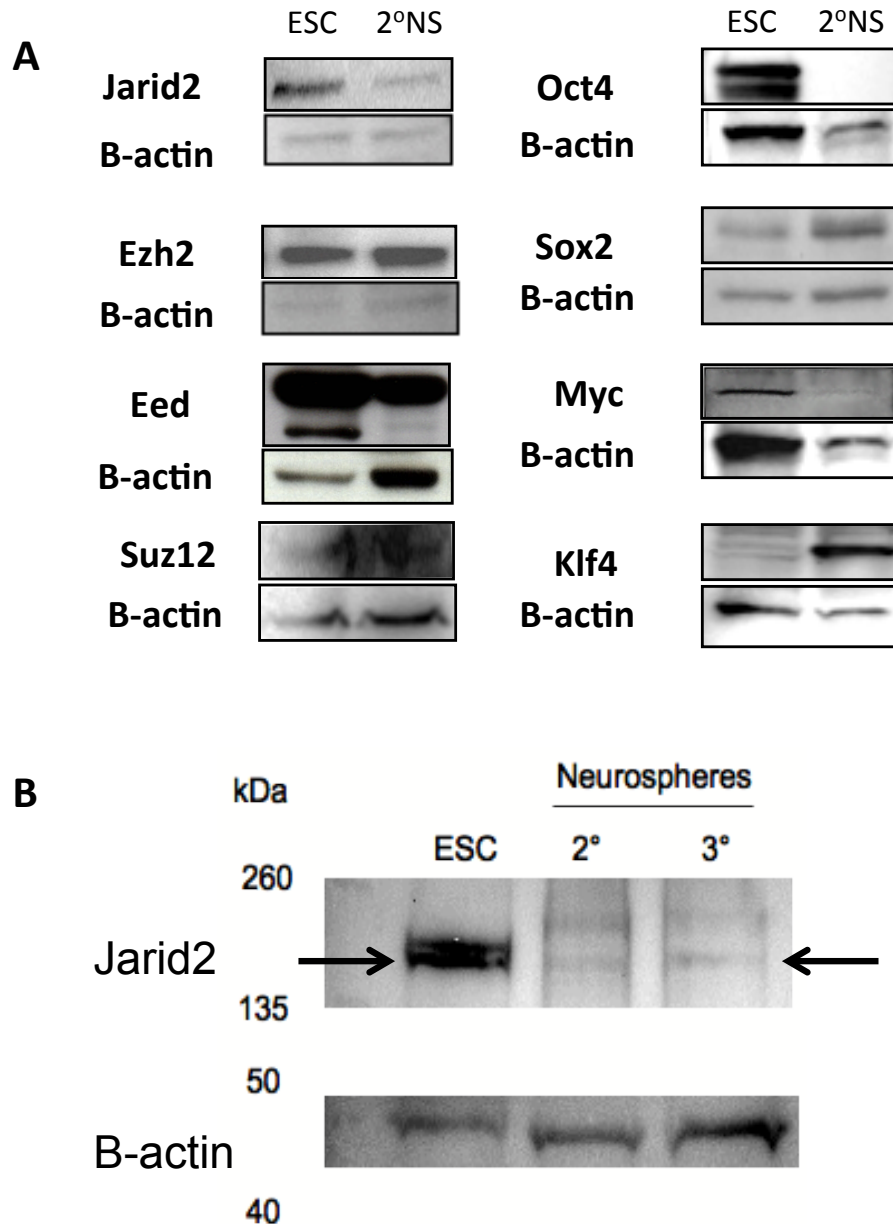


Figure 4-8. Translational level of PRC2 and the transcription factors from ESC and SVZ neurospheres.

(A) Western blot with ESC and SVZ secondary neurospheres (2°NS) proteins showed the endogenous level of Jarid2, Eed, Ezh2, Suz12, Oct4, Sox2, c-Myc and Klf4. (β -actin was used for a positive loading control.) Besides Oct4, all the PRC2 and the transcription factors were expressed in secondary neurospheres (n=3).

(B) Jarid2 protein levels between secondary and tertiary neurospheres did not significantly differ. The Jarid2 band (139KDa) is indicated with arrow. (n=3)

As previously demonstrated in 3.2.1.1. and 3.2.2.2., secondary and tertiary neurospheres showed different phenotypic characteristics. This could be due to gene expression changes, so Jarid2 protein level was examined in secondary and tertiary neurospheres since its function in the PRC2 can be altered based on its subtle expression level changes (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009). However, Jarid2 protein levels in secondary and tertiary neurospheres were not significantly different from one another (p value: 0.78; Figure 4-6B).

4.2.4. Histone 3 Lysine 27 Trimethylation In Brain Diseases

Previously, the Szele laboratory and others have demonstrated that endogenous SVZ NSPCs became activated after the middle cerebral artery occlusion (MCAO) model for stroke and cuprizone model for demyelination in CNS (Goings et al., 2008; Young et al., 2012). The etiology and symptoms of both models were not the same; SVZ NSPCs responded to the cuprizone demyelination model with decreased NSPC proliferation and increased microglial activation, whereas they responded to the MCAO model with increased NSPC proliferation and reactive astrogliosis. Since SVZ as well as parenchymal cells responded dynamically to these models, we speculated that the H3K27me3 signature was also altered in these brains in response to the progression of injury or demyelination.

4.2.4.1. Stroke: Middle cerebral artery occlusion model

Young et al. 2012 generated and characterized the MCAO model in the Szele laboratory (Young et al., 2012). After using intraluminal filaments to create focal ischaemia of MCAO, SVZ phosphohistone 3 (PH3) positive cells were significantly

increased although the proportion of proliferative SVZ cell types did not change after 7 days of MCAO (Young et al., 2012). Also, GFAP immunoreactivity was increased in SVZ and striatum adjacent to SVZ.

After performing immunohistochemistry with H3K27me3 and GFAP antibodies in MCAO and sham mice brains, I confirmed the abnormally increased GFAP immunoreactivity in the ipsilateral SVZ and striatum of stroke brains compared to sham-operated controls whose the GFAP signal is below the level of detection (Figure 4-9A,B) (Young et al., 2012). The H3K27me3 detection was similar to my previous results, that almost all the cells in the SVZ seemed to be H3K27me3 positive in sham controls ($85.22\% \pm 4.78$; Figure 4-9C). In contrast, the contralateral ($43.61\% \pm 4.01$, $p < 0.02$) and ipsilateral ($18.69\% \pm 5.45$, $p < 0.01$) sides of MCAO brains showed significantly less numbers of H3K27me3 positive cells in SVZ (Figure 4-9D,E). Also, the percentage of H3K27me3 positive cells per DAPI positive cells was 83% decreased in the infarct area compared to contralateral side of MCAO brain (Figure 4-9B). Interestingly, majority of striatal cells in the ischaemic penumbra lacked H3K27me3 marks in the center of nucleus (Figure 4-9D,E), and those lacking H3K27me3 cells were not GFAP positive (Figure 4-9C-E). Unlike the GFAP positive cells in sham control, the higher level of H3K27me3 positive cells in ipsilateral SVZ and striatum were all GFAP positive cell population (Figure 4-9 E *yellow arrow*).

4.2.4.2. Demyelinating disease: Cuprizone model

The cuprizone model was established by feeding cuprizone, a copper chelator which attenuates brain copper level to disrupt the mitochondrial metabolism in oligodendrocyte cells (Matsushima and Morell, 2001) and eventually yield the CNS demyelination. This model has demonstrated the plasticity of SVZ NSPCs during

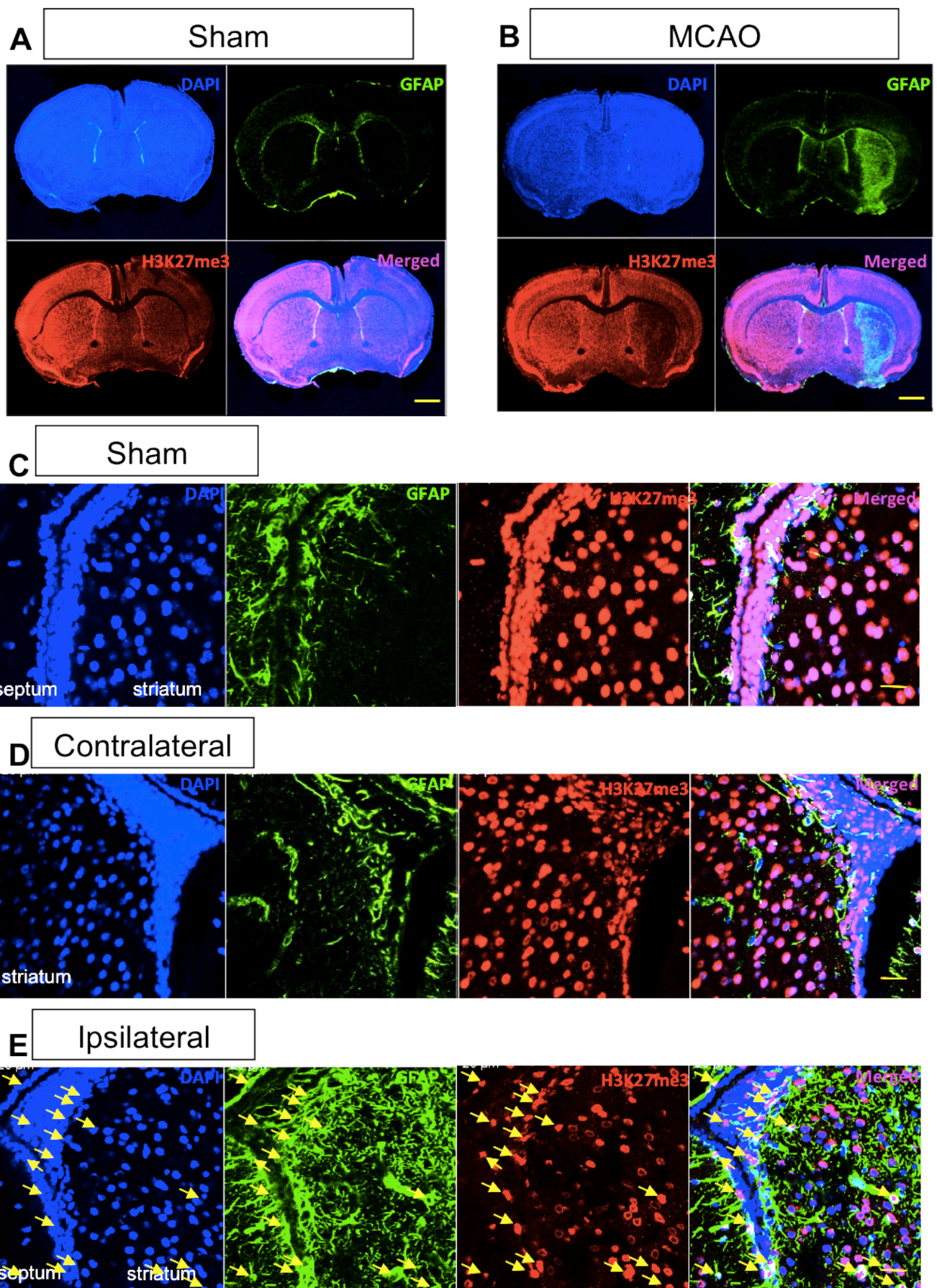
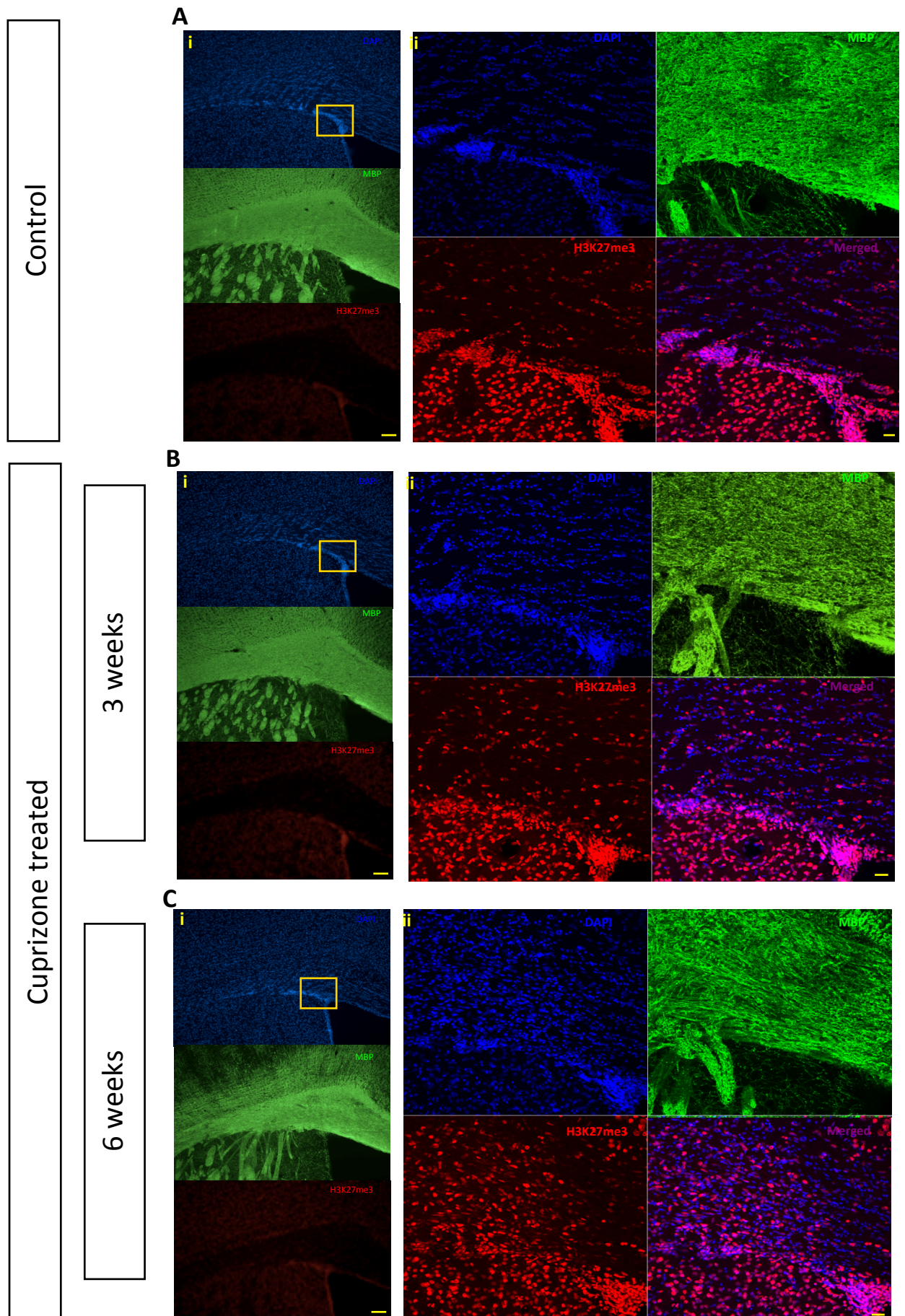


Figure 4-9. H3K27me3 expression in the SVZ after stroke.

Seven days after MCAO, brains H3K27me3 and GFAP double immunohistochemistry was carried out. The number of H3K27me3 immunopositive cells was 83% decreased in the ipsilateral striatum compared to sham mice (A,B). Higher magnification images also indicated the percentage of H3K27me3 positive cells per DAPI positive cells was significantly less in MCAO SVZ compared to the sham (C-E). GFAP positive cells in the ipsilateral to MCAO were significantly increased compared to sham controls, and majority was co-localized with H3K27me3 (*yellow arrows*) Interestingly, non-GFAP positive cells were lacking H3K27me3 signal in the center of the nucleus. Scale bars = 1mm (A,B) and 20 μ m (C-E) (All the data are from n=3 experiments.)

demyelination and remyelination of white matter (Kipp et al., 2009). After feeding cuprizone for 3 weeks, white matter tracks were demyelinated detected using myelin basic protein (MBP) immunostaining (Figure 4-10B). Also, SVZ had fewer PH3 positive cells (data not shown), while BrdU positive cells were increased (Kipp et al., 2009). After 6 weeks of cuprizone treatment, the demyelination process has been taken further in cortex, corpus callosum and striatum (Figure 4-10C). Since these two different time points have shown the significant biological effects in brain demyelination, we attempted to detect H3K27me3 by immunohistochemistry at both time points and quantified the percentage of H3K27me3 positive cells per DAPI positive cells (Figure 4-10D). Compared to the control groups ($27.14\% \pm 4.37$) in corpus callosum, the percentage of H3K27me3 positive cells per DAPI positive cells showed a slight increase at 3 weeks ($29.95\% \pm 1.89$) and a large and significant increase at 6 weeks ($40.56\% \pm 2.82$, $p < 0.05$) whereas SVZ and striatum did not show any obvious difference (Figure 4-10B-D). As the further demyelination was progressed in a brain, the more significant H3K27me3 positive cells were present in the corpus callosum (Figure 4-10A-C).



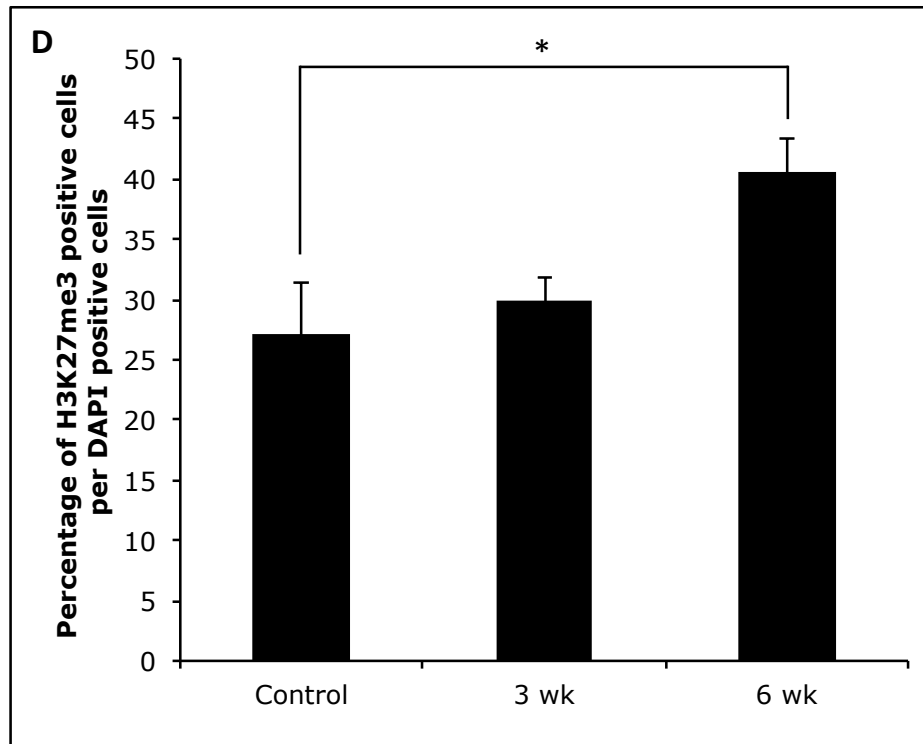


Figure 4-10. H3K27me3 expression in the SVZ after cuprizone induced demyelination.

(A-C) After feeding of cuprizone to young adult mice for 3 and 6 weeks, decreased myelination level was detected with MBP immunoreactivity (B and C respectively). They were compared to wild type control groups (A). (i) images were taken with a 5X objective lens, and (ii) images were taken at higher magnification objective lens at the dorsolateral ventricle of SVZ (yellow box indicated in image i). Merged images excluded MBP staining. Scale bars = 160 μ m (i) and 20 μ m (ii).

(D) More H3K27me3 positive cells were found in corpus callosum after 3 weeks of cuprizone treatment, and the number of H3K27me3 positive cells was significantly increased after 6 weeks. ($p < 0.05$)

(All the data are from n=4 experiments)

4.3. Discussion

4.3.1. H3K27me3 In Postnatal And Adult SVZ And In Disease Models

H3K27me3 was found in various different parts of the postnatal and adult brain. Most brain regions expressed similar levels of H3K27me3 in postnatal compared to adult brains. However the striatum and SVZ contained somewhat fewer H3K27me3 positive cells in postnatal mice compared to adults. After comparing the H3K27me3 marks with different SVZ cell markers, H3K27me3 did not seem to be restricted to a specific cell type. Therefore it is difficult to use it as a marker for identifying individual SVZ cell types within the postnatal and adult SVZ. However, it was consistently seen that neuroblasts in both postnatal and adult SVZ expressed lower levels of H3K27me3.

As mentioned in Chapter 1, many lineage specific genes including neuronal genes (*Dlx*, *Neurog*, *Fabp7*, *Cp*, *Gpr56*) commonly have both H3K4me3 and H3K27me3 in their promoter regions (Azuara et al., 2006; Bernstein et al., 2006; Mikkelsen et al., 2007), and their expression levels are regulated by a bivalent mechanism. Previously Hirabayashi et al. in 2009 demonstrated that loss of H3K27me3 drives SVZ NSPCs to neurogenesis rather than gliogenesis (Hirabayashi et al., 2009). This result was also confirmed by conditionally floxed *Mll1* in postnatal SVZ NSPCs as a H3K4me3 catalase for bivalent mechanism (Lim et al., 2009). Lim et al. in 2009 demonstrated that the *Mll1* normally promotes H3K4me3 on the *Dlx2* gene promoter to enhance neurogenesis in the postnatal SVZ. However, after *Mll1* was depleted, the neuronal differentiation was attenuated as a result of lacking H3K4me3 level. Thus, the regulation of SVZ neurogenesis seems to be associated with the bivalent mechanism, and the reduction of H3K27me3 level in both postnatal

and adult SVZ neuroblasts provides a possible tool for distinguishing the neuronal cells from glial cells.

4.3.1.1. Reactive astrocytes have high levels of H3K27me3 after stroke

In MCAO brains, the infarct area had reduced H3K27me3 marks, whereas GFAP immunoreactivity was highly detected. Although we do not know the identity of cells with lower signal of H3K27me3 marks, we could possibly speculate that loss of H3K27me3 mark positive cell numbers were due to NeuN positive cell death demonstrated by Young et al. 2012 (Young et al., 2012). Also, the rest of the brain including the ischaemic penumbra showed the reduction of H3K27me3 marks after stroke, although the GFAP positive cells in the ischaemic penumbra had higher H3K27me3 marks. This suggests large numbers of cells are potentially de-repress the expression of global or specific genes in the brain in response to brain lesion. Especially, SVZ neuroblasts, after the strokes, migrate toward the infarct area and give rise to neurons (Young et al., 2012), so the loss of H3K27me3 levels in SVZ cells could be for neurogenesis enhancement in the SVZ via bivalent mechanism proposed by previous studies (Hirabayashi et al., 2009; Lim et al., 2009).

4.3.1.2. Increased the number of H3K27me3 positive cells after cuprizone treatment

In the progress of demyelination, I noticed enhanced H3K27me3 positive cells in the corpus callosum (Figure 4-10). I speculate that the identity of the H3K27me3 positive cells could be oligodendrocyte precursor cells (OPCs) based on SVZ neural stem cell (NSC) being oligodendrocyte-genic in deficient Mll1 mice study (Lim et al., 2009). After Mll1 was removed in SVZ NSCs at E13.5, Lim et al. in 2009 showed enhancement of the oligodendrocyte production from SVZ NSCs *in vivo*, due to cell

fate shift from neuronal production *in vitro* (Lim et al., 2009). The immunohistochemical detection of Olig2 in the Mll1 deficient mice brain indicated large numbers of OPCs were present in corpus callosum rather than SVZ and striatum. Since the OPCs were found in corpus callosum in previous cuprizone treated mice (Harsan et al., 2008; Kipp et al., 2009), I speculate the level of H3K27me3 could possibly determine oligodendrocyte production from SVZ NSPCs. Also, the PRC2 and TrxG functions on bivalent gene could be possibly implicated on the postnatal SVZ NSPC oligodendrogenesis.

4.3.2. Polycomb Repressive Complex 2 Expression In The Postnatal SVZ

4.3.2.1. *In vivo* PRC2 and the transcription factors

I have first shown the expression of PRC2 core components including Jarid2 are present in the postnatal SVZ. Yet, some of their regulatory transcription factors are already known to be expressed in postnatal or adult SVZ and have crucial roles for regulating the SVZ NSPCs.

The expression of Oct4 has previously been shown to be diminished during ESC differentiation (Landeira et al., 2010; Pasini et al., 2010; Shen et al., 2009), so it is not a surprise that its expression became very limited in postnatal brains. Although I have shown a small quantity of Oct4 mRNA expression in both FACsorted adult SVZ stem/progenitor cell pure population and with *in situ* hybridization, its protein level could prove to be difficult to detect from postnatal SVZ tissue and neurospheres. By using whole mount technique with Oct4-GFP transgenic mice, van der Kooy's laboratory in University of Toronto have found that 3-4 cells per lateral ventricle side of adult SVZ were GFP positive (the unpublished data presented at the 34th annual meeting of Japan neuroscience society in 2011). Otherwise, Oct4 expression could be

found in embryonic SVZ neurospheres with Nanog as one of the master regulators for stemness (Ziegler et al., 2012). Because the lack of Oct4 expression in the postnatal and adult SVZ NSPCs could be a reason why the stemness is attenuated in SVZ NSPCs, several research groups have overexpressed Oct4 in SVZ neurospheres and possibly generated induced pluripotent stem cells (iPSCs) (Deleidi et al., 2011; Tatsumi et al., 2011).

In contrast, Sox2 was highly expressed its mRNA in SVZ NSPCs. Previously it has been demonstrated that its function in SVZ NSPCs is important for the NSPC self-renewal activity by Sox2 knock-in, knock-out, conditionally knock-out (flox-out), and overexpression experiments (Collignon et al., 1996; Miyagi et al., 2006; Wood and Episkopou, 1999; Zappone et al., 2000).

Although cMyc is known as be an oncogene that acts as a transcription factor, I have shown that its expression was also found in postnatal SVZ tissue and neurospheres as well as all the adult SVZ cell types. Previously Perez-Juste et al. in 2000 found that the endogenous expression of cMyc in SVZ NSCs is regulated by thyroid hormones (Perez-Juste et al., 2000). After knocking out and overexpressing cMyc in the rat embryonic NSCs in forebrain, cMyc was shown to have a critical role for regulating the NSC self-renewal property mediated by p53/p16Ar (Nagao et al., 2008). Also its function in cerebellar NPCs was similar to nMyc, which regulates the NPC growth (Hatton et al., 2006; Knoepfler et al., 2002). Although cMyc function in postnatal and adult SVZ is still unclear, I may speculate its role might be implicated in the process of SVZ NSPC self-renewal and cell cycle regulation, based on the previous findings.

I have shown the mRNA expression of Klf4 in postnatal SVZ neurospheres and adult SVZ neuroblasts but not from postnatal SVZ tissue and adult SVZ NSPC

population. Similar to my results, a recent study has shown that Klf4 protein level was highly expressed in embryonic brain but its expression was gradually decreased postnatally (Qin et al., 2011). By enhancing Klf4 expression during embryonic stage, the NPC proliferation was inhibited and SVZ NSC self-renewal was attenuated *in vivo* and *in vitro*. Therefore, this suggests its role for postnatal and adult SVZ NSPC stemness might be less crucial. However, Klf4 overexpression in MEFs could support to make iPSCs (Takahashi and Yamanaka, 2006; Wernig et al., 2007). In sum, Klf4 function can be different at various cell types and tissues.

Based on the four transcription factors expression and function in the SVZ, it was not surprising to see the expression of PRC2 in the postnatal SVZ. However, those four transcription factors expression levels from SVZ neurospheres *in vitro* could be varied due to the usage of different NSC culture protocol (Kim et al., 2008). The PRC2 transcriptional levels were correlated to the PRC2 translational levels except Jarid2 whose mRNA level was approximately 4.5 folds increased whereas its protein level was 7 folds decreased. Recently, Beck et al. in 2011 found that Jarid2 can be post-transcriptionally modified by 19 different miRNA (Beck et al., 2011). Although the difference between Jarid2 mRNA and protein levels could be possibly explained by endogenous RNA interference mechanism, the identity of the miRNA, that directly regulate Jarid2 expression in SVZ, has yet to be identified.

I also found the PRC2 mRNA expression in postnatal striatum where significantly less than in postnatal SVZ tissue. Previous PRC2 studies have indicated that the normally high expression of PRC2 components in the stem cell population was decreased while the stem cells were differentiating (Peng et al., 2009; Surface et al., 2010). Since there is no stem cell population resident in postnatal striatum, it is

not unexpected that there is a lack of H3K27me3 signals and PRC2 mRNA levels in striatum, as compared to the stem cell niche environment.

4.4. Conclusion

In postnatal and adult brain, the H3K27me3 signal was not restricted to any specific anatomical subregion although its level was found to be different in cortex, striatum, septum, and SVZ. This variation in levels of H3K27me3 seemed to be consistent from one brain to another, but it could be altered after the brain injury and disease. The main components of the H3K27me3 generating protein complex, PRC2 and its regulatory transcription factors were found to be expressed in the postnatal SVZ. It is still unclear whether Eed, Ezh2, Suz12 and Jarid2 are working as PRC2 in the postnatal SVZ cells, yet their expression levels are similar to those observed in ESCs.

Chapter 5

The Function of Jarid2 And Eed In SVZ Neurosphere Self-Renewal And Differentiation

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Chapter 5

The Function Of Jarid2 And Eed In SVZ Neurosphere Self-Renewal And Differentiation

5.1. Introduction

5.1.1. Jarid2: A Novel PRC2 Accessory Gene For The PRC2 Recruitment

The role of PRC2 as a regulator for balance between self-renewal and differentiation in ESCs has been implicated in other tissue specific stem cells including in the cerebral cortex, epidermis, spinal cord, thymocyte, limb, cardiac muscle, hepatocytes, preadipocytes, skeletal muscle, central forgut and small intestine (Akizu et al., 2010; Aoki et al., 2010; Benoit et al., 2012; Caretti et al., 2004; Delgado-Olguin et al., 2012; Ezhkova et al., 2011; Ezhkova et al., 2009; He et al., 2012; Hirabayashi et al., 2009; Juan et al., 2011; Pereira et al., 2010; Richie et al., 2002; Stojic et al., 2011; Wang et al., 2010; Wyngaarden et al., 2011; Xu et al., 2011). In order to regulate these stem cell properties by controlling PRC2 activity, it is necessary to understand how PRC2 recognizes and binds target sites. In *Drosophila*, the first identified PRC2 DNA binding sites were called polycomb response elements (PRE) and were regulatory regions of homeogenes (Chan et al., 1994; Fauvarque and Dura, 1993; Kassis, 1994). Although PRC2 localized to PRE, it was not directly bound to the DNA sequence. The Pho repressive complex (Pho-RC), composed of (1) specific DNA sequence binding protein, Pleiohomeotic (Pho) and (2) Scm-related protein containing four MBT domains (Sfmbt), was directly bound to the PRE and recruited PRC2. This mechanism has not been found in mammalian systems prompting the need to find other ways of recruiting PRC2 to DNA. Three possible mechanisms have been proposed: (1) the Pho homologous gene, called yin yang1 (YY1), could possibly mediate the recruitment of PRC2 (Thomas and Seto, 1999) although YY1 and PRC2

were not directly associated with each other (Li et al., 2010) and did not share their DNA binding sites in ESCs (Squazzo et al., 2006), (2) Jarid2, a member of the Jumonji family, has direct protein-protein interactions with PRC2, and its DNA binding sites were similar to PRC2 target sites in ESCs (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009) and (3) non-coding RNAs could recruit PRC2 to specific target sites (Pandey et al., 2008; Ponting et al., 2009; Rinn et al., 2007; Zhao et al., 2008b), but this represented specific rather than global gene regulation.

With the recent discovery of Jarid2 as a novel accessory gene for PRC2 function in ESCs, it was debated whether Jarid2 dependent PRC2 function was limited to ESCs or also utilized in other cell types (Surface et al., 2010). In fact, a recent study proved that Jarid2 deficiency in postnatal epidermal stem cells could result in the reduction of H3K27me3 (Mejetta et al., 2011). Recently, Jarid2 in *Drosophila* was found to affect PRC2 activity as an enzyme for catalyzing H3K27 trimethylation, and there was also a hint of Jarid2's PRC2 independent function (Herz et al., 2012). Therefore, Jarid2 could possibly mediate PRC2 function in various different mammalian tissues.

5.1.2. Lentiviral Mediated Deliberly Of Short Hairpin RNA For Knockdown Of A Gene Expression In SVZ Neurospheres

RNA interference (RNAi) is one of the major techniques used to suppress expression of target genes (Fire et al., 1998). Originally, it was found as a defense mechanism against viral infections or to suppress risk genes by using endogenous small non-coding RNAs, called micro RNAs (miRNAs) (Baulcombe, 2004; Katze et al., 2002; Voinnet, 2005). Now, it has become a transformational tool for understanding the

function of target genes via introducing chemically synthesized double stranded small or short RNAs into cells.

The endogenous RNAi mechanism is initiated from the host genome miRNA sequence (Figure 5-1). The transcribed miRNA sequence becomes a pre-microRNA (pre-miRNA), whose hairpin structure is cleaved by Drosha. The stem-loop RNA like short-hairpin RNA (shRNA) is exported to the cytoplasm via Exportin-5. The looped configuration in shRNA or miRNA is cleaved by Dicer, an enzyme that cleaves double stranded RNA (Scherer and Rossi, 2003). This eventually results in 21-23 base pair long stranded nucleotides as mature miRNA-like small interfering RNAs (siRNA) in cytoplasm. This is now able to bind with the RNA-induced silencing complex (RISC) to mediate sequence specific degradation (Doench et al., 2003; Hutvagner and Zamore, 2002; Zeng et al., 2002) or post transcriptional repression of complementary mRNA (Fountaine et al., 2005; Manfredsson et al., 2006; Rana, 2007; Tuschl, 2002).

In the general application of RNAi, exogenous siRNA or shRNA is usually delivered into cells by lipid-based transfection and by plasmids or recombinant viruses respectively. SiRNAs could be delivered as a naked siRNA or lipid-complexed transfection to provide sufficient knockdown, but gene silencing is transient due to short siRNA half-lives (Couto and High, 2010). Instead of using endogenous miRNA pathways, siRNA concentrations need to be optimized in order to achieve sufficient knockdown of the target gene as well as to avoid off-target effects (Rao et al., 2009). Another alternative method would be electroporating a DNA plasmid expressing the shRNA sequence, but it is a transient knockdown effect from the transfected cell population.

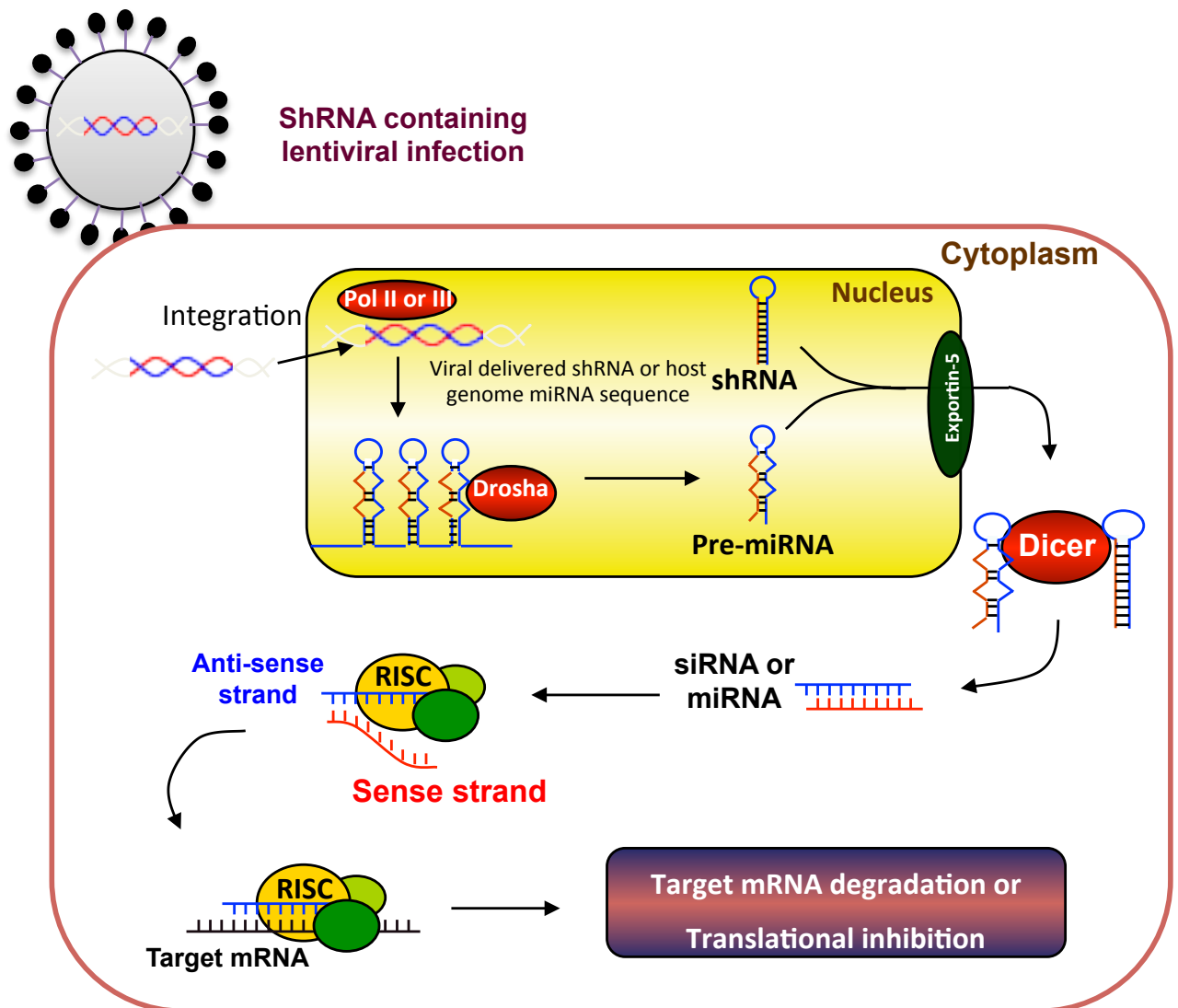


Figure 5-1. RNA interference (RNAi) by lentiviral infection.

Infection of lentivirus containing gene specific shRNA causes viral gene integration into the host genome and shRNA synthesis mediated via endogenous miRNA. The gene specific shRNA sequences bind to the target gene mRNA and either degrades it or inhibits translation. This results in knockdown of the target gene.

However, lentiviral-mediated delivery of shRNA resolves the transient gene silencing problem by integration of shRNA sequence to the host genome. In addition, lentivirus can infect both mitotic and non-mitotic cell populations, so this method allows targeting of not only dividing cells but also differentiated neurons, astrocytes and oligodendrocytes (Manjunath et al., 2009). Lastly, when the lentivirus infects actively dividing cells, the integrated shRNAs are carried on to daughter cells unlike the other viral or plasmid based methods of shRNA delivery (Van den Haute et al., 2003). Therefore, one event of lentiviral infection can result in knockdown effects from all the daughter cells.

Previously several researchers have taken advantage of the lentiviral method to target the heterogeneous cell population of the SVZ *in vivo* in order to understand the function of various genes such as transcription factors, cell receptors, epigenetic regulators, metabolic enzymes, cell adhesion molecules, soluble/secreting molecule, adapter molecule and etc. (Costa et al., 2008; Fasano et al., 2007; Kokovay et al., 2010; Kokovay et al., 2012; Mahairaki et al., 2009; Mao et al., 2009; Mao and Lee, 2005; Niola et al., 2012; Paez-Gonzalez et al., 2011; Patel et al., 2009; Putz et al., 2005).

As SVZ neurospheres are composed of rapidly dividing progenitor cells, quiescent neural stem cells and post-mitotic differentiated cells, transient transfection would not be suitable for obtaining stable and long-term knockdown effects. Thus, lentiviral-mediated delivery of shRNA provides the advantage of potentially infecting all three types of cells in the neurospheres and achieving knockdown in them as well as their progeny, which carry the shRNA sequence in their genome.

5.1.3. Research objectives

Jarid2, a novel gene that leads PRC2 to target sites, could regulate PRC2 mediated methylation of H3K27me3 in *Drosophila*, *Xenopus* and mammalian ESCs (Herz et al., 2012; Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009). This mechanism was also found in epidermal tissue at postnatal stage (Mejetta et al., 2011), yet it has not been shown in other postnatal and adult systems.

As described in Chapter 4, H3K27me3 was present in both postnatal and adult SVZ. Also, both mRNA and protein of PRC2 core components and Jarid2 were found in postnatal SVZ neurospheres. Therefore, the follow-up questions were (1) what could PRC2 function be in the self-renewing and differentiation process of postnatal SVZ neurospheres? (2) is PRC2 function dependent on Jarid2 in the postnatal SVZ neurospheres? (3) what is the possible function of Jarid2 in SVZ neurospheres if PRC2 function is independent from Jarid2? To elucidate these questions, I have utilized lentiviral-based shRNA to knockdown Eed and Jarid2.

5.2. Results

5.2.1. Characterization Of Jarid2 And Eed shRNA Lentivirus

5.2.1.1. Validation of lentiviral construct and Turbo-GFP detection for titration

After confirming that both transcriptional and translational expression of PRC2 were present in postnatal SVZ neurospheres, I investigated the function of PRC2 in neurospheres by down-regulating Jarid2 (an accessory gene for PRC2) and Eed (a core component of PRC2). The Jarid2 and Eed specific shRNAs were obtained from Sigma-Aldrich and from the Brockdorff laboratory in University of Oxford, respectively. Jarid2 and scrambled shRNA (also referred as scramble shRNA in figures) constructs were previously validated in an ESC study (Pasini et al., 2010). The scrambled shRNA is a random combination of nucleotides, which provides no specific targeting effect. Jarid2 shRNA is located in murine exon 15, whereas Eed shRNA sequence is located in exon 12 (Figure 5-2; see detail information for shRNA sequence in table 5.1.). Both shRNA sequences were blasted against the mouse genome, which confirmed that both shRNAs specifically matched their corresponding targets (data not shown). All the shRNA sequences were engineered into a pLKO.1 lentiviral backbone shown in figure 5-3A. To validate the DNA plasmid constructs carrying scrambled, Jarid2 and Eed shRNAs, they were treated with ApaLI and Afl II restriction enzymes. The correct sizes of digested DNA fragments were separated and visualized by agarose gel electrophoresis (Figure 5-3B). In addition, pMD2.G (an envelop plasmid) and psPAX2 (a packaging plasmid) were also digested by EcoRI/KpnI and ApaLI/EcoRI restriction enzymes, respectively (Figure 5-3B). After calcium phosphate transfection of a cocktail of these plasmids, infectious lentiviruses for individual shRNA were produced. These lentiviruses were initially generated from a large quantity of HEK 293 FT cells and ultra-centrifuged to increase viral titer.

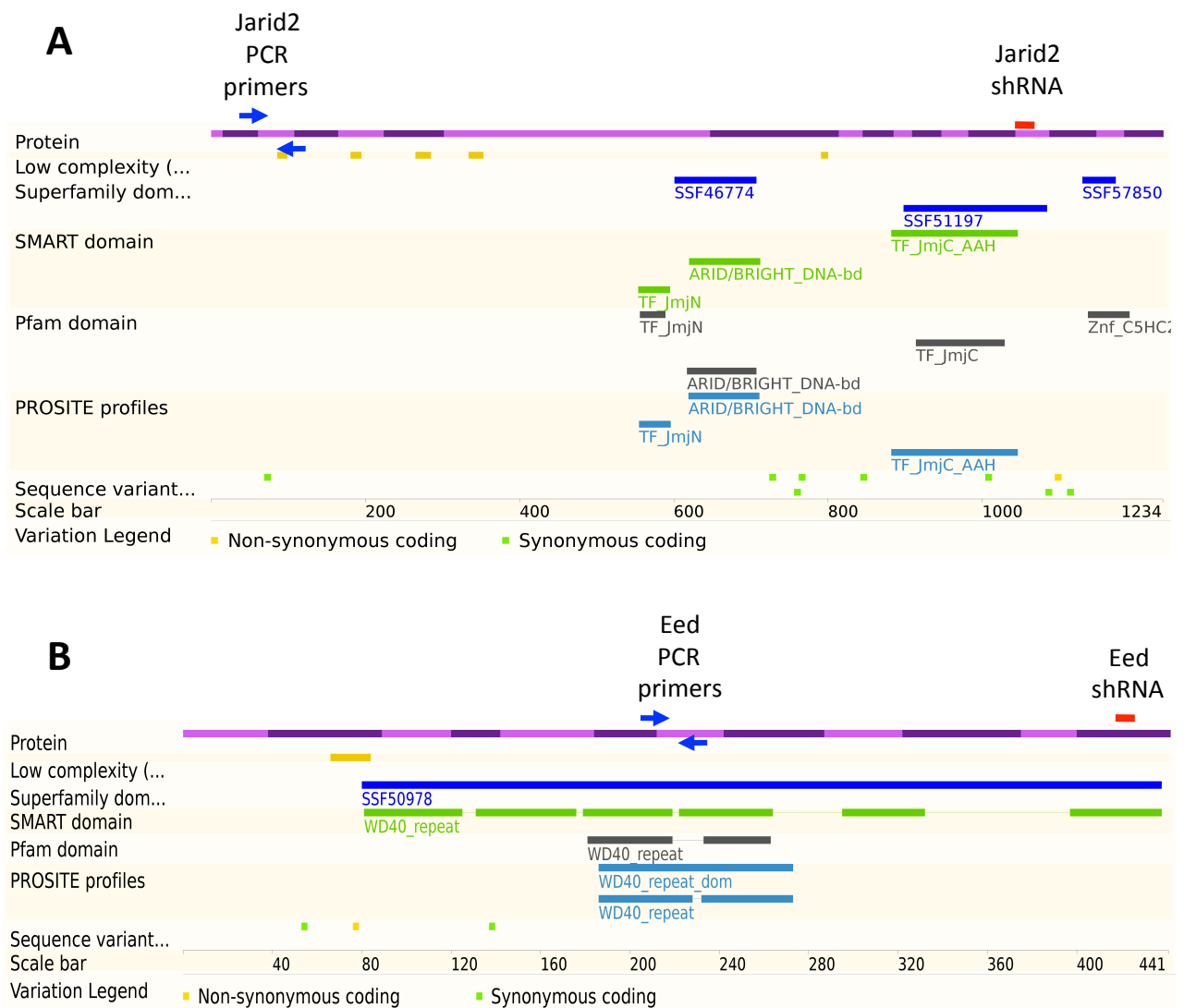


Figure 5-2. Murine Jarid2 and Eed genes with shRNAs target sites and primer sites.

(A,B) Jarid2 and Eed shRNA target Exon 15 and Exon 12 respectively. Both Jarid2 and Eed primers were designed to be bound in exon-exon junctions. The schematics were modified from *Ensembl*.

Table 5.1. shRNA sequences

Gene name	shRNA sequence (5' to 3')
Jarid2 shRNA	CCGGCCGCCATATAGCTAAGCCATTCTCGAGAATGGCTTAGCTATATGGCGGTTTTTG
Eed shRNA	CCGGTCTTGCTAGTAAGGGCACATACTCGAGTATGTGCCCTTACTAGCAAGATTTTTG

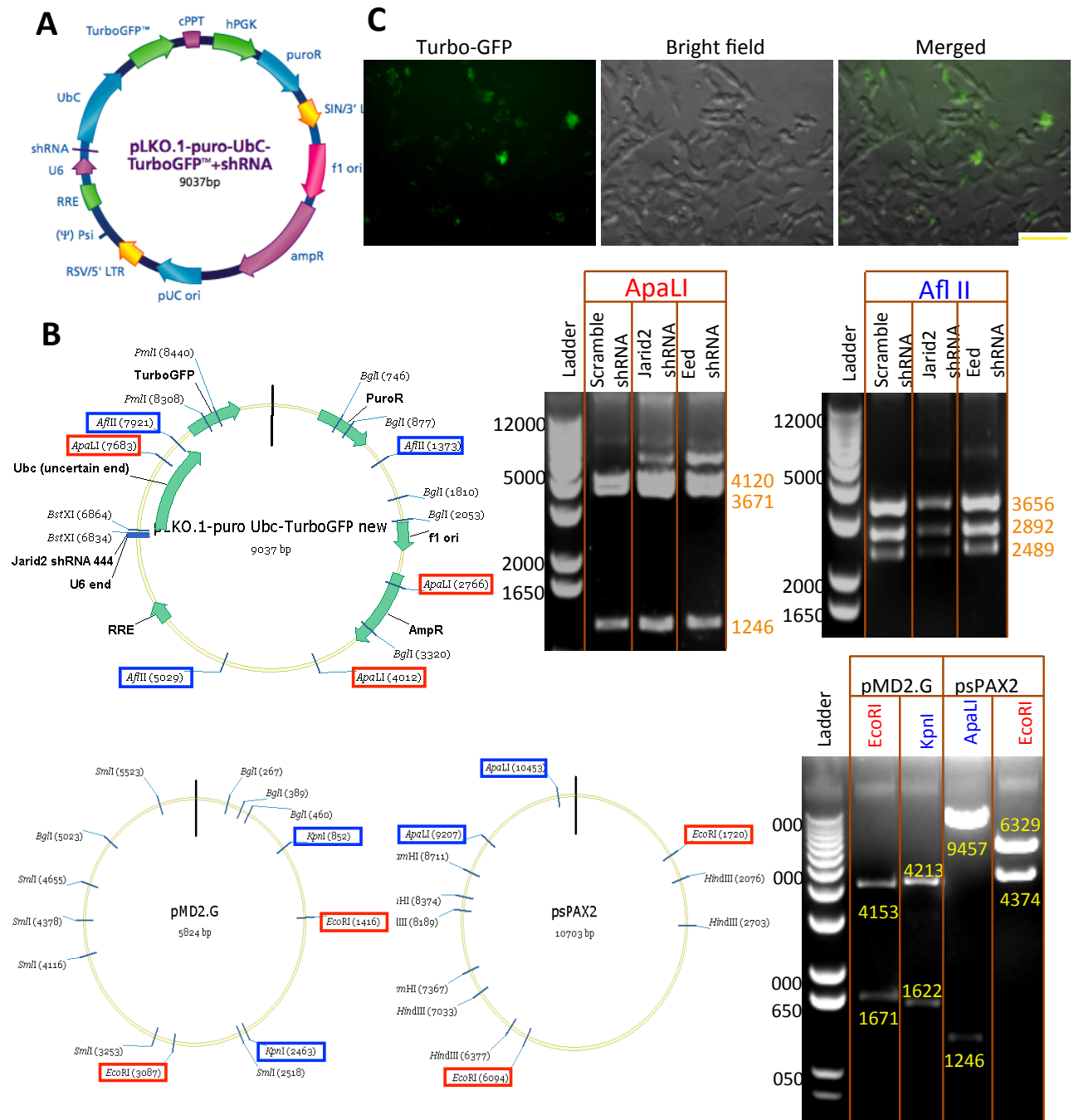


Figure 5-3. Jarid2 and Eed shRNAs construct validation and lentiviral infection.

(A) Schematic of lentiviral construct for shRNA delivery. (Adapted from Sigma-Aldrich.)

(B) Validation of constructs (pLKO.1, pMD2.G, psPAX2) with restriction enzyme digests. ApaLI and Afl II enzymes were used for pLKO.1 plasmids and EcoRI, KpnI and ApaLI were used for pMD2.G and psPAX2 plasmids.

(C) For lentiviral titration, a known amount of virus was used to infect HEK 293 cells and GFP positive cells were counted from the total. Scale bar = 50 μ m (All the data are from n=2 experiments)

After infection of lentiviruses to HEK 293 T cells, $21.5\% \pm 2.6$ of the total number of cells per well were Turbo-GFP positive 4 days post infection (dpi). After calculating these numbers according to the equation mentioned in 2.4.3., the titer of lentivirus was 8.75 million transducing units (TU) per milliliter.

5.2.1.2. Transient knockdown of Jarid2 and Eed in HEPA1-6 cell line

To validate both lentiviral infectivity and shRNA mediated knockdown in murine cells, the endogenous expression of PRC2 was screened with several cell lines including NIH 3T3 and NSC 34 (data not shown). Because primary culture cells such as the neurospheres are fragile and sensitive to external stimuli, it was more suitable to characterize the knockdown of both Jarid2 and Eed in immortalized cell lines, which are more homogeneous cell populations and more resistant to external manipulation.

HEPA 1-6 cells expressed abundant levels of endogenous Jarid2 and Eed, which were similar to ESC in both transcriptional and translational levels (Figure 5-4A). To examine the scrambled, Jarid2 and Eed shRNAs mediated knockdown, multiplicity of infection (MOI) 1 of the lentivirus was used to infect HEPA 1-6 cells but resulted in very few numbers of turbo-GFP positive HEPA 1-6 cells (data not shown). By infecting HEPA 1-6 cells with MOI 2 of each of the lentiviruses, the Turbo-GFP signal was detectable on 3 dpi (Figure 5-4B). Approximately 30-50% of the live cell population was turbo-GFP positive in lentiviral-infected wells. (The non-viral infected wild type control had no GFP positive cells.) In the bright field images of HEPA 1-6 cells after infection, there was obvious amount of cell loss in viral infected conditions, although equal number of single cells was seeded in each well at

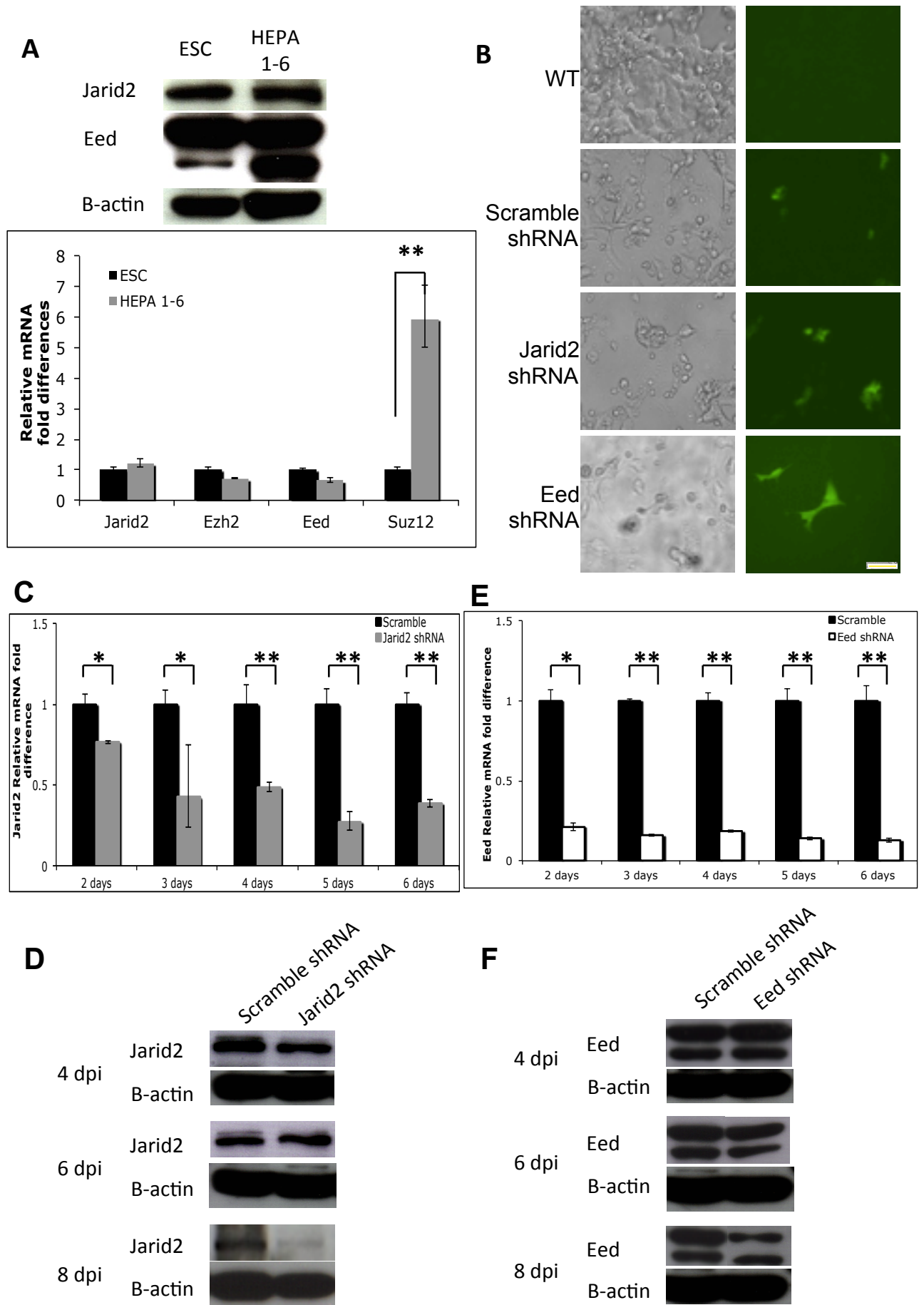


Figure 5-4. Jarid2 and Eed expression in HEPA 1-6 cell line and the efficiency of transient knockdown.

(A) The translational levels of Jarid2 and Eed (*top*) and transcriptional level of PRC2 (*bottom*) in the HEPA 1-6 cell line compared to ESC. ** $p < 0.01$ (n=3)
(B) Lentiviral infected HEPA 1-6 cells expressed Turbo-GFP at 3 dpi. Scale bar = 20 μm (n=3)
(C,E) Jarid2 and Eed transcriptional levels were detected every 24 hours from 2 dpi. * $p < 0.05$, ** $p < 0.01$ (n=3)
(D,F) Jarid2 and Eed translational levels were detected every 48 hours from 4 dpi. Approximately 40% of Jarid2 and 60% of Eed were knocked down in protein level at 8 dpi. (n=1)

the beginning (Figure 5-4B). Without further increasing MOI for lentiviral infection, the amount of gene suppression was measured by using qRT-PCR analysis.

The lentiviral infected HEPA 1-6 cells were harvested every 24 hrs from 2 dpi till 6 dpi, and Jarid2 and Eed transcriptional levels were quantified by qRT-PCR with 18s as an internal control. The Jarid2 knockdown resulted $23.7 \pm 7.0\%$ – $51.6 \pm 11.0\%$ suppression of endogenous Jarid2 mRNA at 2-3 dpi, compared to scrambled shRNA. This suppression was maintained until 6 dpi (Figure 5-4C). In contrast, the Eed knockdown showed $78.7 \pm 2.0\%$ suppression of endogenous Eed mRNA at 2 dpi and also maintained the suppression level until 6 dpi (Figure 5-4E). The protein levels of both Jarid2 and Eed were not noticeably decreased until 8 dpi as compared to scrambled control. The Jarid2 and Eed protein levels were approximately 40% and 60% suppressed respectively (Figure 5-4D,F). Therefore, both gene specific shRNAs knocked down their corresponding endogenous genes at both mRNA and protein levels.

5.2.2. Jarid2 And Eed Knockdown In SVZ Neurospheres

5.2.2.1. Efficient knockdown of Jarid2 and Eed in SVZ neurospheres

As the lentiviral infection increased cell death in HEPA 1-6 cells (Figure 5-4B), it was necessary to find the minimum amount of lentivirus with efficient knockdown of Jarid2 and Eed in neurospheres. After infecting MOI 1 of Jarid2 shRNA lentivirus to single cell dissociated neurospheres, the transcriptional Jarid2 level was not decreased at 4 dpi (Figure 5-5). However, the doubled amount of lentivirus (MOI 2) yielded 50% of Jarid2 knockdown at 4 dpi (Figure 5-5), which was similar to the amount of Jarid2 knockdown demonstrated in HEPA 1-6 cells (Figure 5-4C). Thus, the rest of the experiments were conducted with lentiviral MOI 2. At 3 dpi, Turbo-GFP was detectable in a subset of neurospheres (Figure 5-6A). At the same time, the amount of Jarid2 and Eed knockdown was measured by qRT-PCR, which indicated 77.1 ± 12.0 % and 50.5 ± 19.0 % reductions of Jarid2 and Eed respectively (Figure 5-6B,C).

5.2.2.2. The effects of Jarid2 and Eed knockdown in SVZ neurosphere self-renewal

After successfully knocking down Jarid2 and Eed in secondary neurospheres, neurosphere self-renewal was monitored to evaluate whether neurosphere number, size and cell viability were altered at 3 dpi.

Jarid2 knockdown in neurospheres yielded approximately 50% reduction of individual neurosphere size (56.38 ± 0.9 μ m) compared to the scrambled control (111.12 ± 21.0 μ m), but the number of neurospheres per starting 200,000 cells (4145 ± 153) was not significantly altered (Figure 5-6D,E). On the other hand, Eed knockdown resulted in a 57.1% reduction in the number of neurospheres per starting 200,000 cells compared to the scrambled control (2030 ± 363 and 3552 ± 329 respectively). In contrast the individual size of neurospheres (101.50 ± 5.2 μ m) did

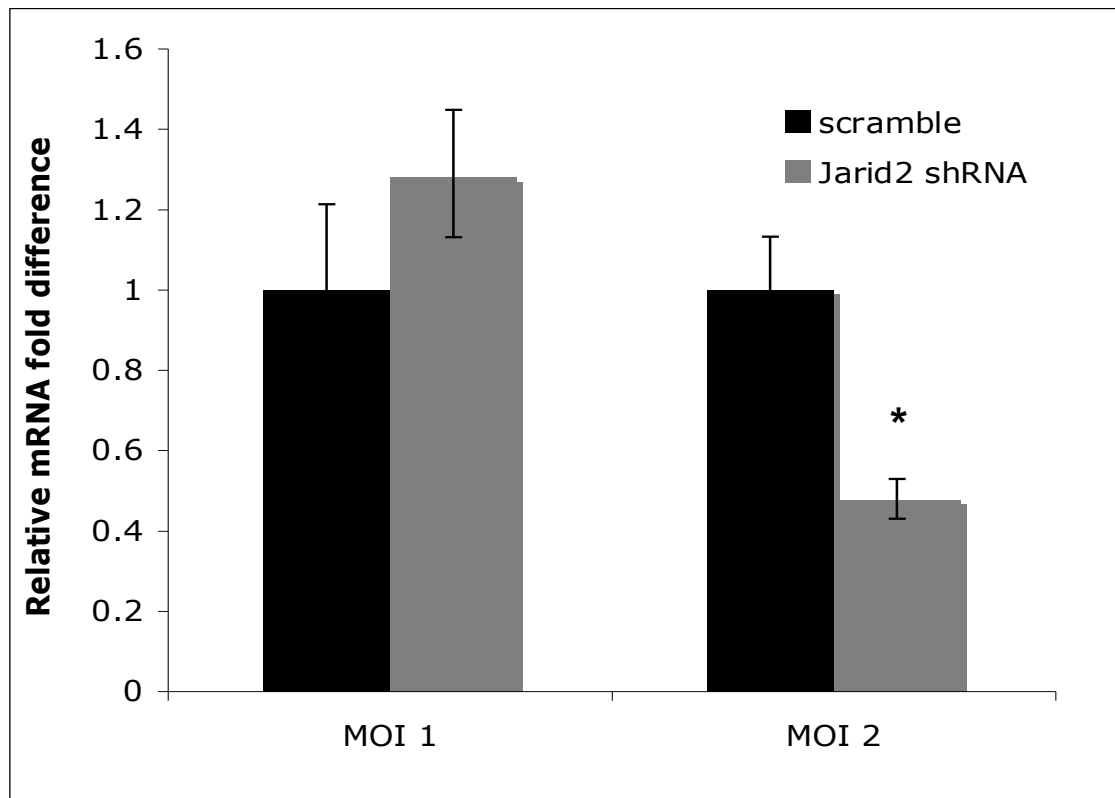


Figure 5-5. Titration of lentivirus in SVZ neurospheres.

Measurement of efficient knockdown with two different concentrations of lentivirus. MOI 1 did not show the significant suppression of Jarid2 mRNA, whereas MOI 2 resulted in 50% Jarid2 knockdown at the transcriptional level. * $p < 0.05$ (n=3)

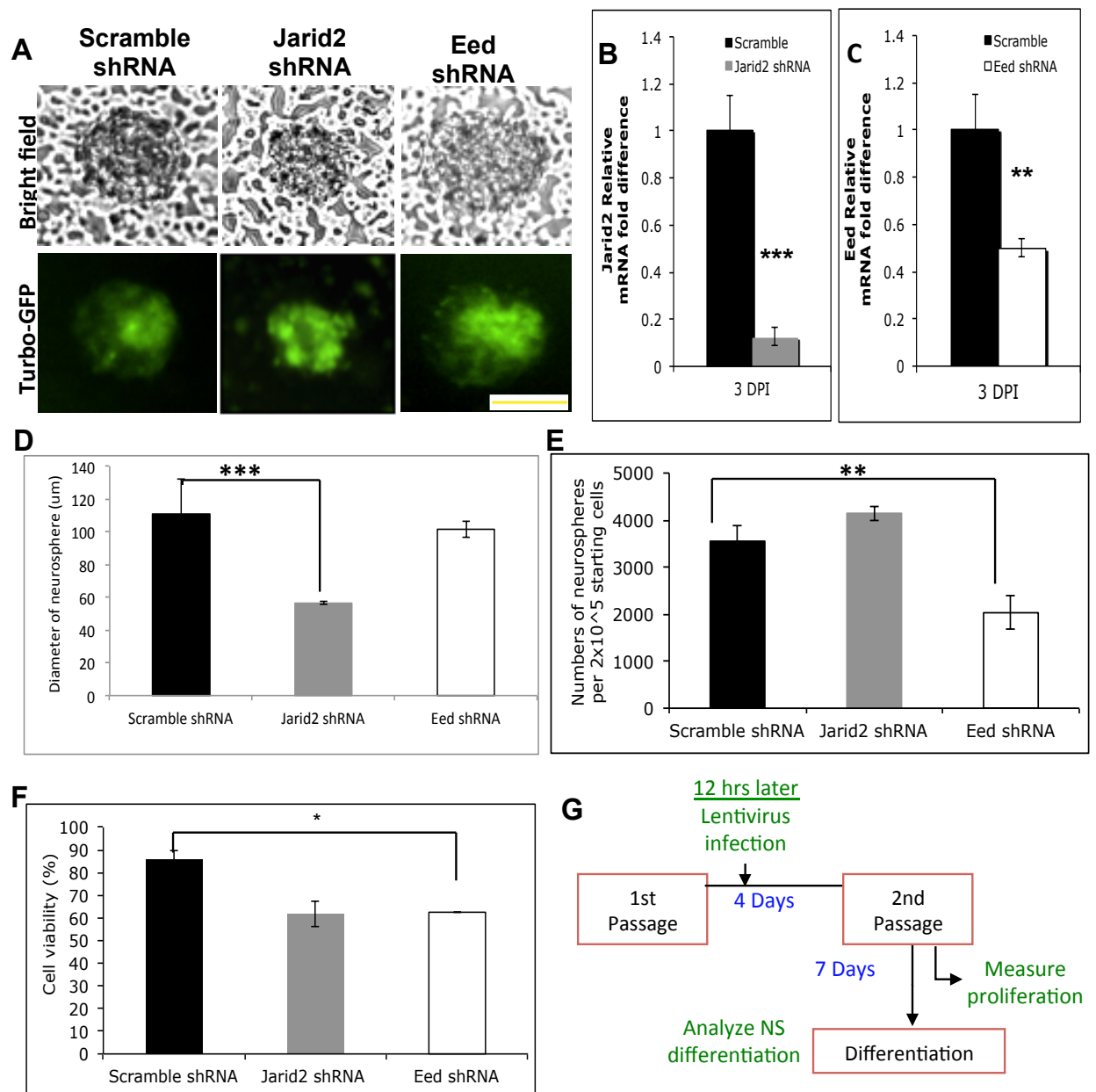


Figure 5-6. The effects of Jarid2 and Eed knockdown on secondary neurosphere self-renewal.

(A) The majority of cells in neurospheres were turbo-GFP positive. Scale bar = 80 μ m

(B,C) The amount of Jarid2 and Eed knockdown was measured at 3 dpi by qRT-PCR. *** $p < 0.001$, ** $p < 0.01$

(D) The diameter of neurospheres after Jarid2 knockdown was significantly decreased. *** $p < 0.001$

(E) The individual number of neurospheres were manually counted and show significant difference between scrambled and Eed knockdown neurospheres. ** $p < 0.01$

(F) Cell viability was measured using trypan blue exclusion. Both Jarid2 and Eed knockdown neurospheres had 20% loss of cell viability, but only difference between scrambled and Eed shRNA groups were statistically significant. * $p < 0.05$

(G) Schematic of experimental design (All the data are from $n = 3$ experiments.)

not change significantly after Eed knockdown (Figure 5-6D,E). By trypan blue exclusion, the scrambled control showed 86.0 ± 3.7 % cell viability, which did not differ from non-viral infected secondary neurosphere cell viability described in 3.2.1.4. (Figure 5-6F). In contrast, Jarid2 knockdown in neurospheres showed a small 24 ± 5.7 % decrease in cell viability compared to scrambled control, whereas Eed knockdown in neurospheres showed a 24 ± 0.4 % statistically significant decrease in cell viability (Figure 5-6F).

5.2.2.3. The effects of Jarid2 and Eed knockdown in SVZ neurosphere differentiation

To examine whether the function of Jarid2 and Eed could influence the process of SVZ neurosphere differentiation, I let neurospheres differentiate on laminin after Jarid2 or Eed knockdown for 7 days by withdrawing both EGF and FGF2. During neurosphere differentiation, they did not show any abnormal phenotypes (Figure 5-7A). After staining with differentiated neurosphere markers: O4, β III-tubulin and GFAP, the individual cell types were quantified. In the scramble control, the ratio of cell fate among three different lineages (O4: 5.6 ± 0.1 %, β III-tubulin: 14.8 ± 3.7 % and GFAP: 49.1 ± 4.9 %) was similar to the untreated neurosphere differentiation described in 3.2.2.2. (Figure 5-7B). Unexpectedly the Jarid2 lacking neurospheres did not seem to differentiate into any of the three different lineages (Figure 5-7A). By closely looking at the morphology of DAPI nucleus staining, they appeared to be ectopic nuclei with enlarged, crumbled and punctate shapes (Figure 5-7A). In contrast, the Eed lacking neurospheres could differentiate into three different lineages. The ratio for the cell fates (O4: 2.5 ± 0.2 %, β III-tubulin: 19.7 ± 4.5 % and GFAP:

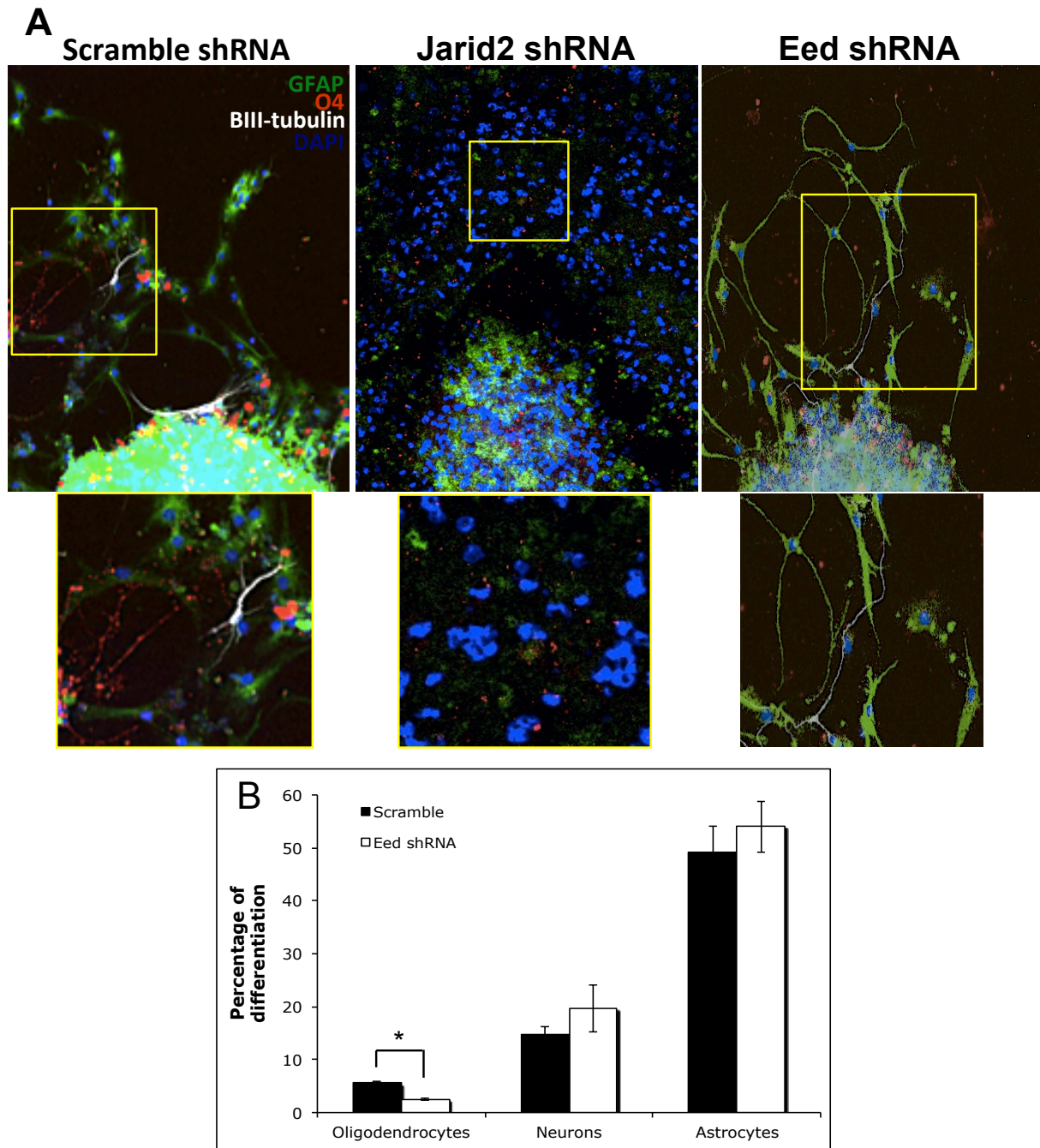


Figure 5-7. The effects of Jarid2 and Eed knockdown on secondary neurosphere differentiation.

(A) Triple immunohistochemistry on differentiated neurospheres that were infected with shRNA at the single cell stage. The higher magnification of the yellow boxes is represented below. Compared to the scrambled control, differentiated Eed knockdown neurospheres showed oligodendrocytes, neurons and astrocytes while Jarid2 (B) Oligodendrocytes (O4; red), neurons (β III-tubulin; white) and astrocytes (GFAP; green) were quantified from differentiated neurospheres. $*p < 0.05$ (All the data are $n=3$ experiments.)

54.0 ± 4.9 %) was similar to the scrambled control. However, the numbers of oligodendrocytes were significantly decreased (55.4% reduction) (Figure 5-7B).

5.2.3. Jarid2 And Eed Long-Term Knockdown In SVZ Neurospheres

5.2.3.1. Jarid2 and Eed knockdown in time course

In order to measure further downstream effects of Jarid2 and Eed knockdown in self-renewing neurospheres, the lentiviral-infected neurospheres were passaged to form tertiary neurospheres. Although it was not technically challenging to make tertiary neurospheres, Jarid2 transcriptional levels were similar to or were enhanced compared to the scrambled controls (Figure 5-8A). Moreover, the Jarid2 mRNA knockdown level seemed to vary within 2.5 – 4 dpi time windows (Figure 5-5 and 5-6B). For these reasons, I measured both Jarid2 and Eed transcriptional levels in secondary neurospheres every 24 or 48 hrs until 7 dpi without passaging. Figure 5-8 indicated that 37.0 ± 4.0 % of Eed mRNA knockdown in neurospheres was consistent without major changes during 7 days (Figure 5-8B). In contrast, Jarid2 transcriptional level was only significantly suppressed at 4 dpi, but its mRNA level gradually increased thereafter. Jarid2 mRNA level was similar to the scrambled control on 5 dpi and was overexpressed at 7 dpi (Figure 5-8A). At 6 dpi, the translational levels of Jarid2 and Eed were measured and their protein levels were correlated with the mRNA results (Figure 5-8C,D). To understand this result, I considered three possibilities: (1) cell selection of the heterogeneous SVZ cell types after Jarid2 suppression in the neurospheres, (2) shRNA translational repression rather than mRNA degradation or (3) external stimulation of Jarid2 transcription from the SVZ neurosphere culture environment.

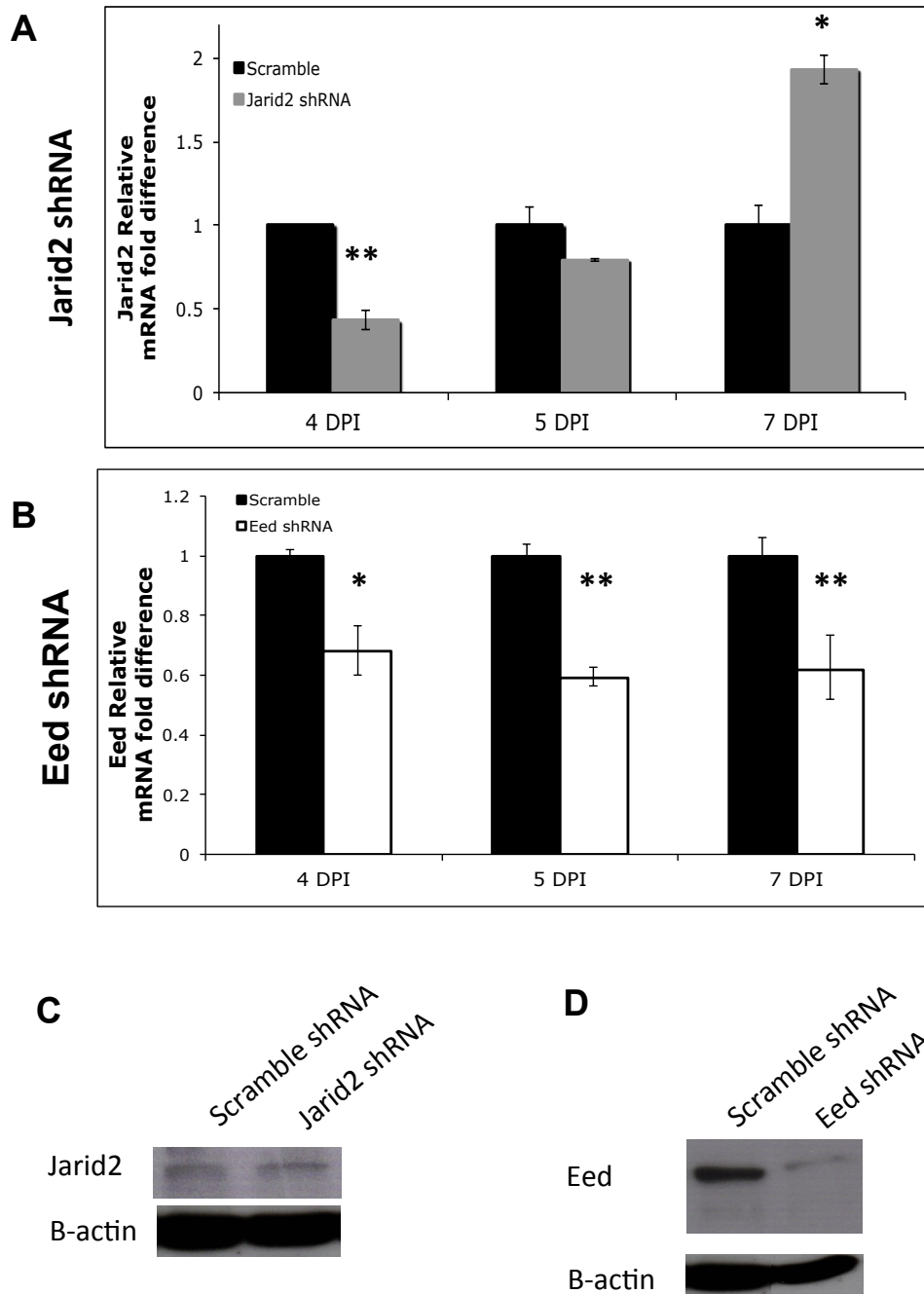


Figure 5-8. Long-term Jarid2 and Eed knockdown in SVZ secondary neurosphere.

The Jarid2 (A) and Eed (B) transcriptional levels were measured in 4, 5 and 7 dpi by using qRT-PCR. Eed shRNA seemed to suppress Eed mRNA level consistently while Jarid2 shRNA provided inconsistent knockdown after 5 dpi. On 7 dpi, the neurospheres infected with Jarid2 shRNA had higher Jarid2 transcriptional level than the scrambled control. * $p < 0.05$, ** $p < 0.01$ (n=3)

(C,D) Jarid2 and Eed protein levels was measured from 6 days post-infected neurospheres. The Jarid2 protein level was not distinctively different from scrambled shRNA control, while Eed protein level was 20% decreased by normalizing with B-actin as a loading control. (n=1)

5.2.3.2. The changes in SVZ cell markers after Jarid2 and Eed knockdown

Jarid2 expression was previously semi-quantitatively examined with FACsorted adult SVZ cell cDNAs obtained from Doetsch's laboratory in Columbia University (Figure 4-7) and found in every SVZ cell subtype. However, the data were not quantitatively analyzed, so it is still unclear if certain postnatal SVZ cell types express more or less Jarid2 and require its expression for different functions. Since SVZ neurospheres were composed of various SVZ cell populations (Figure 3-1K), it was interesting to investigate whether there was any particular cell type selection process among the mixture of neurosphere cell type populations after Jarid2 suppression. If the returning Jarid2 mRNA level after its knockdown was due to the selective cell populations, which can tolerate or overcompensate the loss of Jarid2, it was important to investigate the neurosphere cell type determination after Jarid2 knockdown. In addition, I also measured the different cell type markers' expression after Eed knockdown in neurospheres during self-renewal stage.

In order to measure the different cell type markers, I utilized qRT-PCR to detect the mRNA levels of SVZ cell type markers: Mash1 (transient amplifying cells), EGFR (stem/progenitor cells), GFAP (stem cells/astrocytes) and β III-tubulin (neuronal cells) (Pastrana et al., 2009). This approach has been validated by Pastrana et al. in 2009 in order to estimate the proportion of SVZ cell types in FACsorted single cells and neurospheres (Pastrana et al., 2009). For this experiment, cDNA was generated from lentiviral-infected neurospheres every 24 hrs from 3 dpi till 6 dpi since Jarid2 transcriptional level was dynamically changing during this period. In Jarid2 lacking neurospheres, Mash1 levels oscillated during the 4 days with variable increases at 3 and 5 days and a decrease at 6 days (Figure 5-9A). The EGFR mRNA expression levels in contrast were maintained until 5 dpi, but it was 39.4 ± 0.4 % decreased at 6

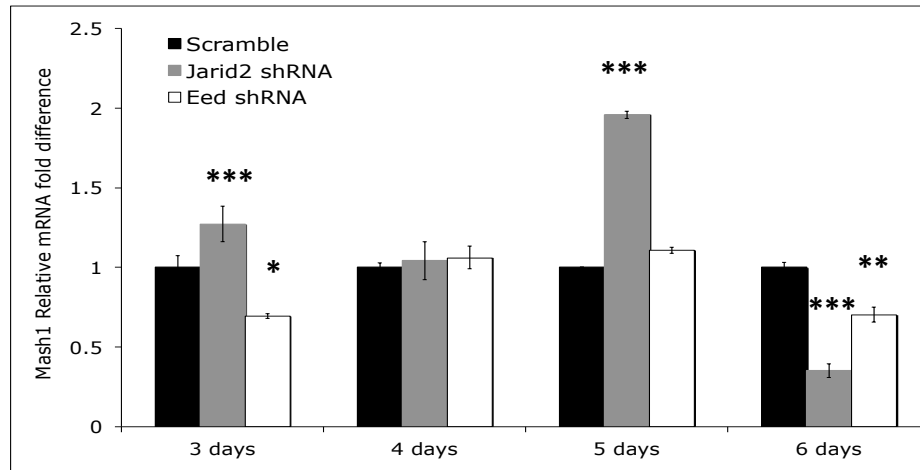
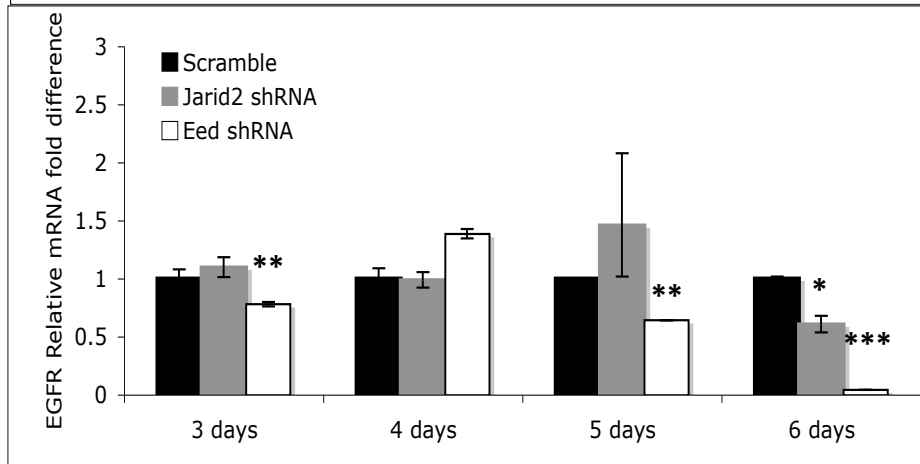
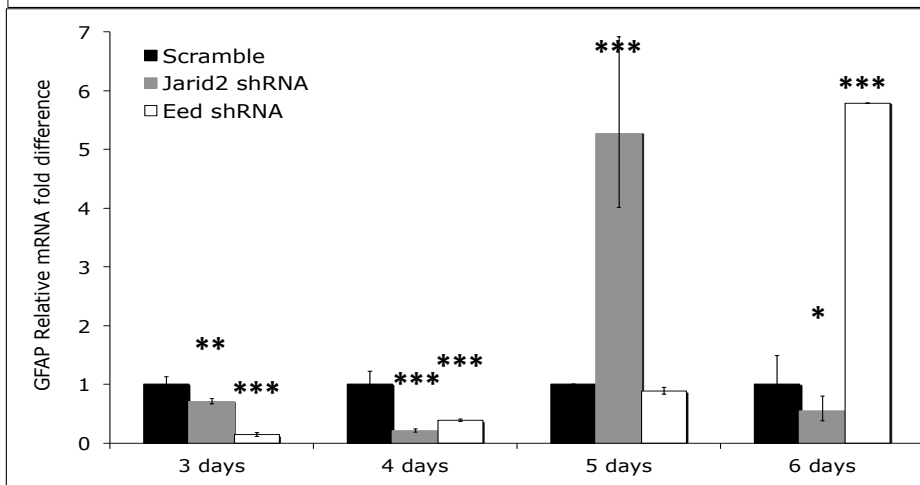
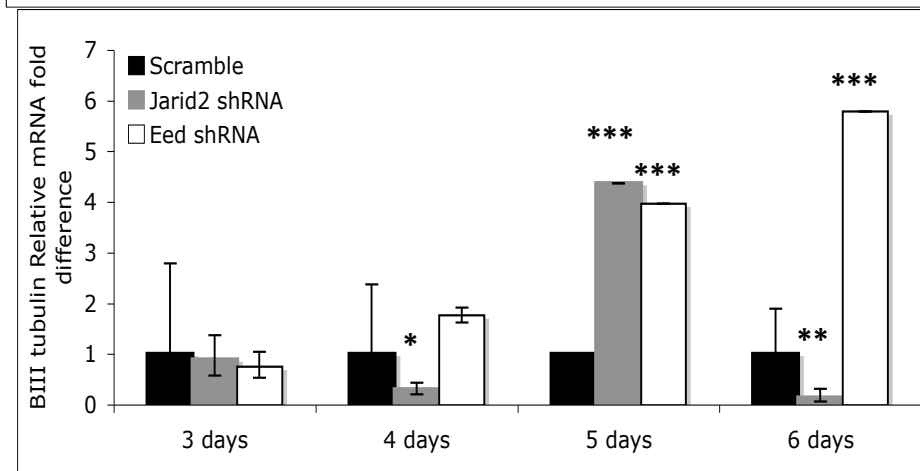
A**B****C****D**

Figure 5-9. Changes in SVZ cell type markers after Jarid2 and Eed knockdown in secondary neurospheres.

(A-D) The transcription level of SVZ cell type markers (Mash1, EGFR, GFAP and β III tubulin) was monitored every 24 hours from 3 until 6 dpi from infected neurospheres. Jarid2 knockdown caused mRNA of Mash1 to be oscillated while EGFR level to be consistently maintained until 5 dpi. For GFAP and β III tubulin, their mRNA was remarkably increased on 5 dpi but decreased dramatically on 6 dpi. Eed knockdown showed the consistent level of Mash1 until 5 dpi but gradually decreased with EGFR mRNA. Without biased fate choice, both GFAP and β III-tubulin mRNAs were gradually increased during 4 days. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (All the data are $n=3$ experiments.)

dpi (Figure 5-9B). Both GFAP and β III-tubulin expression levels were significantly decreased (78.1 ± 2.0 % and 61.4 ± 9.0 % respectively) at 4 dpi, but their levels were significantly increased at 5 dpi (5.54 ± 0.62 fold and 5.13 ± 0.01 fold respectively) but then decreased back down at 6 dpi (48.6 ± 8.0 % and 87.6 ± 7.0 % respectively) (Figure 5-9C,D). Interestingly, all the markers were significantly decreased after Jarid2 knockdown at 6 dpi.

In the Eed lacking neurospheres, Mash1 mRNA level was significantly decreased (26.9 ± 0.03 %) at 6 dpi, whereas EGFR level was gradually decreased from 4 dpi till 6 dpi (Figure 5-8A,B). In contrast, GFAP and β III-tubulin were gradually increased over the 4 days although the GFAP expression was significantly decreased (85.0 ± 0.5 % on 3 dpi and 59.6 ± 0.11 % on 4 dpi) on 3 - 4 dpi, but β III-tubulin expression was not (Figure 5-8C,D).

5.2.3.3. Colony selection and long-term knockdown of Jarid2 and Eed in HEPA

1-6 cell line

To confirm whether the inconsistent knockdown of Jarid2 mRNA was due to the heterogeneity of SVZ cell types or shRNA repressed translational processes rather than mRNA degradation, it was necessary to examine long-term knockdown efficiency from a 100% infected homogenous cell population. Since the lentiviral vector allowed for both the puromycin resistant gene and the shRNA sequence to be integrated into the host cell genome (Figure 5-3A), the infected HEPA 1-6 cells could be selected by adding 6 µg/mL of puromycin, which was the optimized concentration to remove all the non-infected HEPA 1-6 cell population within 14-21 days (data not shown).

After puromycin treatment for 28 days, puromycin resistant HEPA 1-6 cells were all Turbo-GFP positive cells. This was confirmed by immunocytochemistry staining with an anti-Turbo-GFP antibody and imaging the cells with confocal microscopy (Figure 5-10A). The amount of Jarid2 and Eed knockdown was next assessed with qRT-PCR and Western blot (Figure 5-10B,C). Compared to scrambled control, Jarid2 transcriptional level after Jarid2 knockdown did not statistically differ (Figure 5-10B). In addition, Jarid2 knockdown did not affect other PRC2 component transcriptional levels. In contrast to Jarid2 knockdown, Eed knockdown in HEPA 1-6 cells resulted in 64.1 ± 6.0 % suppression of Eed transcriptional level, while the rest of PRC2 genes were not statistically different (Figure 5-10B).

Unlike mRNA, the protein levels of Jarid2 from puromycin selected HEPA 1-6 cells with Jarid2 knock down showed approximately 80% suppression (Figure 5-10 C). Eed also showed decreased protein levels after long-term knockdown (Approximately 30%). Because this long-term knockdown of Jarid2 and Eed could

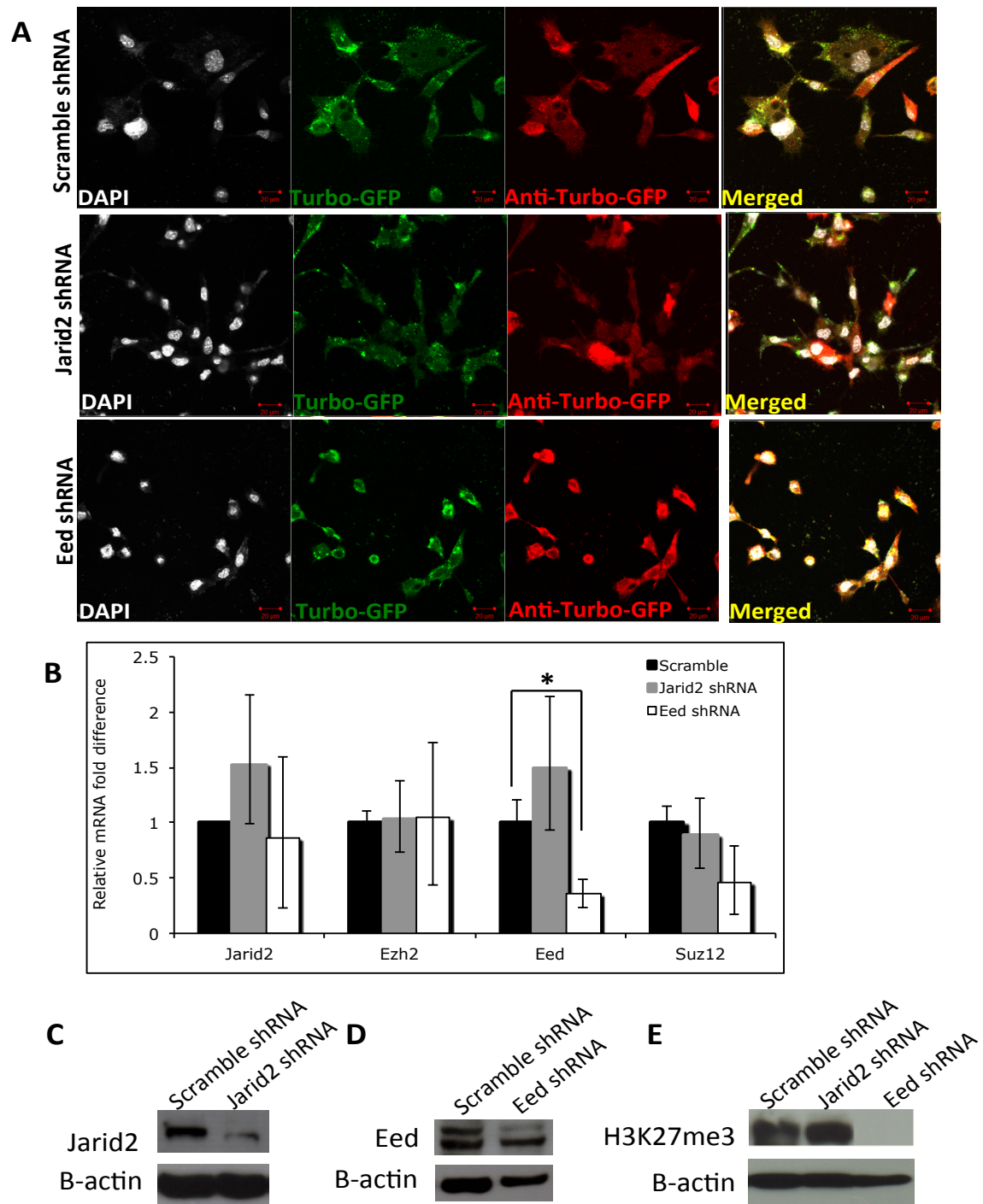


Figure 5-10. Long-term knockdown of Jarid2 and Eed after puromycin selection in HEPA 1-6 cells.

(A) After four weeks of puromycin treatment, those antibiotic resistant colonies were selected and stained with anti-Turbo-GFP to verify the turbo-GFP positive cells. (n=3)

(B) After selection, the PRC2 transcriptional level was measured by using qRT-PCR. Jarid2 mRNA level was not suppressed and statistically different compared to scrambled control. Eed shRNA stably suppressed its transcriptional level in HEPA 1-6 cells. * $p < 0.05$ (n=3)

(C-E) The protein levels of Jarid2, Eed and H3K27me3 were measured after the selection in HEPA 1-6 cells. Compared to the scrambled control, the Jarid2 knockdown HEPA 1-6 cells had 80% suppression while Eed knockdown showed 30% loss. (n=1)

influence the level of H3K27me3, I measured its level in the two different knockdown conditions. Interestingly, the detection of H3K27me3 indicated that even 30% Eed knockdown at the protein level could result in a complete loss of H3K27me3. Surprisingly the approximately 80% of Jarid2 protein knockdown did not reduce H3K27me3 (Figure 5-10E). Together, this experiment showed that Jarid2 and Eed shRNA could stably suppress their protein levels long term, although Jarid2 mRNA levels did not seem to decrease after drug selection of viral-infected HEPA 1-6 cells.

5.2.3.4. Possible effects from growth factors

SVZ neurosphere cells were originally isolated from micro-dissected SVZ tissues. As mentioned in 4.2.2., most of the SVZ transcriptional profile in neurospheres was similar to SVZ tissue, but there was some exceptional genes with different mRNA levels (Figure 4-6B). Since Jarid2 transcriptional levels in SVZ neurospheres were significantly higher than from SVZ tissue, I initially measured the difference of Jarid2 protein levels between SVZ tissue and neurospheres (Figure 5-11A). Similar to the transcriptional level, the neurospheres had distinctively higher amount of Jarid2 protein compared to SVZ tissue. This suggested the neurosphere culture environment could alter Jarid2 expression level.

A recent study showed Ezh2 protein levels could be enhanced by various growth factor stimulation including FGF2 and EGF, while the mRNA levels did not change (Kottakis et al., 2011). Since SVZ neurospheres had been cultured with 20 ng/mL of both EGF and FGF2, I varied the concentrations of both growth factors between 10 ng/mL and 40 ng/mL. After secondary neurospheres were formed, the Jarid2 protein level was measured by Western blot. Using β -actin as loading control, the level of Jarid2 could be compared between different growth factor conditions. Remarkably, the neurospheres cultured in EGF 20 ng/mL and FGF2 40 ng/mL

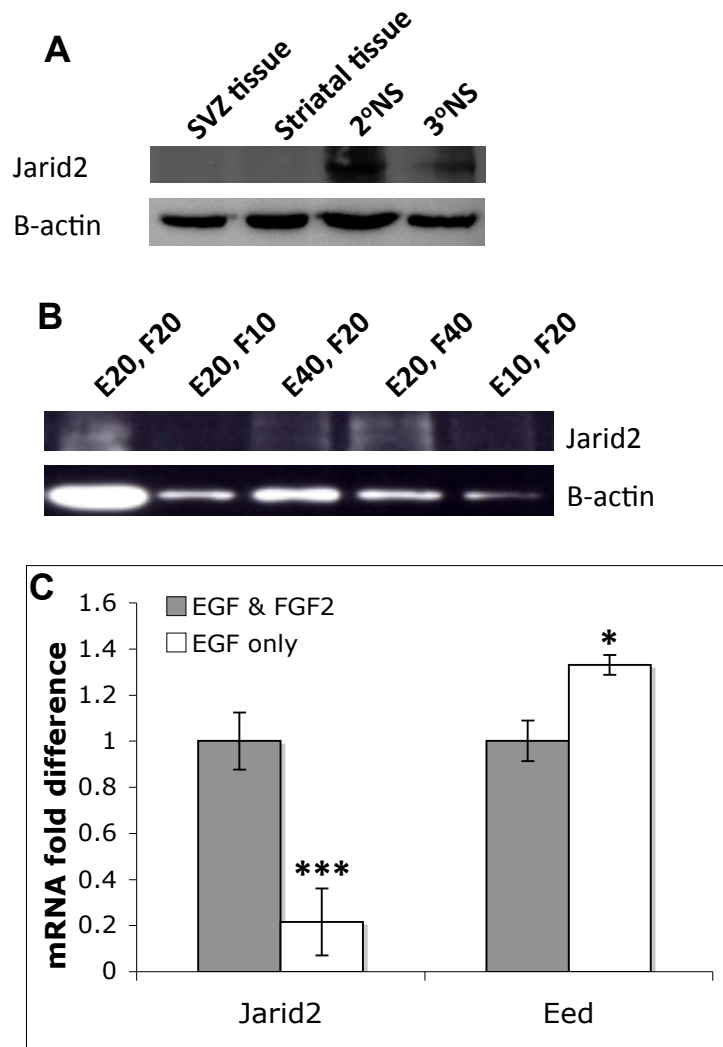


Figure 5-11. Jarid2 expression responds to the amount of FGF2 in culture.

(A) Jarid2 protein level was measured from SVZ tissue, striatal tissue, secondary neurospheres and tertiary neurospheres by western blot. The secondary and tertiary neurospheres had higher Jarid2 expression level compared to SVZ and striatal tissues. (n=3)

(B) Given the various concentration of EGF (E) and FGF2 (F) in the culture condition, Jarid2 protein level was measured from secondary neurospheres by western blot. The highest Jarid2 level was detected from E20,F40 after normalizing the intensity of Jarid2 bands to the B-actin bands which were the loading control. (n=1)

(C) In the absence of FGF2 in neurosphere culture, Jarid2 transcriptional level from secondary neurospheres was detected with qRT-PCR. The Jarid2 mRNA level was significantly decreased ($p < 0.001$) while Eed was significantly increased ($p < 0.05$). (n=3)

condition showed higher Jarid2 protein level compared to the neurospheres from EGF 20 ng/mL and FGF2 10 ng/mL condition (Figure 5-11B). This was also observed at the transcriptional level with qRT-PCR by withdrawing FGF2 only from the culture condition (Figure 5-11C). Interestingly Eed mRNA levels were slightly but significantly increased in the presence of EGF alone (Fig. 5-11C).

Jarid2 and Eed mRNA expression levels were examined after Jarid2 and Eed knockdown in the absence of FGF2 in the culture medium. Jarid2 transcriptional level was significantly decreased (47.5 ± 2.0 % reduction) at 3 dpi but was similar (1.19 ± 0.22 fold difference) to scrambled control at 5 dpi (Figure 5-10B). However, its level was dramatically decreased (73.5 ± 1.0 % reduction) at 7 dpi (Figure 5-12A). In contrast, Eed transcriptional level was stably suppressed during the 7 days after infection (Figure 5-12B). Although Eed mRNA level was slightly but significantly enhanced in the absence of FGF2, its transcriptional level at 7 dpi was significantly further suppressed (74.0 ± 7.0 % decrease) compared to its level from Eed lacking neurospheres cultured with FGF2 (0.62 ± 0.03 fold difference; *p* value: 0.0145; Figure 5-12B).

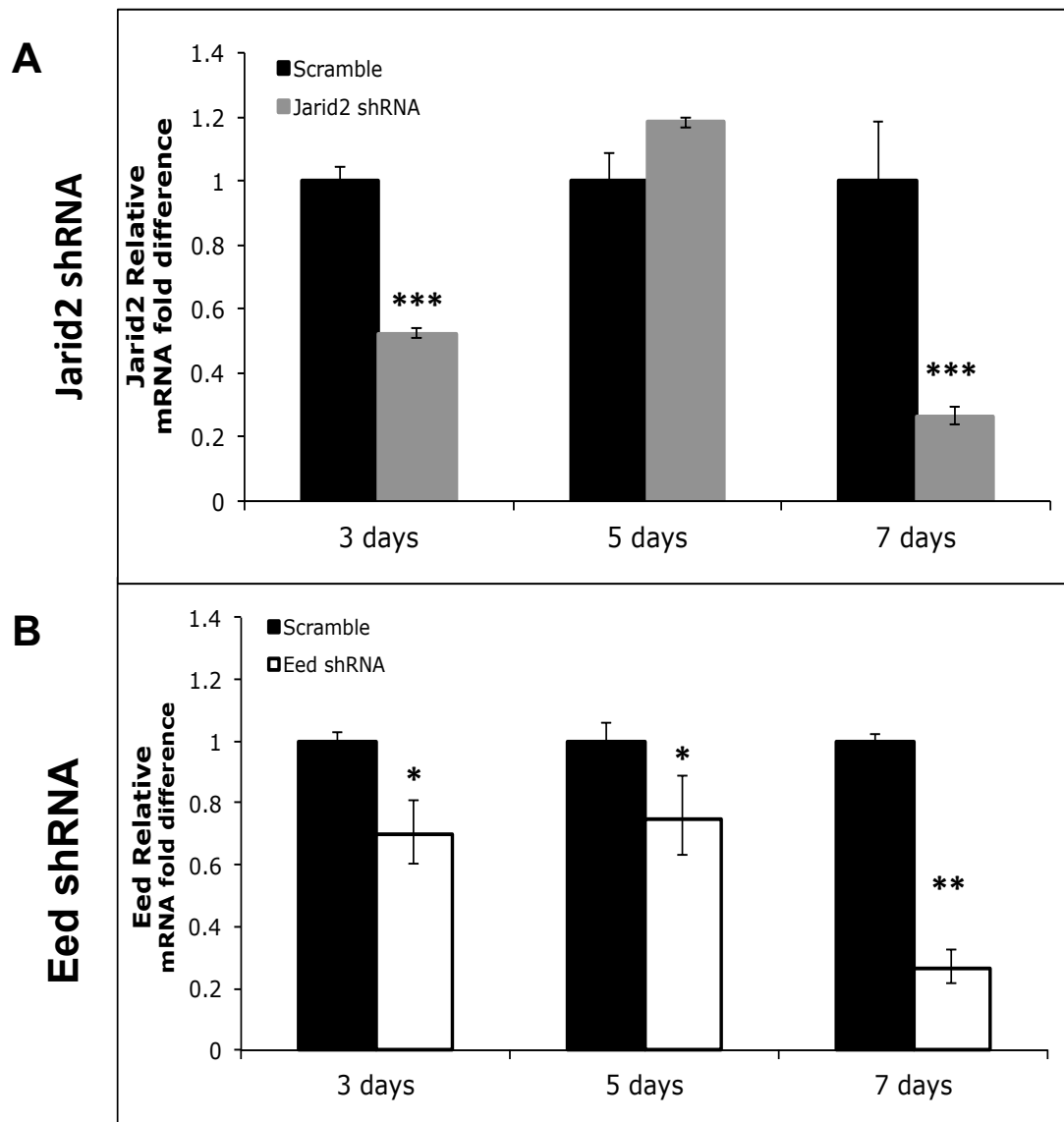


Figure 5-12. Long-term Jarid2 and Eed knockdown in SVZ secondary neurosphere in the absence of FGF2.

After infecting neurospheres with Jarid2 shRNA or Eed shRNA, Jarid2 (A) and Eed (B) mRNAs were measured on 3, 5 and 7 dpi by qRT-PCR. In the absence of FGF2 in neurosphere culture, both Jarid2 and Eed mRNA levels were suppressed on 7 dpi. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (n=3)

5.3. Discussion

5.3.1. Stable And Long-Term Knockdown Of Jarid2 And Eed

By using lentiviral-mediated delivery of shRNA method, the advantage of targeting both mitotic and post-mitotic cell populations with integration and inheritability of the shRNA sequence provided stable and long-term knockdown of Eed mRNA and proteins in SVZ neurospheres as well as HEPA 1-6 cells. However, Jarid2 knockdown did not provide the stable Jarid2 mRNA and protein suppression in SVZ neurospheres but did for only protein level in HEPA 1-6 cells.

The Jarid2 shRNA sequence, I used, had been previously utilized to demonstrate the significant knockdown of Jarid2 protein level in an ESC study (Pasini et al., 2010). By using this validated shRNA sequence, I demonstrated the significant suppression of Jarid2 protein level but not mRNA level after selecting only infected HEPA 1-6 cells. In contrast, the stable knockdown of Jarid2 mRNA and protein levels from SVZ neurospheres was difficult to obtain. In order to explain this outcome as due to the biological effects, here are two possible speculations: The former (HEPA 1-6 cells) is possibly due to that the shRNA acts as a steric blocking antisense molecule (Nguyen and Haenni, 2003), which inhibits translation from mRNA rather than the mRNA degradation of the target gene. The latter (SVZ neurospheres) is Jarid2 is possibly under feedback inhibition, which was relieved upon knockdown although there is no direct evidence demonstrating Jarid2 feedback inhibition. Besides the Jarid2 mRNA and protein level correlation, previous studies have also shown that PRC2 mRNA levels are not directly correlated with their protein levels. For instance, Pasini et al. in 2010 knocked down Suz12 in ESC, which caused the reduction of Jarid2, Ezh2 and Eed protein level. However, the mRNA level for Jarid2 and Ezh2 did not change (Pasini et al., 2010). Also, Kottakis et al. in 2011 have demonstrated

that the same level of Ezh2 proteins could be found in cells with various amounts of Ezh2 mRNA (Kottakis et al., 2011).

Alternatively, it is possible to consider this unstable Jarid2 knockdown could be due to off-target effects of the virus or promoter as technical problems (Rao et al., 2009). However, all the scrambled, Jarid2 and Eed shRNAs share the same U6 promoter and lentiviral construct, so it is difficult to conclude the only the Jarid2 unstable knockdown is due to the lentiviral delivery or vector construct problem. Since Pasini et al. in 2010 showed significant knockdown of Jarid2 protein in ESC (Pasini et al., 2010) similar to my Jarid2 protein knockdown in HEPA 1-6 cells, it is more likely to be a *bona fide* biological knockdown in SVZ neurospheres.

Considering the failure of Jarid2 stable knockdown in SVZ neurospheres was due to the biological effects, I next to examine the SVZ neurosphere cell type selection after Jarid2 knockdown. Since SVZ neurospheres were heterogeneous, it could be the possible scenario, but the ectopic cell marker expression changes did not provide any clues on any particular pattern of cell type selection. As the unstable Jarid2 knockdown was probably not due to cell selection within neurospheres during self-renewal, it could possibly be due to extrinsic factors rather than an intrinsic response. This was supported by the fact that SVZ neurospheres had 4-fold and 8-fold significantly higher Jarid2 mRNA and protein levels respectively compared to equivalent amounts of SVZ tissue demonstrating its labile expression in culture. In fact, Kottakis et al., in 2011 demonstrated that Ezh2 expression is dependent on FGF2 signaling via an FGF2-NDY1/Ezh2-miR-101-Ezh2 pathway (Kottakis et al., 2011). I therefore tested Jarid2 mRNA and protein levels in different concentrations of growth factors and showed FGF2 dependent Jarid2 transcriptional and translational expression. For example, Jarid2 mRNA was suppressed on 7 dpi in the absence of

FGF2 rather than its overexpression in the presence of FGF2. The relationship between FGF2 signaling and Jarid2 expression has not been mentioned before, but the FGF2-NDY1/Ezh2-miR-101 pathway could possibly be utilized for Jarid2 suppression since miR-101 can also bind to the 3'UTR of Jarid2. (Please see further discussion in 6.1.1.4.)

Although Jarid2 protein level was distinctively repressed after puromycin selection of infected HEPA 1-6 cells, Jarid2 mRNA level was similar to the scrambled control level. Also, Jarid2 lacking neurospheres cultured in medium with EGF only showed similar Jarid2 mRNA level to the scrambled control on 5 dpi. The mechanism for this phenomenon is not clear; therefore, more experiments need to be carried out to determine the exact cause or mechanism of Jarid2 regulation. Based on these data, it is possible that FGF2 in neurosphere culture might influence on Jarid2 expression in SVZ neurospheres.

5.3.2. Dissimilar Effects Of Eed And Jarid2 Knockdown In Postnatal Neurospheres

5.3.2.1. Different effects of Jarid2 and Eed in SVZ neurosphere self-renewal

The inconsistent Jarid2 level after its knockdown complicated the investigation of its downstream effects in SVZ neurospheres. Since the acute Jarid2 transcriptional knockdown at 3-4 dpi was significantly higher than other time points, I determined to focus on the implication of Jarid2 mRNA knockdown 3 days after infection.

Previous Jarid2 knockdown and knockout studies in ESCs did not report any significant changes in ESC self-renewal (Chamberlain et al., 2008; Pasini et al., 2007). Yet, the deletion of Jarid2 in epidermal stem cells resulted in reduced proliferation. I showed here that Jarid2 transcriptional knockdown in neurospheres

caused 51.0 % reduction in individual neurosphere size without significantly changing the number of neurospheres at 3 dpi. Because this phenotype could be a result of the lack of cell survival rather than self-renewal defect, I systematically determined cell viability by using trypan blue exclusion. Although Jarid2 lacking neurospheres showed 24% reduction in cell viability compared to scrambled control, the effect of Jarid2 knockdown was specifically shown as a reduction of neurosphere size rather than its number (self-renewal). As only a minority of mitoses in neurospheres are stem cell self-renewal events rather than transit amplifying progenitor proliferation, the most parsimonious explanation was a decrease in proliferation. Also, the Jarid2 suppressed neurospheres had an increased Mash1 mRNA with a lack of differentiated cell marker mRNA (GFAP and β III-tubulin), which indicated that the Jarid2 knockdown cells are more likely to become to the less proliferative stem cell population compared to transient amplifying cells (Pastrana et al., 2009). In sum, I propose that the reduction of Jarid2 at the transcriptional level could reduce neurosphere cell proliferation. To validate this *in vivo*, the Jarid2 shRNA containing lentiviral vector could be delivered to lateral ventricle and monitor the infected cell populations to see what they become to.

In contrast to the Jarid2 effects, Eed knockdown in neurospheres showed the opposite effect on neurospheres at 3 dpi; the number of neurospheres was reduced while the size of neurospheres was unchanged. The decreased number of neurospheres after Eed knockdown could be related to the 24% significant reduction in cell viability. However, the reduced transcriptional levels of Mash1, EGFR and GFAP with no differences in β III-tubulin validated that Eed knockdown in neurospheres were more likely to cause decrease in self-renewal. Interestingly, Eed knockdown continuously influenced Mash1 and EGFR mRNA levels to become

significantly decreased at 6 dpi whereas GFAP and β III-tubulin mRNA levels were significantly enhanced. This feature of enhancing differentiation genes resembled Eed deficient ESCs, which de-repressed differentiation-associated genes to become up regulated for differentiation rather than self-renewal (Boyer et al., 2006; Chamberlain et al., 2008; Ura et al., 2008). In addition overexpression of Sox2 was shown to compensate for the lack of Eed role via a positive feedback loop (Ura et al., 2011), but the high level of endogenous Sox2 did not compensate for the loss of Eed in SVZ neurospheres.

Taken together, I have first demonstrated the data point towards opposite functions of Jarid2 and Eed in postnatal SVZ neurospheres. Although Eed function in neurospheres recapitulated its role in ESCs, Jarid2 knockdown in neurospheres did not. This suggests Jarid2 and Eed in SVZ neurospheres may have independent functions.

5.3.2.2. Different effects of Jarid2 and Eed in SVZ neurosphere differentiation

On 3 dpi, neurospheres lacking Jarid2 or Eed were spontaneously differentiated on laminin for 7 days without the growth factors in the culture medium. After differentiation of neurospheres, the cells were immunohistochemically stained for O4, GFAP and β III-tubulin and cell numbers quantified to determine cell fate choice. Eed lacking neurospheres or a scrambled shRNA control showed proper differentiation, whereas the majority of differentiated neurosphere cells died after Jarid2 knockdown. Although the cell viability of Jarid2 lacking neurospheres was 60% on 3 dpi, which was similar level to the one from Eed suppressed neurospheres. Therefore, the cell death from the differentiated neurosphere after Jarid2 knockdown is more likely due to the downstream effects of Jarid2 suppression in the neurospheres.

So far, different stem cells lacking Jarid2 during development have shown inconsistent effects on their differentiation processes. In ESCs, Shen et al. in 2009 first described Jarid2 deficient ESCs, which improperly differentiated after withdrawing leukemia inhibitory factor (LIF) (Shen et al., 2009). The consecutive three Jarid2 studies in ESCs also showed that Jarid2 knockdown or knockout in ESCs resulted in lack of spontaneous neural lineage induced differentiations (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010). To understand this process, these studies included H3K27me3 signature and microarray data, but they had inconsistent data. Also none of the studies provided reasonable explanations why Jarid2 deficient ESC failed to differentiate properly. Based on the previous Jarid2 knockout mutant mice studies (Berge-LeFranc et al., 1996; Motoyama et al., 1997; Takeuchi et al., 1999; Takeuchi et al., 1995), I could possibly speculate this lack of differentiation is due to ectopic proliferation of ESCs, which is regulated by cell cycle inhibitor such as cyclin-D1 (Shirato et al., 2009; Takahashi et al., 2007). In addition, when Jarid2 function was reduced in epidermal stem cells, improper differentiation continued in postnatal tissues (Mejetta et al., 2011). The role of Jarid2 in stem cell differentiation could be altered in specific tissues at certain time points. Because the Jarid2 downstream genes in SVZ neurospheres are unknown, it is difficult to speculate possible candidate mechanisms for apoptosis during differentiation.

The ectopic morphology of differentiated neurosphere nuclei after Jarid2 suppression could be the result of cell death due to shRNA off-target effects. For example, it could be due to saturation of the endogenous miRNA pathway. When viral-delivered shRNA was overproduced via the endogenous miRNA pathway, the endogenous miRNA plus overexpressed shRNA may not be efficiently exported from the nucleus due to limited activity of Exportin 5 (Grimm and Kay, 2007). Also the

increased amount of both miRNA and shRNA may not be processed efficiently because of the limited activity of RNA-induced silencing complex component Argonaute-2. These two major factors could influence the accumulation of shRNA concentration to reach intracellular toxicity (Rao et al., 2009). The alternative method to alleviate this issue is to use the RNA polymerase II promoter instead of RNA polymerase III promoter, but all the scrambled, Jarid2 and Eed shRNAs were driven by U6 promoter and shared the same lentiviral backbone. Thus, it was probably not due to the promoter or lentiviral backbone. Finally, the non-specific shRNA sequence could randomly target its complementary sites and cause the cell death, but this off-target effect did not occur in the HEPA 1-6 cell line. In sum, the phenotype of differentiated Jarid2 lacking neurospheres is not likely to be the result of shRNA off-target effects. It could however be due to genetic or biochemical changes - Jarid2 downstream effects - which have been previously demonstrated with other genes in neurospheres (Galvin et al., 2008; Maric et al., 2007).

Unlike post Jarid2 lacking neurosphere differentiation, Eed knockdown in neurospheres allowed differentiation into three different lineages with a concomitantly significant reduction of oligodendrogenesis. According to an embryonic cortex study, Eed knockdown in NSPC resulted in extension of neurogenesis with the delay of gliogenesis (Hirabayashi et al., 2009). The mRNA levels of β III-tubulin neuronal differentiation marker was increased a day before the astrocytic differentiation marker (GFAP) expression enhancement. Although delay in GFAP expression by a day could possibly indicate an extension of the neurogenic period during neurosphere spontaneous differentiation, the ratio of astrocytes and neurons was not significantly different compared to scrambled control after 7 days differentiation *in vitro*. Interestingly this result was verified by unpublished *in vivo*

data from Hirabayashi, Y, presented at the International Society of Stem Cell Research annual meeting in 2012.

The reduction of oligodendrogenesis after knockdown of Eed in SVZ neurospheres has not been previously reported. However, the loss of PRC2 activity in primary NSC culture from E14 telencephalon indicated similar results (Sher et al., 2008). Ezh2 suppression in primary NSCs yielded reduction of oligodendrogenesis, whereas Ezh2 overexpression promoted oligodendrogenesis (Sher et al., 2008). In addition, this feature was demonstrated by conditionally floxed out Mll1 in postnatal SVZ mediated by a bivalent mechanism (Lim et al., 2009). In the absence of Mll1, the oligodendrocyte production was promoted as an opposite mechanism for the bivalent. Taken together, Eed knockdown in SVZ neurospheres showed the similar effect of the lack of PRC2 activity (Please see further discussion in 6.1.1.).

In summary, after examining the role of Jarid2 and Eed knockdown in postnatal SVZ neurosphere, their phenotypes were distinctively different from one another during self-renewal and differentiation. The role of Jarid2 in the SVZ neurospheres seemed to be important for cell proliferation and proper differentiation, whereas the Eed function seemed to be a key for initiating differentiation processes, as previously mentioned in the ESC studies with Eed knockout.

5.4. Conclusion

I have demonstrated the efficient knockdown of both genes in HEPA 1-6 cells at the translational level. Although Jarid2 was not stably knocked down in neurospheres, biological read-outs for neurosphere number, size, cell viability and differentiation were measured on 3 dpi when Jarid2 mRNA level was significantly decreased. Based on these measurements, Jarid2 function was found to be important for NSPC proliferation, proper differentiation and cell viability. Unlike Jarid2, the role of Eed was implicated in neurosphere self-renewal and oligodendrogenesis. Interestingly, Jarid2 mRNA suppression was obtained by omitting FGF2 from the culture, which did not affect Eed knockdown in neurospheres. As Jarid2 and Eed knockdown showed distinctively different phenotypes in neurospheres during self-renewal and differentiation, their role may *in vivo* be dissimilar as well.

Chapter 6

Discussion

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Chapter 6

Discussion

6.1. Summary Of The Thesis

This thesis has primarily focused on elucidating the role of Eed and Jarid2, as members of PRC2, in regulating SVZ NSPC stemness *in vitro*. Using a validated neural stem cell culture technique, the neurosphere assay, the capacity of postnatal SVZ NSPC self-renewal and differentiation was examined after Eed and Jarid2 suppression. Because there was no standardized protocol for the neurosphere assay, characterization of neurospheres was required in order to understand how they can act differently under various culture systems (i.e. cell density, degree of adhesiveness to the culture flasks, culture medium/supplement conditions, amount of growth factors and substrates for neurosphere differentiation). Also, this study has begun to elucidate biological changes after Eed and Jarid2 suppression in neurospheres. As H3K27me3 marks were higher in SVZ NSPCs compared to neuroblasts in both postnatal and adult brains, it was important to measure PRC2 core component (Eed, Ezh2 and Suz12) and Jarid2 expression levels in SVZ NSPCs. In the brain injury and disease models examined, H3K27me3 levels were altered in spatiotemporal patterns reflecting SVZ NSPC proliferation, gliosis and cell death. Reducing Eed or Jarid2 in SVZ NSPCs resulted in, either the number of neurospheres, the diameter of neurospheres, cell viability, SVZ cell type marker expression level or FGF2 response to change compared to scrambled controls. Interestingly Eed knockdown in SVZ neurospheres suppressed self-renewal and oligodendrogenesis, whereas Jarid2 knockdown suggested reduced proliferation and inhibition of proper differentiation. Taken together, these results indicate that the roles of Eed and Jarid2 do not seem to

share their functions in SVZ NSPCs suggesting that Jarid2 has PRC2 independent roles.

6.1.1. Integrated view of the work in this thesis

Optimization of the pre-existing protocol for NSA provided me more stable and reproducible neurosphere culture. Although this assay has been numerously optimized to recapitulate the physiological characteristics of NSPCs in SVZ last two decades, it has been technically challenged. Alternatively there are explants or NSC monolayer culture methods, which can allow to examine the SVZ NSPC characteristics, but the sphere-forming assay still has the advantages to easily grow and provide the direct measurement of NSPC self-renewal and proliferation as well as differentiation. Therefore I have chosen to utilize NSA to examine the role of Eed and Jarid2 loss of function *in vitro*.

6.1.1.1. SVZ neurosphere self-renewal/proliferation change after Eed

knockdown

In Chapter 4, I provide data that most SVZ cells had H3K27me3 marks in the postnatal and adult brains. The NSPC population had higher H3K27me3 intensity than neuroblasts in both developmental stages of the SVZ. After Eed (a core component of PRC2) was stably down-regulated in postnatal SVZ NSPC by lentiviral-mediated delivery of shRNA, fewer neurospheres were found when compared to scrambled control but no changes were seen in neurosphere size. Also, the transcriptional levels for stem/progenitor cell markers (EGFR and Mash1) were decreased over time whereas differentiation cell markers (GFAP and BIII tubulin) mRNA levels were increased. Together, these data indicate that NSPC self-renewal

depends on the proper function of PRC2 to trimethylate their downstream target genes. Importantly, I confirmed that loss of Eed resulted in greatly diminished H3K27me3 in a cell line.

In epidermal stem cells from postnatal tissue, the conditional knockout of Ezh2, a core member of PRC2, shows loss of H3K27me3 and decreases in proliferation due to de-repressing cell cycle-dependent kinase inhibitors (Ink4A/Ink4B) (Ezhkova et al., 2009). Besides this study, PRC2 function in cell proliferation has been extensively studied in cancer biology. Ezh2 has been utilized as a marker for aggressive late stage prostate and breast cancer cells (Kleer et al., 2003; Varambally et al., 2002). Reducing PRC2 activities in cancer cells decreased proliferation (Bracken et al., 2003; Varambally et al., 2002) due to the de-repression of tumor suppressor genes: p16, p19 and p15 (Bracken et al., 2007; Ezhkova et al., 2009). As somatic cell proliferation is associated with PRC2 activities and the level of H3K27me3, Lim et al. in 2009 also examined whether postnatal SVZ NSPC proliferation is regulated by a bivalent mechanism. Mll1 depletion in hGFAP positive SVZ NSPC populations caused increased numbers of BrdU positive cells, which accumulated in the postnatal SVZ (Lim et al., 2009). Compared to the previous PRC2 studies and my Eed knockdown data, this opposite phenotypic result confirmed that somatic cell proliferation regulation is governed by a bivalent mechanism. Therefore, PRC2 trimethylation activity is important for regulation of SVZ NSPC self-renewal/proliferation.

Exceptionally, the absence of PcG proteins did not exhibit any changes in ESC self-renewal and proliferation (Chamberlain et al., 2008; Pasini et al., 2007; Shen et al., 2008; van der Stoop et al., 2008) although their differentiation process became activated (Peng et al., 2009; Surface et al., 2010). It is not clear why ESCs were not

affected in their self-renewal/proliferation after the loss of PcG proteins, but it has been speculated they have an alternative way to govern their self-renewal/proliferation. Otherwise, it is due to the difference of the PRC2 target genes from ESCs and SVZ NSPCs.

6.1.1.2. SVZ neurosphere differentiation after Eed knockdown

In the postnatal and adult SVZ, Dcx positive cells (neuroblast) had lesser H3K27me3 intensity compared to GFAP positive cells in the SVZ and striatum. This suggests the amount of H3K27me3 and the role of PRC2 could possibly affect SVZ NSPC fate determination during spontaneous differentiation. As neurospheres with reduced Eed indicated, there were no significant changes in neuron and astrocyte production compared to scrambled control, although oligodendrocyte production was significantly attenuated.

Similar to our data, Sher et al in 2008 demonstrated significant reduction of oligodendrogenesis, a slightly decreased neuron production and significantly increased astrocyte production were obtained after Ezh2 knockdown in E14 NSPCs from telencephalon (Sher et al., 2008). In contrast, the overexpression of Ezh2, resulted in the opposite effect of its knockdown, increased oligodendrocyte genesis, fewer astrocytes but unchanged neuron production (Sher et al., 2008). As a bivalent effect, the conditional knockout of Mll1 in the postnatal SVZ hGFAP positive cell population showed the lack of neurogenesis for SVZ NSPCs but increment of the oligodendrogenesis *in vivo* and *in vitro* (Lim et al., 2009).

Consistently, all four experiments, including our data indicate that oligodendrogenesis is mediated by a H3K4me3/H3K27me3 bivalent mechanism although astrocyte and neuronal differentiations may not be. Ezh2 overexpression and

knockdown in E14 NSPCs indicates astrogenesis occurs rather than oligodendrogenesis. However, our data does not show any change in astrogenesis. Also, Hirabayashi et al. in 2009 showed the loss of Ezh2 and Eed at E13.5 in cortical NSPCs provided increased neuronal production (Hirabayashi et al., 2009). Although there is no other evidence to explain these differences, I speculate it may be due to the difference between NSPC samples, collected from different time points and different areas of brains. As I collected the most postnatal SVZ NSPCs derived from LGE and MGE rather than embryonic cortical NSPCs or entire telencephalon, their intrinsic signatures between three different NSPCs are not the same as described in Chapter 1. Thus, alteration of H3K27me3 signature after loss of PRC2 function may not be the same among these different cell populations.

6.1.1.3. Different effects on SVZ neurosphere self-renewal and differentiation after Jarid2 and Eed suppressions

Neurospheres, infected with lentivirus carrying Jarid2 shRNA, showed significant size decrease with similar number of neurospheres compared to the scrambled control. Also, differentiated neurospheres lacking Jarid2 did not produce cells expressing detectable neural lineage cell markers but showed abnormal nuclear morphology. These data indicate that Jarid2 knockdown in neurospheres induces different phenotypic changes compared to Eed knockdown in neurospheres. Based on this evidence, it is not certain whether Jarid2 acts as a member of PRC2 in the postnatal SVZ NSPC.

Previously published studies showed Jarid2 mutant mice showed neural tube defects and the abnormally proliferating cells in the mantle zone where post-mitotic neurons reside (Takahashi et al., 2007). This abnormal proliferation was mediated by

cyclin-D1, and its expression was regulated by Jarid2 associated H3K9 methyltransferase activity (Shirato et al., 2009). Interestingly, Jarid2 did not show protein-protein interactions with PRC2 within these cell populations (Shirato et al., 2009). This role of Jarid2 is also found in endothelial cells during cardiac development where it directly regulates Notch gene expression (Mysliwiec et al., 2011; Mysliwiec et al., 2012). Therefore, Jarid2 can potentially have a completely PRC2-independent function, or it could work as both dependent and independent dual mechanisms.

In contrast, ESC lacking Jarid2 showed no significant abnormal phenotypes during self-renewal/proliferation, but showed improper differentiation (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009). Similarly, Ezh2, Eed or Suz12 depleted ESCs showed the same phenotypic result as Jarid2 depleted ESCs in both proliferation and differentiation, so Jarid2 is considered as a member of PRC2 (Surface et al., 2010). Biochemically, Jarid2 protein binding partners in ESCs are PRC2 components rather than H3K9 methyltransferase (Shen et al., 2009).

However, all the PRC2 core component knockout mice are consistently lethal around E7.5-8.5 (Surface et al., 2010) whereas the Jarid2 knockout mice survives until E11.5-15.5 (Takeuchi et al., 1995). The particular reasons for the death of Jarid2 knockout mice are a combination of neural tube defects and/or heart defects based on individual mice and strain differences (Lee et al., 2000; Motoyama et al., 1997; Takeuchi et al., 1999; Takeuchi et al., 1995). As the role of Jarid2 is implicated in both brain and heart development at different levels, it is difficult to define its clear role during embryonic development. Due to this reason, Jarid2 knockdown and knockout effects in ESCs are significantly different, reminiscent of the inconsistent

H3K27me3 signatures. In contrast, ESCs lacking Ezh2, Eed or Suz12 by knockdown or knockout provide similar phenotype changes with the similar pattern of the H3K27me3 signature alteration (Surface et al., 2010). Taken together, PRC2 function is partially mediated by Jarid2 activity since other proteins and ncRNA are associated with PRC2 in ESCs (Kaneko et al., 2010; Surface et al., 2010; Wu et al., 2011). This suggests that the role of Jarid2 in SVZ NSPCs can be implicated as non-PRC2 pathway.

Unlike in knockout mice, the conditionally knockout Ezh2 or Jarid2 in postnatal epidermal stem cells provided similar results to each other. At the protein level, Jarid2 directly interacts with the rest of PRC2 core components (Eed and Ezh2), so the conditional knockout of Ezh2 or Jarid2 mice show the same feature as the loss of H3K27me3, reduction of proliferation and promotion of differentiation during postnatal stage (Ezhkova et al., 2009; Mejetta et al., 2011). Therefore, it is important to understand the dynamic change in Jarid2 function has been demonstrated in various tissues at several different time points.

6.1.1.4. FGF2 dependent Jarid2 expression

In Chapter 3, I showed that FGF2 induction decreases neurosphere differentiation while others have shown it promotes neurosphere proliferation (Gritti et al., 1999; Vescovi et al., 1993). I also showed, I showed FGF2 induces Jarid2 expression, which is necessary for neurosphere proliferation and proper differentiation. There is no clear evidence to explain a possible mechanism for FGF2-Jarid2 regulation, yet it has been speculated that FGF2 induces Jarid2 gene regulatory transcription factors. Since Oct4, Sox2, cMyc and Nanog genes are known to bind to the Jarid2 upstream

promoter to drive gene expression (Ben-Porath et al., 2008; Peng et al., 2009), these genes could have been induced by FGF2 stimulation.

In the neurosphere assay or other FGF2-induced neural stem cell cultures, Oct4 and Nanog expression levels have not been found in NSPCs as similar to our neurospheres (Kim et al., 2008). According to a study where FGF2 was induced in human ESCs, it caused down-regulation of Nanog and Oct4 (Eiselleova et al., 2009). Therefore, FGF2 induction in SVZ neurospheres could be negatively affected for Oct4 expression.

In contrast, it has been demonstrated in mouse adrenocortical tumor cells that FGF2 promotes cMyc transcription via the MEK/ERK pathway (Lepique et al., 2004). As cMyc has a critical role to govern NSC self-renewal mediated by p53/p16Arf (Nagao et al., 2008), cMyc could be an FGF2-Jarid2 intermediate. Also, Sox2 expression enhancement has been demonstrated by FGF2 stimulation in rat neural stem cells, iris-derived lens regeneration and fibroblast reprogramming (Hayashi et al., 2004; Page et al., 2009; Sun et al., 2011). Together, the evidence suggests that Sox2 and cMyc could be the possible intermediate in FGF2-Jarid2 regulation in SVZ NSPCs.

Another possible mechanism for FGF2-Jarid2 regulation could be mediated by FGF2 – NDY1/Ezh2-miR-101 pathway: the induction of FGF2 can up-regulate NDY1 expression, which binds with Ezh2 to suppress miR-101 expression (Kottakis et al., 2011). Normally miR-101 binds the Ezh2 gene and suppresses its translation, but FGF2 induction can inhibit this mechanism. Based on the miR-101 binding sequence, it can also bind to Jarid2. After the statistical analysis (pre-programmed analysis website: <http://mirnamap.mbc.nctu.edu.tw/index.php>), the *p* value for the positive correlation between miR-101 and Jarid2 was 0.02 ($p < 0.05$). Since miR-101 is

specifically expressed in a brain along with miR-124, miR-128 and miR-132, Jarid2 could also be regulated via the FGF2 – NDY1/Ezh2-miR-101 pathway.

6.1.2. Limitations

6.1.2.1. Protein level of PRC2 components for the SVZ specific cell types

After H3K27me3 staining of postnatal and adult SVZ brain sections, I found various cell types in the SVZ had different levels of H3K27me3 mark intensity. This suggests different cell types have different level of PRC2 activity. In order to identify the amount of PRC2 core components from individual SVZ cell types I tried immunohistochemistry but it was not possible to determine them with the specific antibodies on brain sections due to lack of antibody specificity. Therefore, I attempted to quantitatively measure PRC2 expression in postnatal SVZ NSPC by using qRT-PCR and Western blots, but some genes have a lack of correlation between mRNA and protein expression levels. In order to understand PRC2 expression levels for individual cell types in the SVZ, Western blots of FACsorted individual SVZ cell types could resolve this question until new antibodies for PRC2 component genes are produced that could possibly work in immunohistochemistry staining with brain sections. An alternative method would be to perform *in situ* hybridization of these genes followed by co-immunohistochemistry staining for SVZ cell type markers. Although this is technically challenging, *in situ* hybridization could be performed on transgenic mice brains such as DCX-GFP, CSH-nestin-GFP, Mash1-GFP and hGFAP-GFP (Kim et al., 2009). Because mRNA levels are not always correlated with protein levels, this would provide a hint of PRC2 expression pattern.

6.1.2.2. Selection of the infected cell population in SVZ neurospheres

The infectivity of lentivirus has been optimized by using the appropriate amount of polybrene to neutralize cell-viral membrane charges. However, it was difficult to obtain 100% lentiviral-infected cell populations within a neurosphere as well as HEPA 1-6 cell line. During the validation of both Jarid2 and Eed shRNAs in HEPA 1-6 cell line, the mRNA and protein levels of Eed and Jarid2 were gradually decreased 2-6 days after the lentiviral infection. However, the significant reduction of H3K27me3 level was not obtained until the non-infected cells were removed by drug screening. In order to measure the suppression of H3K27me3 as a result of Jarid2 and Eed knockdown in neurospheres, it requires the selection of those viral-infected neurosphere cells. Because neurospheres have limited life span with characteristic changes by multiple passaging (as mentioned in Chapter 3), the FACsorting of lentiviral-infected Turbo-GFP positive cells from different time points could possibly indicate when and how much H3K27me3 marks get lost after Eed and Jarid2 knockdown in SVZ neurospheres. Also, Turbo-GFP FACsorted cells could allow accurate measurements of Eed and Jarid2 downstream gene effects.

6.1.2.3. Validation of Jarid2 and Eed knockdown function as PRC2 dependent or independent effect

Kim et al. in 2007 have demonstrated that Eed protein from the postnatal mice brain is directly associated with Ezh2 and functions in the PRC2 complex (Kim et al., 2007). However, it is not clear whether these molecules have the same functions in SVZ NSPC. Compared to previous studies on the loss of Ezh2 in E14 SVZ NSPCs, Eed knockdown in neurospheres showed similar phenotypic changes, whereas Jarid2 knockdown did not. Based on these observations, it is possible that Jarid2 function

can be partially associated or not related with PRC2. In order to demonstrate direct interactions between Jarid2 and Eed or other PRC2 core components at the protein level co-immunoprecipitation (Co-IP) experiments with SVZ NSPCs should be performed. However, the endogenous protein level of Jarid2 in SVZ NSPC is 8-fold lesser than in ESCs. Also, the Jarid2 antibody is not entirely specific for the Jarid2 protein, so it could be technically challenging to pull-down with only endogenous Jarid2 in the neurospheres. An alternative way to perform Co-IP could be by first generating a vector containing Jarid2 full-length cDNA with flag-tag. By overexpressing this flag-tag-Jarid2 in neurospheres, Jarid2 associated proteins can be pulled down by using flag-tag as well as overexpressed Jarid2.

6.1.2.4. Possibly different effects from Jarid2/PRC2 knockdown and knockout in SVZ NSPCs

In order to support the effects of Jarid2 knockdown in SVZ neurospheres, the Jarid2 knockout study in SVZ NSPC *in vivo* has been considered. By spatiotemporally removing Jarid2 in SVZ NSPC, it could help to clarify the role of Jarid2 in the SVZ. Jarid2 floxed mice were generated by Lee, Y.S. in University of Wisconsin Madison, and they are already applied to various different research purposes (Mejetta et al., 2011; Mysliwiec et al., 2011; Mysliwiec et al., 2012; Shen et al., 2009; Zhang et al., 2011). To further elucidate the physiological PRC2 function in postnatal SVZ the Szele laboratory has acquired Ezh2 flox mice originally generated by Tarakhovsky's laboratory in Rockefeller University. While time restraints did not allowed investigations on these mice to be included in my thesis, ongoing work in the laboratory will use these Ezh2 flox/flox mice to ask specific questions about the role of Ezh2 in the postnatal SVZ.

6.1.3. Future direction

As this thesis has demonstrated, the function of Eed and Jarid2 are important in regulating NSPC self-renewal/proliferation and differentiation *in vitro*. Based on the phenotypic changes found, the future direction of further work should include screening Eed and Jarid2 target genes by ChiP-on-ChiP assay or ChiP-seq. Although the Co-IP could provide evidence of Jarid2 binding with other PRC2 proteins, it does not demonstrate whether they share target genes. Thus, ChiP-on-ChiP or ChiP-seq can resolve this particular question. Also, ChiP-seq data could indicate downstream target genes, which have not been demonstrated in SVZ cells. This experiment could possibly clarify the particular phenotypic changes in Jarid2 and Eed lacking neurospheres.

The neurosphere assay has provided the relevant *bona fide* SVZ NSPC conditions, but it cannot completely represent the physiological condition in the absence of stem cell niche environment. In order to examine the role of PRC2 in the SVZ NSPCs at the physiological level, the direct injection of lentivirus containing shRNA or electroporation of the DNA plasmid, which contains shRNA sequence, into lateral ventricle to target the postnatal SVZ NSPC population must be carried out. The advantage of lentivirus as integration and inheritance of shRNA sequence can allow studying the Eed and Jarid2 knockdown effects on not only the NSPC population but also their progeny including olfactory bulb interneurons. Otherwise, Ezh2 floxed mice could be crossed with tamoxifen inducible cre mice such as Glast-cre^{ER/T2} to temporally control the Ezh2 gene deletion in Glast positive cells. As Glast is one of the SVZ NSC markers, this experiment could strongly support Eed and Jarid2 knockdown in neurospheres as well as directly provide the of PRC2 function in SVZ NSC at different time points.

A major objective of this thesis, was to discover how PRC2 function regulates the maintenance of NSC properties from postnatal to adult SVZ. After characterizing Eed and Jarid2 function in postnatal NSC population (mainly RGCs) at P4, Jarid2 was found to be necessary for NSPC proliferation and proper differentiation, whereas Eed was required for NSPC self-renewal and oligodendrogenesis. However, it was not known whether PRC2 function remained in the adult SVZ. If it is present, what is the function? How does it influence NSPC function? Recent studies suggest PRC2 function and H3K27me3 levels in adult SVZ neurogenesis are mediated via BMP signaling. In the adult SVZ, Noggin is expressed in ependymal cells and acts as an antagonist to BMP, which is produced by the rest of SVZ cells and inhibits neurogenesis (Lim et al., 2000). Interestingly, Noggin expression in spinal cord has been found to be regulated by H3K27me3 on its promoter (Akizu et al., 2010). In addition, the expression of Smad4, an essential gene for SVZ adult neurogenesis down-regulated by BMP signaling (Colak et al., 2008), is also regulated by a H3K4me3/H3K27me3 bivalent mechanism (Fei et al., 2010). Taken together, the level of H3K27me3 becomes more important for regulating adult neurogenesis in the SVZ. It is likely that PRC2 regulates this activity in adult neurogenesis, however, much remains to be discovered about the mechanistic details. Thus, this study needs to be carried out further in order to understand PRC2 function in adult SVZ neurogenesis better.

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Appendix

Table 2.2. Antibody list for Western blot

Antigen	Host	Company/Reference	Cat#/Code	Dilution Factor
Actin	Mouse	Sigma	A4700	1:1500
βactin	Rabbit	Abcam	ab8227	1:1000
cMyc	Mouse	Santa Cruz	sc-40	1:200
EED	Mouse	Millipore	AA19	1:2500
EZH2	Mouse	Diagenode	pAb-039-050	1:1000
H3K27me3	Rabbit	Cell Signaling	07-449	1:2500
JARID2	Rabbit	Abcam	ab48137	1:500
Klf4	Rabbit	Santa Cruz	sc-20691	1:1000
Oct4	Rabbit	Santa Cruz	sc-9081	1:200
Sox2	Rabbit	Millipore	AB5770	1:200
SUZ12	Goat	Dagenode	CS-029-100	1:1000

Table 2.3. Antibody list for Immunohistochemistry

Antigen	Host	Company/Reference	Cat#/Code	Dilution Factor
DCX	Goat	Santa Cruz	sc-8066	1:100
EGFR	Rabbit	Santa Cruz	sc-03	1:200
GFAP	Rat	Invitrogen	13-0300	1:400
H3K27me3	Rabbit	Millipore	07-449	1:5000
Nestin	Mouse	Millipore	MAB353	1:500
O4	Mouse	Millipore	MAB345	1:100
S100B	Mouse	Sigma	s2657	1:100
Sox2	Rabbit	Millipore	AB5603	1:200
Tuj1	Rabbit	Covance	MMS-435P	1:1000
Turbo-GFP	Rabbit	Evrogen	AB513	1:200
Vimentin	Goat	Santa Cruz	sc-7558	1:200

Table 2.4. Recipe for common solutions

PB 0.1 M pH 7.4

Monobasic Solution A (0.2 M)	140 mL
Dibasic Solution B (0.2 M)	360 mL
Distilled water	Up to 1 L
Monobasic Solution A (0.2 M)	
Sodium phosphate monobasic	27.8 g
Distilled waater	Up to 1 L
Dibasic Solution B (0.2 M)	
Sodium phosphate dibasic anhydrous	28.4 g
Distilled water	Up to 1 L

PBS 10X 1M pH 7.4

Sodium chloride	80 g
Potassium choloride	2 g
Potassium dihydrogen phosphate	2.4 g
Sodium phosphate dibasic anhydrous	14.4 g
Distilled water	Up to 1L

TAE buffer 50X

Tris-base	242 g
Glacial acetic acid	57.1 mL
EDTA 0.5 M	100 mL
Distilled water	Up to 1L