Role of a Novel microRNA in Adult Neurogenesis

by

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Truth is ever to be found in simplicity, and not in the multiplicity and confusion of things.

- *Sir Issac Newton, 1643-1727*

To Mother and Father. Thank you for bringing me to this magical world and teaching me to respect life. And to my dearest wife, Shih-han. Without you I would never make it.

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Table of contents

List of of Figures

List of Tables

Abstract

Multipotent, self-renewing stem cells which are present throughout the developing nervous system remain in discrete regions of the adult brain. In the subventricular zone (SVZ) signaling molecules, including the bone morphogenetic proteins and their secreted inhibitor, Noggin appear to play a critical role in controlling neural stem cell (NSC) behavior. To examine the function of this signaling pathway in the intact nervous system, we have developed a transgenic mouse model in which Noggin expression can be induced specifically in NSC via a *Nestin* promoter-driven reverse tetracycline-controlled transactivator (rtTA). In adult animals, induction of Noggin expression promotes proliferation of NSC in the SVZ, and shifts the differentiation of NSC from mature astrocytes to transit amplifying cells and oligodendrocyte precursor cells without depleting the NSC population.

Interestingly, over-expression of Noggin in the adult SVZ neural stem cells also inhibits the expression of a novel microRNA-410 (miR-410). miR-410 is expressed in the developing nervous system, remaining in the germinal zones of the adult brain. Over-expression of miR-410 in SVZ derived neurospheres consistently inhibited neuronal and oligodendrocyte differentiation while promoting the formation of astrocytes. Conversely, inhibition of miR-410 activity in NSC promoted neuronal and decreased astroglial differentiation. In addition, over-expression of miR-410 rescued the increase in neuronal differentiation and the decrease in astroglial differentiation caused by Noggin over-expression. Using computer prediction algorithms and luciferase reporter assays, we identified multiple neurogenic genes including *Elavl4* as one of the downstream targets of miR-410 via the canonical miRNA-3'UTR interaction. Over-expression of *Elavl4* transcripts without the endogenous 3' UTR rescued the decrease in neuronal differentiation caused by miR-410 over-expression. Interestingly, we also observed that miR-410 affected neurite morphology. Over-expression of miR-410 resulted in the formation of short, unbranched neurites. These results demonstrate that miR-410 controls the crucial lineage choice of adult

neural stem cells between neurons and glial cells by controlling the expression of neurogenic genes, and suggest a method to regulate NSC differentiation following disease, injury or aging.

Chapter One

Introduction

Neural Induction

The formation of the nervous system is one of the most tightly regulated processes of early vertebrate development. Neurulation begins on embryonic day 7 (E7) in the mouse embryo, when the primitive node secretes bone morphogenetic protein (BMP) inhibitors such as Noggin, Chordin, and Follistatin (Smith and Harland, 1992; Lamb et al., 1993; Hemmati-Brivanlou et al., 1994; Sasai et al., 1995). Antagonizing BMP signaling causes the ectodermal cells to begin to express neural gene transcripts to form the neural plate (Kiecker and Lumsden, 2012). The thickened neural plate later folds and fuses, forming a closed neural tube (Copp et al., 2003). At the same time, the extending axial mesoderm forms the notochord and acts as a ventral organizer.

After signaling from the node establishes the neural plate, the anterior visceral endoderm (AVE) which secretes inhibitors of Wnt, BMP and Nodal signaling such as cerebrus, induces differentiation of the head (Levine and Brivanlou, 2007).

Patterning

Once established, the nervous system is patterned along its dorsal-ventral, anterior-posterior and radial axes. After neural tube closure, the non-neural epidermal ectoderm lies above/dorsal to the

neural tube, and continues to secrete BMPs. In addition, the roof plate of the neural tube also secretes Wnts (Roelink and Nusse, 1991). Ventrally, the notochord and the floor plate of the neural tube secrete Sonic hedgehog (Shh). This two pole gradient of morphogenic factors (dorsal: Wnt/BMP-high, Shh-low; ventral: Wnt/BMP-low, Shh-high) establishes the dorsoventral axis and neuronal subtype specification along the neural tube (Kiecker and Lumsden, 2012).

Wnt and retinoic acid (RA) signaling play an important role in the establishment of the anteroposterior (AP) axis of the nervous system. Both Wnt and RA are secreted by the paraxial mesoderm underlying the neural plate, with a posterior-high/anterior-low gradient (Maden 2002, Nordstrom 2002). This gradient is crucial for initial axial establishment which is then translated by the Hox gene code.

Hox genes are a family of helix-turn-helix transcription factors. In most vertebrates there are 39 members organized in four separate clusters on different chromosomes (Alexander et al., 2009). For example, during vertebrate hindbrain development Hox genes are expressed in a nested fashion along the anterior-posterior axis, where the expression borders of each gene coincides with the boundaries of the segment-like rhombomeres (Wilkinson et al., 1989). Retinoic acid (RA), fibroblast growth factor (FGF), and Wnt signaling are crucial for initiating and define the expression of Hox genes (Reviewed in (Alexander et al., 2009). Between the rhombomeres Hox genes are expressed in two-segment periodicity. Together with other segmental cues such as Eph/Ephrin signaling Hox genes determine the identities of each segments and instruct the development of neuron types and neural circuitry in the hindbrain and neuraxis (Narita and Rijli, 2009; Tumpel et al., 2009).

Secondary organizers are required for further programing the AP axis. One such organizer is the anterior neural boundary (ANB) which is located between the most anterior end of the neural plate and adjacent non-neural epidermal ectoderm (Houart et al., 1998). After neural tube closure the ANB eventually forms the commissural plate (CP). The ANB/CP secrets fibroblast growth factor (FGF)-8 which is crucial for forebrain patterning (Shimamura and Rubenstein, 1997). In addition, in zebra fish it was shown that the ANB secretes Wnt inhibitors to maintain and further enhance the Wnt gradient (Houart et al., 2002).

Another important AP organizer is the midbrain-hindbrain boundary (MHB) or isthmus. Transplantation of the MHB to the posterior forebrain induces ectopic midbrain/hindbrain tissue in the chick embryo (Gardner and Barald, 1991; Bloch-Gallego et al., 1996). MHB asserts its organizer function by secreting FGF8 and Wnt1 (Kiecker and Lumsden, 2012) as shown by gene targeting in transgenic mice (Chi et al., 2003).

Differentiation

The newly formed neural tube is a long cylindrical structure composed of a pseudostratified neuroepithelium. With differentiation, the anterior part of the neural tube undergoes a series of expansions, constrictions and bends which become the future forebrain, midbrain, and the hindbrain, while the posterior part of the neural tube develops into the spinal cord. Multiple factors are crucial in these morphological changes, including cytoskeletal rearrangement (Hildebrand and Soriano, 1999; Lee et al., 2007; Roffers-Agarwal et al., 2008), regional cell growth (Xuan et al., 1995; Kahane and Kalcheim, 1998; Lowery and Sive, 2005), apoptosis (Keino et al., 1994; Kuida et al., 1996), and increased intraluminal pressure to form the brain vesicles (Desmond, 1982; Schoenwolf and Desmond, 1984).

The developing neuroepithelium undergoes a series of symmetrical and asymmetrical cell divisions which is controlled by the orientation of the mitotic spindle (Fish et al., 2006; Roszko et al., 2006; Morin et al., 2007). The initial newly formed neurons migrate radially toward the basement membrane of the neural tube by somal dislocation and later by glial-guided cell translocation (Nadarajah et al., 2001), and the developing neural tube becomes a stratified structure. The inner-most layer (near the lumen) of the developing forebrain is termed the ventricular zone (VZ). The radial glia cells residing in the VZ act as stem cells during neural development (Noctor et al., 2001). The cell bodies of RG cells reside in the VZ and have long ascending processes, the radial fibers, that reach the basement membrane located beneath the pia mater (Figure 1.1).

Cortical neurons migrate from their birthplace near the ventricle via two distinct cell migration pathways. For the excitatory glutamatergic neurons, which originate in the pallium of the developing cortex, radial migration is the dominant pattern. The RG cells in the ventricular zone undergo interkinetic nuclear movement during cell cycle, and the nucleus migrates to the apical side (the luminal side) before entering the M phase. The RG cells then undergo a round of asymmetric cell division and produce two daughter cells: one inherits the radial fiber and the identity of stem cells, the other daughter cell becomes the immediate progenitor cell, which undergoes a second round of symmetric cell division in the subventricular zone (SVZ) before committing to the neuronal fate (LaMonica et al., 2012; Tabata et al., 2012). The newly formed neurons then migrate radially toward the pial surface along the radial fiber and populate the cortical plate of the developing neocortex. The radial fibers thus function as the scaffold for the migrating neurons. The outward migration of neurons occurs in a “inside-out” fashion, as the later-born neurons progressively migrate farther toward the pia passing earlier formed neurons

(Rakic, 1974). This method is proposed to be the main pattern building the layered cytoarchitecture of the cortex.

Inhibitory gamma-aminobutyric acid (GABA) -ergic interneurons are born in the ventricular zone of the subpallium in the ventral forebrain (Marin and Rubenstein, 2003). Most of the cortical interneurons are born in the medial ganglion eminence (MGE) (Marin and Rubenstein, 2003). Interneurons from the MGE follow two routes toward the pallium. At embryonic day 12 (E12) the interneurons avoid the striatum and travel superficially through the cortex via the marginal zone or the subplate (Lavdas et al., 1999). At E13.5 and later stages, the majority of the interneurons migrate deeply through the lower intermediate zone and the subventricular zone of the striatum primordium/lateral ganglion eminence (LGE) (Del Rio et al., 1992; DeDiego et al., 1994; Wichterle et al., 2001). It has been suggested these interneurons may use corticofugal axons as the guidance substrate toward the cortex (Denaxa et al., 2001), termed tangential migration. Expression of hepatocyte growth factor (HGF), brain derived neurotrophic factor (BDNF), and neurotrophin-4 in the subpallium have been shown to promote the interneuron migration while TrkB signaling is required for interneuron outward migration (Powell et al., 2001; Polleux et al., 2002).

The lateral ganglionic eminence (LGE) is the birthplace of the olfactory bulb interneurons (Marin and Rubenstein, 2003). Post-mitotic interneuron precursors originate in the dorsal part of the LGE and migrate rostrally via the rostral migratory stream (RMS) to integrate into the periglomerular and granular layers of the olfactory bulb (Wichterle et al., 2001). Generation of new interneurons persists throughout adulthood with interneuron precursors born in the subventricular zone along the lateral wall of the lateral ventricles (Lois and Alvarez-Buylla, 1994). However, unlike their postnatal counterparts, during embryonic development migrating

neurons do not form chain like structures, but appear to rather travel individually (Kishi et al., 1990). The composition of the extracellular matrix of the RMS is crucial during embryonic stages. α1, β8 , and β1 integrins as well as α5 and γ5 chains of the laminin molecule are expressed in the RMS, suggesting that the migrating cells utilize the integrins to move on the laminin substrate (Murase and Horwitz, 2002). Chemotactic signals are also important in the embryonic RMS. The mitral cells in the olfactory bulb express Netrin1 whereas the migrating neurons express the netrin receptors Neogenin and Deleted in Colorectal Carcinoma (DCC) (Murase and Horwitz, 2002). Moreover, the chemorepulsive secreted proteins Slit-1 and Slit-2 are also expressed in the septum and are proposed to direct interneuron migration toward the olfactory bulb (Hu, 1999; Wu et al., 1999). Progenitor cells from the neuroepithelium of the LGE and adjacent regions of the embryonic telencephalon, such as the medial ganglion eminence and the cerebral cortex, later form the adult subventricular zone (SVZ) along the lateral wall of the lateral ventricles (Young et al., 2007). There has been considerable debate regarding the cellular origin of the proliferating neural stem cells in the adult SVZ. Conventionally it was thought that the germinal ventricular zone (VZ) of the developing cortex disappears during the perinatal period and the remnants become the ependymal layer lining the adult lateral ventricles (1970). However, it has been argued that at least a subset of the radial glial cells become the GFAP-positive astrocytes in the postnatal subventricular zone thus linking the germinal ventricular zone in the developing forebrain with the adult subventricular zone (Tramontin et al., 2003; Merkle et al., 2004).

Gliogenesis in early development

In general, gliogenesis follows neurogenesis in the developing mammalian CNS (Rowitch and Kriegstein, 2010). The switch from production of neurons to oligodendrocytes and astrocytes is

regulated at several levels. During early developmental stages the promoters of pro-glial genes such as GFAP are methylated and inactive toward extrinsic signals, but are demethylated later during gliogenesis (Takizawa et al., 2001). Other cell intrinsic factors are also involved. Homeobox proteins DLX1 and DLX2 have been shown to regulate the lineage switch between neurons and oligodendrocytes (Petryniak et al., 2007). In addition, epigenetic control is suggested to play an important role. For example, the polycomb group complex represses the promoter of the pro-neural activator Neurogenin-1 in a stage-dependent manner (Hirabayashi et al., 2009). The neuron-glial switch is also controlled by cell extrinsic factors. During neurogenesis the expression of Fgf2 and Neuregulin-1 have been shown to repress pro-glial gene expression (Hermanson et al., 2002; Sardi et al., 2006). Conversely, gliogenesis can be promoted by neuronal signals. It has been shown that the neuronal intermediate progenitors and newly formed neurons express Notch signaling ligands Dll1 and Dll3, which in turn activate gliogenesis and inhibit neurogenesis in the nearby neural stem cells (Campos et al., 2001; Namihira et al., 2009). The differentiated neurons also express and secret pro-glial cytokines such as leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF) and Cardiotrophin 1 and thus promote gliogenesis (Bonni et al., 1997; Ochiai et al., 2001; Barnabe-Heider et al., 2005). In addition, astrocyte secreted platelet-derived growth factor (PDGF) signaling has been shown to be crucial for oligodendrocyte lineage specification, as PDGF promotes the differentiation of oligodendrocytes-type-2-astrocyte (O-2A) progenitor cells (Richardson et al., 1988; Robinson and Miller, 1996; Marmur et al., 1998), and PDGFα knockout results in defective oligodendrocyte development and hypomyelination (Fruttiger et al., 1999).

Post-natal/adult neurogenesis

Before the 1960s, neurogenesis, the generation of neural tissue, was believed to only occur during embryogenesis and perinatal stages in mammals. The adult CNS was thought to lack regenerative capability and no new neurons were thought to form in adulthood (Cajal, 1928; Rakic, 1974). However, in the late 1960s Joseph Altman and Shirley Bayer first demonstrated that cell proliferation continues in various regions in the post-natal rat brain including the hippocampus, the olfactory bulb, and the cortex using tritium-thymidine (^{3}H-T) labeling (Altman, 1962, 1963, 1969). In the late 1970s this novel idea of adult neurogenesis was further confirmed by Kaplan and colleagues. By using ^{3}H-T-labeling and electron microscopy they showed the ^{3}H-T-labeled, newly formed cells indeed have neuronal identity in rodent brains (Kaplan and Hinds, 1977; Kaplan and Bell, 1984). Around the same time adult neurogenesis was also confirmed in the avian hyperstriatum ventrale pars caudalis (HVc) in song birds (Goldman and Nottebohm, 1983). Then in the early 1990s, the Weiss group first reported that cells isolated from the mouse striatum (later identified as the subventricular zone (SVZ) along the lateral wall of the lateral ventricles) could be expanded in suspension culture and could differentiate into neurons and astrocytes *in vitro*, indicating the presence of multipotent stem cells in the adult rodent brain (Reynolds and Weiss, 1992). Later a second regional source of neural stem cells was identified in the hippocampus, where neurons born in the dentate gyrus migrate and integrate into the granule cell layer (Stanfield and Trice, 1988) in response to learning and exercise. The newly formed neurons in the subgranular zone (SGZ) migrate and integrate locally into the circuitry in the hippocampus, and are important for the functions of hippocampus such as memory formation and learning.

The SVZ neural stem cell niche

In the adult SVZ, poly-ciliated ependymal cells line the wall along the lateral ventricles (Spassky et al., 2005). They form a unique pinwheel architecture surrounding the apical endings of reactive astrocytes on the ventricular surface (Mirzadeh et al., 2008). Although the ependymal cells, as well as the multi-potent stem cells, are derived from radial glial cells, ependymal cells remain quiescent in normal conditions. They apparently only enter cell cycle and generate neuroblasts that migrate to the olfactory bulb when responding to injury (Carlen et al., 2009). In addition to serving as a reservoir, ependymal cells has been shown to secrete Noggin and are important in promoting neurogenesis in the SVZ (Lim et al., 2000). Furthermore, the beating of motile cilia by ependymal cells generate cerebrospinal fluid flow, which in turn has been suggested to play a significant role in directing neuroblast migration (Sawamoto et al., 2006). Ependymal cells express CD24 (Mirzadeh et al., 2008; Pastrana et al., 2009), S100β (Raponi et al., 2007), and CD133/Prominin (Coskun et al., 2008), although none of these markers are exclusive to ependymal cells (Figure 1.2).

The neural stem cells, or "B cells" in adult SVZ

Proliferative cells in the adult SVZ can be divided into two subgroups: the slowly dividing, BrdU retaining neural stem cells (B cells) and the rapidly dividing transit amplifying cells (TAC, or C cells) (Doetsch et al., 1997; Doetsch et al., 1999b). B cells express glial fibrillary acidic protein (GFAP) and have an astrocyte morphology (Doetsch et al., 1999b). The B cells can be further categorized into two groups based on their location and morphology (Doetsch et al., 1997). The B1 cells locate near the ventricular surface and directly contact the ependymal cells. They extend cell processes toward the ventricular surface and have primary cilia extruding into the ventricle, which directly contact the CSF and have been suggested to play an essential role in cell signaling (Doetsch et al., 1999a; Mirzadeh et al., 2008). B2 cells, on the other hand, locate more deeply

and close to the underlying striatal parenchyma (Doetsch et al., 1997). B cells also form a glial sheath surrounding the migrating neuroblasts as they migrate anteriorly toward the OB (Lois and Alvarez-Buylla, 1994; Doetsch et al., 1999b). Moreover B cells extend long endfeet which directly contact blood vessels (Shen et al., 2008; Tavazoie et al., 2008). In addition to GFAP (Figure Two), B cells also express several astroglia or neural progenitor markers, such as brain-lipid-binding protein (BLBP) (Platel et al., 2009), astrocyte-specific glutamate transporter (GLAST) (Pastrana et al., 2009), PDGFRα (Jackson et al., 2006), Vimentin (Doetsch et al., 1999b), SSEA1 (Capela and Temple, 2002), Nestin (Doetsch et al., 1997), and CD133/Prominin (Coskun et al., 2008), although Nestin is also expressed at low levels in other cell types in the SVZ (Doetsch et al., 1997). In mice, the human glial fibrillary acidic protein promoter has been used extensively to target genes to B cells, since it is not expressed in other lineages. In addition, onset of hGFAP-Cre transgene expression starts in the forebrain by E13.5, so the hGFAP promoter is active in multi-potential neural stem cells (Zhuo et al., 2001).

The transit amplifying cells, or "C cells" in adult SVZ.

The transit amplifying cells (TAC), or C cells, are rapid proliferating, immediate progeny of B cells (Doetsch et al., 1999b). They have been shown to express EGFR (Pastrana et al., 2009). Indeed the C cells are proposed to be the major population that responds to EGF in the neurosphere assay *in vitro* (Doetsch et al., 2002; Pastrana et al., 2009). However, a small population of B cells also express EGFR, thus EGFR is not an exclusive C cell marker (Pastrana et al., 2009). C cells typically locate close to the vessels, suggesting signals from the vasculature play an important role in C cell regulation (Shen et al., 2008; Tavazoie et al., 2008). In addition to EGFR, commonly used markers expressed by transit amplifying C cells also include Mash1/Ascl1 and Dlx2. However, just as EGFR, both Mash1 and Dlx2 are not exclusive to C

cells. Mash1 is also expressed in a population of B cells (Kim et al., 2011a) while some doublecortin (Dcx) and polysialylated neural cell adhesion molecule (PSA-NCAM) positive neuroblasts also express Dlx2 (de Chevigny et al., 2012).

The neuroblasts, or "A cells" in adult SVZ

The C cells further differentiate to the neuroblasts, or A cells (Doetsch et al., 1999b). In the SVZ, neuroblasts migrate anteriorly and form a chain-like structure which is surrounded by B cells (Lois and Alvarez-Buylla, 1994). Their cell bodies are typically round or elongated, with a thin leading process tipped by a growth cone and occasionally with a trailing process (Doetsch et al., 1999b). Neuroblasts ultimately join the rostral migratory stream (RMS) and enter the olfactory bulb (OB). After reaching the olfactory bulb, the neuroblasts differentiate and form interneurons which integrate into the periglomerular and granular layers (Lois and Alvarez-Buylla, 1994). This phenomenon is crucial for odor discrimination and learning, as reduced neurogenesis in mice impairs discrimination between discrete odors (Gheusi et al., 2000). The neuroblasts express a series of markers such as doublecortin (Dcx) (Yang et al., 2004), type-III-β-tubulin (Tuj1) (Doetsch et al., 1997), polysialylated-neural cell adhesion molecule (PSA-NCAM) (Seki and Arai, 1993), and low levels of CD24 (Pastrana et al., 2009).

Gliogenesis in adult SVZ

Gliogenesis in the adult SVZ is less well understood. It has been reported that both some B cells and a subpopulation of C cells express the oligodendrocyte progenitor cell (OPC) marker oligodendrocyte lineage transcription factor-2 (Olig-2). These cells originating from the adult SVZ migrate to the corpus callosum, striatum and fimbria fornix to differentiate to NG-2-positive oligodendrocyte precursors and mature myelinating oligodendrocytes (Hack et al., 2005;

Menn et al., 2006). However, it has been controversial whether the astroglial versus neuronal lineage decision happens at the B cell stage or C cell stage, and if all OPCs share the same origin as neuronal precursor cells as mutation of Mash-1, a proneuronal gene which is also highly expressed in C cells, leads to loss of both neuronal and oligodendrocytes lineages in a cell-autonomous fashion.

The extracellular matrix in adult SVZ

The extracellular matrix (ECM) in the adult SVZ is rich in multiple components such as laminin, collagen, nidogen, perlecan, and proteoglycans (Gates et al., 1995; Kerever et al., 2007). N-sulfate heparin sulfate proteoglycan (HSPG) in the adult SVZ can directly bind to FGF-2 and therefore modulate local mitogen availability (Kerever et al., 2007). Another proteoglycan, Tenascin-C, which is highly expressed during embryonic neural development, is also highly expressed in the adult SVZ (Gates et al., 1995). Tenascin-C can directly bind to specific cell surface receptors and thus modulate cell behavior (Jones and Jones, 2000). In perinatal mice, knockout of Tenascin-C results in fewer NSCs and altered NSC response toward growth factors (Garcion et al., 2004). It has also been shown that dermatan sulfate-dependent (DSD)-1-proteoglycan, a chondrointin sulfate proteoglycan (CSPG), and apolipoprotein E (ApoE) promote NSC survival and proliferation while treatment of neurospheres with the enzyme chondroitinase ABC (ChABC) results in diminished cell proliferation and impaired neuronal differentiation (Gates et al., 1995; Sirko et al., 2007; Tham et al., 2010).

Vasculature in adult SVZ

The close proximity of blood vessels and the sites of adult neurogenesis in both adult SVZ and SGZ has suggested the importance of the vasculature in neurogenesis (Leventhal et al., 1999;

Palmer et al., 2000; Gotts and Chesselet, 2005). In the SGZ in the dente gyrus of the adult hippocampus, the majority of dividing neural precursors are closely associated with the vasculature and dividing endothelial cells, suggesting that neurogenesis occurs within an angiogenic niche (Palmer et al., 2000). In the adult SVZ, the BrdU retaining neural stem cells extend long endfeet and directly contact blood vessels (Shen et al., 2008; Tavazoie et al., 2008). In addition, proliferating transit amplifying cells also lie near blood vessels (Shen et al., 2008; Tavazoie et al., 2008). The laminin receptor α6β1 integrins are important in NSCs binding to endothelial cells (Shen et al., 2008). Furthermore, the blood-brain barrier (BBB) in the SVZ is altered so that the blood vessel walls are permissive to small molecular weight molecules (<400Da), which is not observed in other parts of the brain (Tavazoie et al., 2008). C-X-C ligand 12 (CXCL12/SDF1), expressed by the endothelial cells, has been proposed to induce B cells activation and translocation from near the ependyma toward the blood vessels (Kokovay et al., 2010). In addition, transplanted neural progenitors home to blood vessels, which is mediated by the interaction of CXCL12/SDF1 and its receptor CXCR4 expressed by the neuroblasts (Kokovay et al., 2010). Furthermore, pigmented epithelium derived factor (PEDF), which is secreted by both ependymal cells and endothelial cells, can enhance the activation of B cells (Ramirez-Castillejo et al., 2006). Finally, vascular endothelial growth factor (VEGF), which is expressed by the astrocytes in the adult SVZ and SGZ as well as the choroid plexus (Licht and Keshet, 2013), has been shown to play an important role in regulating NSCs. (Detailed below)

Many cytokine and growth factor signaling family members have been implicated in maintaining the integrity of the niche or alternatively to promote lineage differentiation (Faigle and Song, 2013).

The BMP signaling pathway

Bone Morphogenetic Proteins (BMPs) are a group of secreted glycoproteins that belong to the Transforming Growth Factor-beta (TGF-β) superfamily (Zimmerman et al., 1996). To date, 15 members of this subfamily have been identified. BMPs act by binding to the receptor tyrosine kinase BMP receptors type I and type II. After ligand binding, two type I and two type II receptors form a heterotetramer (Figure 1.3). The type II receptors then autophosphorylate and phosphorylate the type I receptors, thus creating binding sites for the downstream effector SMAD proteins. After activating phosphorylation by the BMPR two receptor-activated SMADs (R-SMADs, SMAD-1, 5, 8) form a trimer with an additional common-mediator SMAD (Co-SMAD, SMAD-4). The trimerized SMADs then translocate into the nucleus and activate target gene transcription. The inhibitory SMADs (I-SMADs, SMAD-6, 7) can interfere with signaling by binding to SMAD-4 (Mueller and Nickel, 2012). In addition BMP signaling can be antagonized by binding of BMPs by extracellular proteins Noggin and Chordin (Piccolo et al., 1996; Zimmerman et al., 1996). (Figure 1.3)

BMP2, BMP4, and the BMPRs are expressed in the adult SVZ (Lim et al., 2000). BMPs are expressed by the GFAP+ astrocytes, while Noggin is expressed by the ependymal cells lining the ventricular wall (Lim et al., 2000). Transgenic over-expression of BMPs in the SVZ has been shown to promote glial differentiation (Lim et al., 2000; Bonaguidi et al., 2005); while either infusion of noggin protein into the SVZ or ectopic expression of Noggin in the adjacent striatum increases neuronal differentiation (Lim et al., 2000). In the SGZ of the dentate gyrus BMP signaling has been proposed to be important in NSC aging while Noggin expression is crucial for

the maintenance of NSC both *in vitro* and *in vivo* (Bonaguidi et al., 2008). Blockade of the BMP signaling by either Noggin infusion or SMAD-4 conditional knockout transiently increases, but later reduces the number of proliferating neural precursors and thus limits the production of mature neurons (Mira et al., 2010).

The Notch signaling pathway

The Notch receptors Notch1-4 are single pass transmembrane proteins. There are six ligands, which are also transmembrane proteins: Jagged-1, 2 (Jag-1, 2) , Delta-like-1 through 4 (Dll-1, 2, 3, 4) (Figure 1.4). The receptor and ligand are typically expressed by adjacent cells (Greenwald and Kovall, 2013). Upon ligand binding, the Notch protein undergoes two separate cleavage events. First the ADAM protease cuts at the base of the Notch extracellular domain. The second cleavage occurs at the base of the Notch intracellular domain by the γ-secretase complex thereby releasing the Notch intracellular domain (NICD). The NICD then migrates into the nucleus and forms a transcription regulating complex with two other proteins, Mastermind and CSL protein (CBF-1/RBP-Jk in vertebrates, Su(H) in flies, and LAG-1 in worms) to regulate target gene expression (Greenwald, 2012; Greenwald and Kovall, 2013) (Figure 1.4).

In the adult SVZ, it has been shown that Notch signaling is required for the maintenance of the NSC pool. Conditional knockout of RBP-Jk in the adult SVZ leads to a transient increase in the number of type C cells and later depletion of the BrdU retaining stem cells (Imayoshi et al., 2010). Similarly, conditional knockout of Notch-1 in the adult hippocampal SGZ results in the loss of GFAP+ type-1 cells and transit amplifying cells (Ables et al., 2010). It has also been shown that the NSC marker Sox2 is a direct downstream target of the Notch signaling and

deletion of the RBP-Jk in the SGZ leads to premature neuronal differentiation and subsequent depletion of Sox2+ cells (Ehm et al., 2010).

The Sonic Hedgehog (Shh) signaling pathway

Shh is a member of the Hedgehog secreted protein family, which also includes Desert and Indian Hedgehog. In the absence of Shh, the receptor Patched-1, a twelve-pass transmembrane protein, localized in the primary cilium of the cell inhibits Smoothened (Smo), a member of the seven-pass G-protein coupled receptor family, by accumulation in the cilium (Figure 1.5). The Gli proteins are then phosphorylated by the protein kinase A (PKA) and targeted for proteasome-mediated proteolysis. The cleaved, truncated form of Gli (GliR) then migrates into the nucleus and acts as transcriptional repressor. Upon binding of Shh, Patched-1 leaves the primary cilium allowing Smo to enter. The active Smo then inhibits PKA and the proteolysis of Gli. Full-length Gli then enter the nucleus and promotes its target gene transcription (Rohatgi et al., 2007).

Ectopic expression of Shh in the hippocampus results in an increase in the number of proliferating cells and newborn neurons (Lai et al., 2003). In addition, the Nestin::Cre-Smo$^{null/flox}$ conditional knockout animals have decreased numbers of BrdU retaining cells and increased apoptosis in both the postnatal SVZ and SGZ, suggesting Shh is required for stem cell maintenance (Machold et al., 2003; Balordi and Fishell, 2007). More interestingly, loss of Smo in the SVZ leads to abnormalities of A cell migration into the OB and accumulation of A cells in the SVZ, which is non-cell autonomous (Balordi and Fishell, 2007).

The Wnt signaling pathway

The Wnt signaling pathway is one of the most complex pathways, with 19 ligands and more than 15 receptors/co-receptors identified to date (Niehrs, 2012). In addition, there are multiple

pathways within the pathway. A) The canonical Wnt signaling pathway: In the absence of Wnt ligand, glycogen synthase kinase-3 (GSK3) forms a destruction complex with casein kinase Iα (CKIα), Axin, and adenomatosis polyposis coli (APC) protein, phosphorylates β-catenin leading to its ubiquitination and degradation (Figure 1.6). When Wnt ligand binds the receptor, the Frizzled/Dishevelled/LRP-5/6 complex, the destruction complex is inhibited and relieves β-catenin from degradation. β-catenin then translocates into the nucleus, binds to T-cell factors (TCF), and induces its target gene expression. B) The non-canonical, β-catenin independent Wnt signaling pathway: Wnt binding to the receptor can also activate small GTPases, which in turn activate RHO kinase (ROCK) and JUN-N-terminal kinase (JNK). This pathway, the PCP (planar cell polarity) pathway, is particularly important in regulating cell polarity and migration (Niehrs, 2012). Wnt can also activate phospholipase C, which induces the increase of cytosolic Ca^{2+} concentration. High Ca^{2+} concentration subsequently leads to the activation CAMKII, PKC and Calcineurin (Niehrs, 2012) (Figure 1.6).

In the adult SVZ, it has been shown that induction of the Wnt signaling pathway by retrovirus-mediated expression of β-catenin or GSK-3 inhibition by drugs promotes proliferation of Mash-1+ type-C cells and subsequent increased numbers of newly integrated neurons in the OB (Adachi et al., 2007). Wnt-3a and Wnt-5a also promote neuronal differentiation of adult SVZ neural progenitor cells *in vitro* (Yu et al., 2006). Expression of a dominant-negative Wnt in the adult dente gyrus inhibits cell proliferation and newborn neuron formation in the SGZ (Lie et al., 2005). It has also been shown that Wnt-β-catenin signaling can directly activate NeuroD1 transcription to promote neuronal differentiation (Gao et al., 2009; Hsieh et al., 2009).

The Retinoic acid (RA) signaling pathway

Retinoic acid is a non-peptide, small lipophilic signaling molecule that is either synthesized from retinol (Vitamin A) from the diet or diffuses from the surrounding extracellular environment. Uptake of extracellular retinol, bound by retinol binding protein 4 (RBP4), is aided by a transmembrane protein STRA6. Once in the cytosol, retinol is bound by cellular retinol binding protein (CRBP) and oxidized by retinol dehydrogeneses (RDHs) becoming retinaldehyde. Retinaldehyde is further oxidized by retinaldehyde dehydrogenases (RALDHs) becoming retinoic acid (RA). Cytosolic RA is bound by cellular retinoic acid binding proteins (CRABPs) and transported to the nucleus. In the nucleus, the free RA binds to the heterodimers of the nuclear RA receptors RARs (RARα, RARβ, and RARγ) and the retinoid X receptors RXRs (RXRα, RXRβ, and RXRγ). The active nuclear receptors then bind to the retinoic acid-responsive elements (RAREs) and regulate gene transcription. Cytosolic RA can also be exported to the extracellular environment and act in autocrine/paracrine fashions. (Reviewed in (Niederreither and Dolle, 2008; Rhinn and Dolle, 2012)) (Figure 1.7).

In the adult SVZ, a subset of the slow dividing B cells respond to RA signaling (Haskell and LaMantia, 2005). RA exposure increases proliferation of postnatal SVZ neuroblasts and neuronal differentiation; while blocking RA signaling impairs the migration of the neuroblasts (Wang et al., 2005). In addition, RA application after stroke has been shown to promote neurogenesis and decrease infarct volume in the striatum (Plane et al., 2008). In the adult hippocampal SGZ, RA promotes neuronal differentiation by up-regulating NeuroD expression (Takahashi et al., 1999). Conversely, depletion of RA significantly decreases neuronal differentiation and cell survival while, interestingly, proliferation was not affected (Jacobs et al., 2006).

RTK Signaling: The Fibroblast growth factor (FGF), Platelet-derived growth factor (PDGF), Vascular-endothelial growth factor (VEGF), and Epidermal growth factor (EGF) signaling pathway

Receptors for FGF, PDGF, VEGF, and EGF belong to the receptor tyrosine kinase (RTK) superfamily. Upon ligand binding, receptors dimerize and the intracellular tyrosine kinase domains activate and transphosphorylate each other (Figure 1.8). The phosphorylated tyrosine residues thus create binding sites for the adapter protein Grb2. This is followed by the activation of the small GTPase Ras, and subsequent activation of the Raf/MEK/ERK pathway. The activated RTKs can also activate phosphatidylinositol-4,5-bisphosphate-3 kinase (PI3K), which in turn activates its downstream Akt/mTor signaling pathway. Phospholipase C-γ also binds to the phosphotyrosine residues on RTKs and activates the downstream signaling pathways. (Reviewed in (Hausott et al., 2009; Pownall and Isaacs, 2010; Casaletto and McClatchey, 2012)). (Figure 1.8)

NSCs from the dente gyrus of the adult hippocampus and SVZ respond to FGF-2, and these FGF-2 responsive cells can be isolated and maintained *in vitro* (Gage et al., 1995; Palmer et al., 1995). FGFR1 and FGFR2 have been shown to be expressed in the adult rat SVZ (Frinchi et al., 2008), and intracerebroventricular infusion of FGF-2 and EGF increase cell proliferation in both adult SVZ and SGZ (Wagner et al., 1999; Jin et al., 2003). Disruption of FGF-2 signaling by either injection of neutralizing antibody to FGF-2 or conditional knockout of FGFR1 inhibits cell proliferation and newborn neuron production in the adult DG (Tao et al., 1997; Zhao et al., 2007). In addition, FGF-2 expression is elevated after either chemical induced seizure or middle cerebral artery occlusion (MCAO), a condition that increases neurogenesis; while proliferation is

hindered in the hippocampus of FGF-2 knockout animals (Lin et al., 1997; Yoshimura et al., 2001).

PDGF signaling has been shown to be required for oligodendrocyte lineage specification during embryonic development (Fruttiger et al., 1999). In the adult SVZ a subset of the B cells express PDGFRα, and it is required for oligodendrogenesis but not neurogenesis (Jackson et al., 2006). In addition intraventricular infusion of PDGF is sufficient to induce B cell proliferation and the generation of hyperplasia, suggesting PDGF signaling is important for tumor formation (Jackson et al., 2006).

In the adult rodent brain including SVZ and SGZ, VEGF is extensively expressed by astrocytes as well as in the choroid plexus (Licht and Keshet, 2013). In the SVZ both the GFAP+ B cells and Dcx+ neuroblasts express receptors for VEGF (Wittko et al., 2009). It has been shown VEGF promotes SVZ cell proliferation both *in vitro* and *in vivo* (Jin et al., 2002). In neonatal animals VEGF is crucial for forming the vascular scaffolding for RMS migration (Bozoyan et al., 2012). However VEGF is not required for the RMS migration in adults (Licht et al., 2010), but is suggested to accelerate neuroblast migration through the RMS (Wittko et al., 2009). Detail functions of VEGF in the adult SGZ are less clear. VEGF loss of function does not impair basal neurogenesis (Cao et al., 2004; Licht et al., 2011), but it is required for the increased neurogenesis induced by exercise or enriched environment (Fabel et al., 2003; Cao et al., 2004).

EGF has been shown to promote proliferation of neurosphere forming cells isolated from the adult SVZ (Reynolds and Weiss, 1992). It has been shown that EGFR is expressed by the C cells in the adult SVZ (Enwere et al., 2004; Pastrana et al., 2009), and infusion of EGF into the adult lateral ventricles promotes proliferation (Craig et al., 1996; Kuhn et al., 1997). Although EGF

expression is low in the SVZ and hippocampus (Fallon et al., 1984), the other endogenous EGFR ligand, TGFα, is highly expressed in both the striatum and the dente gyrus (Wilcox and Derynck, 1988; Seroogy et al., 1993). TGFα knockout animals have reduced constitutively proliferating cells in the SVZ and decreased migration of neuroblasts to the OB, suggesting that the EGF signaling pathway is crucial for the transit amplifying C cells in the adult SVZ (Tropepe et al., 1997).

The Leukemia inhibitory factor (LIF)/ Ciliary neurotrophic factor (CNTF) signaling pathway

Both LIF and CNTF are polypeptide cytokines belonging to the interleukin-6 family. LIF binds to a heterodimeric receptor complex consisting of one LIF receptor β (LIFRβ) and one co-receptor gp130; while CNTF binds to a trimeric receptor complex consisting of one LIFR β, one gp130, and one additional CNTF receptor α (CNTFRα) (Bauer et al., 2007) (Figure 1.9). Upon ligand binding, the receptor associated Janus-activated kinase (JAK) autophosphorylates and also phosphorylates the tyrosine residues on the receptors, thus creating binding sites for the signal transducing and activator of transcription (STAT). JAK also phosphorylates receptor bound STATs. Phosphorylated STATs then dimerize and translocate into the nucleus and activate gene transcription (Figure 1.10). In addition, the phosphorylated tyrosine residues also create binding sites and activate the MAPK and PI3K signaling pathways. (Reviewed in (Bauer et al., 2007).) (Figure 1.9).

LIF/CNTF signaling is required for astrocyte differentiation, as GFAP+ astrocyte formation is halted in LIFR null mice during embryonic development (Koblar et al., 1998). In addition, activation of the JAK-STAT signaling pathway by constitutive expression of STAT3 leads to

precocious differentiation of astrocytes from isolated neural progenitors (He et al., 2005). In adult animals, injection of CNTF in the forebrain increases the number of BrdU labeled cells in both SVZ and SGZ (Emsley and Hagg, 2003). It has further been shown that only a subset of B cells respond to CNTF signaling. Conditional knockout of CNTFRα using hGFAP:Cre increases neurogenesis and neuroblast number in the OB, while NSC maintenance is not affected (Lee et al., 2013). On the other hand, over-expression of LIF in the adult SVZ inhibits neurogenesis, promotes proliferation or formation of glial progenitors in the SVZ, while expands the NSC pool (Bauer and Patterson, 2006).

Thus, many signaling pathways, extracellular matrix and cell types combine to play a critical role in maintaining/controlling adult neurogenesis.

microRNA

microRNAs (miRNAs) are 20 to 24 nucleotides long, single stranded non-coding RNAs (Lee et al., 1993). To date more than 2,500 and nearly 3,000 microRNAs have been identified in human and mice, respectively . microRNAs can be located within coding genes, either intronically or exonically, or between coding genes, and can be either transcribed by RNA polymerase II or polymerase III, (Lee et al., 2004; Borchert et al., 2006). The 150-200 nucleotide long primary transcripts of microRNA (pri-miRNAs) are processed, or "cropped", in the nucleus by the microprocessor, which includes the nuclear RNase II enzyme Drosha and DiGeorge syndrome critical region gene 8 (DGCR8), to release the 60-100 nucleotides long hairpin-structured precursor-microRNAs (pre-miRNAs) (Lee et al., 2003). Alternatively, pre-miRNAs of microRNAs located within introns can be generated through the mRNA splicing step and bypass

the Drosha-mediated processing (Okamura et al., 2007; Ruby et al., 2007). Pre-miRNAs then are transported by exportin-5 out of the nucleus to the cytoplasm (Yi et al., 2003). In the cytoplasm, pre-miRNAs are then further cut, or "diced", to release the 20-24 nucleotide long duplexes by Dicer (Bernstein et al., 2001; Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). Typically, one strand of the duplex is degraded while the other strand, the mature miRNA, survives (Khvorova et al., 2003; Schwarz et al., 2003). (miRNA biogenesis is reviewed in (Kim, 2005).) The mature miRNA then is incorporated into the miRNA loaded-RNA induce silencing complex (miRISC), which also contains an Argonaute family member. miRISCs target the 3' untranslated region (3' UTR) of target gene transcripts by an imperfect match between the miRNA and the mRNA transcript. The "seed" sequence, position 2-8 from the 5' end of the miRNA, has been shown to be crucial in the target recognition. (miRNA target recognition is reviewed in (Bartel, 2009).) Binding of miRISC to the 3' UTR leads to translational repression, mRNA destabilization, and/or degradation, thus resulting in the down-regulation of target gene expression. (Mechanisms of miRNA mediated post-transcriptional regulation are reviewed in (Filipowicz et al., 2008).)

microRNAs play an important role in regulating neurogenesis. Complete knock-out of the RNase III Dicer1 results in embryonic lethality as early as E7.5 and the depletion of multi-potent stem cells (Bernstein et al., 2003). Conditional deletion of Dicer1 in the nervous system using Nestin:;Cre also leads to embryonic lethality (Kawase-Koga et al., 2009). Furthermore, conditional ablation of Dicer1 in different neural progenitor populations demonstrate that miRNAs are crucial for both neuronal and glial development (Davis et al., 2008; De Pietri Tonelli et al., 2008; Kawase-Koga et al., 2009; Huang et al., 2010). Each miRNA, has different functions in the CNS. One of the most well-understood microRNAs is miR-124. miR-124 is one

of the most abundant miRNA in the brain (Lagos-Quintana et al., 2002). During embryonic neural development, miR-124 promotes neuronal differentiation by suppressing the small C-terminal domain phosphatase 1 (SCP-1) and the repressor element-1 silencing transcription factor (REST) pathway (Visvanathan et al., 2007), in addition to the neural progenitor-specific BAF complex 53 kDa subunit (BAF53a) (Yoo et al., 2009). In the adult SVZ, miR-124 expression level is low in B cells but is up-regulated during the C cell to A cell transition (Cheng et al., 2009). Functional inhibition of miR-124 maintains the neural progenitors as dividing precursor cells, while over-expression of miR-124 promotes precocious neuronal differentiation (Cheng et al., 2009; Akerblom et al., 2012). miR-124 asserts its function by repressing the SRY-box transcription factor Sox-9 (Cheng et al., 2009) and Notch ligand Jagged-1 (Liu et al., 2011). In addition, miR-124 targets PTB/hnRNP I (PTBP1) mRNA, which encodes a global repressor of alternative pre-mRNA slicing in non-neuronal cells. Down-regulation of PTBP1 in turn leads to the accumulation of PTBP2 promoting neuronal differentiation (Makeyev et al., 2007).

miR-9, another highly expressed miRNA in the brain, directly targets the orphan nuclear receptor TLX, inhibiting cell proliferation and promoting neuronal differentiation in adult SGZ NSCs. TLX itself represses expression of miR-9, forming a negative regulatory loop (Zhao et al., 2009). In addition, miR-9 also targets the REST pathway thereby promoting neuronal differentiation (Packer et al., 2008). The evolutionarily conserved let-7 family of miRNAs are also important in regulating neurogenesis. let-7b targets TLX, cyclin D1, and high mobility group-AT-hook 2 (HMGA2). Overexpression of let-7b reduces NSC proliferation and increases neural differentiation, while antisense knockdown of let-7b leads to enhanced proliferation (Nishino et al., 2008; Zhao et al., 2010). miR-184, on the other hand, targets Numb-like (Numbl), promoting self-renewal and inhibiting differentiation in the adult SGZ NSCs. Expression of miR-184 itself

is then controlled by the methyl-CpG binding protein 1 (MBD1) (Liu et al., 2010). miR-137 also inhibits NSC proliferation and promotes neuronal differentiation in both the adult SGZ and embryonic NSCs by repressing histone lysine-specific demethylase 1 (LSD1) and Ezh2, a histone methyltransferase and Polycomb group protein (Szulwach et al., 2010; Sun et al., 2011). The miR-106b-25 cluster has also been reported to regulate adult NSCs. Ectopic expression of miR-25 promotes cell proliferation, while the expression of the cluster itself is regulated by FoxO3 (Brett et al., 2011). In addition, it has been shown that miR-34a promotes astrocyte differentiation of embryonic NSCs (Aranha et al., 2011).

We have observed that the neurons derived from the miR-410 over-expressing neural stem cells have shorter and fewer numbers of neurites (Chapter 1). Neurons derived from adult subventricular neurogenesis typically takes 21 to 42 day to fully mature (Whitman and Greer, 2009). Considering the early stage these neurons were at (in vitro differentiation day 7), the phenotype may possibly be attributed to the difference in neurite initiation and elongation. Neurite initiation and elongation are complex processes and several pathways have been reported to promote or inhibit the formation of neurites (reviewed in (Hall and Lalli, 2010; Polleux and Snider, 2010)).

Small GTPases

Small GTPase family proteins such as Ras and Rho have been shown to play a major role in regulation of neurite growth (reviewed in (Hall and Lalli, 2010)). Small GTPases are activated by GEFs and inactivated by GAPs (Hall and Lalli, 2010). Ras has been shown to be present in the newly formed axon (Fivaz et al., 2008). Over-expression of constitutively active R-Ras in the

hippocampal neurons in vitro induced multiple axon formation while inactivation of the endogenous R-Ras by siRNA abolished normal axon formation (Oinuma et al., 2007). In addition, Oinuma et al also suggested the function of R-Ras on axon outgrowth relays through PI3K. Furthermore, PI3K in turn activates Ras, thus forming a positive feedback loop, and another small GTPase Rap1B and its downstream effector Cdc42 (Schwamborn and Puschel, 2004). Cdc42, yet another small GTPase, regulates the actin filament/microtubule structure and axon outgrowth through its downstream effector IQGAP3 (Wang et al., 2007), PAK4 (Qu et al., 2003), and N-WASP(Banzai et al., 2000; Strasser et al., 2004). Additionally Cdc42 also acts through the Par6/aPKC-APC (adenomatous polyposis coli) and regulates microtubule assembly (Shi et al., 2004; Goldstein and Macara, 2007). In parallel another small GTPase Rac1 promotes neurite outgrowth by increasing the phosphorylation and inactivation of the microtubule destabilizing protein Stathmin/Op18 (Watabe-Uchida et al., 2006). The Rho small GTPase functions antagonistically to Rac/Cdc42. Constitutively active Rho prevents neurite outgrowth (Schwamborn and Puschel, 2004). RhoA asserts its negative regulation by activating its downstream kinase ROCKII ROCK phosphorylates Profilin-IIa and thus destabilizes actin filaments (Da Silva et al., 2003). However, Rho is required in axon elongation induced by the chemokine Stromal cell-derived factor (SDF)-1α in cerebellar granule neurons through a distinct downstream effector mDia (Arakawa et al., 2003).

PI3K-AKT-mTor and PTEN

The phosphatidylinositol-3 kinases (PI3Ks) are kinases that phosphorylate inositol lipids at the 3' position and are responsible for generating its downstream signaling transducer phosphatidylinositol-(3,4,5)-triphosphate (PIP3) and can be directly activated by Ras (Leevers et al., 1999). Activation of the PI3K pathway promotes axon formation, as inhibition of PI3K by

small molecules such as LY294002 or Wortmannin impedes axon formation (Shi et al., 2003; Menager et al., 2004; Jiang et al., 2005; Yoshimura et al., 2006). Furthermore, constitutively activation of one of the PI3K downstream effector AKT/protein kinase B by myristoylation in neurons leads to multiple axon formation (Yoshimura et al., 2006). Likewise, inhibition of mTor, which is activated by AKT, by Rapamycin in hippocampal neurons severely inhibited neurite formation (Li et al., 2008).

Phosphatase and Tensin homolog deleted on chromosome 10 (PTEN) directly antagonizes the function of PI3K by dephosphorylating PIP3 and thus turning off its downstream pathways. Consequently over-expression of PTEN in the hippocampal neurons results in the inhibition of axon formation (Shi et al., 2003; Jiang et al., 2005). In addition knock-down of PTEN by siRNA leads to multiple axon formation (Jiang et al., 2005). Interestingly, PTEN also represses injury-induced axon regrowth by suppressing the Rapamycin sensitive mTor pathway (Park et al., 2008).

MAPK

The classic MAPK pathway, the RAF-MEK-ERK cascade, is activated by the tyrosine receptor kinase and its downstream small GTPase Ras (Casaletto and McClatchey, 2012). More importantly over-expression of dominant negative RAF in the dorsal root ganglion neurons abolished the axon elongation induced by NGF while constitutively active RAF led to elongation and axon growth (Markus et al., 2002). Furthermore, it has been shown that inhibition of MEK by small molecule U0126 is sufficient to induce growth cone collapse in sympathetic axons (Atwal et al., 2003). In addition using of the ERK inhibitor PD98059 reduced nascent protein

synthesis in the growth cones, which may provide partial explanations for the underlying mechanism (Campbell and Holt, 2003).

Another family of MAPK, the c-Jun N-terminal kinase (JNK), which can be activated by both the tyrosine receptor kinase pathway (Chiariello et al., 1998) and the Wnt pathway (Rosso et al., 2005), has also been shown to play an important role in axon growth. JNK1 is involved in regulating microtubule assembly. In JNK1 knock-out animals, the anterior commissure tract formation is disrupted and both the axons and dendrites showed progressive loss of microtubules (Chang et al., 2003). In addition, activation of JNK has been shown to be required for dopaminergic neuron neurite outgrowth (Eom et al., 2005). Although ubiquitously present in the whole cell, the phosphorylated, activated form of JNK is enriched in the axon. And treatment of JNK specific inhibitor SP600125 prevented axon formation (Oliva et al., 2006). Furthermore JNK phosphorylates various cytoskeleton associated proteins such as SCG10 (Tararuk et al., 2006) and Paxillin (Yamauchi et al., 2006) to regulate cytoskeleton assembly.

GSK-3

Glycogen synthase kinase-3 (GSK-3) family kinases are serine/threonine kinases that are regulated by several pathways including Wnt and RTK signaling. Unlike most kinases, GSK-3 kinase activity is constitutively active at normal stage and turned off by phosphorylation from other kinases such as AKT and atypical kinase C (aPKC) (Etienne-Manneville and Hall, 2003). Transfection of constitutively active GSK-3β that cannot be phosphorylated and inhibited to hippocampal neurons prevents axon formation (Jiang et al., 2005). On the other hand, inhibition of the endogenous GSK-3 either by small molecule inhibitors SB216763 or SB415286 (Jiang et al., 2005) or siRNA (Yoshimura et al., 2005) led to multiple axon formation. One downstream

substrate and effector of GSK-3, Collapsin response mediator protein-2 (CRMP-2), is a microtubule binding protein and is phosphorylated and inhibited by GSK-3. Over-expression of nonphosphorylatable, constitutively active form of CRMP-2 also led to the formation of multiple axon in hippocampal neurons (Yoshimura et al., 2005). Moreover, several other GSK-3 substrate proteins such as APC, CLASP2, Map1B, and Tau are also microtubule associated proteins which are also located in the axons (Zhou and Snider, 2006). However, a more complete inhibition of GSK-3 led to a inhibition of axon inhibition (Kim et al., 2006), suggesting a fine balance of GSK-3 activities is required for proper axon growth (reviewed in (Kim et al., 2011b)). CLASP is proposed to mediate this suppression on axon growth upon severe GSK-3 inhibition as suppression of GSK-3 does not block axon growth in the absence of CLASP2 (Hur et al., 2011).

Extracellular signaling molecules

Research in C. elegans has identified several key genes that regulate axon formation. UNC-6/Netrin binds to its receptor UNC-40/DCC were originally identified to regulate axon guidance. However recently it has been shown that Netrin-DCC also induce axon formation through the AGE-1/PI3K-DAF-18/PTEN pathway (Adler et al., 2006). In addition, Wnt protein and its receptor Frizzled have also been reported to determine neuron polarity and axon initiation in C. elegans mechanosensory neurons (Hilliard and Bargmann, 2006; Prasad and Clark, 2006). In mouse hippocampal neurons, BDNF has been shown to direct axon determination through cAMP-dependent kinase (PKA) (Shelly et al., 2007). On the other hand, the class 3 secreted Semaphorin (Sema3A) is able to repulse the axon initiation in cortical neurons (Polleux et al., 1998). Another group of extracellular signaling molecules, the Neurotrohphins (NGF, NT3, BDNF) , promote axon growth and elongation through their receptors, the Trk receptor tyrosine receptor family proteins, and the downstream MAPK/PI3K/phospholipase C (PLC) pathways.

Another Neurotrophin receptor is the p75 neurotrophin receptor (p75NTR) which activates the nuclear factor κB (NF-κB) pathway (Reichardt, 2006). In addition, insulin-like growth factor-1 (IGF-1) has been shown to promote mouse corticospinal motor neuron axon (CSMN) elongation via the IGF-1R and its downstream signaling pathway as inhibition of IGF-1R with neutralizing antibodies abolished the axon elongation in these CSMN neurons (Ozdinler and Macklis, 2006).

Since the discovery of adult neurogenesis four decades ago, remarkable progress has been made in understanding the molecular signaling mechanisms and regulation of adult neural stem cell self-renewal and differentiation. In addition, it has been suggested more than 60% of total human genes are regulated by microRNAs (Friedman et al., 2009). However, much of the interplay between the extracellular signaling pathways and the intracellular regulation of neural stem cells remains elusive. Further understanding of the interactions that control the proliferation or differentiation of neural stem cells may help us to decode the regulation of the cancer stem cells and cancer biology, and to improvise cell-based therapy toward brain/spinal cord injury.

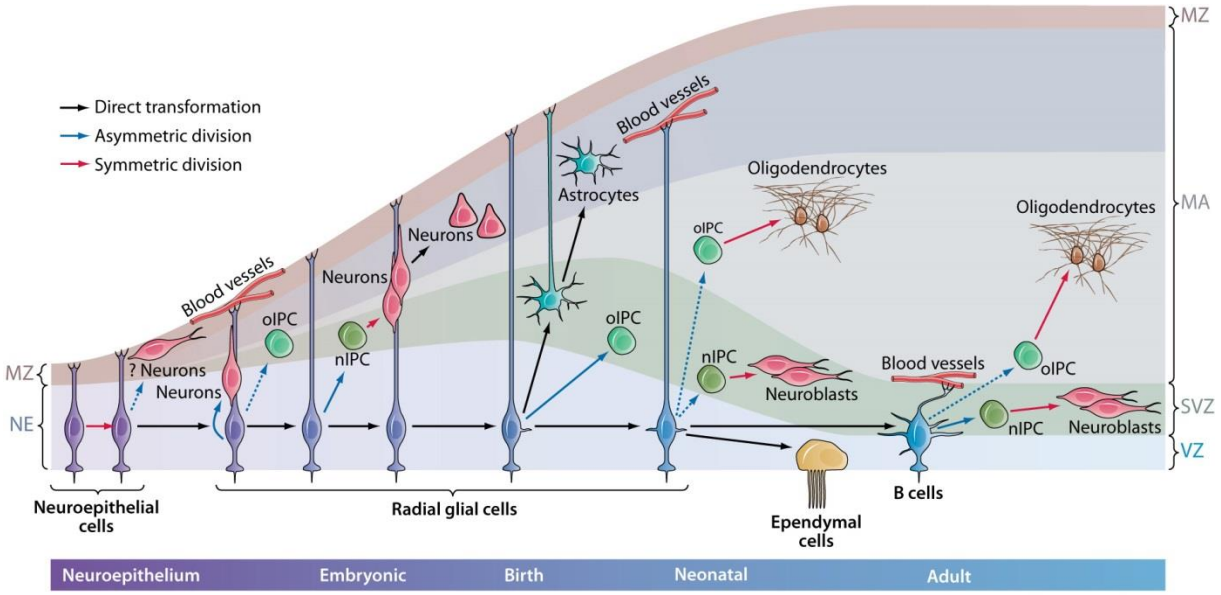

Figure 1.1 The development from neural epithelium to cortex (Kriegstein and Alvarez-Buylla, 2009).

The developing neuroepithelium undergoes a series of symmetrical and asymmetrical cell divisions which is controlled by the orientation of the mitotic spindle (Fish et al., 2006; Roszko et al., 2006; Morin et al., 2007). The initial newly formed neurons migrate radially toward the basement membrane of the neural tube by somal dislocation and later by glial-guided cell translocation (Nadarajah et al., 2001), and the developing neural tube becomes a stratified structure. The inner-most layer (near the lumen) of the developing forebrain is termed the ventricular zone (VZ). The radial glia cells residing in the VZ act as stem cells during neural development (Noctor et al., 2001). The cell bodies of RG cells reside in the VZ and have long ascending processes, the radial fibers, that reach the basement membrane located beneath the pia mater.

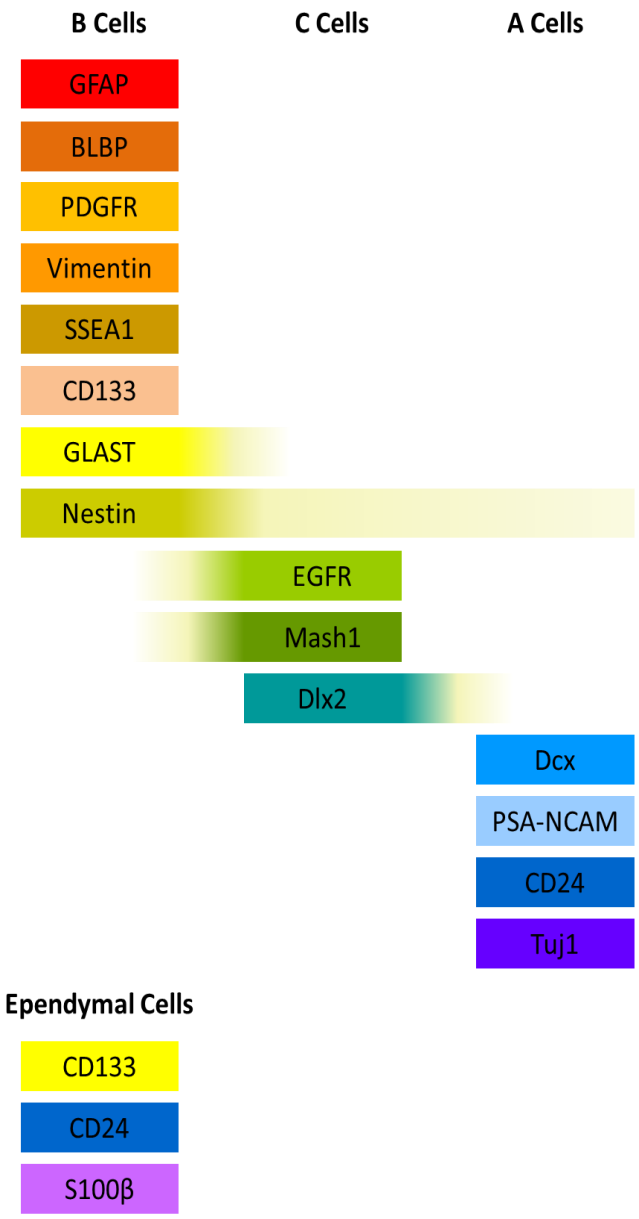

Figure 1.2 Markers expressed by different cell types in adult SVZ.

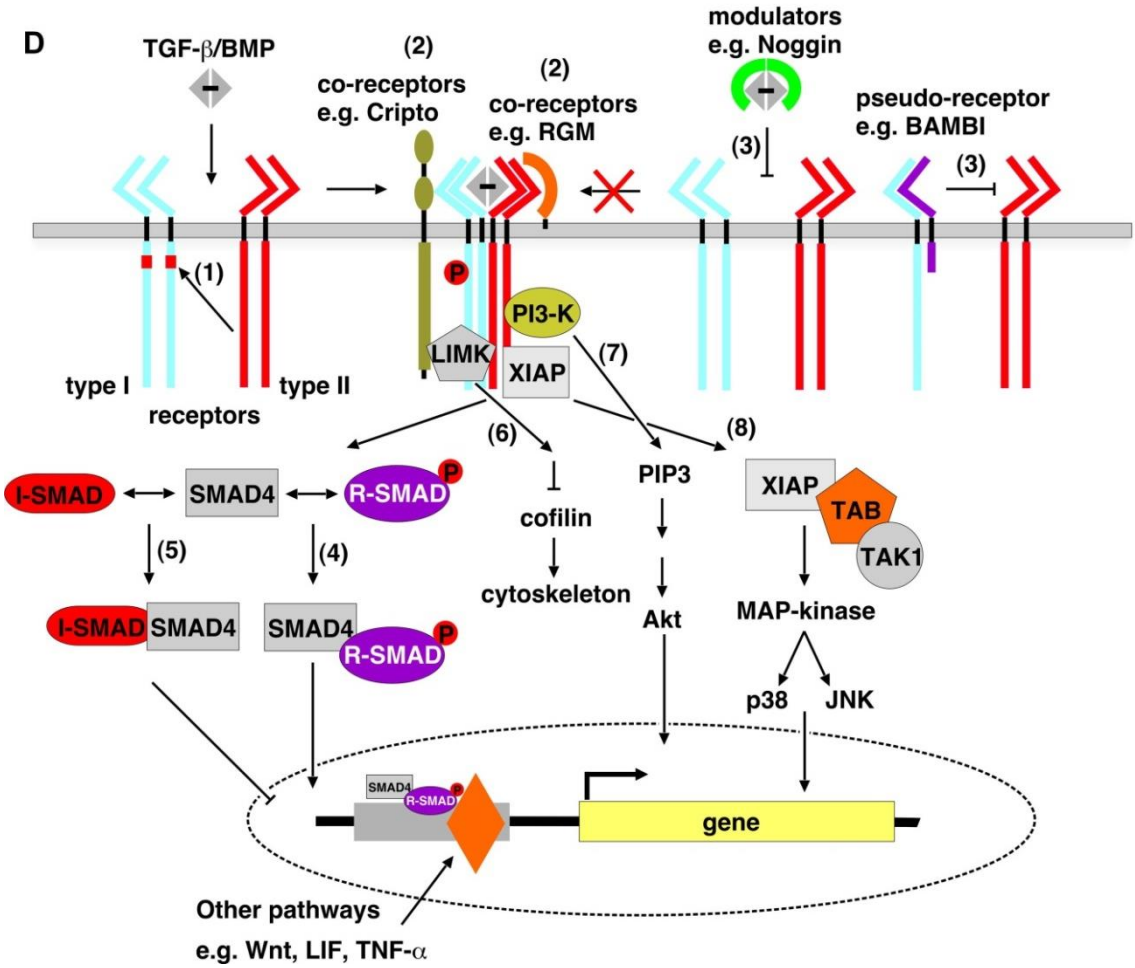

Figure 1.3 BMP signaling pathway (Mueller and Nickel, 2012).

Bone Morphogenetic Proteins (BMPs) are a group of secreted glycoproteins that belong to the Transforming Growth Factor-beta (TGF-β) superfamily. To date, 15 members of this subfamily have been identified. BMPs act by binding to the receptor tyrosine kinase BMP receptors type I and type II. After ligand binding, two type I and two type II receptors form a heterotetramer. The type II receptors then autophosphorylate and phosphorylate the type I receptors, thus creating binding sites for the downstream effector SMAD proteins. After activating phosphorylation by the BMPR two receptor-activated SMADs (R-SMADs, SMAD-1, 5, 8) form a trimer with an additional common-mediator SMAD (Co-SMAD, SMAD-4). The trimerized SMADs then translocate into the nucleus and activate target gene transcription. The inhibitory SMADs (I-SMADs, SMAD-6, 7) can interfere with signaling by binding to SMAD-4 (Mueller and Nickel, 2012). In addition BMP signaling can be antagonized by binding of BMPs by extracellular proteins Noggin and Chordin.

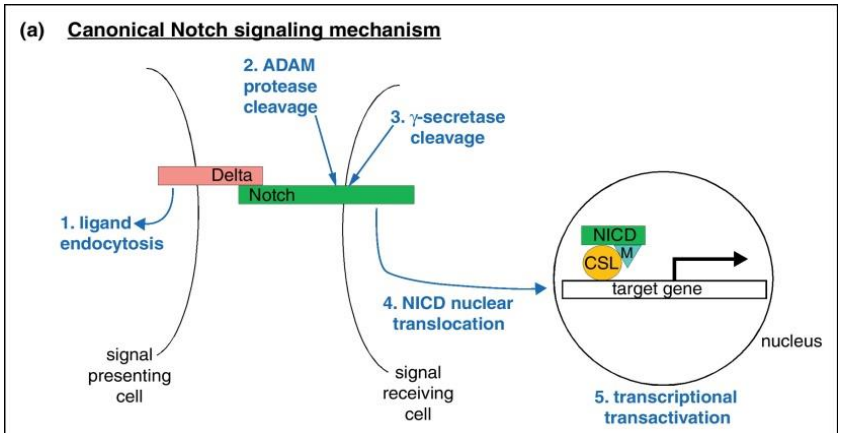

Figure 1.4 Notch signaling pathway (Giniger, 2012).

The Notch receptors Notch1-4 are single pass transmembrane proteins. There are six ligands, which are also transmembrane proteins: Jagged-1, 2 (Jag-1, 2) , Delta-like-1 through 4 (Dll-1, 2, 3, 4). The receptor and ligand are typically expressed by adjacent cells. Upon ligand binding, the Notch protein undergoes two separate cleavage events. First the ADAM protease cuts at the base of the Notch extracellular domain. The second cleavage occurs at the base of the Notch intracellular domain by the γ-secretase complex thereby releasing the Notch intracellular domain (NICD). The NICD then migrates into the nucleus and forms a transcription regulating complex with two other proteins, Mastermind and CSL protein (CBF-1/RBP-Jk in vertebrates, Su(H) in flies, and LAG-1 in worms) to regulate target gene expression.

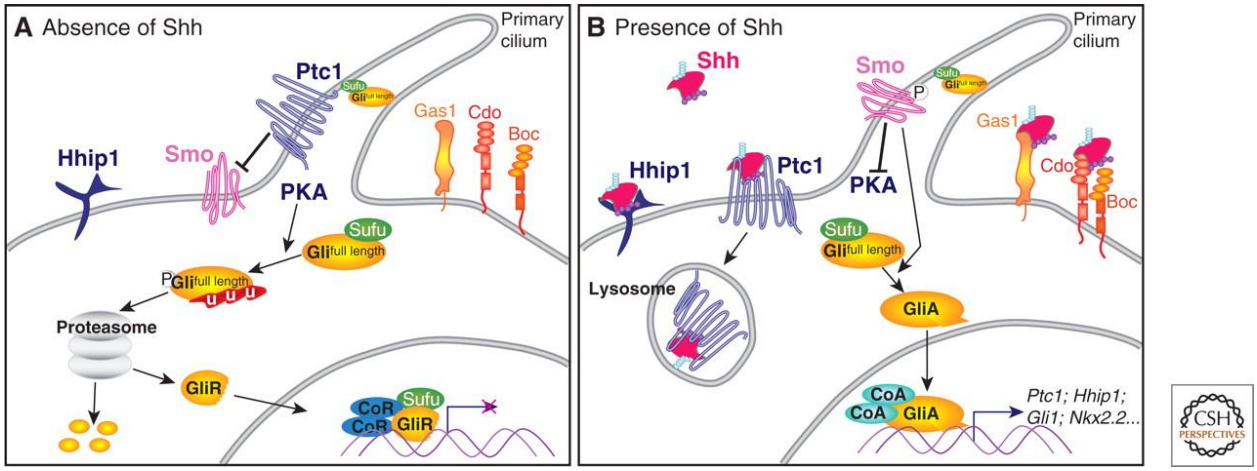

Figure 1.5 Sonic Hedgehog signaling pathway (Ribes and Briscoe, 2009).

Sonic Hedgehog (Shh) is a member of the Hedgehog secreted protein family, which also includes Desert and Indian Hedgehog. In the absence of Shh, the receptor Patched-1, a twelve-pass transmembrane protein, localized in the primary cilium of the cell inhibits Smoothened (Smo), a member of the seven-pass G-protein coupled receptor family, by accumulation in the cilium. The Gli proteins are then phosphorylated by the protein kinase A (PKA) and targeted for proteasome-mediated proteolysis. The cleaved, truncated form of Gli (GliR) then migrates into the nucleus and acts as transcriptional repressor. Upon binding of Shh, Patched-1 leaves the primary cilium allowing Smo to enter. The active Smo then inhibits PKA and the proteolysis of Gli. Full-length Gli then enter the nucleus and promotes its target gene transcription.

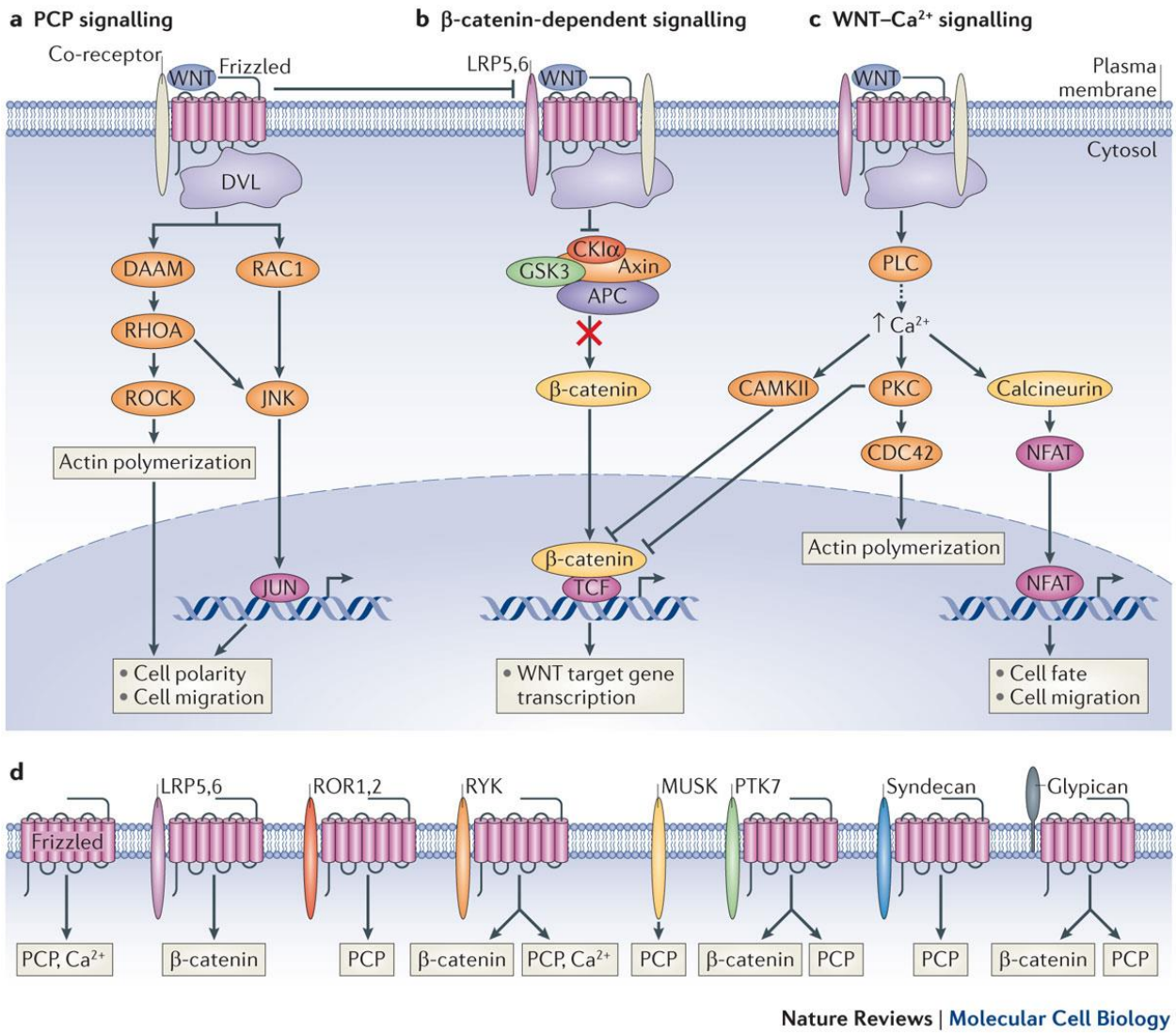

Figure 1.6 Wnt signaling pathway (Niehrs, 2012).

The canonical Wnt signaling pathway: In the absence of Wnt ligand, glycogen synthase kinase-3 (GSK3) forms a destruction complex with casein kinase Iα (CKIα), Axin, and adenomatosis polyposis coli (APC) protein, phosphorylates β-catenin leading to its ubiquitination and degradation. When Wnt ligand binds the receptor, the Frizzled/Dishevelled/LRP-5/6 complex, the destruction complex is inhibited and relieves β-catenin from degradation. β-catenin then translocates into the nucleus, binds to T-cell factors (TCF), and induces its target gene expression. B) The non-canonical, β-catenin independent Wnt signaling pathway: Wnt binding to the receptor can also activate small GTPases, which in turn activate RHO kinase (ROCK) and JUN-N-terminal kinase (JNK). This pathway, the PCP (planar cell polarity) pathway, is particularly important in regulating cell polarity and migration. Wnt can also activate phospholipase C,

which induces the increase of cytosolic Ca^{2+} concentration. High Ca^{2+} concentration subsequently leads to the activation CAMKII, PKC and Calcineurin.

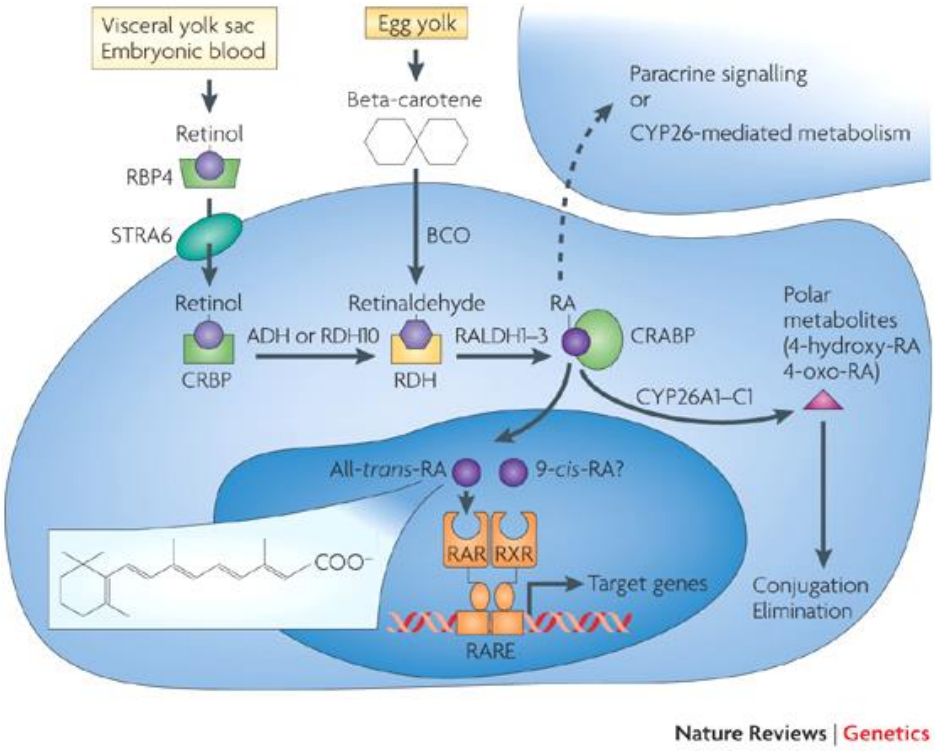

Figure 1.7 Retinoic acid signaling pathway (Niederreither and Dolle, 2008).

Retinoic acid is a non-peptide, small lipophilic signaling molecule that is either synthesized from retinol (Vitamin A) from the diet or diffuses from the surrounding extracellular environment. Uptake of extracellular retinol, bound by retinol binding protein 4 (RBP4), is aided by a transmembrane protein STRA6. Once in the cytosol, retinol is bound by cellular retinol binding protein (CRBP) and oxidized by retinol dehydrogeneses (RDHs) becoming retinaldehyde. Retinaldehyde is further oxidized by retinaldehyde dehydrogenases (RALDHs) becoming retinoic acid (RA). Cytosolic RA is bound by cellular retinoic acid binding proteins (CRABPs) and transported to the nucleus. In the nucleus, the free RA binds to the heterodimers of the nuclear RA receptors RARs (RARα, RARβ, and RARγ) and the retinoid X receptors RXRs (RXRα, RXRβ, and RXRγ). The active nuclear receptors then bind to the retinoic acid-responsive elements (RAREs) and regulate gene transcription. Cytosolic RA can also be exported to the extracellular environment and act in autocrine/paracrine fashions.

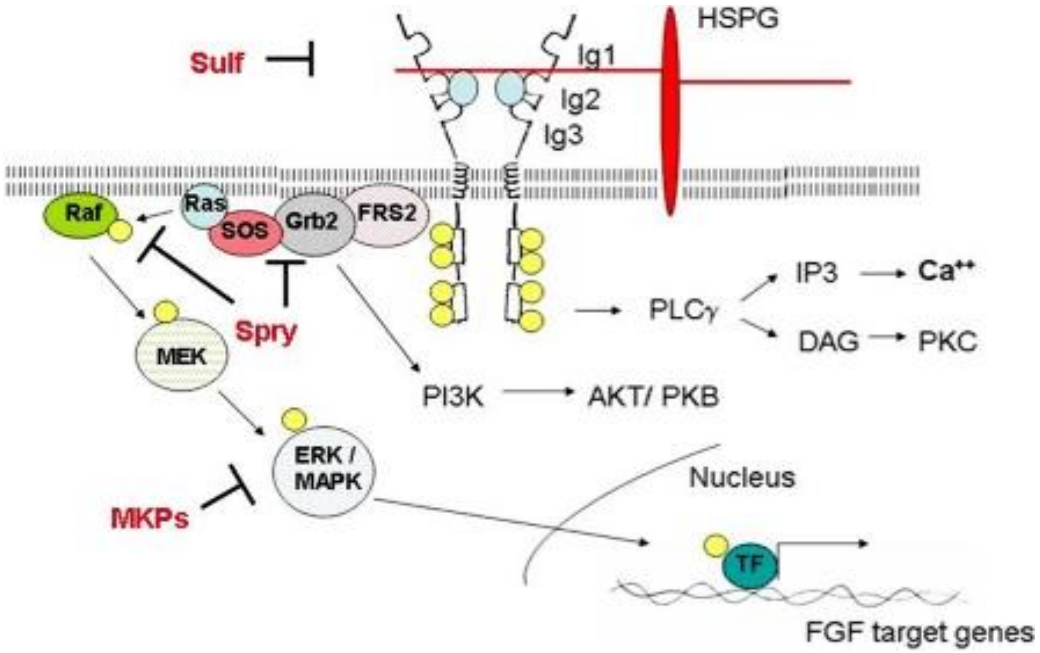

Figure 1.8 Receptor tyrosine kinase (RTK) signaling pathway (Pownall and Isaacs, 2010).

Receptors for FGF, PDGF, VEGF, and EGF belong to the receptor tyrosine kinase (RTK) superfamily. Upon ligand binding, receptors dimerize and the intracellular tyrosine kinase domains activate and transphosphorylate each other. The phosphorylated tyrosine residues thus create binding sites for the adapter protein Grb2. This is followed by the activation of the small GTPase Ras, and subsequent activation of the Raf/MEK/ERK pathway. The activated RTKs can also activate phosphatidylinositol-4,5-bisphosphate-3 kinase (PI3K), which in turn activates its downstream Akt/mTor signaling pathway. Phospholipase C-γ also binds to the phosphotyrosine residues on RTKs and activates the downstream signaling pathways.

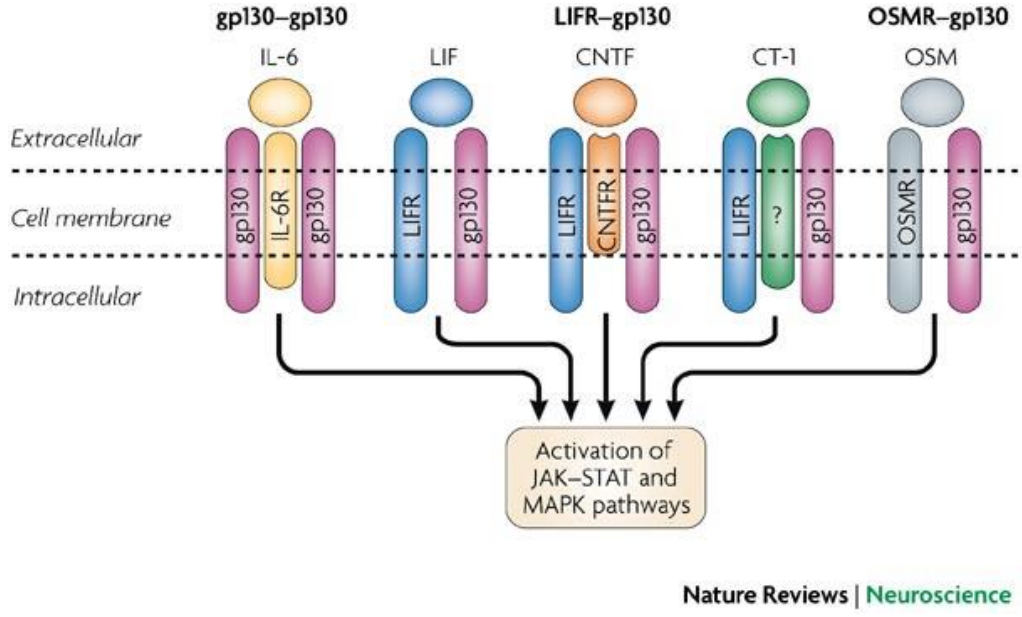

Figure 1.9 LIF/CNTF signaling pathway (Bauer et al., 2007).

Both LIF and CNTF are polypeptide cytokines belonging to the interleukin-6 family. LIF binds to a heterodimeric receptor complex consisting of one LIF receptor β (LIFRβ) and one co-receptor gp130; while CNTF binds to a trimeric receptor complex consisting of one LIFR β, one gp130, and one additional CNTF receptor α (CNTFRα). Upon ligand binding, the receptor associated Janus-activated kinase (JAK) autophosphorylates and also phosphorylates the tyrosine residues on the receptors, thus creating binding sites for the signal transducing and activator of transcription (STAT). JAK also phosphorylates receptor bound STATs. Phosphorylated STATs then dimerize and translocate into the nucleus and activate gene transcription. In addition, the phosphorylated tyrosine residues also create binding sites and activate the MAPK and PI3K signaling pathways.

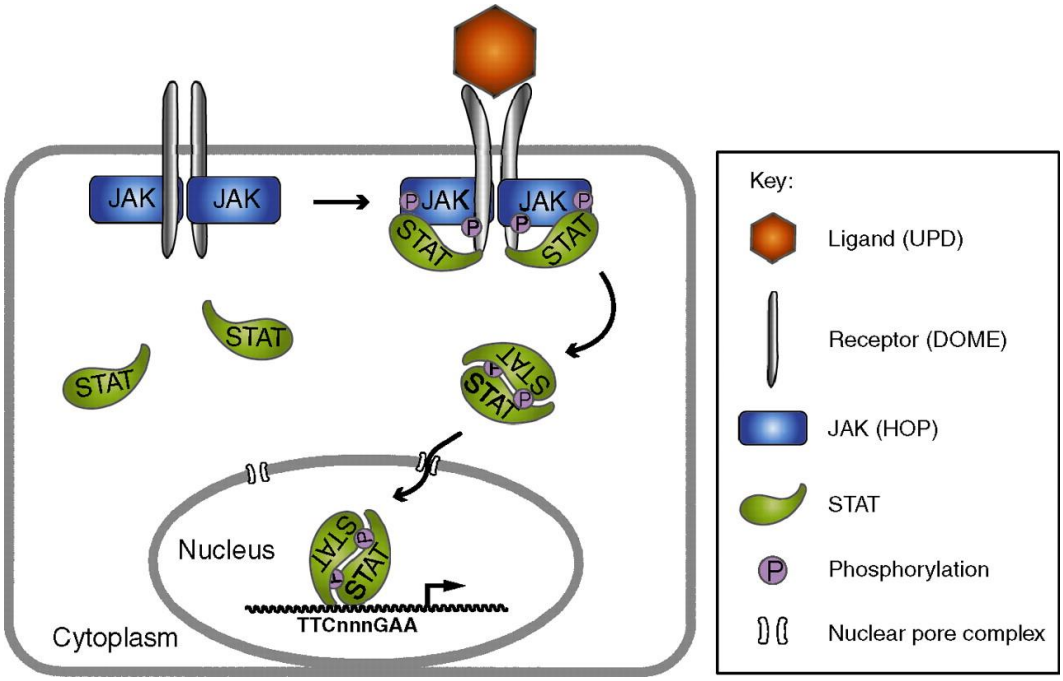

Figure 1.10 JAK-STAT signaling pathway (Arbouzova and Zeidler, 2006).

Upon ligand binding, the receptor associated Janus-activated kinase (JAK) autophosphorylates and also phosphorylates the tyrosine residues on the receptors, thus creating binding sites for the signal transducing and activator of transcription (STAT). JAK also phosphorylates receptor bound STATs. Phosphorylated STATs then dimerize and translocate into the nucleus and activate gene transcription. In addition, the phosphorylated tyrosine residues also create binding sites and activate the MAPK and PI3K signaling pathways.

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Chapter Two

miR-410 Controls Adult Neurogenesis by Targeting Neurogenic Genes

Introduction

Both intracellular and extracellular mechanisms have been shown to maintain the neurogenic niches in the adult brain (review, (Ming and Song, 2011)). In fact, considerable work has demonstrated that dynamic interactions between these compartments regulate cell behaviors within the niche and ultimately its neuronal vs glial output. In addition to transcriptional regulators, intracellular modulators including non-coding microRNAs have been shown to control proliferation, self-renewal and cell fate choice in the nervous system via their ability to bind and inhibit translation (or to promote degradation) of critical target genes (Yao #13).

Cell type-specific microRNAs (He et al., 2012; Jovicic et al., 2013), which bind and suppress lineage specifying genes or groups of genes, are thought to play a critical role in maintaining the niche and controlling its response to injury. For example, one of the most abundant microRNAs in the CNS, miR-124, is up-regulated at the transition from subventricular zone (SVZ) transit amplifying C cells to neuroblasts (A cells), thereby promoting neurogenesis (Bian and Sun, 2011; Bian et al., 2013b; Bian et al., 2013a). Regulatory networks are beginning to be identified—i.e., miR-25 can promote proliferation of neural stem cells (NSC) via its ability to regulate the IGF signaling pathway (Brett et al., 2011), while Sox2 regulates miR-137, which in turn targets Ezh2

to inhibit differentiation and promote NSC proliferation (Szulwach et al., 2010). Although many genes and epigenetic mechanisms that regulate miRNAs have been identified (Ji et al., 2013), extracellular regulation of miRNAs, particularly within the CNS, has been less well studied.

Despite the potential importance of microRNAs in understanding CNS development (Lang and Shi, 2012), disease (Eacker et al., 2009), in identifying molecular markers (Di Leva and Croce, 2013), and potentially in therapeutic approaches (Pers and Jorgensen, 2013), the critical link between the extracellular compartment and microRNA expression and function is poorly understood (Bian et al., 2013a). In the companion paper (Morell et al., 2013), we describe a transgenic mouse in which expression of the BMP signaling pathway inhibitor, noggin, in SVZ NSC promotes neuronal and oligodendroglial differentiation, while decreasing astrocyte differentiation both *in vivo* and *in vitro*. Microarray analysis of RNAs from noggin over-expressing and control SVZs identified a novel microRNA, miR-410, which was up-regulated with BMP pathway inhibition. We have determined that miR-410 is expressed in the SVZ NSC niche, and in mESC as they differentiate into neurons, where it inhibits neuronal differentiation and reverses the increase in neuronal differentiation produced by noggin expression. Predicted targets of miR-410: *Elavl4*, *Sox1*, *Smad7*, *Tcf4* and *Fgf7* were validated in luciferase assays, and expression of *Elavl4* rescued the inhibitory effects of miR-410 on neuronal differentiation.

Materials and Methods

Animals: Noggin inducible transgenic mice were generated as described in(Morell et al., 2013). All animals were handled according to protocols approved by University of Michigan UCUCA.

Cell culture: the mouse embryonic cell line D3 was cultured in 10% FBS/DMEM containing 4.5mM HEPES, 1.5mM L-Glutamine, and 0.00038% (v/v) β-mercaptoethanol. The cells were grown on 0.1% gelatin coated tissue culture flasks and passaged one to three every other day. For neural differentiation, 1 x 10^5 cells per well were plated on gelatin coated 6-well plates in 80% N2 medium: 20% B27 medium with 1μM retinoic acid. The medium was change every other day. After 6 days of differentiation, the cells were fixed for immunocytochemistry or RNA extraction

For mouse neurosphere culture: 8 to 10 week old mice were euthanized by cervical dislocation ($n \geq 5$ per group). The subventricular zone was then microdissected, tissue pooled and dissociated in 0.133% (w/v) trypsin, 0.067% (w/v) hyaluronidase and 0.69mM kynurenic acid in artificial cerebral spinal fluid (ACSF) (124mM NaCl, 5mM KCl, 3.2mM MgCl, 26.2mM $NaHCO_3$, 10mM glucose, and 0.098 mM $CaCl_2$) at 37℃ for 30 minutes. Trypsin was stopped by adding 1:1 trypsin inhibitor solution (0.022% (w/v) trypsin inhibitor and 0.001% (w/v) DNaseI in N2 medium). The cell clumps were broken to single cells by trituration using fire-polished glass pipets. Cells were then cultured in N2 medium supplemented with 10 ng/ml FGF2, 20 ng/ml EGF, and 2 μg/ml heparin. Medium was changed twice each week.

Plasmid transfection: two weeks after isolation, neurospheres were disaggregated by pipetting with fire-polished Pasteur glass pipettes, transfected with the miR-410 over-expression, miR-410 sponge, with Elavl4 over-expression, or control plasmids using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. They were grown in N2 medium supplemented with FGF2, EGF, and heparin for an additional week. For differentiation, neurospheres were disaggregated by trypsinization in 0.25% trypsin / 1mM EDTA at 37℃ for 90 seconds followed by addition of trypsin inhibitor solution and trituration. Cell number was determined and $1.5x10^4$cells per well plated in N2 medium supplemented with 1% FBS. 48-well tissue culture

plates were pre-coated with 6.67μg/ml poly-ornithine/water solution for 2 hours at room temperature, then rinsed once in water. Medium was changed on day 4 and cells were fixed on day 7 for further analysis. To generate miR-410 over-expressing mES cell lines, D3 cells were transfected with pPUS2-miR-410, as described below. Selection in 10 μM puromycin was started 24 hours after transfection. 4 days after transfection the cells were passaged 1:10 and single colonies picked and expanded.

For lentiviral transduction: 7 days after isolation, primary neurospheres were pooled and dissociated by repeated pipetting,then transduced with 1 x 10^6 moi lentiviral particles per group. 3 days post-transduction the neurospheres were plated for differentiation as described above.

For luciferase assays: HEK293T cells were cultured in 10% FBS/DMEM and passaged 1:5 every other day. 0.4 μg of either miR-410 over-expression or control vector and 0.4 μg of either pmirGLO-wt-3'UTR or pmirGLO-mutated-3'UTR were transfected into 5x 10^4 HEK293 cells in one well of a 24-well-plate. Cell lysates were harvested 48 hours after transfection for further analysis.

DNA constructs: the miRNA expression vector pPUS2C was made by inserting the mCherry coding sequence (R.Y. Tsien, UCSD) into the pUS2 plasmid (D. Turner, University of Michigan) between the BamHI and EcoRI sites. A separate puromycin resistance cassette was cloned into the second multiple cloning site. To make the miR-410 over-expression vector, 360 bp of mouse genomic sequence containing the full pre-miR-410 was cloned by PCR (FW: TAGAATTCGTGCTGCCTGTGTCAACCCTACTC; REV: TATCTAGAATCTGGCCAATGCTTCGTG) into pPUS2C between the EcoRI and XbaI sites. The miRNA sponge targeting miR-410 was made as described previously (Ebert et al., 2007) and

inserted into the pPUS2C plasmid between the EcoRI and XbaI sties. The efficacy of the miR-410 over-expression vector, pPUS2C-miR410, and the miR-410 sponge vector, pPUS2C-410SP, was tested in the mouse ES cell line D3. The fulllength mouse Elavl4 coding region without the 3'UTR was cloned by PCR and inserted into the pCIG plasmid (Ben Allen, University of Michigan).

Lentivirus: miR-410 over-expression, miR-410 sponge, or scrambled control sequence were cloned into the pLentilox-eGFP backbone vector (UofM Vector Core). Functional virus particles were packaged in 293FT cells (Invitrogen) by co-transfecting the expression vectors with packaging plasmid pMD2.G and psPAX2 (Addgene) as previously described (Barde et al., 2010).

For luciferase assays:the fulllength or truncated 3'UTR of miR-410 target candidate genes was amplified by PCR and cloned into the pmirGLO plasmid (Promega, WI) between NheI and SalI sites according to the manufacturer's protocol. For genes with a 3'UTR shorter than 800 bp, the full length 3'UTR was cloned into the vector. For genes with a 3'UTR longer than 800 bp, a region at least 800 bp long containing the miR-410 site in the center was cloned. Vectors containing a 3'UTR with a mutated miR-410 site (TTAATTAA) were made by PCR based site-directed mutagenesis. Luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's protocol and using a Lumat LB 9507 luminometer (Berthold Technologies).

In situ hybridization and immunocytochemistry: For *in situ* hybridization, anti-sense RNA probe against the mature form miR-410 was synthesized (Invitrogen, CA). Eight week old wildtype animals were anesthetized using Ketamine and Xylazine and perfused with PBS followed by 4% PFA. Brains were then dissected and embedded in OCT followed by cryosectioning at 10 µm. *In*

situ hybridization was performed as described previously (Deo et al., 2006). For immunohistochemistry, cells were fixed in 2% PFA at room temperature for 15 minutes, permeabilized in 0.1% Triton X-100/0.1% sodium citrate in PBS for 10 minutes, blocked with 10% normal donkey serum / 0.5% Triton X-100/0.1% sodium azide in PBS. The cells were then incubated with primary antibodies at 4°C overnight. The next day the cells were washed in PBS and incubated with secondary antibodies for 30 minutes at room temperature. Nuclear staining was done by incubating the cells in 1 μM Hoechst 22358 at room temperature for 5 min. Antibodies included: βIII tubulin (Tuj1 antibody; 1:1000, Covance); GFAP (1:500, Santa Cruz); and Olig2 (1:1000, Millipore). Secondary antibodies were obtained from Jackson labs and used at 1:400-1:1000.

Protein extraction and western blotting: Total cell lysates were collected in RIPA buffer from neurospheres 7 days post transfection with either control or miR-410 over-expression plasmids as described (Sambrook et al.). After electrophoresis, β-actin (1:1000, Sigma) and Elavl4 (1:200, Millipore) were blotted with antibodies respectively.

RNA extraction and RT-PCR: Total RNA was extracted from microdissected SVZ or cells in culture using Trizol (Invitrogen) following the manufacturer's protocol. 1 μg of DNase-treated RNA was used for RT-PCR either using miScript Reverse Transcription kit (Qiagen) for miR-410 or Verso cDNA kit (Thermo Scientific) for other genes.

Quantitative PCR and primer design: Quantification of miR-410 expression was done using the miScript quantification PCR system (Qiagen) following the manufacturer's protocol. For qPCR on other genes, primers were designed using Lasergene software (DNASTAR) and verified by the NCBI primer-BLAST program (http://blast.ncbi.nlm.nih.gov). qPCR was done in triplicate

using the Abgene SYBR system (Thermo Scientific) on Bio-rad iCycler qPCR system (Bio-rad). Data were then analyzed by the ΔΔCt method. Primers on request.

Quantification: To assess the number of neurons and astrocytes present following neurosphere and ESC differentiation, photomicrographs (20X) were taken along two perpendicular lines bisecting the culture dish: from 0 to 180° and along a second line from 90 to 270°. Numbers of neurons, astrocytes and total cell numbers were counted from at least three wells each from three independent experiments ($n \geq 9$ wells) and analyzed using Students t-test. The nuclear expression of oligodendrocyte precursor maker Olig2 was measured and quantified using a custom written MATLAB (MathWorks Inc., MA) script. Images of Hoechst stained cells were loaded and a binary image of nuclear regions was created employing a threshold found by Otsu's method (Otsu, 1979). The binary image was cleaned up by morphological image operations creating a mask image. The average intensity of Olig2 fluorescence was measured over each nuclear mask.Total numbers of Olig2-positive oligodendrocyte precursors were counted from at least two wells each from three independent experiments ($n \geq 6$) and the percentage of Olig2+ nuclei were analyzed as described. To assess neurite differentiation, processes stained using β-III tubulin were scored as: lacking a neurite, having a neurite less than the length of the cell body, having a neurite twice or greater in length than the cell body. At least 50 neurons were counted from three replicate wells with three biological replicates (i.e., 450 cells per experimental group: miR-410 over-expression, Scrambled control, GFAP control, microRNA sponge).

Computational analysis: Potential miR-410 targets were identified by searching four databases: TargetScan (http://www.targetscan.org), ElMMo (http://www.mirz.unibas.ch/ElMMo2), PicTar (http://pictar.mdc-berlin.de) and MicroCosm (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5).

Results

Noggin controls miR-410 expression. We previously identified this previously uncharacterized microRNA, miR-410, in RNAs from the SVZ NSC zone of a transgenic mouse in which Noggin expression can be inducibly driven in Nestin positive NSCs (Morell et al., 2013). Over-expression of Noggin in NSC increased neuronal and oligodendrocyte differentiation while down-regulated the expression of miR-410. miR-410 is a 21 nt microRNA embedded in the miRNA-encoding gene *Mirg*. miR-410 was originally described as restricted to the developing central nervous system (Wheeler et al., 2006; Han et al., 2012), suggesting it may have important roles in neurogenesis. To examine expression of miR-410 in the adult SVZ we carried out *in situ* hybridization using an antisense probe against the mature miR-410 on sections of 8-week mouse SVZ. miR-410 was highly expressed in the SVZ, with low expression in scattered cells in the parenchyma of the septum (Figure 2.1.A). We also compared the expression levels of miR-410 in Noggin induced and un-induced control adult SVZ using quantitative-PCR against the mature form of miR-410. MiR-410 was down-regulated 16-fold in the Noggin induced SVZ, suggesting miR-410 expression may be regulated by BMP signaling, and validating our microarray analyses.

Over-expression of mir410 reduces neuronal differentiation and the number of Olig2 positive cells in neurosphere differentiation assays. To test the function of miR-410 in neuronal and glial differentiation, we over-expressed and inhibited 410 function in primary neurospheres obtained from adult NSC. We generated lentivirus expressing miR-410 and eGFP, a miR-410 sponge also expressing eGFP, a scrambled miRNA control with eGFP, or eGFP alone.

To obtain neurospheres, the SVZ was micro-dissected from 8 to 12 week old FVB/N wildtype mice, dissociated, pooled and cultured in N2 medium in the presence of FGF-2, EGF, and heparin. We cultured primary neurospheres from SVZ and on day 7, the neurospheres were transduced with lentivirus described above; 72 hrs later more than 90% of the cells expressed eGFP (not shown). Transduced neurospheres were differentiated and stained for GFAP or β-III tubulin antibody (Tuj1, Figure 2.2.A). Neurospheres transduced with lentivirus carrying either eGFP alone or miR-Scrambled did not show significant differences in neuronal differentiation (23.6 ± 3.5% vs 21.5 ± 1.1%, respectively, $p \leq 0.24$), and were combined as a single control group. Control neurospheres yielded 22.5 ± 2.8% neurons, and over-expression (OE) of miR-410 significantly reduced that percentage to 14.9 ± 1.3% ($p \leq 7.5 \times 10^{-4}$, Control vs OE). Exposure to the miR-410 sponge significantly increased the number of Tuj1+ neurons to 33.8 ± 1.8% ($p \leq 4.9 \times 10^{-5}$compared to the control group). As expected, the majority of the cells formed in the neurosphere differentiation assay in control conditions were astrocytes (77.5 ± 3.5%; eGFP alone: 76.4 ± 3.5%; scramble: 78.5 ± 1.1%). Over-expression of miR-410 significantly increased the percentage of astrocytes to 85.1 ± 1.3%($p \leq 7.5 \times 10^{-4}$ compared to the control group), while expression of the miR-410 sponge decreased the percentage of astrocytes to 66.2 ± 1.8% ($p \leq 4.9 \times 10^{-5}$ compared to the control group) (Figure 2.2.B).

We also examined the function of miR-410 in oligodendrocyte differentiation. Over-expression of miR-410 in NSC significantly reduced the Olig2-positive cell number from 7.7 ± 1.1% in the control group to 4.9 ± 2.5% ($p \leq 0.023$). However, miR-410 loss of function via lentiviral miR-410 sponge vectors did not significantly affect OPC commitment (7.7 ± 1.1% vs 6.0 ± 2.0%, $p \leq 0.08$) (Figure 2.2.D), suggesting that regulation of oligodendrocyte differentiation by miR-410 may be asserted through another mechanism or with different kinetics.

Over-expression of miR-410 inhibits neural differention of mESC. To examine the function of miR-410 in neural differentiation in a pluripotent stem cell model, we generated two additional mouse embryonic stem cell lines (#20 and #24) over-expressing miR-410. The miR-410 over-expressing cells and the parental D3 cells were plated and differentiated in defined neural induction media for 6 days before fixation and staining for β-III-Tubulin (Tuj1). Over-expression of miR-410 inhibited the differentiation of neurons (β-III-Tubulin/Tuj1 positive after 6 days of neural differentiation compared with the control cell line D3 (Figure 2.3.A). To obtain additional quantitative data, neuronal numbers were quantified using q-RT-PCR. Over-expression of miR-410 reduced the expression of the early neuron marker, β-III-Tubulin, 4.3 fold and the neural progenitor cell marker, Sox3, 15.3 fold compared to the parental cell line D3, while the expression of the astroglial marker GFAP was increased 2.5 fold in the miR-410 over-expressing line. The two cell lines showed no difference in the expression of markers of pluripotency (Oct4), endoderm (FoxA2), mesoderm (Brachyury), (Figure 2.3.B) while the trophectoderm marker Cdx2 was not detected in either cell lines.

miR-410 acts downstream of Noggin/BMP. To determine the relationship of miR-410 to Noggin expression in BMP signaling, we transfected mouse primary neurospheres with miR-410 expression vectors while Noggin protein was added to the culture media (Figure 2.4). As expected(Morell et al., 2013)Noggin alone promoted neuronal differentiation (from 27.3 ± 1.5% to 37.5 ± 1.1%, $p \leq 3.3 \times 10^{-4}$), while co-overexpression of miR-410 with Noggin treatment partially reduced the increase in Tuj1+ neurons observed in Noggin alone group (29.6 ± 0.9%, $p \leq 2.9 \times 10^{-4}$) (Figure 2.4.A). Similarly, Noggin treatment alone decreased astrocyte number (from 72.6 ± 1.6% to 62.5 ±1.1%, $p \leq 4.3 \times 10^{-4}$), while over-expression of miR-410 partially reversed the decrease in astrocyte cell number (to 70.3 ± 1.0%, $p \leq 3.7 \times 10^{-4}$). These results suggest that

miR-410 functions downstream of the BMP signaling pathway. However since over-expression of miR-410 only partially rescues the phenotypes caused by Noggin, other factors, or timing, may play a role in the ability of Noggin to promote neuronal differentiation.

mir410 down-regulates Elavl4 expression. To examine the mechanism(s) underlying the ability of miR-410 to affect NSC lineage differentiation, we analyzed potential target candidates of miR-410 using four databases: TargetScan (http://targetscan.org), ElMMo (http://www.mirz.unibas.ch/ElMMo), Miranda (http://www.microrna.org), and Pictar (http://pictar.mdc-berlin.de). Of the predicted candidates, we selected seven genes known to function in neurogenesis or in self-renewal to test in luciferase assays. The full length 3'UTR of the candidate targets was cloned after a luciferase coding region into a test plasmid. As an unbiased control, the predicted seed-binding regions of the 3'UTRs were mutated to a PacI restriction enzyme site. The luciferase test plasmids were then co-transfected with a miR-410 expression vector into HEK293T cells, which do not express miR-410 (unpublished data). Over-expressed miR-410 should target the wildtype 3'UTR and down-regulate luciferase expression, sparing the mutated 3'UTR. The efficiency of miR-410 induced gene down-regulation was quantified by luciferase activity. Among the genes tested, 3'UTR of *Zfx* was not targeted by miR-410, the 3'UTR of *Musashi-2* actually up-regulated luciferase expression, while miR-410 down-regulated luciferase expression from *Elavl4, Sox1, Tcf4, Fgf7,* and *Smad7* (Figure 2.5.A).

mi-410 controls neuronal differentiation via Elavl4. Given its striking role in *Drosophila* neurogenesis, and our luciferase results, we chose to determine if Elavl4 was functionally targeted by miR-410 in neurosphere assays. In western blot, Elavl4 protein expression level was decreased approximately 45% in neurospheres over-expressing miR-410 compared to control neurospheres (Figure 2.5.B). To determine if miR-410 affects NSC lineage decision by

regulating Elavl4 expression, we co-transfected neurospheres with the miR-410 expression vectors and Elavl4 vectors which contain the Elavl4 coding sequence without the wildtype 3'UTR. The transfected neurospheres were then differentiated as described above and stained to identify βIII-tubulin+ neurons (Figure 2.6.A). Neurospheres transfected with control vectors generated 25.2 ± 1.6% neurons. Over-expression of miR-410 decreased the percentage of neurons to 20.0 ± 1.1% ($p \leq 4.1 \times 10^{-3}$), over-expression of both miR-410 and Elavl4 restored neuronal differentiation to control levels (27.6 ± 1.4%; $p \leq 0.1$). Over-expression of Elavl4 alone increased the percentage of neurons to 39.7 ± 1.7% (Figure 2.6.B).These data suggest that miR-410 inhibits neuronal fate by down-regulating the expression of a crucial neuronal gene *Elavl4*, as over-expression of a miR-410-resistent Elavl4 rescued the reduction in neuronal fate produced by miR-410.

miR-410 alters neuronal morphology . Neurons differentiated from adult neurospheres typically have multiple long, highly branched cell processes (Figure 2.7.A, lower right), while many neurons exposed to miR-410 often had very short, unbranched processes. To quantify these observations, we grouped the neurons into three categories: neurons with processes longer than the soma (Group 1), those with a second neurite that was shorter than the cell body (Group 2), and cells lacking a second process (Group 3). Quantified in Figure 2.7.B, over-expression of miR-410 led to a significant increase in the number of neurons without obvious neurites compared with pooled GFP/scrambled controls (Type 3, 50.8 $\pm$ 11.3% vs 10.3 $\pm$ 0.7%, $p \leq 3.8 \times 10^{-4}$) and a decrease in the number of neurons with neurites longer than the length of the cell body (Type 1, 29.8 $\pm$ 8.3% vs 75.8 $\pm$ 2.0%, $p \leq 3.7 \times 10^{-5}$). There was no significant differences between control and sponge groups (Type 1, 75.8 $\pm$ 2.0 vs 81.0 $\pm$ 4.0, $p \leq 0.06$; Type 3, 10.3 $\pm$

0.7% vs 6.9 $\pm$ 4.5%, $p \leq 0.2$), and no significant differences in the number of neurons with short processes (Group 2).

Discussion

We have identified and begun to characterize a novel miRNA, miR-410, which on inhibition of BMP signaling by Noggin, is down-regulated in the adult SVZ. Over-expression of miR-410 in SVZ neurospheres inhibited neuronal differentiation and increased the number of astrocytes produced. Loss of function of miR-410 had the opposite effect – promoting neuronal differentiation at the expense of astrocyte formation. While co-expression of miR-410 with Noggin rescued the increase in neuronal differentiation caused by Noggin, suggesting that miR-410 functions downstream of BMP signaling. To understand the mechanisms underlying these effects, we tested multiple candidate targets of miR-410: *Elavl4*, *Sox1*, *Smad7*, *Tcf4*, and *Fgf7* were down-regulated by miR-410. In fact, co-expression of *Elavl4* (lacking the 3'UTR) also reversed the decrease in neuronal differentiation caused by miR-410. Surprisingly, we also observed that over-expression of miR-410 had an impact on neuronal morphology, with miR-410 over-expressing neurons characterized by processes that were shorter and less branched, possibly via its ability to affect Pumilio proteins (Fiore et al., 2009).

Although the functions of miRNAs in neurogenesis have been widely studied (De Pietri Tonelli et al., 2008; Cheng et al., 2009; Zhao et al., 2009; Szulwach et al., 2010), there is little information regarding crosstalk between exogenous growth factors and miRNA function (Terao et al., 2011; Wang et al., 2012; Kao et al., 2013). Here we demonstrate that the BMP signaling pathway plays a role in NSC lineage decision via regulation of miR-410 and its target genes.

miR-410 is encoded in the miRNA containing gene *Mirg*, a member of the maternally imprinted *Dlk2-Gtl2* gene cluster, which is enriched in the brain (Tierling et al., 2006; Han et al., 2012). To identify binding sites that might control its expression, we analyzed the 1.2 kb enhancer region encompassing from 1000 bp upstream to 200 bp downstream of the *Mirg* transcription start site (Figure 2.8.A). We identified three GG-C/A-GCC GC-rich BMP-specific Smad binding elements (Morikawa et al., 2011), in addition to 10 Smad consensus binding motifs GTCT/AGAC (Massague et al., 2005). Current investigations are in progress to determine if BMP-specific Smad 1/5/8 signaling directly regulates the expression of *Mirg* in luciferase reporter assays.

Although the astrocyte has been assumed to be the predominant cell fate of adult SVZ neurospheres (Li et al., 2010), few miRNAs have been shown to regulate astrocyte differentiation (Zhang et al., 2013), unlike the many miRNAs that appear to regulate neuronal cell fate (Cheng et al., 2009; Aranha et al., 2011). BMP signaling also plays an important role in oligodendrocyte differentiation, as interfering with Smad signal transduction (Colak et al., 2008) or application of another BMP inhibitor, Chordin, can redirect neuronal progenitors to an oligodendrocyte fate (Jablonska et al., 2010).We have observed that miR-410 over-expression leads to a reduction in OPC numbers. However miR-410 loss of function did not alter OPC differentiation. This suggests that miR-410 may inhibit oligodendrocyte lineage commitment through a second mechanism, and/or there are additional controls on OPC differentiation, since removing miR-410 alone was not sufficient to promote oligodendrocyte differentiation.

miR-410 expression was down-regulated in the adult SVZ after Noggin over-expression in our Noggin inducible transgenic animals (Morell et al., 2013). Noggin was initially characterized by its ability to rescue the phenotype of UV light-dorsalized embryos (Smith and Harland, 1992;

Lamb et al., 1993; Hemmati-Brivanlou et al., 1994; Sasai et al., 1995). In the adult SVZ, Noggin has previously been reported to be expressed in ependymal cells where it has been suggested to act as a "brake" on neurogenesis (Lim et al., 2000; Colak et al., 2008), and to control lineage progression during development (Smith and Harland, 1992; Lamb et al., 1993), following injury (Cate et al., 2010), and in aging (Bonaguidi et al., 2008). In addition, inactivation of BMP signaling by small molecule inhibitors such as SB-431542, LY-364947, and Dorsomorphin are widely used in the generation of neural progenitor cells from ESCs and hiPS cells (Vogt et al., 2011; Mak et al., 2012). It is likely that one mechanism involved in the ability of Noggin to regulate lineage differentiation may be via its ability to control miR-410 expression.

Elavl4/*HuD* is a member of the Elav-like RNA binding protein family. *Elav*, or embryonic lethal abnormal vision, was originally identified in *Drosophila*. Of the four mammalian Elav homologs, *Elavl1*, or *HuA/R*, is ubiquitously expressed and has been proposed to play a role in mRNA stability (Brennan and Steitz, 2001). The other three family members, *Elavl2* (*HuB/Hel-N1*), *Elavl3* (*HuC*), and *Elavl4* (*HuD*) are restricted in their expression to neurons (Okano and Darnell, 1997), and *Elavl4* is expressed in NSC isolated from adult SVZ (Figure 2.5.B). Recently the neuron-specific Elav-like proteins have been shown to recognize and bind GU and AU rich sequences in the 3'UTR and intronic regions of target mRNA transcripts (Ince-Dunn et al., 2012). These proteins are also crucial in regulation of mRNA stability and alternative splicing. AU-rich sequences are common in the 3'UTR; 10% of total cellular mRNAs are estimated to have AU-rich elements in the 3'UTR (Halees et al., 2008). *Elavl4* has been reported to stabilize several genes crucial in proliferation and neuronal differentiation including: *p21* (Joseph et al., 1998), *N-Myc* (Manohar et al., 2002), *Musashi1* (Ratti et al., 2006), *NGF*, *Neurotrophin 3*, and *BDNF* (Lim and Alkon, 2012). Several groups have employed CLIP/microarray approaches to identify

other downstream targets of *Elavl4* (Bolognani et al., 2010; Perrone-Bizzozero et al., 2011; Ince-Dunn et al., 2012). Interestingly, in addition to transcripts involved in neural development, *Elavl4* regulates a wide range of genes that control RNA processing, cell signaling, vesicle transport and neurotransmitter biosynthesis, suggesting that miR-410 may be involved in many other crucial cellular functions. A preliminary FACS sort of hGFAP-EGFP+ cells from uninduced SVZ determined that miR-410 expression was highest in hGFAP+ (B cells) (unpublished data); understanding the miR-410 expression kinetics in different cell types of the SVZ will inform *in vivo* approaches and may suggest novel therapeutic strategies..

In addition to *Elavl4*, *Sox1, Smad7, Tcf4,* and *Fgf7* were also identified as targets of miR-410. All have been implicated in neurogenesis. We have previously reported that inhibition of *Tcf4* prevents the terminal differentiation of mESC to β-III tubulin+ neurons (Slawny and O'Shea, 2011), suggesting miR-410 may also control a NSC lineage decision through the Wnt signaling pathway. The fibroblast growth factor family has long been known to regulate adult neurogenesis (Gage et al., 1995; Palmer et al., 1995; Tao et al., 1997; Jin et al., 2003). Although *Fgf7*-null mice are viable, *Fgf7* is essential for proper inhibitory synapse formation in hippocampal CA3 neurons (Terauchi et al., 2010), and *Fgf7*- null animals exhibit increased dentate neurogenesis (Lee et al., 2012). Smad7 is one of the two inhibitory Smad proteins (Massague et al., 2005). Consistent with our results, NSC isolated from adult *Smad7*-null animals exhibited decreased neuronal differentiation (Krampert et al., 2010). *Sox1*, a member of the SoxB1 family of HMG-box DNA binding proteins, is one of the earliest markers of the neural ectoderm (Pevny et al., 1998). NPCs isolated from *Sox1*-null mice form neurospheres, but are deficient in neuronal differentiation (Kan et al., 2007), while over-expression of *Sox1* in E17 neurospheres promotes neuronal differentiation (Kan et al., 2004). These findings suggest that miR-410 may also control

NSC neuronal fate through the regulation of *Sox1* or other critical gene transcripts. The detailed interaction of these factors with miR-410 will require further clarification.

We propose that in the adult SVZ, in the absence or low levels of Noggin, BMP proteins expressed by astrocytes and NSC bind and activate the BMPR, presumably BMPR-1A, activating its downstream SMAD proteins. Activated SMADs up-regulate the expression of the miRNA coding gene *Mirg* which encodes miR-410. Higher levels of miR-410 then bind the transcripts of its targets *Elavl4, Sox1, Tcf4, Fgf7* and *Smad7* to decrease their expression levels. Attenuated expression of *Elavl4*, and likely *Sox1*, directly inhibits neuronal lineage differentiation. In addition, down-regulation of *Smad7* may further enhance Smad signal transduction, forming a positive feedback loop and strengthening lineage choice. Noggin, produced by the ependymal cells in the SVZ, binds BMP proteins and inhibits downstream signaling, reducing expression of miR-410. The reduction in miR-410 de-represses expression of its target genes, increases levels of Elavl4, which promotes neuronal differentiation of NSC. At the same time higher levels of Smad7 further tunes down BMP signaling through a positive feedback loop enhancing/reinforcing the neuronal fate decision (Figure 2.8.B).

miR-410 has recently been suggested to be a prognostic marker in neuroblastoma (Gattolliat et al., 2011); patients with high miR-410 expression levels having higher survival rates. miR-410 also targets the hepatocyte growth factor receptor MET to regulate proliferation and invasion of glioma cells (Chen et al., 2012). We have observed that primary human glioblastoma cells express low levels of miR-410 (YCT, XF, KM, SO, unpublished data), implying that miR-410, in addition to regulating lineage commitment, may also control proliferation and cancer stem cell behavior. Thus miR-410 may provide a new mechanism involved in the essential choice by NSC

between self-renewal and differentiation. Finally, further elucidation of miR-410 function may identify novel approaches to CNS injury and cancers.

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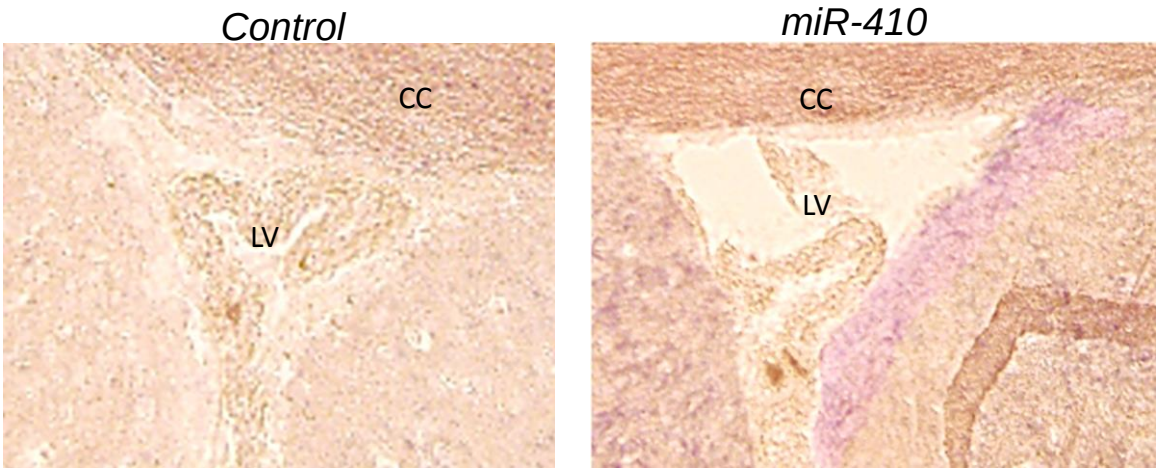

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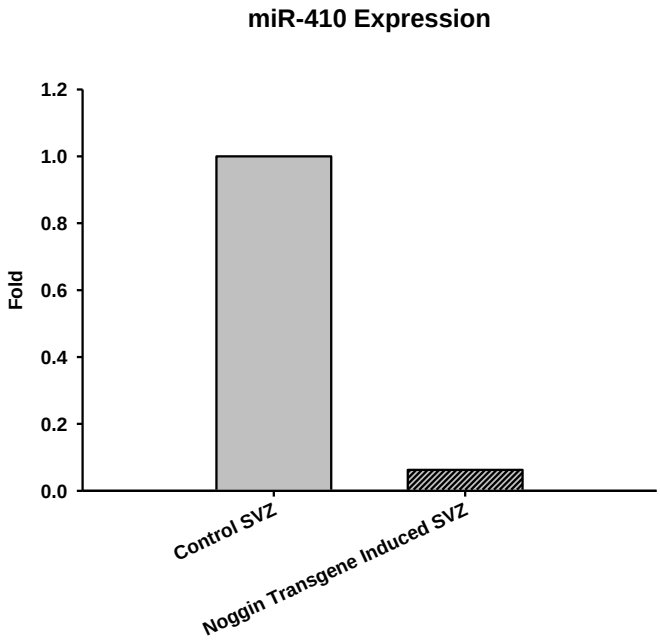


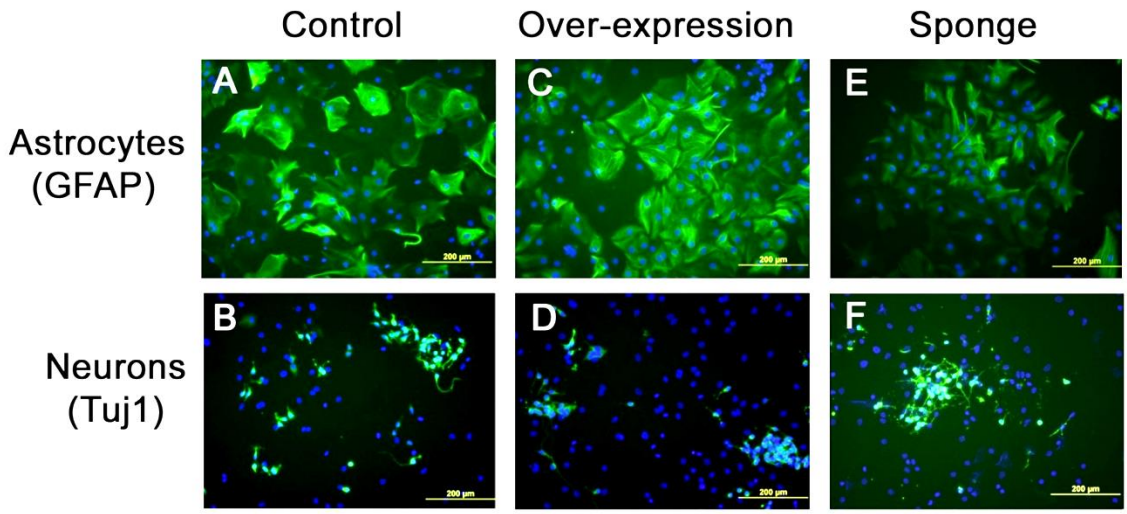
Figure 2.1 miR-410 expression.

A. *In situ* hybridization localization of miR-410 in transverse sections from the SVZ of an 8-wk old mouse. miR-410 is expressed diffusely in the anterior SVZ and in scattered cells in the parenchyma of the striatum. Control = no probe. LV = lateral ventricle, cc = corpus callosum.

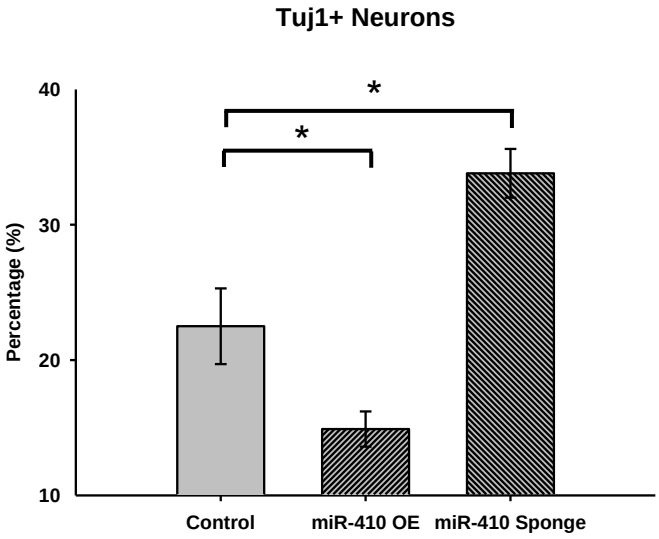
B. miR-410 expression was down-regulated 16-fold following *in vivo* induction of noggin expression. After 8 days of Noggin transgene induction, the SVZ was microsurgically

dissected from 5 induced animals and 5 un-induced mice, then total RNA extracted for q-RT-PCR.

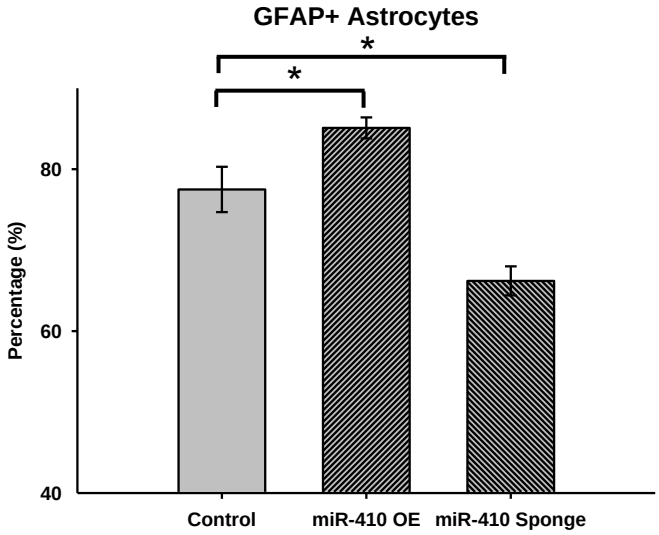
A


Control
Over-expression
Sponge
Astrocytes (GFAP)
Neurons (Tuj1)
A
B
C
D
E
F
200 μm

B


Tuj1+ Neurons
Percentage (%)
40
30
20
10
*
*
Control
miR-410 OE
miR-410 Sponge

C


GFAP+ Astrocytes
Percentage (%)
80
60
40
*
*
Control
miR-410 OE
miR-410 Sponge

D

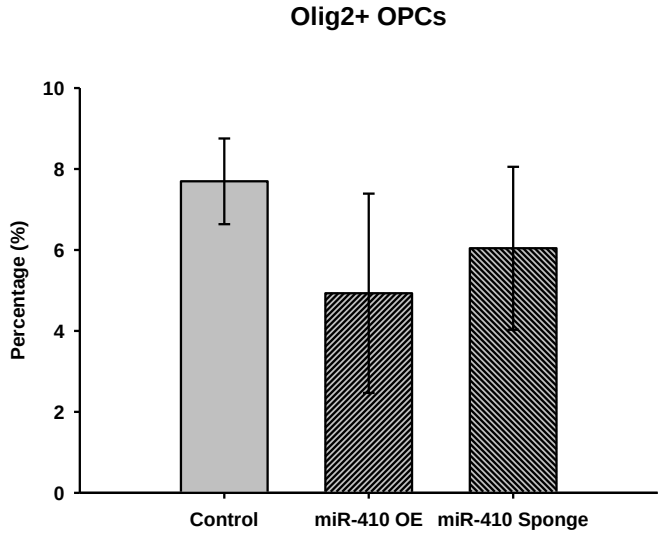
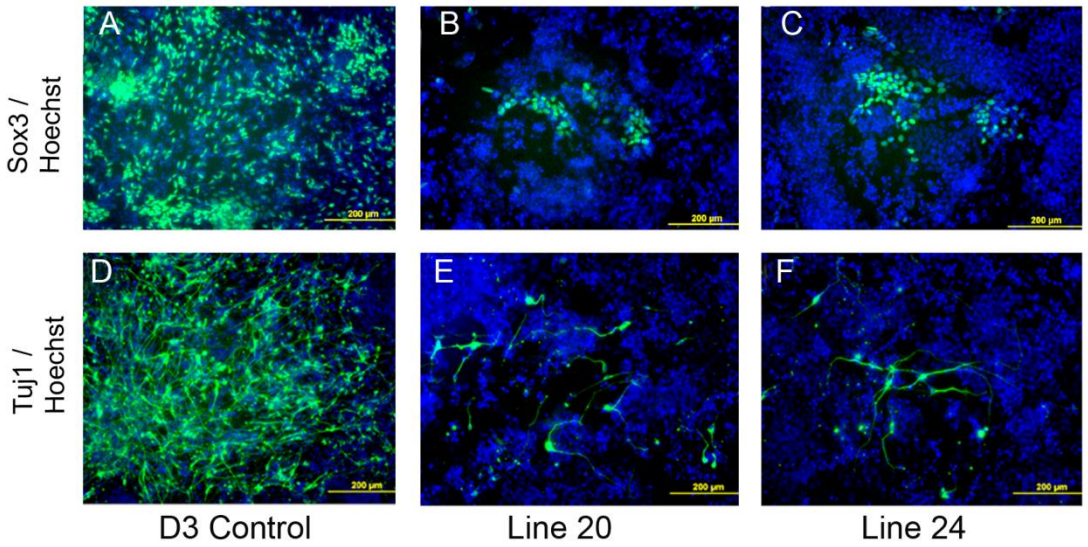


Figure 2.2.Effects of miR 410 over-expression and inhibition on neuron, astrocyte and oligodendrocyte differentiation.

A. Primary neurospheres isolated from 8 week old mice were transduced with vectors carrying either eGFP alone, a scrambled miRNA control, miR-410, or the miR-410 sponge. After 72h, the neurospheres were dissociated and plated for7 days of differentiation. Cells were fixed and immunohistochemical localization of β-III tubulin (Tuj1 antibody, neurons) and GFAP (astrocytes) carried out. There was widespread differentiation of GFAP positive astrocytes in all culture conditions (A,C,E), and differentiation of Tuj1 + neurons in control cultures (B) and in the presence of the microRNA sponge (F), but few neurons differentiated when miR-410 was over-expressed (D).

B,C,D. Quantitative analysis indicated that over-expression of miR-410 significantly inhibited neuronal differentiation (Figure 2.2.B), promoted astrocyte differentiation (Figure 2.2.C), and reduced the number of Olig2-positive cells (Figure 2.2.D). Conversely,miR-410 loss of function via the miR-410 sponge increased neuronal differentiation (Figure 2.2.B) and reduced astrocyte differentiation (Figure 2.2.C), but did not affect OPC commitment (Figure 2.2.D). Percentages of positive cells are expressed as mean $\pm$ SD, * = $p < 0.01$

A



B

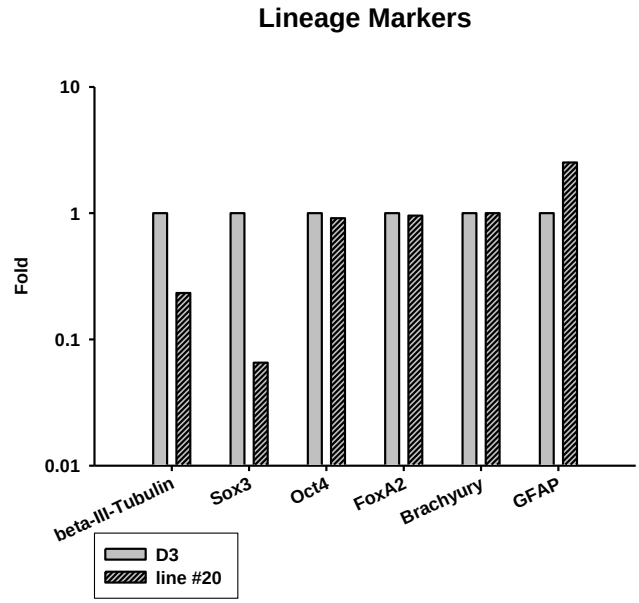


Figure 2.3 Effects of miR-410 over-expression on mouse ESC differentiation.

A. Two mouse embryonic cell lines (#20 and #24) that over-express miR-410 (20- and 6-fold respectively) were generated. The miR-410 over-expressing cells and the parental D3 cells were plated and differentiated in defined neural media for 6 days before fixation and localization of Sox3 or β-III-Tubulin (Tuj1 antibody). Over-expression of miR-410 inhibited

the differentiation of Sox3+ neural precursors and neurons (Tuj1/β-III-Tubulin positive) after 6 days of neural differentiation compared with the control cell line D3.

B. The reduction in neural differentiation in line #20 compare to the parental line D3 was quantified by q-RT-PCR. Over-expression of miR-410 reduced the expression of the early neuron marker, β-III-Tubulin 4.3 fold compared to the parental cell line D3. There was no significant difference in the expression of the pluripotency marker Oct4, the endoderm marker FoxA2, or the mesoderm marker Brachyury while the astroglial marker GFAP was increased 2.5 fold in line 20. The trophoblast marker Cdx2 was not detected in either cell line.

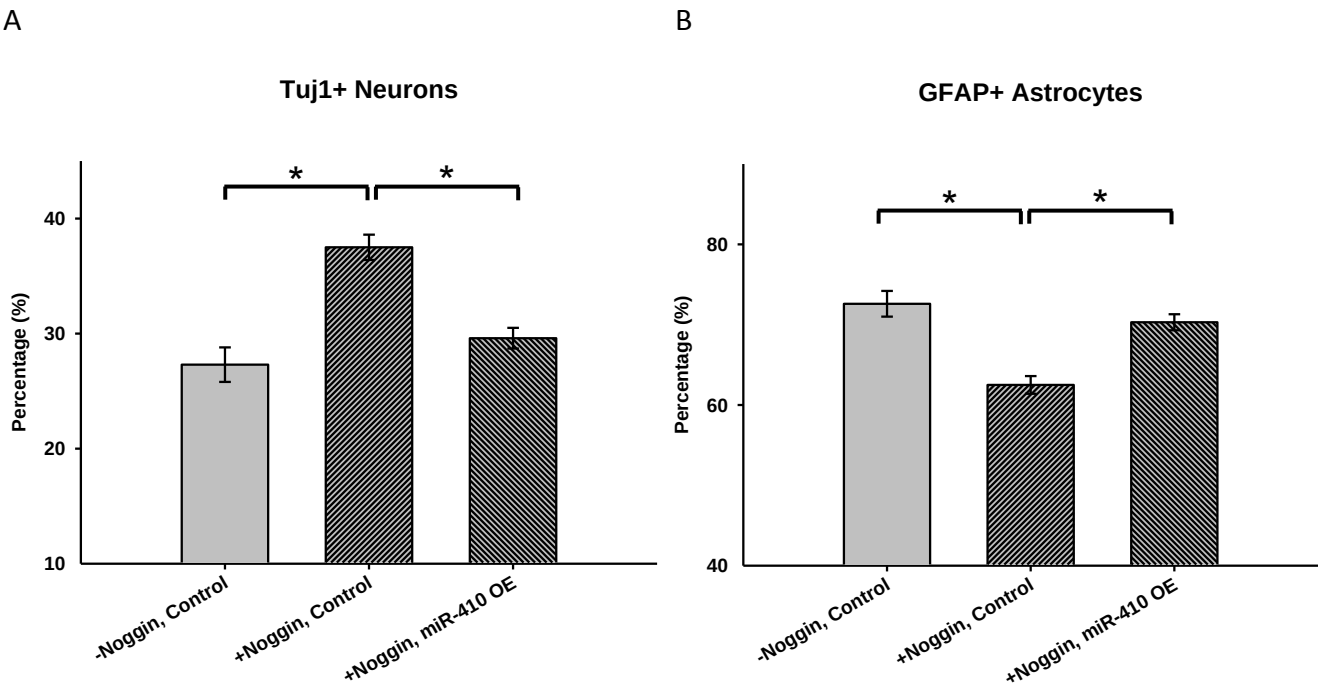


Figure 2.4 Effects of exogenous noggin protein on neuronal differentiation of miR-410 over-expressing NSC.

Neurospheres were transfected with miR-410 over-expression vectors ± Noggin protein and grown in suspension for 7 days. The neurospheres were then dissociated and plated for 7 days of differentiation, then fixed and stained for β-III tubulin (Tuj1 antibody, neurons) and GFAP (astrocytes) and cell numbers counted. Percentages of positive cells are expressed as mean ± SD, * = $p \leq 0.01$.

A. Noggin treatment strongly promoted neuronal differentiation. However miR-410 over-expression partially rescued the increase in Tuj1-positive neurons caused by Noggin, suggesting miR-410 functions downstream of BMP signaling.

B. Noggin treatment alone inhibited astrocyte differentiation. miR-410 over-expression partially rescued the decrease in GFAP-positive astrocytes caused by Noggin.

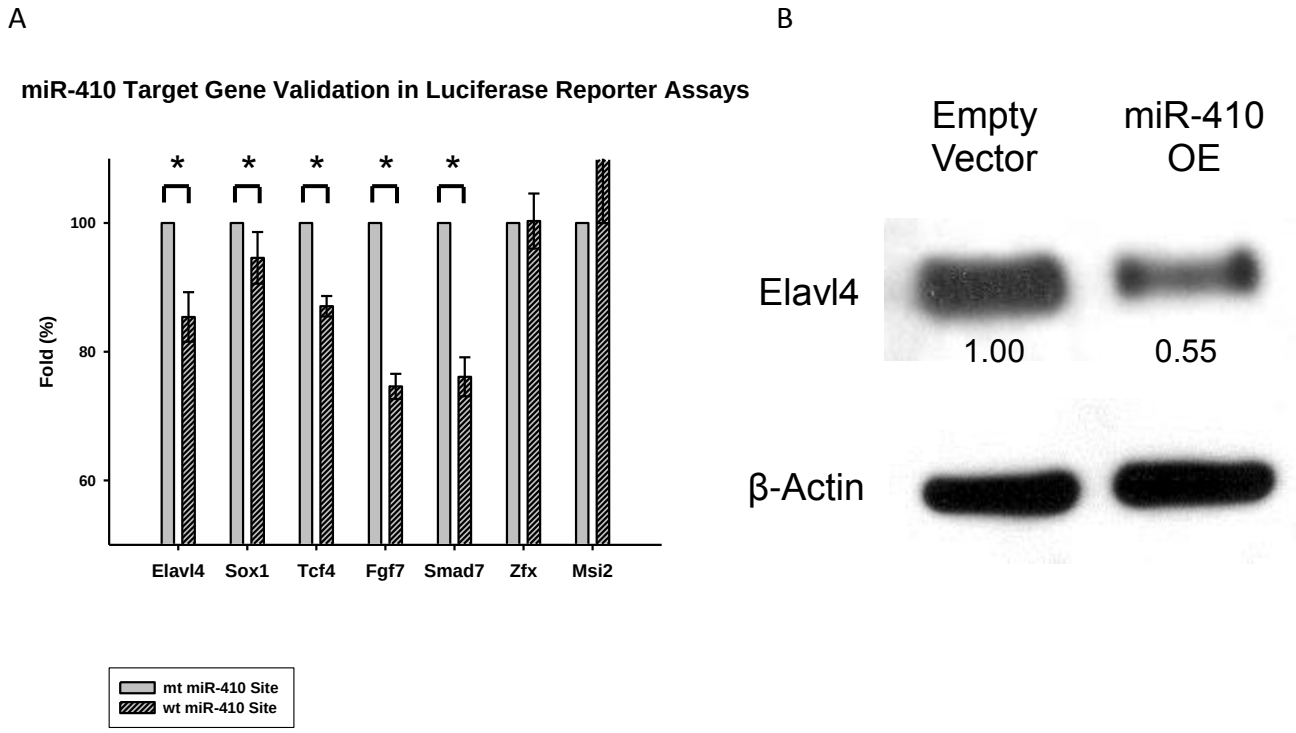
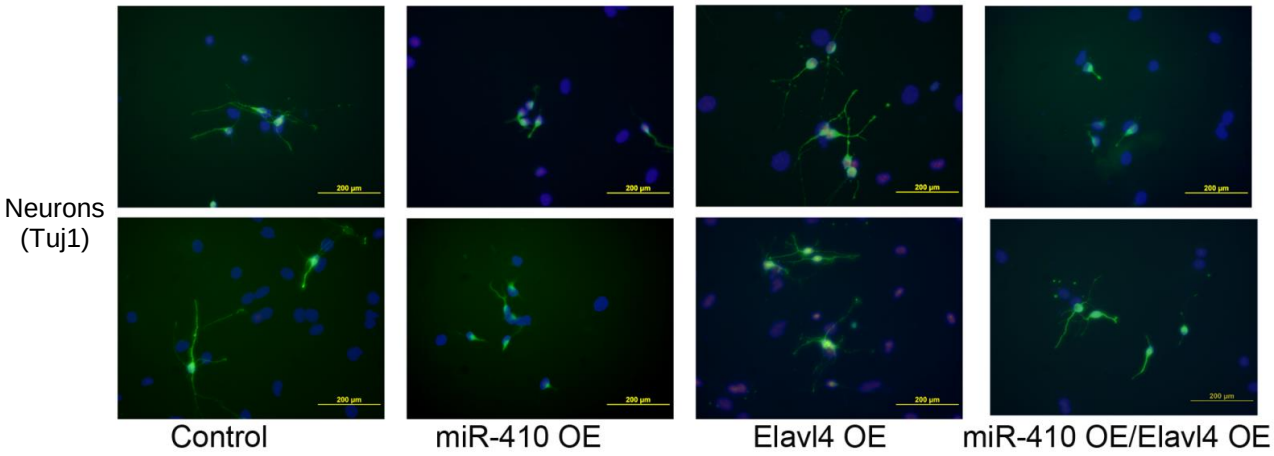


Figure 2.5 Target validation.

A. Computer predicted miR-410 target candidates were examined using luciferase reporter assays. The 3'UTR of the candidate genes was cloned and inserted after the luciferase reporter. The predicted seed sequence region in the 3'UTR was mutated to create the mutant control. Among the genes tested, *Elavl4*, *Sox1*, *Tcf4*, *Fgf7*, and *Smad7* were verified as targets of miR-410, while *Zfx* and *Msi2* were not. Values are expressed as mean + SD, ** = $p \leq 0.01$.

B. Neurospheres over-expressing miR-410 or a control vector were lysed and the whole cell lysate was blotted against Elavl4. Elavl4 protein expression was down-regulated approximately 45% compared to the control, confirming that Elavl4 is a downstream target of miR-410.

A



B

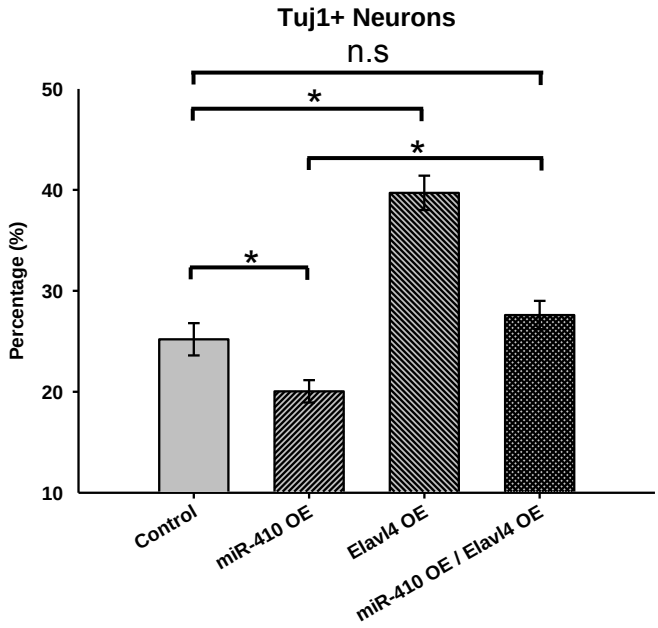
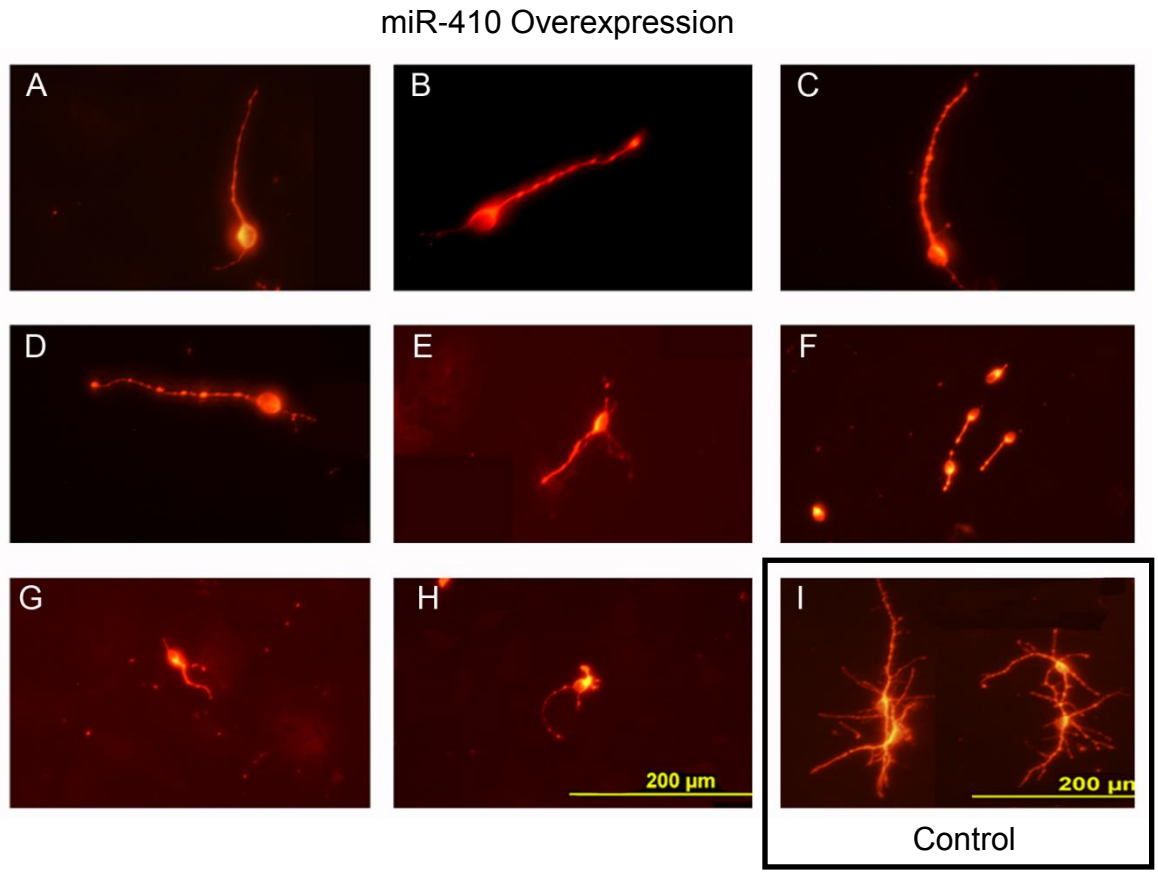


Figure 2.6 Elavl4 rescues the neuronal phenotype caused by miR-410 over-expression.
Neurospheres were transfected with miR-410 over-expression and Elavl4Δ3'UTR vectors. After 7 days, the neurospheres were dissociated and plated for differentiation, then after 7 additional days in culture the cells were fixed and localization ofβ-III tubulin (Tuj1 antibody, neurons) carried out and positive cells counted.

A, B. Compared to controls, over-expression of miR-410 alone reduced the numbers of neurons formed, while over-expression of Elavl4 without the endogenous 3'UTR with miR-410 rescued the decrease in the number of Tuj1 + neurons caused by miR-410. Over-expression of Elavl4 alone strongly promoted neuronal differentiation. There was no significant

difference between the empty control vector and the miR-scrambled vector. Data are expressed as mean percentage $\pm$ SD. * = $p \leq 0.01$.

A


miR-410 Overexpression
A
B
C
D
E
F
G
H
200 µm
I
200 µm
Control

B

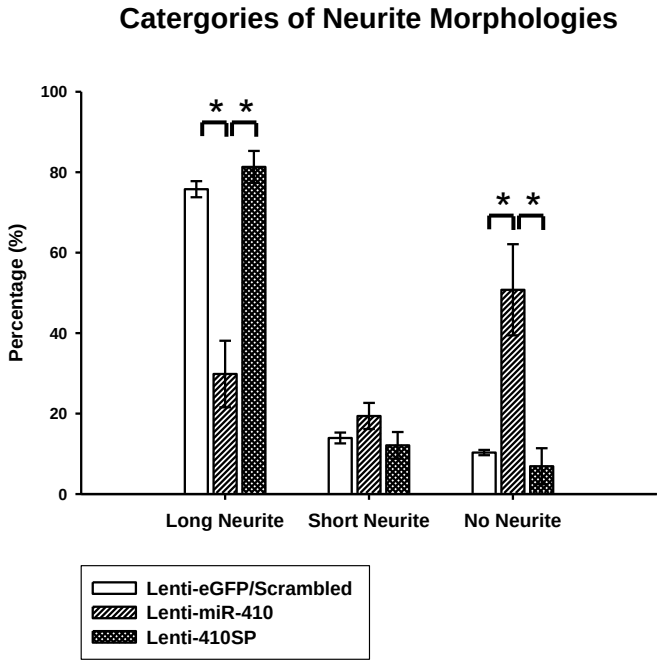

Catergories of Neurite Morphologies
Percentage (%)
100
80
60
40
20
0
* *
* *
Long Neurite
Short Neurite
No Neurite
Lenti-eGFP/Scrambled
Lenti-miR-410
Lenti-410SP

Figure 2.7 miR-410 over-expression produces alterations in neurite morphology.

A. Neurospheres were transduced with lentivectors carrying miR-410 and control vectors. After 72 hours the neurospheres were dissociated and plated for differentiation. After 7 days of differentiation the cells were fixed and immunohistochemical localization of β-III tubulin (Tuj1 antibody, neurons) carried out. Neurons over-expressing miR-410, compared to the control neurons, were characterized by shorter, unbranched neurites. A-H. Neurons over-expressing miR-410. I. Control neurons.

B. Neurites from each group were characterized and counted from at least 100 neurons per well, 3 wells per group. Type I: neurons with no processes. Type II: cells with neurites shorter than the length of the soma. Type III: neurons with processes longer than the cell body. Data are presented as mean $\pm$ SD, ** $p \leq 0.01$.

A

CATCACCTTTGG**GTCT**CTGCCTGT**GGAGCC**AGCTTGGCACAGAGGCCGA**GGCGCC**TTTCAACATTCTGTTTCCTCTGCCTGA
GAAGCGGGGATTTTTTTTTTTTTTTTT**GTCT**TGTAATCTGTTTCAGATGAGCCAAGCAGCAAGCCTGGTCCTTCCCGGAACT
CAGTCCTTCTTTGGTATTTAAAAGGTGGATATTCCTTCTATGGTTACGTGCTTCCTGGATAATCATAGAGGAACATCCACTT
TTTCAGTATCAAATGTCGCTCTGGAACTATTTCTGGAAGATTCTGTAGG**GTCT**GGGGCTGGGGGGGGAAGATCATGTAATAAT
GAGTGGGGCGTGGCCAGAAATGGCCTTAAGAAGCCAACTGAGTGTTA**GTCT**GATCTGG**GTCT**GATATACCGCCTCTTTGGGC
AGCCTGCTCACTCTGCCCAGTGGACTTCCATTTCTGTCGACACAAGCACAAGCTCACAGCATCCTGGATCGAGTTGTTCTTT
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ATGCTGCTTGGGAAACCACCGAGGACATGACATCTTCACCGAAGAAAGCGTGCTATCCGTC**GTCT**TCCTGGGCTTGGCGGGG
ATAGATAGATTTGTGACTTGTGTGATGTGTGCCTATATGCCAGCCTCTCCCAGTGATGTGTTCTG**AGAC**CACTGTGTTCTTT
ATATCCCTTCTCTGACCTCAAATGAAGTTGTTTCTCTGTGTGCTCACCATGTGTATGTTTTCTGGAGTCGGCTTCTTTTTTC
TGTAGCGATAT**GTCT**TGGAGAGCTCCTGGGTGGGCCATCATTTTGGGTAAG**GGCGCC**TTGGGTAACCCTTTGGGGGGTGTGC
GGAGAAGGGGGGGCATGTGAATCATCTGGAAGTGTAACCTCCAAATGTGCCCCCTCAGGCTCACATCCGGACCGTCATCGCA
TCTGTCATCGCACCGGACCAGCTCTCCCCAGCGCTCCTTATCGTTTGCTACTTGAAGAGAGGTTATCCTTTGTGTGTTTGCT
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TAGCAGAAGGGTGTG**GTCT**CTCCAGGGTAAGTAAATGCATGGTGGGCCACCGAGCTTGAGCTCCTTCTTC**GTCT**GACCCTAC
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B

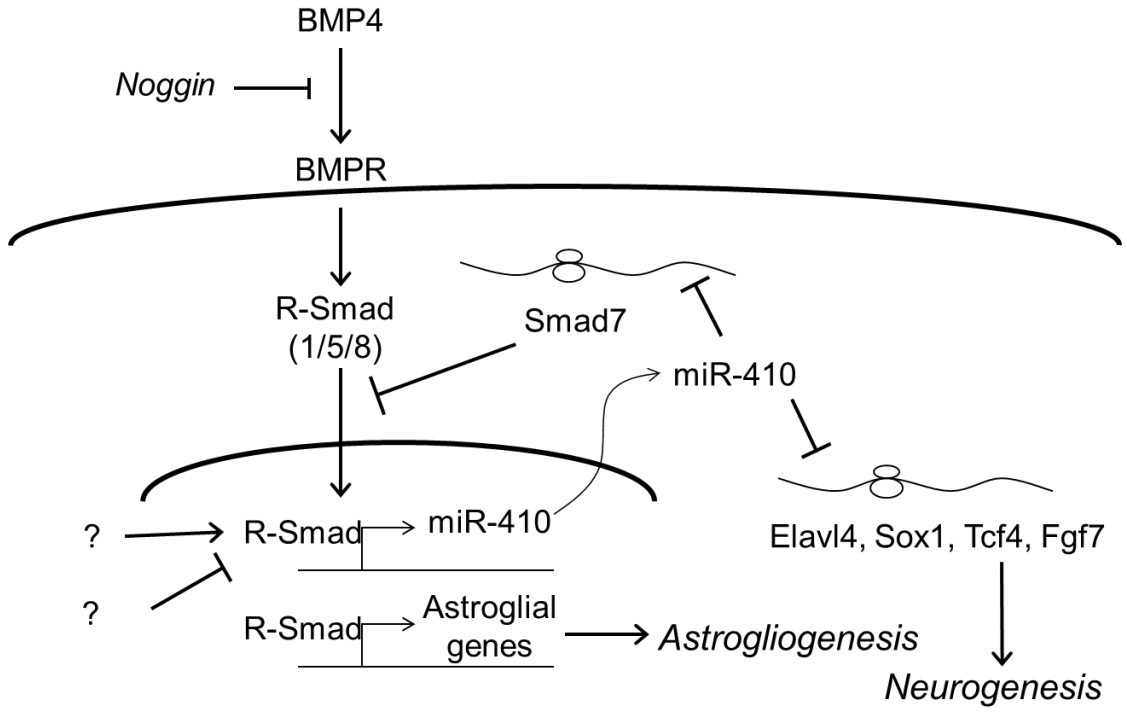

Figure 2.8 Modeling 410 behavior

A. The Mirg promoter (-1000 to +299bp from the transcription start site) was analyzed to identify possible Smad binding sites. Three GG-C/A-GCC GC-rich BMP specific Smad binding elements (highlighted bold) in addition to 10 Smad consensus binding motifs GTCT/AGAC (underlined bold) were identified in the region.
B. Proposed model of how Noggin, BMP, and miR-410 may regulate NSC differentiation. BMP signaling activates the expression of miR-410 (and other astroglial genes). miR-410 down-regulates Smad7, in turn creating a positive feedback loop, and other pro-neuronal genes including Elavl4, Sox1, Tcf4, and Fgf7. When Noggin is present it directly binds and inhibits BMP proteins from binding BMPRs. Inactive BMP signaling leads to low level expression of miR-410 and astrocyte-restricted genes. The pro-neuronal genes are thus de-repressed and promote neurogenesis.

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Chapter Three

Significance and Future directions

Significance

The goal of my initial research was to understand the function of BMP signaling in lineage decisions in adult SVZ neurogenesis. BMP signaling is vital during early embryogenesis and neurogenesis (Jones et al., 1996). In addition, the BMP inhibitor, Noggin, is crucial in the induction of neuroectoderm from ectoderm (Smith and Harland, 1992; Lamb et al., 1993; Sasai et al., 1995; Zimmerman et al., 1996). In the first part of the research, I describe the generation and validation of an inducible transgenic mouse in which Noggin can be inducibly expressed in the neural stem cell population of the adult SVZ. In this animal, the CNS restricted enhancer in the *Nestin* promoter drives reverse tetracycline-controlled transactivator (rtTA) expression to neural stem cells. Using this animal as a model, we demonstrated that Noggin promoted neuronal differentiation of the neural stem cell population (B cells) toward transient amplifying C cells. In addition we observed increased numbers of oligodendrocyte progenitor cells in the adult SVZ both *in vivo* and *in vitro*. Noggin also promoted proliferation of the NSC population, thus the prolonged stimulation by Noggin did not deplete the stem cell population. In a microarray to identify differences between the transcriptome of the Noggin over-expressing versus control SVZ, multiple differentially expressed genes were identified. Among the genes which were

down-regulated in the Noggin over-expressing cells is a microRNA (miRNA) encoding gene *Mirg,* which encodes multiple previously uncharacterized miRNAs, and which is located in the imprinted *Dlk1-Gtl2* locus in mice (Tierling et al., 2006; Hagan et al., 2009).

In the second part of the research, I followed up one of the miRNAs encoded by *Mirg*, miR-410, and its function in adult neurogenesis in the SVZ. miR-410 was previously reported to be expressed in the central nervous system during embryonic development (Wheeler et al., 2006), but our studies determined much of that "localization" was due to trapping within the lumen of the neural tube (not shown). The microarray result was verified by quantitative PCR, and the expression of miR-410 in the adult SVZ was confirmed by *in situ* hybridization. Over-expression of miR-410 via lentiviral vectors in neurospheres derived from the adult SVZ, as expected, led to a reduction in neuronal and oligodendroglial differentiation and increased astroglial differentiation. More importantly, over-expression of miR-410 partially rescued the differentiation phenotypes caused by Noggin, suggesting miR-410 functioned downstream of Noggin-BMP signaling. To further understand the molecular mechanism of miR-410 functions, computer algorithm predicted miR-410 target candidates were validated by luciferase reporter assays. Among the candidates tested, five genes were verified as true targets of miR-410: *Elavl4*, *Sox1, Tcf4, Fgf7*, and *Smad7*, while computer predicted genes such as *Zfx* and *Msi2* were shown to be independent of miR-410 regulation. In addition, *Elavl4* was down-regulated at the protein level in the miR-410 over-expressing neurospheres. Over-expression of miR-410 resistant Elavl4 also rescued the decrease in neuronal differentiation induced by miR-410 over-expression, indicating that miR-410 inhibited neuronal differentiation by down-regulating pro-neuronal genes such as *Elavl4*. Unexpectedly, over-expression of miR-410 also led to a change in

neuronal morphology. Neurons over-expressing miR-410 typically had short, unbranched neurites compared to the long, highly branched neurites characteristic of the control groups.

Active BMP signaling has previously been shown to promote astroglial differentiation while inhibiting both neuronal and oligodendroglial differentiation (Gross et al., 1996; Lim et al., 2000). However the underlying molecular mechanisms are poorly understood. BMP signaling has been shown to activate the transcription of GFAP, which is an early and strong inducer of astroglial differentiation, via canonical Smad signaling and the PI3K pathway (Dore et al., 2009). In addition, BMP4 treatment has been shown to directly activate the inhibitor of differentiation (ID) family of helix-loop-helix factors, which in turn inhibit oligodendrocyte commitment (Samanta and Kessler, 2004). Here, I propose a novel mechanism by which the BMP signaling pathway may regulate the NSC lineage decision in adult SVZ neurogenesis. In the absence of the BMP inhibitor Noggin, BMP protein activates Smad signaling and directly promotes the expression of miR-410. miR-410 down-regulates neurogenic genes including *Elavl4*, *Sox1*, *Tcf4*, and *Fgf7*, which results in the suppression of neuronal differentiation. In addition miR-410 also down-regulates Smad7, an inhibitory Smad protein, forming a positive feedback loop to further strengthen the lineage commitment. Conversely, the presence of Noggin, which directly binds and inhibits BMP proteins from binding their receptors, would turn off this pathway. The neurogenic genes listed above are therefore de-repressed and promote neuronal differentiation.

There have been few reports demonstrating a role of extrinsic signaling pathways in controlling miRNA expression or activation (Terao et al., 2011; Han et al., 2012; Wang et al., 2012; Kao et al., 2013; Park et al., 2013). In this research I proposed that one such pathway, BMP signaling, regulates the activation of a miRNA. Interestingly, among the other miRNAs also encoded by the *Mirg* gene, miR-382 (Milosevic et al., 2012) and miR-377 (Lan and Chung, 2011) have

previously been shown to be up-regulated by TGF-β signaling, suggesting that this pathway plays a central role in regulating the expression of this cluster of miRNAs. Indeed, by bioinformatics analysis, there are ten consensus Smad binding motifs identified in the promoter region of *Mirg*, which may explain the regulation of expression by both the TGF-β and the BMP signaling pathways. Furthermore, three additional BMP specific GC-rich Smad binding sites were also found in the promoter of *Mirg*. This implies that in addition to the TGF-β signaling, BMP may also regulate other miRNAs encoded by the *Mirg* gene.

Future directions

miR-410 expression kinetics in different cell types.

Although we have shown that miR-410 is expressed in the adult SVZ, understanding its expression kinetics in different SVZ cell types could be very informative regarding the detailed control of lineage decision by miR-410. I have determined that the over-expression of miR-410 inhibited oligodendroglial differentiation, but antagonizing its function by the miRNA sponge did not significantly affect the lineage commitment. A possible explanation is that since oligodendrocyte progenitor cells are derived from the transient amplifying C cells (Ming and Song, 2011), different expression levels of miR-410 in different cells may contribute to this result. Moverover, in preliminary data (Figure 3.1) the hGFAP:GFP-positive B cells in the neurospheres expressed higher levels of miR-410 compared to other cells. I hypothesize that the lower level of miR-410 in the C cells may de-repress oligodendroglial differentiation. Pastrana et al (Pastrana et al., 2009) have proposed a method to purify the different cell types residing in the SVZ by fluorescence activated cell sorting (FACS). The activated B cells can be isolated by

GFAP::GFP/EGFR-double positive, CD24-negative labeling. The transient amplifying C cells can then be purified as they are GFAP::GFP-, EGFR+, CD24-, while the neuroblasts (A cells) can be purified by their GFAP:GFP-, EGFR-, $CD24^{low}$ labeling. Identifying cell-type specific miR-410 expression kinetics would answer this question. I would predict that B cells would have the highest expression level of miR-410 expression and it would be the lowest in A cells, as they are committed neuroblasts, while the transient amplifying C cells would have intermediate levels of miR-410. Alternatively, the identification of a cell-specific miR-410 expression pattern could also be achieved by locked nucleotide-fluorescence *in situ* hybridization (LNA-FISH) (Exiqon, MA) (Silahtaroglu, 2010) with cell markers (B cells: GFAP+BrdU+, C cells: Mash1+, A cells: Dcx+).

***in vivo* function of miR-410 in adult SVZ.**

In the second part of my research, I demonstrated miR-410's function in regulating adult NSC lineage *in vitro*. The next logical step would be to test the function of miR-410 *in vivo* in the adult SVZ. The lentiviral vector over-expressing either miR-410 with eGFP, the miR-410 sponge with eGFP, scrambled miRNA control sequence with eGFP, or eGFP alone, would be injected directly into the adult SVZ in either wildtype or our Nestin-driven Noggin inducible transgenic animals. Similar to the *in vitro* data, I expect miR-410 over-expression will inhibit both neuronal and oligodendroglial differentiation while the miR-410 sponge will promote neuronal, and possibly oligodendroglial, differentiation. Moreover, I also predict that miR-410 over-expression will rescue the increase in neuronal and oligodendroglial differentiation caused by Noggin in the Noggin inducible mouse. To study the long term effects of miR-410, a miR-410 flox/flox animal model would be ideal. Unlike other miRNAs, miR-410 is located in the 12^{th} exon of the *Mirg* gene. Handily, this exon only encodes miR-410, thus making it ideal and easy for targeting and

generation of knockout animals. In combination with specific promoter driven Cre recombinase animals such as the Nestin-Cre/ERT2 (Lagace et al., 2007), hGFAP-Cre (Zhuo et al., 2001), and Ascl1/Mash1-CreERT2 (Kim et al., 2011) the function of miR-410 in each specific cell types in the adult SVZ could be further studied.

Study of miR-410 in neonatal SVZ neurogenesis would also be of great interest, since there are many more neurons formed at this stage compared to the adult (Wang et al., 2011). Thus, the expression pattern and the function of miR-410 in neonates may provide further information on how this neuron to astrocyte transition is regulated. The function of miR-410 in the SGZ of the hippocampus is also of considerable interest. In my preliminary experiments we determined that miR-410 is expressed in the SGZ, but was not regulated by Noggin/BMP signaling pathway. The actual function and regulation of miR-410 in SGZ remain to be elucidated.

***In vivo* function of miR-410 in embryo development.**

miR-410 was originally identified in the developing CNS (Wheeler et al., 2006). In my preliminary experiments, miR410 was expressed in the hindbrain region and the rhombic lip (Figure 3.2) at embryonic day 11.5. Interestingly miR-410 is expressed in the Purkinje layer in the adult cerebellum (Pena et al., 2009), suggesting miR-410 may play an important role in cerebellum formation. However the detailed functions of miR-410 during cerebellum differentiation were never studied. The plasmid vector encoding miR-410 with eGFP, miR-410 sponge, or control vectors could be delivered through tail vein injection at different time points (E10.5 to E15.5) to pregnant female mice (Gratsch et al., 2003), or injected via *in utero* surgical approaches. The formation of cerebellum of the pups then will be examined at different stages to test the function of miR-410 in cerebellum differentiation. Alternatively, the miR-410 floxed

animals described above can be crossed with Math1-CreERT2 (Machold and Fishell, 2005) to create an inducible knockout animal model to study the loss of function of miR-410 in cerebellum formation.

miR-410 function in glioblastoma/neuroblastoma.

Recently miR-410 was shown to target and down-regulate the hepatocyte growth factor receptor MET in glioma cells (Chen et al., 2012). Over-expression of miR-410 led to reduced proliferation and decreased invasive capability. Furthermore low expression of miR-410 was identified as a prognostic biomarker for high risk neuroblastoma (Gattolliat et al., 2011). Interestingly, I have also made similar observations in miR-410 over-expressing mES cells (Figure 3.3). mES cell lines over-expressing miR-410 (lines #20 and #24) have significantly slower proliferation rates compared to the parental D3 cell line. Moreover, in collaboration with Dr. Xin Fang (Neurosurgery, University of Michigan), I have observed that in all four samples of human glioblastoma (GBM) we tested, miR-410 expression levels were dramatically lower than the normal brain control tissue, although there was great variance among the tumor samples which might correlate with invasive phenotype (Figure 3.4). These data suggest that miR-410 is important in regulating tumor progression. However large scale screening would be required to establish the correlation between miR-410 expression level and the prognosis and invasiveness of GBM. Recently Aldaz et al (Aldaz et al., 2013) also have shown that over-expression of miR-21 in the stem cell-like cells of glioblastoma, the GBM inducing cells (GIC), led to differentiation of these cells. Considering the preliminary data, the hypothesis would be that over-expression of miR-410 would be expected to lead to quiescence and differentiation toward an astroglial lineage in these GICs. To test this hypothesis, isolated GICs could be either transfected by plasmid vectors or transduced by lentiviral vectors carrying either miR-410,

scrambled miRNA control, or empty/eGFP control sequence. The cell proliferation rate, differentiation of the three lineages could then be measured and tested to learn more about miR-410 function. In addition, cell migration assays such as transwell migration or scratch wound healing could be also used to study the invasion capability of the transfected/transduced cells.

miR-410 in other stem cell populations.

In addition to the developing CNS (Wheeler et al., 2006) and regions of adult neurogenesis, miR-410 was also identified in the scalp dermal papilla where up-regulation of miR-410 was linked to male pattern baldness (Goodarzi et al., 2010). However, whether antagonizing miR-410 by miR-410 sponge in the hair follicle stem cells may reverse this phenotype and the detailed underlying molecular mechanisms remain unclear.

Signaling pathways regulating *Mirg*.

In my research, I showed that miR-410 expression is regulated by Noggin/BMP signaling. In addition, multiple Smad binding sites were found in the promoter region of the miR-410 encoding gene Mirg. To directly test whether Smad proteins bind to this region, I propose to do electrophoresis mobility shift assays (EMSA). Alternatively, chromatin immunoprecipitation (ChIP) would be a good approach. Antibodies against activated phosphorylated Smad-1/5/8 are commercially available and have been tested for this purpose (Cell Signaling, MA) (Wang et al., 2013), indicating this experiment should be feasible. On the other hand, to further identify upstream regulation, the *Mirg* promoter region should be examined to identify additional transcription factor binding motifs. Online databases/algorithms such as TRED (http://rulai.cshl.edu/TRED) provide a platform to identify putative binding sites. However one major limitation of this approach is that the algorithm itself may not include all known protein-

DNA binding pairs. Results from multiple databases/algorithms should be pooled and analyzed to obtain a more broad coverage. The *in silico* prediction will then subjected to validation by either EMSA or ChIP.

Other miRNAs encoded by *Mirg*.

In addition to miR-410, *Mirg* encodes 13 additional miRNAs: miR-382, miR-134, miR-668, miR-485, miR-453, miR-154, miR-496, miR-377, miR-541, miR-409, miR-412, miR-369, and miR-3072. Most interestingly, among the 13 miRNAs, six miRNAs have been reported to either control cell cycle arrest (miR-377 (Maes et al., 2009)), directly inhibit proliferation in cancer cells (miR-668 (Shin et al., 2011), miR-134 (Niu et al., 2013; Yin et al., 2013), miR-154 (Xin et al., 2013)), induce apoptosis (miR-382 (Thayanithy et al., 2012)), or their expression level inversely correlated to bad prognosis (miR-485 (Costa et al., 2011)). One would hypothesize the cluster of miRNAs encoded by *Mirg* as a whole may be important in cell proliferation. I propose to test whether the 6 remaining miRNAs with unclear function also inhibit cell proliferation. Using mouse embryonic stem cells as a model, their ability to alter cell proliferation will be tested systematically by transfecting the cells with individual chemically synthesized miRNAs. In addition, since around half of the miRNAs in the cluster have been shown to act as tumor suppressors in cancer cells, one would expect that restoring these miRNAs in cancer cells would inhibit tumor progression. Using glioblastoma cells as an *in vitro* model, I propose to transfect the cells with a cocktail of chemically synthesized miRNAs with proliferation inhibitory function to test if they synergistically affect tumor cell growth. The next step would be to design a cell type specific delivery system, either promoter driven or via mechanical injection, to carry the cocktail *in vivo* and to test their therapeutic value in mouse models.

Fascinatingly, even though they are all encoded by the *Mirg* gene, different miRNAs in this cluster have different cell type expression patterns and are linked to different type of tumors (Maes et al., 2009; Costa et al., 2011; Shin et al., 2011; Thayanithy et al., 2012; Niu et al., 2013; Xin et al., 2013; Yin et al., 2013). This may suggest a novel regulation of miRNA biosynthesis. Among the 14 miRNAs encoded by *Mirg*, miR-134, miR-485, and miR-410 are located in exons while the rest are located on introns (MGI mouse genome build). I propose that the location and surrounding sequence may provide addition information about the expression pattern of each miRNA. To test this hypothesis, I propose to generate a bacteria artificial chromosome (BAC) carrying the 20kb region of chromosome 12 containing *Mirg*. The position of each miRNA then can be mutated and switched between different locations. Each mutated BAC then will be transfected to different cell types *in vitro* and the expression level of each miRNA then can be measured by q-RT-PCR. If the expression pattern of the miRNA is independent of the location/surrounding sequence, this cell type specific information then must be carried by the primary/pre-miRNA scaffold structure sequence. To test this, the mature miRNA sequence would then be cloned and switched between each primary miRNA scaffold. The mutated miRNA will then be transfected and the expression measured in different cell types.

Noggin/miR-410 function in animal behavior.

Alterations in adult SVZ neurogenesis have been shown to cause behavioral consequences (Pan et al., 2012). In my research, induced Noggin over-expression promoted neurogenesis and increased neuroblast formation in the SVZ, while over-expression of miR-410 had the opposite effect -- reducing neuronal differentiation. I would hypothesize the Noggin and miR-410 would affect olfactory-associated behaviors. The Noggin inducible animal and the miR-410 floxed animals described above would be subjected to the behavioral tests. I expect the both Noggin

induced and the miR-410 conditional knock out animals, with enhanced SVZ neurogenesis, would perform better in the olfactory short term memory test and odor-cued associative olfactory learning.

Using miR-410 in bioengineering applications.

In my research I have determined that miR-410 inhibits neuronal and oligodendroglial differentiation while promoting astroglial differentiation. This function may be useful in bioengineering approaches. Recent advances in induced pluripotent stem (iPS) cell differentiation (Takahashi and Yamanaka, 2006; Takahashi et al., 2007) has provided an approach for personalized cell based regeneration therapy in diseases such as stroke or spinal cord injury. However a major challenge is to generate neural progenitor cells, oligodendrocyte progenitor cells (OPC), or astroglial precursor cells with high efficiency for transplantation. The lineage controlling miR-410 could be manipulated during differentiation to acquire the lineage intended. If neural or OPC are intended, chemically synthesized miR-410 antagonists such as locked nucleotide (LNA) (Exiqon, MA) can be transfected into the differentiating iPS cell to suppress the function of miR-410. In contrast, to increase the yield of astroglial progenitor cells, synthesized mature miR-410 RNA could be delivered to the cells to promote astroglial differentiation. This application is protected under US patent pending number 133/213,848.

A B

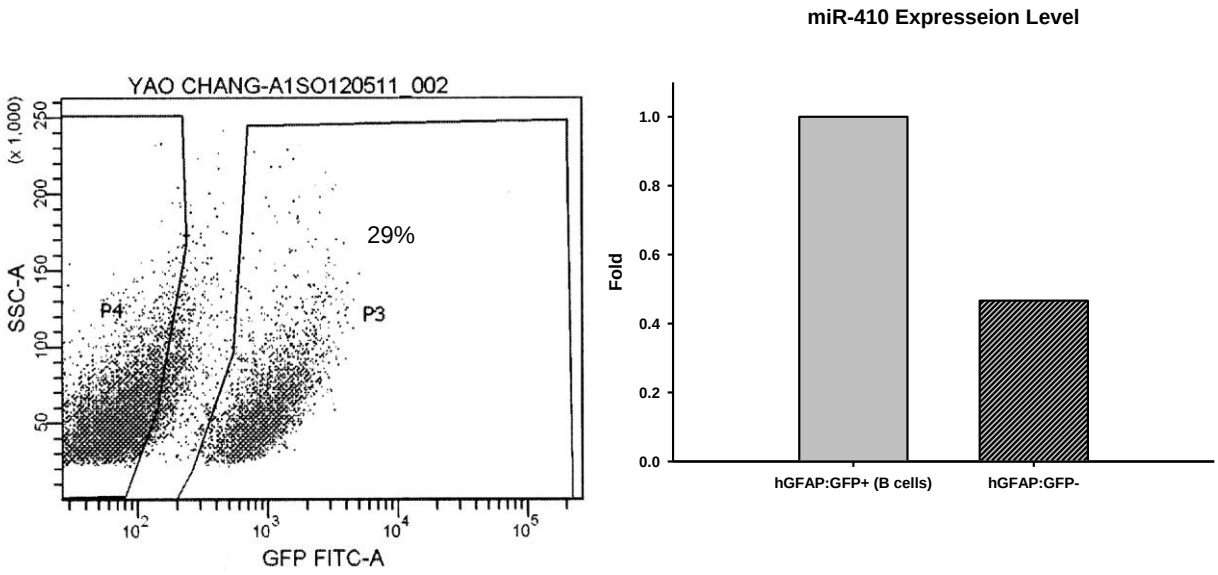

Figure 3.1

A. hGFAP:GFP-positive cells (B cells) purified by FACS from neurospheres derived from adult SVZ.
B. miR-410 expression is higher in the hGFAP+ B cells compared to the rest of cells in the neurospheres.

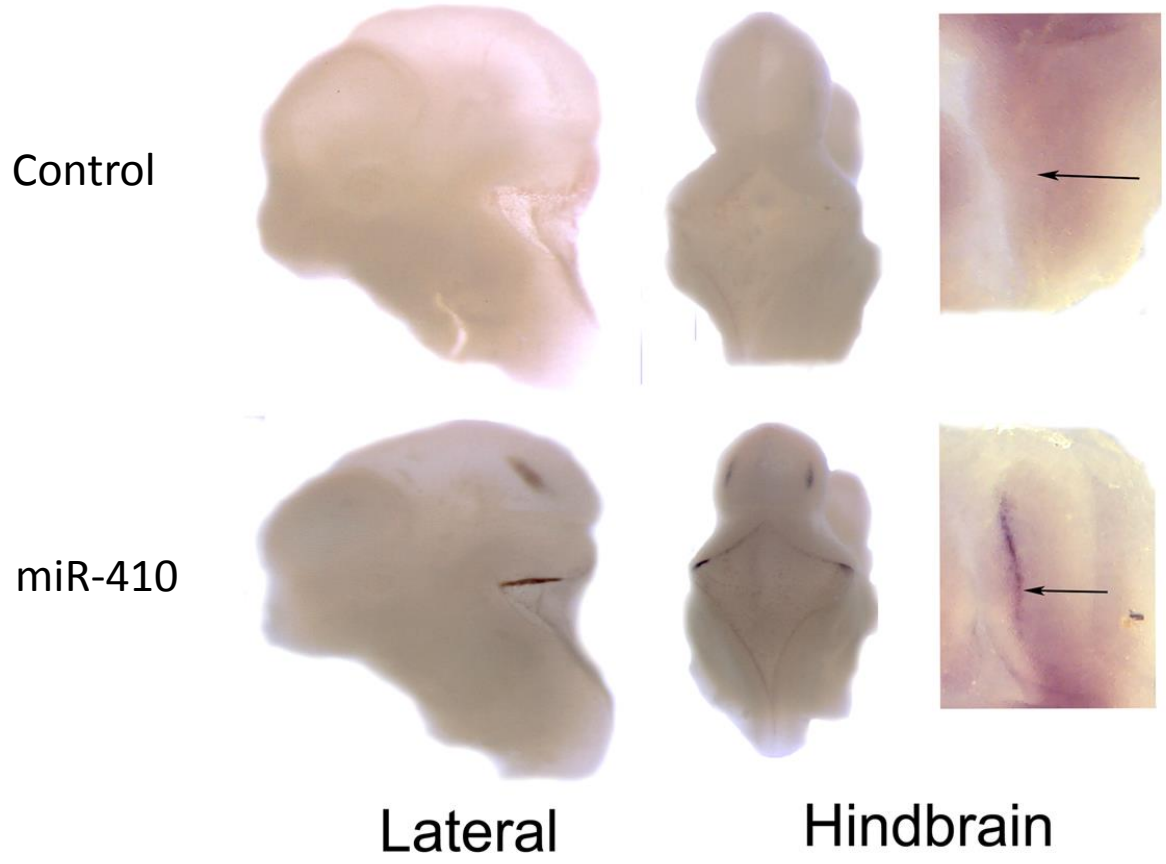

Figure 3.2

miR-410 is expressed in the midbrain and the rhombic lip on E11.5. Arrow: neuroepithelium at the rhombic lip. (Lisa DeBoer Emmet).

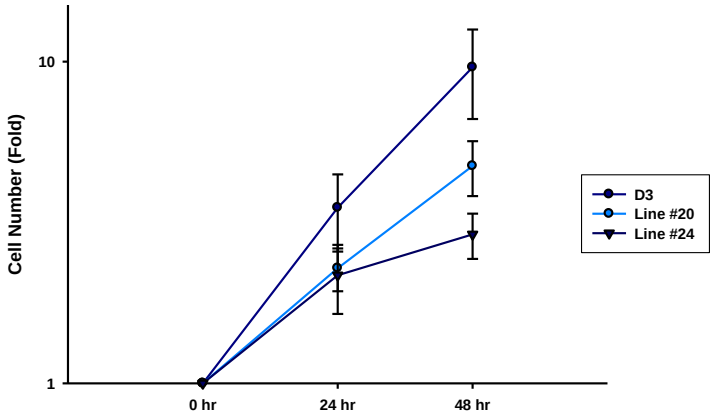

Figure 3.3

Proliferation curves of two mouse embryonic cell lines (Line #20 and #24) over-expressing miR-410 and the parental line D3 grow in the absence of leukemia inhibitory factor (LIF). The two miR-410 over-expressing lines grow at significantly slower rate compare to D3.

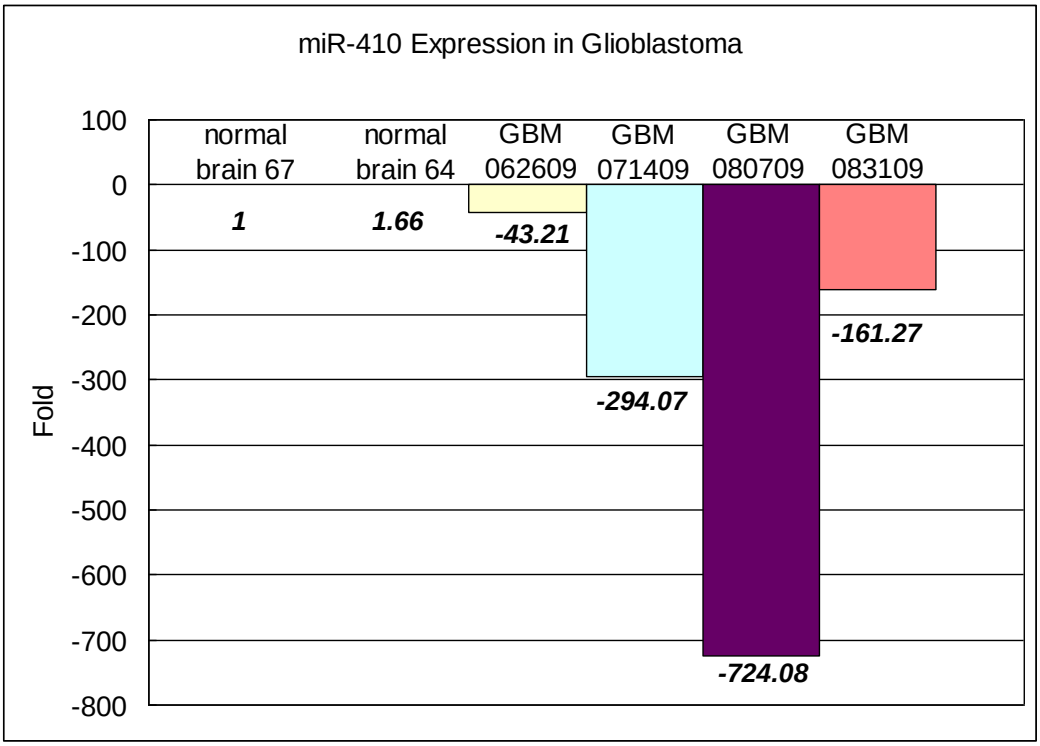

Figure 3.4

miR-410 expression in human glioblastoma (GBM). miR-410 was down-regulated in all four GBM samples tested compared to normal brain control tissue.

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Appendix

Inducible Expression of Noggin Selectively Expands Neural Progenitors in the Adult SVZ

Introduction

Understanding the normal function and homeostasis of the adult brain is one of the major challenges in biology, and is key in developing cell replacement strategies for a number of human neurodegenerative diseases. Neurogenesis is a multifactorial process that appears to be largely restricted to two main regions of the adult CNS: the subgranular zone (SGZ) of the dentate gyrus of the hippocampus (Gage, 2000) and the lateral wall of the lateral ventricles, the subventricular zone (SVZ) (Gage, 2000; Alvarez-Buylla and Lim, 2004).

In the SVZ niche, the balance between quiescence, self-renewal and differentiation is thought to be determined by diffusible molecules, growth factors, neurotransmitters, cell-to-cell contacts, and components of the extracellular matrix (Riquelme et al., 2008); which regulate expression of transcription factors that control neurogenesis (Ayoub et al., 2011; Hsieh, 2012). BMPs, BMP receptors and the secreted BMP inhibitor, noggin are widely expressed during development, remaining in discrete locations in the adult SGZ, olfactory bulb and SVZ; sites of adult neurogenesis (Peretto et al., 2004). BMP signaling

plays multiple key roles in CNS morphogenesis from neural induction to patterning of the nervous system, proliferation and lineage differentiation (Chen and Panchision, 2007). Since the effects of BMP signaling appear to depend on signal strength, inhibitors such as noggin may be critically required in modulating signaling by pathway members (Bonaguidi et al., 2008; Colak et al., 2008; Gajera et al., 2010; Mira et al., 2010). In the adult SVZ, BMP signaling is active as indicated by the phosophorylation of SMAD1/5/8 (Colak et al., 2008); transit amplifying cells (C cells, activated astrocytes (B cells), and endothelial cells (Mathieu et al., 2008) express BMPs, while noggin is produced by ependymal and B cells (Lim et al., 2000; Peretto et al., 2004). In fact, recent evidence suggests that BMP signaling is one of the earliest events that regulates quiescence vs lineage differentiation of SVZ NSC (Bonaguidi et al., 2008; Colak et al., 2008; Mira et al., 2010), but the responsive cells and kinetics within the niche are largely unknown.

To address the role of BMP signaling in the behavior of neural progenitors in the adult brain, we generated a transgenic mouse model in which noggin can be inducibly expressed in NSC populations. Even short bursts of noggin expression were sufficient to increase proliferation of progenitors and shift SVZ lineage progression from mature astrocytes to transit amplifying cells and oligodendrocyte precursors. In *vitro*, noggin did not affect self-renewal of neurospheres (NS), but promoted differentiation of both oligodendrocytes and neurons, which was inhibited by BMP4. Our results indicate that transient expression of noggin expands progenitor populations without depleting the NSC population, promotes neuronal and oligodendrocyte differentiation of adult NSCs *in vivo* and *in vitro,* and suggest that controlled expression of noggin may modulate stem cell fate within the niche.

Material and Methods

Double transgenic mice: construction and genotyping

The FVB/N nestin-reverse transactivator (rtTA) mice have been described previously (Mitsuhashi et al., 2001), and were kindly provided by Dr. Steven Reeves. A new transgenic animal was generated by the University of Michigan Transgenic Core by injecting linearized plasmid DNA pBi-Noggin-EGFP into fertilized oocytes from Nestin-rtTA female mice mated to FVB/N males. To generate the plasmid pBi-Noggin-EGFP, the coding region of the Enhanced Green Fluorescent Protein (EGFP, obtained from plasmid pEGFP-N1 (Clontech) was cloned into the Multiple Cloning Site (MCS) I of plasmid pBi (Clontech). Next, a mouse noggin cDNA (Gratsch and O'Shea, 2002) was cloned into MCSII of pBi to generate the plasmid pBi-Noggin-EGFP. A linear DNA fragment of approximately 5-kb was obtained upon *Ase* I restriction, purified, and injected into fertilized oocytes. After 5 independent injections (total of 12 recipient females) 11 different transgenic lines were obtained.

The presence of the transgenes was determined in PCR using DNA isolated from tail biopsieswith primers and conditions as follows: rtTA Fw: AGAGCTGCTTAATGAGGTCG, rtTA Rev: GTCCAGATCGAAATCGTCTAG, GFP Fw: CGGCCACAAGTTCAGCGTGTC, GFP Rev: CGTCCTCGATGTTGTGGCGGA (Tm 54º C and 53º C). Double transgenic animals from crosses of nestin-rtTA mice with pBi-noggin-EGFP mice were tested to determine their response to doxycycline (Dox),

and three lines of mice in which the noggin transgene was reliably up-regulated by Dox administration with little background were selected for subsequent experiments.

Transgene induction *in vivo*

Transgene expression was induced in 8-10 week old animals for 2-16 days by administration of 2 mg/ml of doxycycline (Dox, Sigma) and 5% sucrose in the drinking water. Controls were double transgenic animals not exposed to Dox and nestin-rtTA single transgenic mice.

Bromodeoxyuridine (BrdU) exposure

5-Bromo-2'-deoxyuridine (Sigma) was dissolved at 10 mg/ml in 0.9% NaCl, and a single injection of 50 mg/kg administrated by intraperitoneal injection. Animals were injected on day four of an eight-day induction paradigm to label recently divided cells in the SVZ at a time of high noggin expression. In other experiments, two doses of BrdU were injected 24 hours apart, at the end of the induction period.

Tissue preparation

Adult transgenic mice were sacrificed by carbon dioxide overdose or cervical dislocation following guidelines provided by the Unit for Laboratory Animal Medicine (ULAM) and protocols previously approved by the University Committee on the Use and Care of Animals (UCUCA). Brains were removed and embedded in OCT (Sakura, Tissue-Teck), and ten micron coronal frozen sections were cut. Alternatively, animals were anesthetized with phenobarbital and perfused transcardially with 20 ml of cold PBS pH 7.4 followed by 30 ml of 4% paraformaldehyde (PFA). Brains were removed, fixed overnight in 4% PFA followed by equilibration in 30% sucrose and after extensive

washes were embedded in OCT. Additional SVZ were fixed in 2% glutaraldehyde, post-fixed in 1% OsO4, embedded in Araldite 812, then one micron sections cut and stained with toluidine blue for light microscopy.

Neurosphere culture

Neurosphere cultures were prepared as previously described (Gritti et al., 1996) with minor modifications (Wang et al., 2005). After eight-day transgene induction with Dox, 4-5 animals per group (8-10 w old females) were sacrificed, brains removed and a 2mm thick coronal block cut through the lateral ventricles. The lateral SVZ walls were microdissected from surrounding tissue and dissociated in trypsin. 2-4 x 10^4 cells/well were plated in 6-well plates in Dulbecco's modified Eagle's medium/F12 (DMEM/F12, Invitrogen) containing 20 ng/ml EGF (Sigma), 10 ng/ml FGF2 (Sigma) and 2 µg/ml heparin (Sigma). Primary neurospheres (NS) were cultured for 7-9 days *in vitro* (DIV), mechanically dissociated with a fire-polished Pasteur pipette, and grown until they formed secondary NS or were plated for differentiation at 1.8 x 10^4 cells/well in poly-ornithine (Sigma) coated 48-well plates. For differentiation, NS were grown in DMEM/F12 (Invitrogen) supplemented with 1% fetal bovine serum (FBS, Atlanta Biologicals). To generate secondary NS, triplicate wells in 12-well plates were seeded with 3 x 10^3 cells, and the number of secondary spheres was determined after 7 DIV. Media was partially replaced every 3 days. BMP4 (R&D) at 20 ng/ml or doxycycline (Sigma) at 2 µg/ml, were added as indicated. For clonal analyses, cells were plated at 20 cells/well in 96-well plates in a final volume of 200 µl of medium. After 10 DIV, NS from wells containing a single sphere were expanded in media containing FGF and EGF. After additional growth *in vitro* (7-10 DIV) spheres were dissociated with a fire-polished

Pasteur pipette and seeded to 12-well plates to develop secondary neurospheres or to 48-well polyornithine-coated plates for differentiation.

Immunohistochemistry

Fresh frozen sections were fixed in 4% paraformaldehyde (PFA) for 10 minutes, permeabilized with 0.2% Triton X-100 for 10 min, then non-specific binding blocked for 1 hour in phosphate buffered saline (PBS) containing 0.2% Triton X-100 and 10% normal goat or donkey serum (PBST). Primary antibodies were diluted in PBST containing 1% serum and applied to sections overnight in a humidified chamber at 4°C. After three washes in PBS, sections were exposed to secondary antibodies diluted in PBST for 30 min at room temperature. Hoescht 33258 (Sigma) was employed to stain nuclear DNA. Primary antibodies and dilutions were: rabbit anti-EGFP (1/500, Molecular Probes), goat anti-noggin (1/200, Santa Cruz), rabbit anti-doublecortin (DCX; 1/200, Cell Signalling), mouse monoclonal anti-Mash1 (1/500, BD Bioscience), rabbit anti-caspase 3 (1/250, Cell Signaling), rabbit anti-Dlx-2 for early neuroblasts (1/200, Chemicon), mouse monoclonal anti-nestin (1/200, Chemicon), guinea pig anti-glial fibrillary acidic protein (GFAP; 1/1000, Advance ImmunoChemical), rabbit anti-SOX3 (1/1000, generous gift from M. Klymkowsky, University of Colorado), rat anti-BrdU (1/100, Serotech), rabbit anti-Ki67 (1/500, Novocastra), and guinea pig anti-Olig2 (1/100, generous gift of B. Novitch, UCLA). Secondary antibodies were conjugated to FITC, Cy3, Alexa 488, or Alexa 555, and used at a 1/200 dilution (FITC or Cy3 conjugated, from Jackson ImmunoResearch) and 1/1000 (Alexa conjugated antibodies, from Invitrogen). To determine the expression pattern of the rtTA transgene, detection of β-galactosidase was carried out using the X-gal substrate (Invitrogen).

For immunohistochemical localization of cell type restricted proteins following NS differentiation, cells were fixed for 15 min in 2% PFA, permeabilized with 0.2% Triton X-100 for 10 min, then treated as described above. Primary antibodies were: rabbit anti-β-III tubulin for neurons (Tuj1; 1/1000, Covance), guinea pig anti-glial fibrillary acidic protein for astrocytes (GFAP; 1/1000, Advance ImmunoChemical) and rat anti-myelin basic protein for oligodendrocytes (MBP; 1/1000, Chemicon). Secondary antibodies were purchased from Jackson ImmunoResearch and used at a 1/200 dilution.

Quantitative-PCR

The level of noggin transgene expression in the SVZ was determined using quantitative RT-PCR. After 2, 4, 8, and 16 days of Dox administration, SVZ from induced and control animals were micro-dissected, homogenized in Trizol (Invitrogen), and RNA extracted. Total RNA from control or induced animals was DNAsed (DNAse I, Sigma), pooled (n= 4 mice/ per group 8 SVZ), and 1 µg was used in Reverse Transcription reactions using PowerScript reverse transcriptase (Clontech) following the manufacturer's instructions. The resulting cDNAs were employed as templates in triplicate for real-time PCR reactions using primers and conditions available on request. We employed the SyBR Green system (ABgene) and a BioradiCyclerPCR machine. Samples were normalized to β-actin, and noggin fold change estimated using the ΔΔ ct value method (Pfaffl, 2001).

Image analysis and quantification

Images were acquired using a Leica CMIRB, Zeiss Axioplan, or Zeiss LSM510 and imported into Photoshop for analysis and to construct figures. In coronal sections the number of cells expressing a cell type restricted protein (GFAP, MASH1, DCX, Dlx-2,

SOX3 or Olig2), co-labeled with BrdU (GFAP/BrdU, DCX/BrdU, SOX3/BrdU) or Ki67 (Mash1/Ki67) were counted along the dorsolateral extent of the SVZ lateral wall, in 4 animals per group (~8 SVZ/animal, n = 32). Because of the considerable regional pattering of the SVZ, sections used in quantitative analysis were restricted to regions with coordinates 0.26-0.98 mm from Bregma. For the *in vitro* differentiation experiments, the percentage of positive cells was estimated by counting at least 200 nuclei per well (5 random fields) in duplicate wells from at least three independent experiments (six wells per group). Means were calculated and analyzed using unpaired Student t-test and additional statistical analyses carried out using SPSS and GraphPadPrism 5.

MicroArray Analysis

RNA was isolated from 8 SVZ microdissected from 4 uninduced and 4 noggin-transgenic mice exposed to doxycycline for 8 days using Trizol as described above. The RNAs were analyzed in the UM Comprehensive Cancer Center Microarraying Core to assess quality control (perfect match; PM chip densities), and regarding mRNA degradation. The RNAs were hybridized to Affymetrix 2.0 arrays (Santa Clara CA, http://www.affymetric.com) microarrays, the signal intensity of each array was normalized using robust multiarray averaging (RMA), and an initial analysis of fold change carried out using Bioconductor. Functional annotation cluster analysis (David) was then employed to identify significant clusters of transcripts associated with noggin over-expression.

Results

Noggin can be inducibly expressed in nestin positive cells of the CNS

Previous studies of the effects of noggin on SVZ NSC have employed local injection of recombinant protein with unknown activity or viral delivery to the ventricular system, with the attendant concerns of local injury, and cytokine release. To determine the role of noggin in the adult NSC population *in vivo* absent these concerns, we developed mice in which the expression of noggin and EGFP is controlled by a bidirectional tetracycline-responsive promoter. When crossed with existing mice in which expression of the reverse tetracycline transactivator (rtTA) fused to β-galactosidase is directed by the CNS-restricted nestin promoter/enhancer in intron 2 (Mitsuhashi et al., 2001) transgenic noggin expression is driven exclusively to nestin positive cells of the CNS.

The CNS specific intronic enhancer element has been widely employed to drive reporter genes or to target transgene expression to neural stem cells (Lardelli et al., 1996; Panchision et al., 2001; Magdaleno et al., 2002; McFarland et al., 2006; Mills et al., 2006; Walker et al., 2010). Expression of the tetracycline reverse transactivator (rtTA) was examined by X-gal staining and confirmed to be expressed in GFAP positive cells along the lateral wall of the ventricles (Figure A.1.A). Immunohistochemical localization of nestin and EGFP in sections of doxycycline-induced brain (Figure A.1.B) also showed complete overlap, indicating that transgenic noggin expression is restricted to nestin positive cells in the SVZ, hippocampus, the glomerular layer of the olfactory bulb, the Purkinje cell layer of the cerebellum, and scattered cells throughout the cortex (data not shown). In the adult SVZ A, B, and C cells all have been reported to express nestin (Doetsch et al., 1997; Nakamura et al., 2003), and co-staining of nestin and cell type

restricted antigens, e.g., GFAP (B cells) or DCX (A cells) identified considerable overlap in B cells (Figure A.1.C a-e) and some double positive A cells (Figure A.1.C d-f) as previously reported. In sum, B, C and A cells will express noggin upon transgene induction.

In adult, control animals (single transgenic or double transgenic mice exposed to water alone), endogenous noggin was expressed by ependymal cells lining the lateral ventricles, as previously reported (Lim et al., 2000; Peretto et al., 2002; Peretto et al., 2004; Colak et al., 2008). Following eight days of doxycycline mediated transgene induction, a clear increase in noggin expression along the lateral ventricle was observed using immunohistochemistry (Figure A.2.A). To quantify the increase in noggin expression, we carried out quantitative RT-PCR analysis on microdissected SVZ of animals induced for 0, 2, 4, 8 and 16 days (pooled, 4 animals per group). We observed a 2, 3, 7 and 8 fold increase in noggin expression in the induced SVZ compared with controls (Figure A.2.B). Double transgenic animals exposed to water alone (not induced, NI) showed similar levels of noggin as single transgenic animals, indicating that the transgene was not over-expressed in the absence of doxycycline, i.e., did not "leak". These results indicate that noggin can be reliably induced in cells in the SVZ-NSC niche in a regulated and robust manner by doxycycline administration.

To determine if transgene-driven noggin was active and inhibited BMP signal transduction, we carried out immunohistochemical localization of phospho-Smad1/5/8. After BMP binding and receptor dimerization, the Smad 1/5/8 complex is phosphorylated, associates with Smad4 and moves to the nucleus, where pSmads act as transcription factors and activate BMP target genes (Massague, 2000). In control animals there were

many positive nuclei; however, few pSmad1/5/8+ nuclei were observed in the SVZ of induced animals (Figure A.2.C), indicating that noggin actively inhibited the transduction of BMP signals. To insure that the transgene was not indirectly affecting BMP production the local levels of BMP4 were determined by qRT-PCR. There was no significant difference in the level of BMP4 in the SVZ after noggin induction (Figure A.2.B). High resolution microscopy using 1 μm sections indicated that the overall organization and morphology of the SVZ was not altered after as long as 30 days of noggin expression. The only observed alterations were in the ependymal cells, which were cuboidal in noggin-exposed compared to the more flattened morphology typical of control animals. Thus, doxycycline-induced noggin expression is reliably observed in nestin positive cells in the SVZ where it abrogates BMP signaling, without altering the overall organization of the niche.

Noggin promotes proliferation in the SVZ

To determine the effects of noggin over-expression on cell behavior in the SVZ, we first examined whether proliferation was altered. BrdUwas injected i.p. on day 4 of the 8 day induction period, a point when Noggin expression was increased three-fold. Noggin over-expression produced a statistically significant increase in the mean number of BrdU positive cells in the SVZ of induced animals compared with uninduced controls (12.6 ± 3 vs 9.4 ± 2.8, $p \leq 0.001$, Student's t test). Similar results were obtained in two independent experiments (8 animals per group) using two different double transgenic lines (32 animals/group, 64 SVZ analyzed/each case), demonstrating that cells in the SVZ respond to noggin by increasing proliferation.

Increased numbers of transit amplifying cells and neuroblasts differentiate at the expense of GFAP+ cells

In the adult SVZ, a subset of GFAP+ SVZ astrocytes are the slowly self-renewing multipotent neural stem cells (type B1) (Doetsch et al., 1997; Garcia-Verdugo et al., 1998; Doetsch et al., 1999a) that give rise to rapidly proliferating intermediate progenitor cells (transit amplifying precursor; TAP, type C) which expand the progenitor pool producing neuroblasts (type A cells) that divide and migrate through the rostral migratory stream to the olfactory bulb (Doetsch et al., 1999b; Garcia et al., 2004). To determine if there were cell type-specific effects of noggin over-expression, we carried out immunohistochemical localization of cell lineage-restricted markers: GFAP (B), MASH1 (C) and DCX (A) or SOX3 (neural precursor cells) in 8 animals per group, induced and uninduced controls. Noggin over-expression significantly decreased the number of GFAP+ cells in the induced SVZ compared with controls (48.8 ± 7.7 vs 64.1 ± 7.6; $p \leq 0.001$; Figure A.3.A). However, the number of MASH1 positive cells (type C) was increased by noggin expression (14.2 ± 3.2 vs 11.4 ± 2.4; $p \leq 0.001$; Figure A.3.A), as was the mean number of DCX positive neuroblasts (A cells) (54.3 ± 7.6 vs 41.3 ± 7.6; $p \leq 0.001$; Figure A.3. A). We also employed the SOXB1 family member SOX3 to examine neural precursor cell number. There was also a significant increase in the number of SOX3 positive cells after induction compared to controls (45 ± 7.8 vs 38.2 ± 3.8; $p \leq 0.001$; Figure A.3.A). SOX3 is one of the earliest pan-neural markers (Brunelli et al., 2003) expressed throughout the neuroepithelium, becoming enriched in neural precursors in the adult CNS (Wang et al., 2006) where it has been implicated in self-renewal (Saarimaki-Vire et al., 2007), consistent with these observations. Since there were no differences in the expression of

activated caspase 3, and the overall level of cell death was low (not shown) it appears that the reduced number of GFAP+ cells is due to a shift in lineage allocation, with noggin favoring C and A cells at the expense of mature astrocytes present deeper in the SVZ.

Noggin promotes differentiation of oligodendrocyte precursor cells

Careful mapping studies have demonstrated that B cells present in the adult dorsolateral SVZ can form oligodendrocyte precursor cells (OPC) that migrate to the fornix, striatum and corpus callosum (Menn et al., 2006). Since the short-term induction paradigm we employed was not expected to produce mature oligodendrocytes, we quantified OPC numbers based on their expression of the transcription factor Olig2 (Dimou et al., 2008). Noggin expression produced a significant increase in the number of Olig2 + cells in the SVZ compared to controls (5.3 ± 1.1 vs 3.2 ± 0.7; $p \leq 0.001$; Figure A.3. A,B). Olig2+ cells were present in the dorsolateral zone where C cells are typically found, but Olig2 and MASH1 expression overlapped only in a small proportion of these cells (data not shown). Increased numbers of Olig2 positive cells were also detected in the corpus callosum and striatum (Figure A.3.B) following noggin expression. Since inhibition of BMP signaling has been suggested to re-direct differentiation from neuroblasts to OPC (Colak et al., 2008; Jablonska et al., 2010), we also examined expression of key downstream regulators of neurogenesis, Dlx2 and Olig2. We did not observe significant differences in the number of cells positive for both Dlx2/Olig2 between induced and non-induced animals, suggesting that the OPCs may differentiate from B cells. Additional paradigms of transgene induction and labeling will be required to determine if these cells are generated *in situ* or are progeny of the SVZ (see discussion), as well as their disposition.

Noggin promotes proliferation of multiple precursor populations in the SVZ

To determine if individual progenitor cell types respond to increased levels of noggin protein, BrdU was administered at day 4 of the 8 day induction period and double-labeling analyses of markers of B cells (GFAP+), neuroblasts (DCX+) and neural precursor cells (SOX3+) and BrdU were carried out (Figure A.4.A). There was a small, but significant increase in the mean number of GFAP+ / BrdU+ cells in the dorsal region of the SVZ in noggin over-expressing animals compared with uninduced controls (5.9 ± 1.8 vs 4.1 ± 1.3; $p \leq 0.05$; Figure A.4. A), and an increase in the mean number of DCX+ / BrdU+ neuroblasts in the SVZ of induced animals (14.9 ± 4.2 vs 10.8 ± 3.9; $p \leq 0.0007$; Figure A.4.B). The number of SOX3+ / BrdU+ precursor cells was also significantly increased (9.1 ± 2.9 vs 7.2 ± 2.3; $p \leq 0.05$; Figure A.4.C). These results indicate that noggin over-expression stimulates proliferation of both progenitor cells and neuroblasts in the SVZ. Due to the innate higher proliferation rate of transit amplifying cells we also quantified the number of proliferating C cells after 8 days of induction using the cell division marker Ki67. The percentage of Ki67 /MASH1 double positive cells was strikingly increased in the induced animals compared to controls (17.0 ± 3.8 vs 10.1 ± 4; $p \leq 0.001$; Figure A.4.C). Since the dividing GFAP+ cells are likely B cells, these data suggest that noggin promotes proliferation of B cells within the SVZ and promotes their differentiation to C cells at the expense of mature GFAP+/BrdU- astrocytes. In addition, noggin stimulates proliferation of both C and A cell populations. Our results suggest a model in which lineage differentiation is shifted from mature astrocytes, producing more C cells which then form both neurons and OPC, or promoting differentiation of both OPC and C cells from the B cell.

Noggin promotes selective differentiation of SVZ neural stem cell populations

To discriminate between these models and to determine where in the differentiation cascade noggin had its major effects, we carried out additional statistical analyses. As described above, the mean numbers of all four groups of cells were significantly different between induced and control animals (A, C, and OPC increased, while GFAP+ cell numbers were decreased by noggin expression (Figure A.3.A). However, when we compared the population distributions of induced and uninduced animals using Chi-squared analysis (Table 1.A), there was no change in the B and A cell populations, suggesting that the underlying characteristics of B cell self-renewal and A cell production did not change. However, the standard deviations in the C and OPC cell groups were significantly altered, suggesting that noggin expression altered the behavior of these cells. We next examined the change in the proportion of cells in each group relative to the B cell (Table 1.B).

Noggin expression nearly doubled the proportion of both C cells (1.8X) and OPC cells (2.2X) in the SVZ, while having little effect on the ratio of A/C cells (0.96X), and only a slight effect on C/OPC (0.8X). The major treatment effect therefore, was on the number of OPC and C cells differentiating from B cells, while the proportion of neuroblasts (A) present scaled with the number of C cells (0.96). In other words, the increase in OPC cell number could not be explained by an increase in C cell numbers, while the increase in A cells was directly attributable to an increased number of C cells; i.e., there was not an additive/independent effect of noggin expression on the lineage differentiation of C cells to neuroblasts.

The strongest effect of noggin over-expression was to increase OPC cell number in the population by 2.2 fold. When the ratio of C/OPC was compared between groups there was a slight (0.8X) decrease in the noggin-exposed group suggesting that the C cell is not likely the source of the new OPC. These data do not preclude a C cell origin of some Olig2+ cells, but suggest that most of the Olig2+ cells formed from B cells, consistent with lineage tracing studies (Gonzalez-Perez et al., 2009).

Overall, these data indicate that expression of noggin in the SVZ does not deplete the NSC population, but promotes differentiation of transit amplifying C cells and OPC at the expense of mature astrocytes. Our results suggest a model in which B1 cells are actively inhibited from forming mature astrocytes and are stimulated to produce OPC and C cells that differentiate proportionally into neuroblasts, and a population of OPC.

Noggin does not affect self-renewal of NSC *in vitro*

Although cell fate can be altered by simply removing cells from the microenvironment of the SVZ (Kokovay et al., 2010; Kusek et al., 2012), neurosphere assays are useful to study population dynamics *in vitro*, allowing analysis of the response of different cell types to specific growth factors. To distinguish the effects of noggin over-expression on neural progenitors vs. neuronal precursors, we examined the effects of noggin on self-renewal using neurosphere (NS) assays. Induction of noggin expression *in vivo* followed by NS culture in control medium (no Dox) had no effect on the proliferation, size or number of primary or secondary NS (Figure A.6). Nor did combined *in vivo* and *in vitro* induction alter proliferation, secondary neurosphere size or number (Figure A.6, and data not shown). These results suggest that mitogens present in the culture medium may override the effects of noggin over-expression on self-renewal. Consistent with our *in*

vivo results; these data suggest that noggin does not interfere with self-renewal of GFAP+ progenitors in the SVZ.

Noggin promotes differentiation of neurons and oligodendrocytes from neurospheres

Neurospheres derived from uninduced animals differentiated into astrocytes (≈68%), neurons (≈30%) and oligodendrocytes (≈2%) (Figure A.5.B). *In vivo* induction of noggin expression prior to NS culture strikingly decreased the percentage of GFAP+ astrocytes to 56.4% ($p \leq 6.6 \times 10^{-7}$; NI vs +8), increased the proportion of Tuj1+ neurons to 36.9% ($p \leq 7.0 \times 10^{-7}$; NI vs +8) and the number of MBP+ oligodendrocytes to 7% ($p \leq 1.0 \times 10^{-5}$; NI vs +8). When *in vivo* induction was combined with *in vitro* induction, the number of astrocytes decreased further (to 51%, $p \leq 2.0 \times 10^{-8}$; NI vs +8/DOX+8), the number of neurons increased to 41% ($p \leq 1.5 \times 10^{-7}$; NI vs +8/DOX+8) and the percentage of oligodendrocytes present in the cultures increased to 8% ($p \leq 4.1 \times 10^{-6}$; NI vs +8/DOX+8). In addition, oligodendrocytes differentiated in the presence of noggin often appeared more mature, with multiple branched, longer processes than controls (Figure A.5. c,f). Noggin stimulation of neurogenesis and oligodendrogenesis was strongly inhibited by addition of BMP4 to the cultures (+8/DOX+8/+BMP4), indicating a direct effect on differentiation. Both induced and control groups showed a striking increase in the number of astrocytes (85%) with reductions in the number of neurons (11%) and oligodendrocytes to 2% in the presence of BMP4.

To rule out the possibility that neuronal precursor cells were over-represented in the noggin NS cultures, we carried out clonal analyses, and found that there was no substantial difference in the percentage of astrocytes, neurons or oligodendrocytes

between individual clones and the population of NS (Table 2). These data indicate that single clones behave as the population, and that preferential differentiation toward neuronal and oligodendrocyte lineages is a direct effect of noggin and is not related to *in vitro* selection artifacts. Overall, our data indicate that expression of noggin promotes neurogenesis and oligodendrogenesis at the expense of mature GFAP+/BrdU- astrocytes.

MicroarrayAnalysis

To identify alterations in gene expression in the SVZ following eight days of noggin induction, we carried out microarray analysis of RNAs from induced and control animals (n=4 each). Robust multi-array averaging (RMA) was used to calculate expression values for each gene, then transcripts with at least a 1.5 fold expression difference, with the added criterion that at least one sample had an expression value of 2^6 or greater, were selected for additional analysis. This produced 436 differentially expressed probesets, of which 420 were unique genes; 221 were expressed at higher levels in control compared with noggin expressing SVZ; 199 expressed at higher levels following noggin expression. Transcripts were analyzed using DAVID to identify Gene Ontology (GO) terms that were associated with noggin over-expression ($p \leq 0.05$, Benjamini correction). These included: feeding behavior; synapse; insulin-like growth factor binding protein, N-terminal; cytoplasmic vesicle; cell junction; signal; and membrane. Functional annotation cluster analysis identified four significant clusters of transcripts. Cluster One: synapse (n = 10, $p \leq 1.9 \times 10^{-2,}$ Benjamini corrected), Cluster Two: Cell junction (n = 13, $p \leq 1.5 \times 10^{-2)}$, Cluster Three: Signal (n = 43, $p \leq 2.8 \times 10^{-2}$), Cluster Four: membrane (n = 67, $p \leq 3.2 \times 10^{-2}$). Genes involved in synaptic vesicle behavior and cell-cell junctions were identified in Clusters One and Two, including: *Arc, Gabrb2, Gad2, Gjb1, Jam2, Syt10,* and *Vamp1*.

Transcripts involved in signaling included extracellular matrix proteins/receptors: *Adamtsl4, Col11a1, Itgb4, Lama2, Mcam, Thbs4.* Additional transcripts present in Custer 3 included: myelin associated proteins: *Lama2, Mog, Mag, Ugt8a*; growth and signaling factors: *Aplp2, Cck, Dkk3, Gh, Igfbp2, Igfbp6, Lrp4, Nts, and Pthlp*. Cluster 4 was composed of membrane receptors, transporters and channel genes including: *Htr1a, Htr2c, Tub, Cacng5, Gabrg2, Mib, Nkd1, Ntrk2, Ntrk3, Kcne3, Prom1, and Sel1l.*

When we analyzed transcripts that were expressed at higher levels in control vs noggin-expressing SVZ, we identified genes involved in: CNS development: *Med1, Per2, Sox6, Top2b*, in cilia: *Whrn*; in cell cycle: *Cdkn1a, Fosb;* in dendrites: *Mtap2,* and*Opa1*. There were transcripts that encode ECM/cell surface molecules: *Cbln1, Dscam, Plxnc1, Srgap3;* and ion channels: *Kcne2.* There were also genes that encode factors involved in synaptic/neurotransmitter function: *Lin7a, Ntrk2, Ntrk3, Syt10;* signaling pathway members: *Igfbp2, Prickle1, Lfng, Pdgfra.* There was a novel microRNA-containing gene: *Mirg*; and the helicase *Ddx3y*.

Transcripts expressed at lower levels in control vs in the noggin-exposed SVZ included genes involved in lineage differentiation of oligodendrocytes: *Mag, MBP, Mog, PMP22, Sox8;* in forebrain development: *Foxp1, Fezf2, Nfib;* in C cells: *Egfr*; in neurogenesis: *Sox11;* specifically in olfactory bulb neurogenesis: *Fezf2, POMC, and Epha7.* There were a number of transcripts for matrix/cell surface/cytoskeleton elements: *Cdh9, Gsn, Itgb4, Plxnd1, Rtn);* and *Zeb2.* Signaling pathway members included: *Acvr1c, Cck, Ctgf, Gli3, Hes5, Igfbp6, Rora, Tcf4*; cell cycle regulators: *Cdca7, Cdkn1b*; and a membrane transporter: *Slc17a7.*

Discussions

Considerable evidence suggests that it is the inhibitory microenvironment of the adult CNS that limits proliferation and differentiation of neural progenitors. BMP signaling has previously been shown to act as a brake on proliferation, promoting cell cycle exit and astrocyte differentiation (Gross et al., 1996; Mabie et al., 1999; Lim et al., 2000; Mekki-Dauriac et al., 2002; Gomes et al., 2003; Wagner, 2007), while inhibition has the opposite effect, promoting neuronal differentiation and cell cycle entry (Lim et al., 2000; Mira et al., 2010).

In the SVZ, quiescence may be attained simply by titrating the generation of BMP-producing B cells until the effects of ependyma-derived noggin are balanced and proliferation stops (Bonaguidi et al., 2008). Factors secreted by the ependyma may be particularly critical in the SVZ niche, as NSC scale proportionally to the number, rather than volume of ependymal cells (Kazanis and Ffrench-Constant, 2012). Unlike other growth factor treatments that target one step in the differentiation cascade of the B cell, and often deplete the NSC niche (e.g., Shh, Egf, Wnt, Lif), by promoting differentiation (Encinas et al., 2011), or disorganization of the niche (Kokovay et al., 2010), noggin promoted proliferation of adult SVZ progenitors at multiple stages of their differentiation cascade without altering the organization of the SVZ after 30 days of transgene induction. In fact, noggin expression stimulated a small but significant increase in the proliferation of GFAP+ cells located near the ventricle, while decreasing the number of mature GFAP+ astrocytes. Controlled expression of noggin may therefore be useful in

stimulating proliferation within the niche without altering its essential characteristics, which will be critical in expanding cells for replacement strategies (Breunig et al., 2007).

These results are consistent with those obtained in the adult hippocampus where noggin over-expression promoted (Bonaguidi et al., 2005; Bonaguidi et al., 2008), and antisense noggin oligonucleotide exposure reduced (Fan et al., 2004) NSC proliferation and neuronal differentiation. However, inhibition of BMP signaling by noggin infusion, targeted deletion of Smad4, or deletion of BMPRIA, initially stimulated then depleted precursors and newborn neurons in the dentate gyrus, although as in our model, the number of radial progenitors remained constant and non-radial astrocytes declined (Mira et al., 2010). However, in the most posterior region of the SVZ, Bonaguidi et al. (Bonaguidi et al., 2008) did not detect changes in proliferation following NSE transgene driven noggin expression, while Guo et al. (Guo et al., 2011), demonstrated that infusion of noggin did not alter proliferation, but shifted lineage differentiation. Overall, these results suggest that as in the SVZ, BMP signaling is crucial in regulating both the initial differentiation of the NSC and subsequent differentiation of precursors. Each adult germinal niche is unique in its relationship to the ventricular system, its geometry and cellular composition. This inducible model will particularly useful in examining the effects of controlled noggin expression on progenitor behavior in these complex niches.

Our results differ somewhat from an earlier report that interfering with BMP signaling by deleting the common Smad, Smad4 in NSC, or infusing noggin into the ventricle, promotes oligodendrogenesis by redirecting neuroblast differentiation (Colak et al., 2008). In addition to differences in experimental design: e.g., transgene driven vs recombinant protein, deletion rather than down-regulation, effects of injury following catheter

placement, delivery to the ventricle vs to NSC, targeted deletion of Smad4 in NSC would be expected to abrogate signaling by multiple pathways (Fgf, Nodal, TGFβ, Lif, and Wnt) also involved in NSC behavior (Varga and Wrana, 2005). In fact, Colak et al. (Colak et al., 2008) make many of the same final conclusions: that the initial differentiation of the B cell is most sensitive to BMP signaling, abrogation of BMP signaling does not affect NSC self-renewal *in vivo* or *in vitro*, and also conclude that the decision to form OPC vs C (and therefore A) cells depends on the level of BMP signaling.

Noggin stimulates OPC differentiation both *in vivo* and *in vitro*. Until recently the extent to which the adult SVZ is capable of *in vivo* oligodendrogenesis was unappreciated. It now appears that demyelination promotes changes in lineage commitment to favor production of OPC in a manner influenced by BMP signaling (Cate et al., 2010; Jablonska et al., 2010). OPC differentiate from the SVZ following injury (Pluchino et al., 2003; Parent et al., 2006), over-expression of Olig2 (Hack et al., 2005; Marshall et al., 2005), abrogation of Smad4 signaling (Colak et al., 2008) or over-expression of noggin (Bonaguidi et al., 2008; Irvin et al., 2008), or of chordin (Jablonska et al., 2010). In fact, oligodendrocyte lineage elaboration requires active inhibition of BMP signaling (Mehler et al., 2000; Kondo and Raff, 2004; Cheng et al., 2007; Imura et al., 2008); BMP modulation of Olig2 may control OPC lineage differentiation in the SVZ (Gabay et al., 2003; Hack et al., 2005; Menn et al., 2006; Fukuda et al., 2007; Colak et al., 2008), but the mechanisms and target cell(s) remain to be conclusively determined.

Lineage tracing studies have demonstrated that B cells form OPC, but whether direct or via an Olig2+ C cell intermediate is unclear (Gonzalez-Perez and Alvarez-Buylla, 2011). Expression of Olig2 in A or C cells actively inhibits neuroblast lineage differentiation

(Colak et al., 2008) and promotes OPC differentiation. Olig2 promotes oligodendrocyte and inhibits neuronal differentiation of SVZ derived progenitors (Hack et al., 2005); and mice deficient for Olig1 and 2 lack cortical OPC, while neurons and astrocytes are unaffected (Zhou and Anderson, 2002), supporting a primary effect on the C/B cell. This is similar to the model proposed for EGFr stimulation of SVZ NSC, where in the presence of EGF, B cells (and/or C cells) can be directed to form an Olig2+ intermediate progenitor cell, a "like-C" cell (Gonzalez-Perez and Alvarez-Buylla, 2011). Careful analysis of cell number and behavior suggests that it is more likely that B cells are the source of OPC in this investigation. In our model the number of Dlx2/Olig2 (C/OPC double labeling) was similar in control and induced animals, but the number of C cells was higher in noggin induced SVZ. We also detected a significant increase in the number of Olig2+ cells in the SVZ and surrounding areas. Finally, our *in vitro* results also support a role for noggin in oligodendrocyte differentiation where we observed a significant increase in oligodendrocyte cell number after neurosphere differentiation. The ability to specifically stimulate proliferation, tangential migration and differentiation of OPC would have major benefits in treating dysmyelinating diseases of the CNS and may ultimately be achieved by controlling the level of noggin expression in the SVZ, the major adult source of OPC.

Microarray analysis identified four significant clusters of transcripts involved in: synapses, cell-cell junctions, signaling, and the cell membrane. Among those increased in controls were genes involved in CNS development, in dendrite differentiation, in neurotransmitter function, as well as a microRNA-containing gene *Mirg*, which is expressed in the developing nervous system (Wheeler et al., 2006) and critically involved

in lineage differentiation (Tsan et al. , accompanying). The noggin-exposed group was enriched in transcripts involved in neuronal and oligodendrocyte lineage differentiation. Consistent with our qPCR analysis and immunohistochemical studies, transcripts characteristic of oligodendrocytes (*Mag, MBP, Mog, PMP22, Sox8*), as well as *Lama2,* which regulates oligodendrocyte survival in the SVZ (Relucio et al., 2012), were increased in the noggin-exposed cells. *Sox11,* which promotes neurogenesis and can convert mature glia to neurons (Ninkovic et al., 2013); and *Egfr* (C cells) were also increased by noggin exposure.

This model will be useful in modulating BMP signaling during development, in models of neurodegeneration, aging and following injury.

Since injury associated with the delivery of growth factors to the SVZ can redirect cell migration (Goings et al., 2004) and activate STAT signaling (Fuller et al., 2007), this model makes it possible to supply desired levels/schedules of noggin to the niche without confounding injury. This paradigm will therefore be useful in analyzing the molecular histogenesis of germinal zones, and in modifying the local growth factor milieu to expand progenitor populations following injury, disease, normal and pathological aging. Aging brain is characterized by both ependymal alterations (Conover et al., 2000; Marshall et al., 2003; Maslov et al., 2004; Luo et al., 2006; Conover and Notti, 2008) and decreased NSC production (Craig et al., 1996; Maslov et al., 2004; Luo et al., 2008). These animals should allow us to tease out the optimal kinetics and levels of noggin expression that best promote the regenerative response of the adult and aged CNS. Ultimately mating with mouse models of aging (Kawahara et al., 2009), models of neurodegenerative disease where noggin levels decrease, such as Alzheimer's disease (Tang et al., 2009), as well as

in models of ischemic brain injury (Samanta et al., 2010), or demyelination (Cate et al., 2010) should determine the optimal paradigm of noggin expression that stimulates recovery of function. It will also be important in promoting plasticity in regions refractory to regeneration or NSC derivation.

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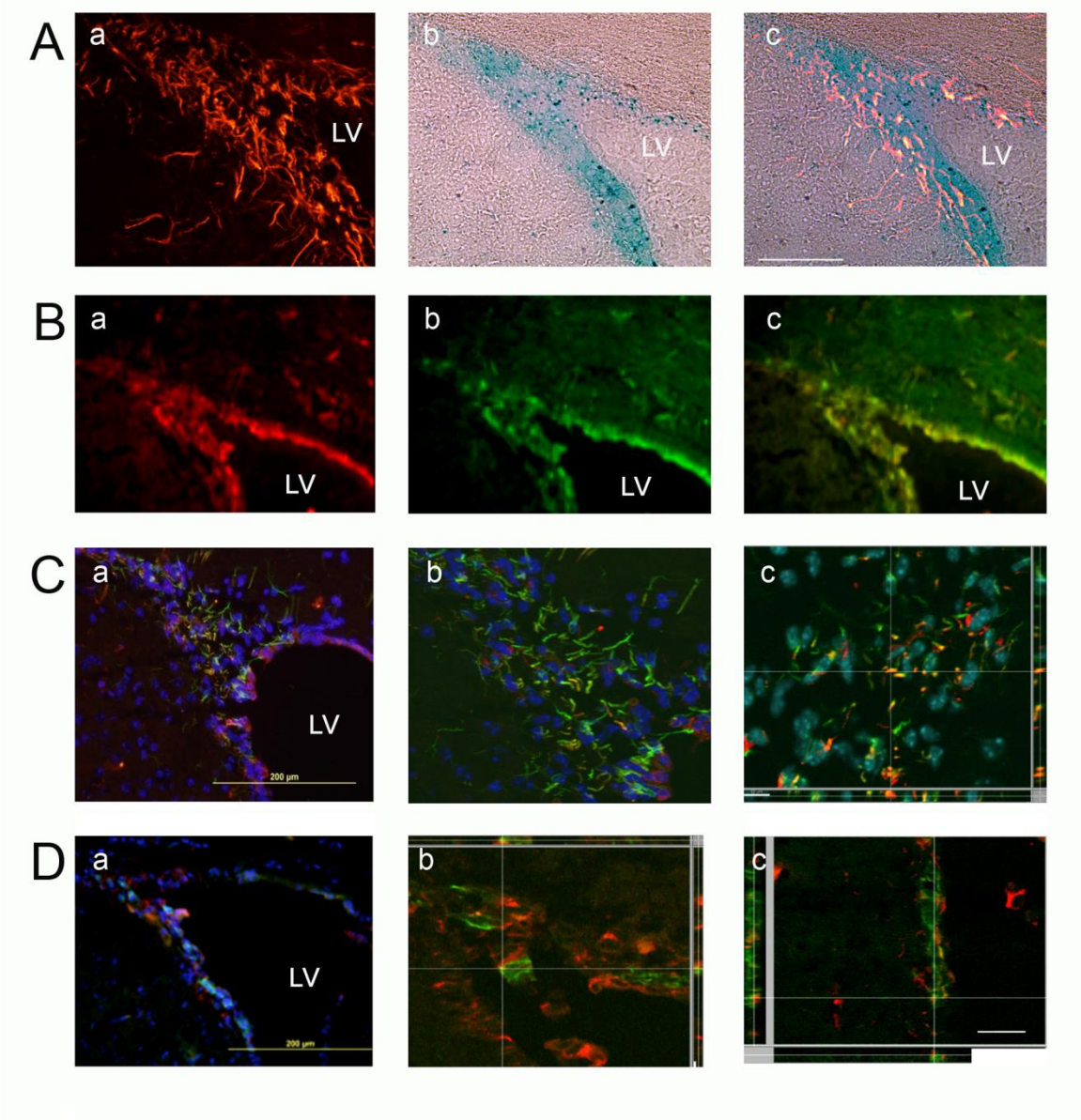

Figure A.1. Fidelity of transgene expression

A. β-gal expression (a, X-gal staining) completely overlaps with Nestin positive cells (b, Cy3 secondary, red) lining the SVZ, indicating that rtTA transgene expression is restricted to cells expressing Nestin. Hoechst identifies nuclei in b. LV = lateral ventricle. Scale bars = 200µm.

B. After 8 days of transgene induction, Noggin over-expression is restricted to Nestin positive cells (a, Cy3, red) as marked by eGFP expression (b), indicating fidelity of transgene expression. c = overlay. Scale bar = 200µm.

C. a-c. Nestin (Cy3 secondary, red) is expressed in B cells (GFAP positive cells, FITC secondary).

D. a-c. Nestin (Cy3 secondary, red) is also present in some DCX+ A cells (FITC secondary).
LV = lateral ventricle. Scale bars = 200 µm A-D, 20 µm C.c, D.b,c.

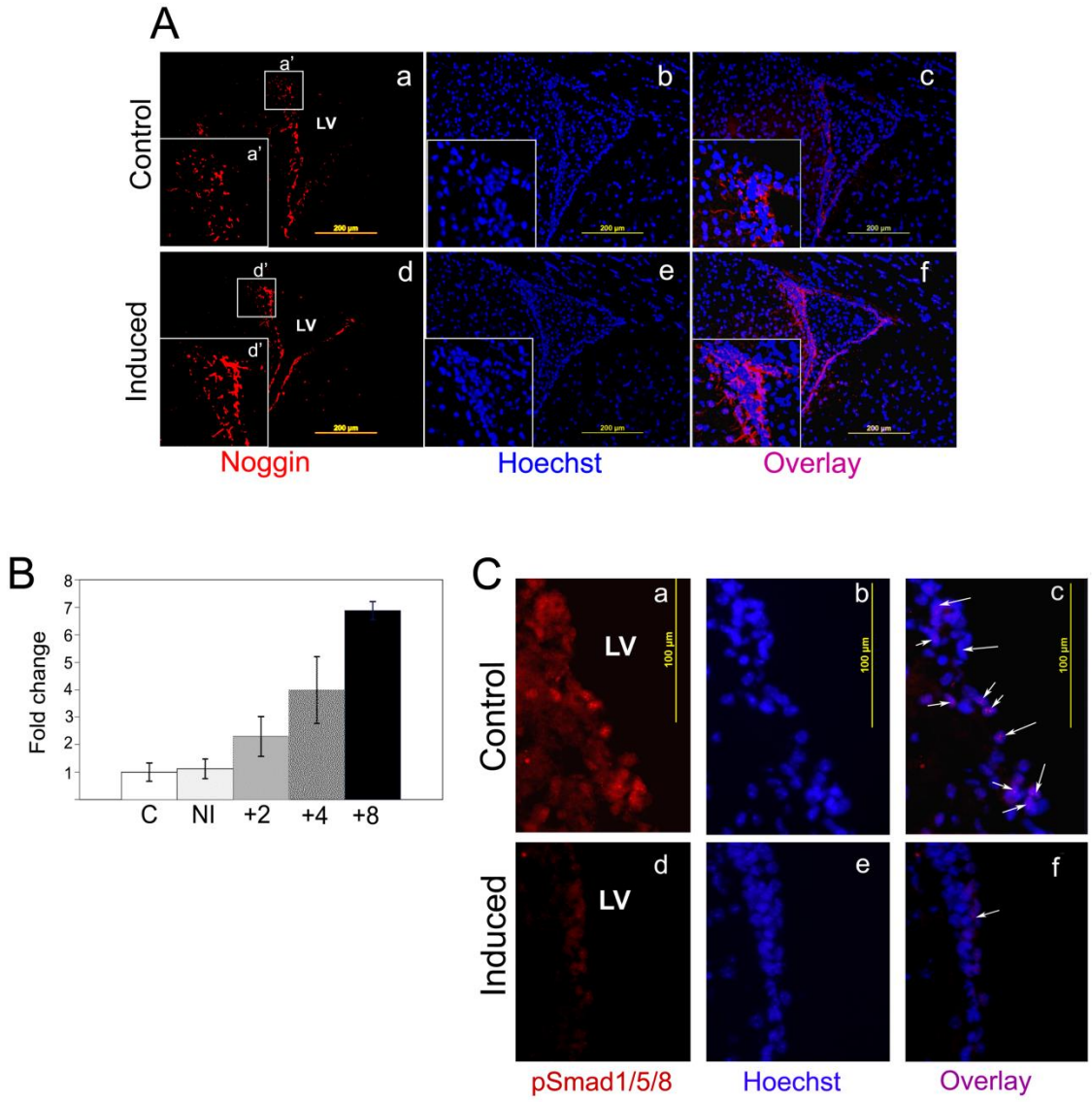

Figure A.2. Expression of Noggin in the SVZ is tightly controlled by doxycycline treatment. Expression of both Noggin protein (A) and mRNA (B) in the SVZ of double transgenic animals were up-regulated by doxycycline.

A. Noggin is expressed in the ependymal zone in the uninduced SVZ (a-c) and is strikingly increased by 8 days of transgene induction (d-f). a-f are coronal sections, a,d = anti-Noggin antibody (Cy3 secondary, red), Hoechst 33258 identifies nuclei (b,e). Inserts show higher magnification views of indicated regions and c,f are overlays. LV = lateral ventricle. Scale bars = 200 µm. In all figures, medial is oriented to the right, lateral to the left.

B. Quantitative RT-PCR analysis of Noggin mRNA from microdissected SVZ. Similar levels of mRNA were expressed in control SVZ (C, single transgenic), and double transgenic mice not exposed to doxycycline (NI; not induced), indicating that the

transgene was not expressed in the absence of doxycycline, i.e., did not "leak". The transgene was highly responsive to doxycycline induction; Noggin mRNA was upregulated 2-fold after two days of induction (+2), 3-fold by four day induction (+4), and 7-fold after 8 days (+8). β-actin was employed for normalization, fold-change was estimated using the ΔΔct method. Samples were analyzed in triplicate and fold-change represented as mean ± SD.

C. Noggin inhibits BMP signal transduction at the level of Smad1/5/8. Immunohistochemical localization of phospho-Smad1/5/8 indicates that in control animals (a-c) there is active BMP signaling as phosphorylated Smad protein (a, Cy3 secondary, red) is present in the nuclei (b, Hoechst, blue) of cells lining the LV (c, arrows, overlay). In animals induced for 8-days to express noggin (d-f), pSmad1/5/8 (d) is rarely present in nuclei (e), indicating that BMP signaling has been abrogated (f, overlay) in induced animals compared with controls. LV = lateral ventricle. Scale bars = 100 μm.

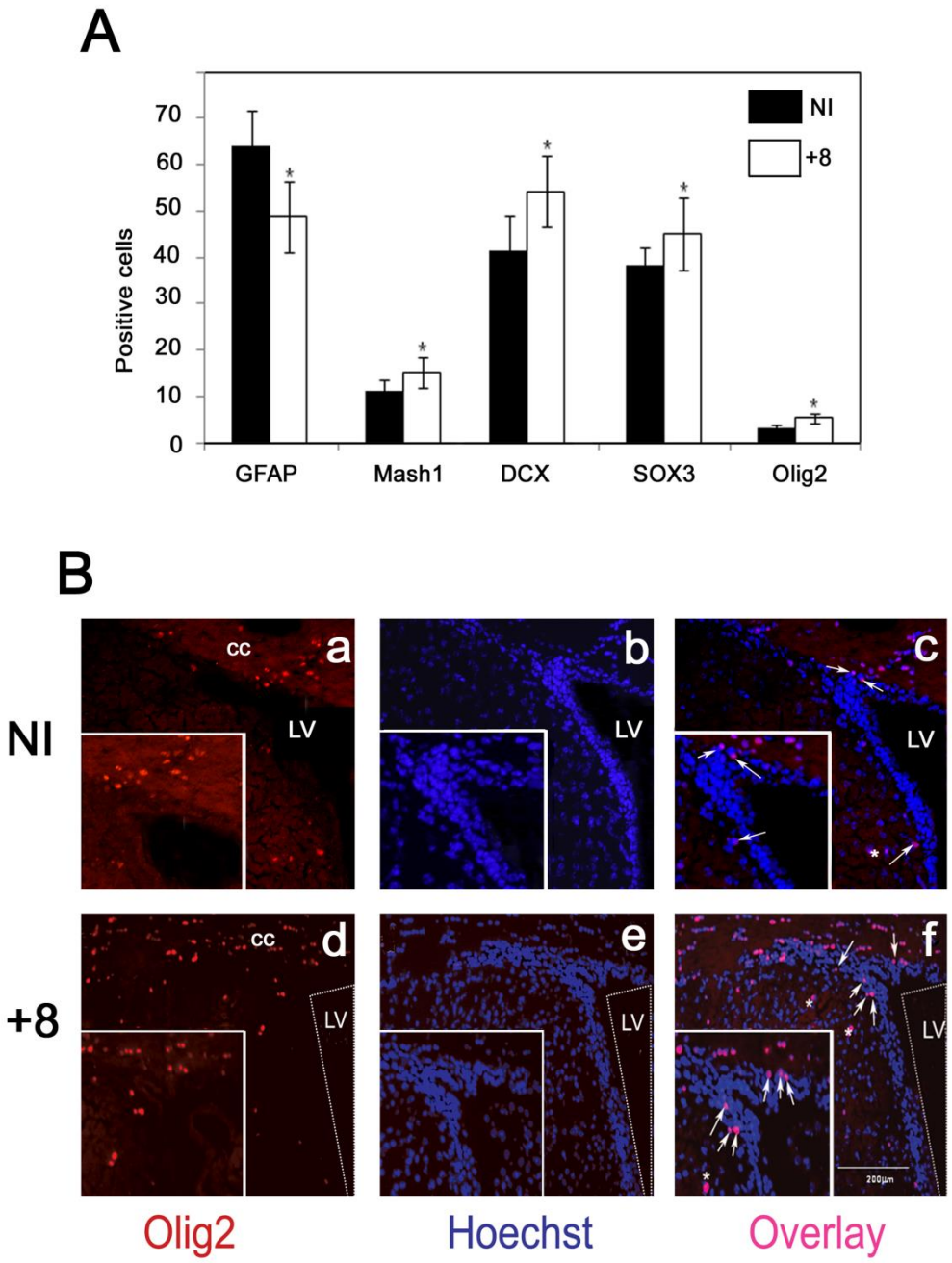

Figure A.3. Noggin promotes differentiation of SVZ neural stem cells.

A. Noggin over-expression significantly increases the number of Mash1+ transit amplifying C cells, DCX+ neuroblasts, SOX3+ neural precursor cells, and Olig2+ OPC, and decreases the number of GFAP+ astrocytes in the SVZ of double transgenic animals exposed to doxycycline for 8 days (+8) compared with non-induced controls (NI). Data

are represented as mean $\pm$ SD, n = four animals per group, 8 SVZ. * indicates $p \leq 0.003$, Students t test.

B. Noggin increases the number of oligodendrocyte precursor cells present in the SVZ. Cells expressing the transcription factor Olig2 (a,d, Cy3 secondary, red) were examined in coronal sections of uninduced controls (NI, a-c), and 8-day induced animals (+8, d-f). In uninduced controls there are very few Olig2+ cells in the SVZ (a-c), however, Noggin induction strikingly increased the Olig2+ cells in the SVZ, striatum and corpus callosum (d-f). Hoechst staining identifies nuclei (b,e); arrows in the overlays (c,f) indicate Olig2 positive cells in the SVZ, * identifies positive cells in the striatum. cc = corpus callosum, Scale bar = 200 μm.

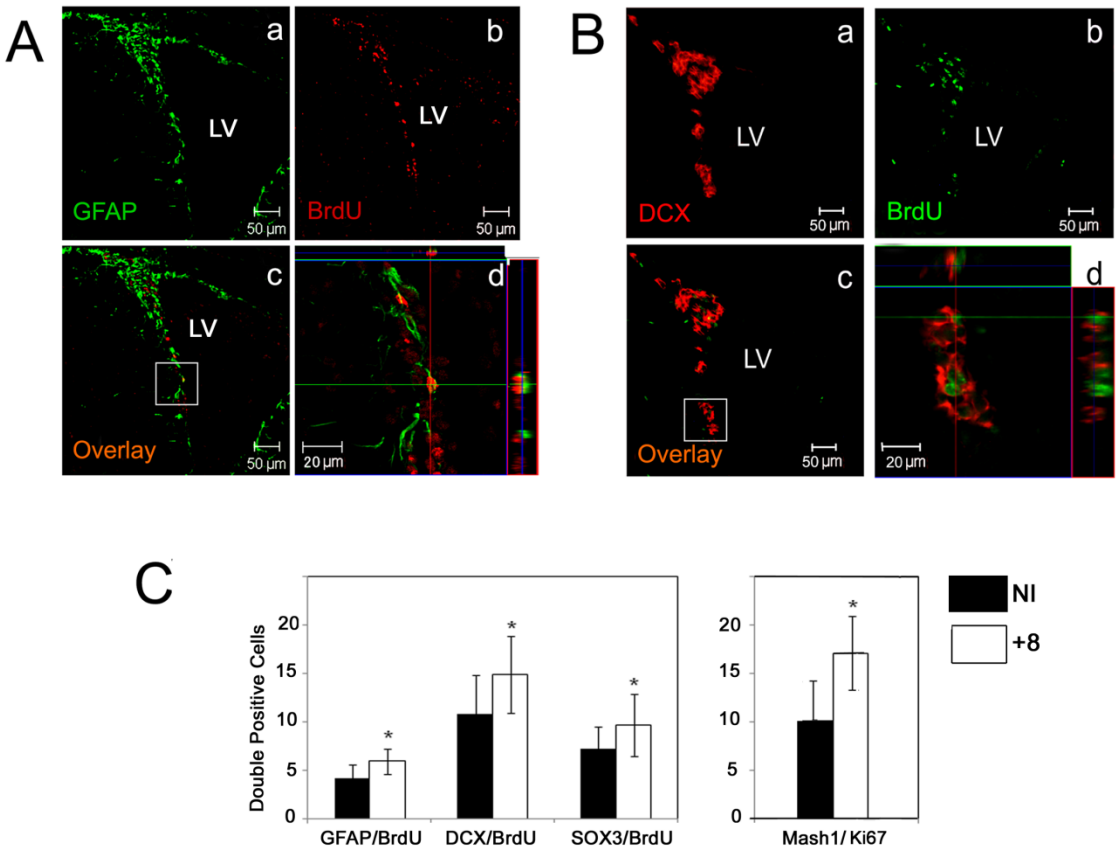

Figure A.4. Noggin promotes proliferation in the SVZ.

A. Confocal sections illustrating co-staining of GFAP (a, secondary antibody=FITC, green) and BrdU (b, Cy3 secondary, red) in a coronal section of the SVZ following 8-day induction. Insert (white box in overlay c) is shown at higher magnification with orthogonal views employed for quantification of double positive cells in d. Bars a,b,c = 50 µm; d = 20 µm.

B. Confocal image of a coronal section through 8-day induced SVZ illustrating co-localization of the neuroblast marker DCX (a, Cy3, red) and BrdU (b, green). The overlay is illustrated in c and the orthogonal view is shown at higher magnification in d. LV = lateral ventricle. Scale bars a,b,c = 50 µm; d = 20 µm.

C. Noggin induced proliferation of GFAP+ astrocytes, Mash1+ transit amplifying C cells SOX3 neural precursors, and DCX+ neuroblasts. Quantification of BrdU+ and cell type specific antigen+ cells (GFAP, DCX or SOX3) was carried out in the SVZ of non-induced (NI) and mice induced for 8 days (+8). Ki67 was also used to quantify the number of dividing Mash1+ C cells. Double positive cells were counted when the nuclear signal was central to cytoplasmic GFAP or DCX, or overlapping with nuclear Mash1 or SOX3 in n = 4 animals, 8 SVZ per group. Values are expressed as mean $\pm$ SD, * = $p \leq 0.05$, NI vs +8, Students t test.

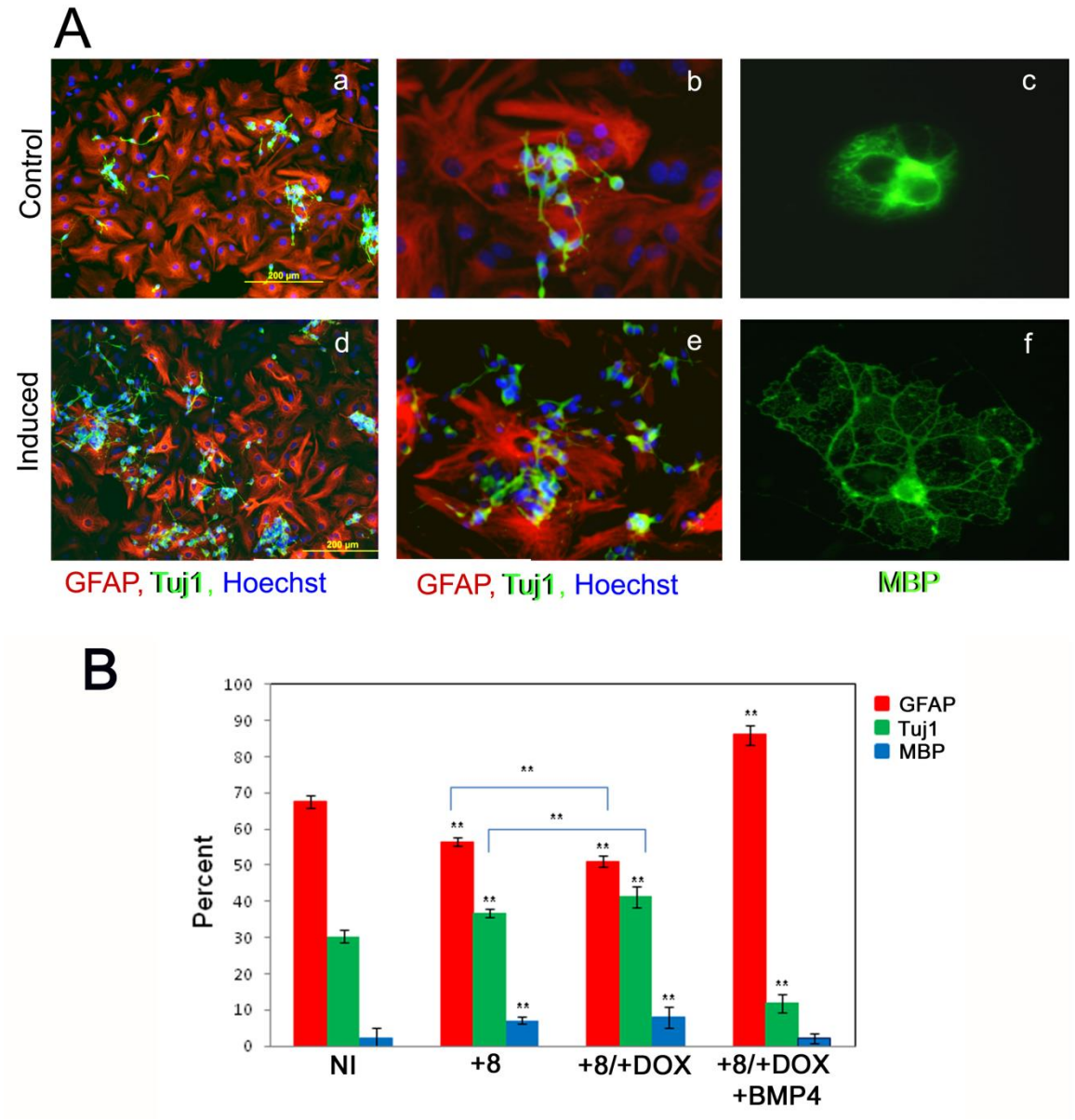

Figure A.5. Noggin promotes differentiation of neurons and oligodendrocytes from neurospheres.

A. Immunohistochemical localization of GFAP+ astrocytes (Cy3 secondary, red), and Tuj1+ neurons (FITC, green) in differentiated neurosphere cultures from control (a,b) and 8-day induced mice (d,e) illustrating the increased numbers of neurons and fewer mature astrocytes differentiated in the presence of Noggin. Noggin expression also increased both the number and maturity of MBP+ oligodendrocytes (c,f, FITC secondary).
B. Percentage of GFAP+ astrocytes (red bars), TUJ1+ neurons (green) and MBP+ oligodendrocytes (blue) in cultures from non-induced controls (NI), animals induced for 8-days *in vivo* (+8), and animals induced both in vivo and *in vitro* (+8/+DOX). Addition of BMP4 to the cultures (+8/+DOX/+BMP4) significantly suppressed differentiation of

both neurons and oligodendrocytes. Mean numbers of GFAP+, Tuj1+, and MBP+ cells in NI vs +8 cultures, between NI vs +8/+DOX, and between NI vs +8/+DOX+BMP4 cultures (except MPB+ cell numbers) were significantly different, ** indicates $p \leq 0.005$, bars indicate the comparison of +8 vs +8/+DOX.

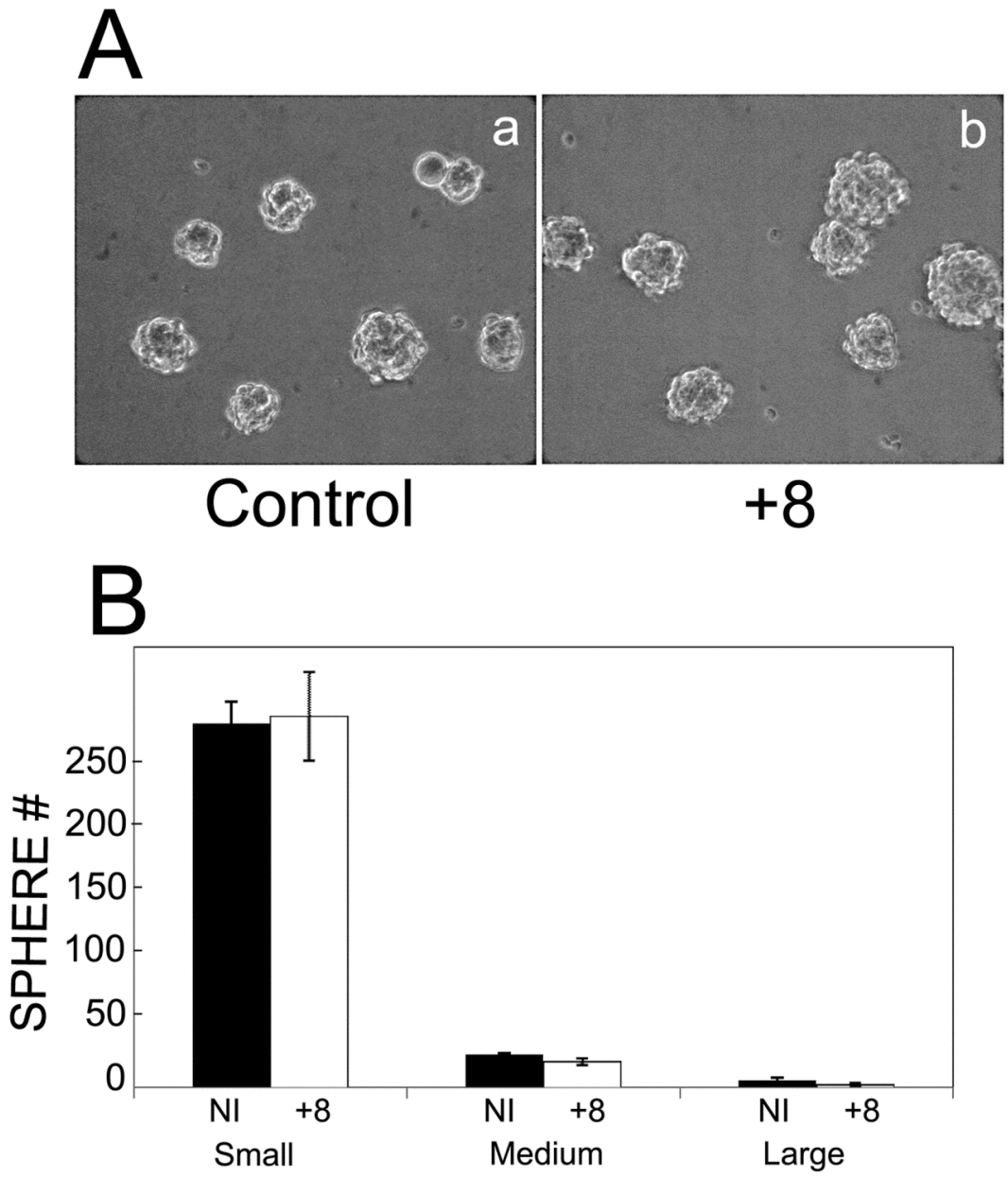

Figure A.6. Noggin does not alter self-renewal of neurospheres.

A. Noggin over-expression did not affect the number, morphology or size of secondary neurospheres from control (a) or mice induced *in vivo* for 8 days to express Noggin (b, +8).

B. After 7 DIV, neurospheres were classified as small (< 4 units), medium (4-8 units) and large (> 8 units) using an optic micrometer, then counted. There was no significant difference in the mean number (± SD) of neurospheres from control (NI) or induced (+8) groups in n = 3 experiments.

Table A.1. Cell type specific effects of Noggin over-expression in the SVZ

To determine if there were changes in the distributions of each population, we compared the standard deviations of induced and uninduced animals using Chi-squared analysis. The standard deviations were not significantly different in B or A cell populations, but changed significantly (*) in response to Noggin exposure in C and OPC cells. To examine changes in individual cell populations by Noggin over-expression, we first determined the ratio of individual cell types relative to the B cell (set at 1) within the in non-induced (NI) control group and in animals induced for 8 days (+8), and to each other. We then compared the change in +8 relative to NI (+8/NI). ** = actual percentage of B cells.

Chi-squared analysis of the change in distributions of NSC in non-induced and induced animals			
B	C*	A	OPC*
$\chi 2=28.2$, $p \leq 0.8$	$\chi 2 = 51.6$, $p \leq 0.006$	$\chi 2 = 29.0$, $p \leq 0.9$	$\chi 2 = 25.2$, $p \leq 0.01$

Percentage Change						
	B	**C**	**A**	**OPC**	**C/OPC**	**A/C**
Non-induced (NI)	1.0	.17	.64	0.05	3.57	3.68
Induced (+8)	1.0	.31	1.1	0.11	2.86	3.54
Ratio +8/NI	0.76** (.489/.647)	1.8	1.7	2.2	0.8	0.96

Table A.2. There was no difference in the percentage of GFAP+ astrocytes, Tuj1+ neurons, or MBP+ oligodendrocytes formed from clonal spheres compared with traditional neurospheres.

Individual clones were expanded and differentiated *in vitro* in serum containing media following mitogen withdrawal. Five representative clones per group from non-induced control (NI) vs animals induced *in vivo* (+8) were exposed to three different conditions *in vitro*: no treatment (NI), addition of doxycycline (+8) or addition of recombinant BMP4 protein (+BMP4). Percentage of astrocytes (GFAP+) and neurons (Tuj1+) and number of oligodendrocytes (MBP+) were quantified as described previously. Means for individual clones were the same as those obtained for the populations (Figure A.5. B), with increased numbers of neurons (36.5 ± 1.7 vs 30.6 ± 1.0; $p \leq 5.4 \times 10^{-6}$) and oligodendrocytes ($8.4 \pm 3.6$ vs 2.0 ± 1.1; $p \leq 0.006$) after Noggin induction accompanied by a decrease in the number of GFAP+ cells ($55.0 + 2.0$ vs 67.4 ± 1.4; $p \leq 4.0 \times 10^{-6}$). Combined *in vivo and in vitro* induction of the transgene further decreased the number of GFAP+ cells (49.7 ± 3.4 vs 55.0 ± 2.0; $p \leq 0.008$), and increased the number of neurons and oligodendrocytes compared with *in vivo* induction alone (40.2 ± 1.1 vs 36.5 ± 1.7; $p \leq 0.001$ for Tuj1+ cells and 10.0 ± 2.9 vs 8.4 ± 3.6; $p \leq 0.005$ for MBP+ cells). Addition of BMP4 to the cultures reverted the effects of Noggin on OPC numbers similar to those in uninduced cultures. There was a resulting increased number of GFAP+ cells (~ 89% independent of Noggin induction) and a drastic decrease in the number of neurons in all cases (i.e., from 40.2 ± 1.1 to 10.7 ± 2.8 in induced animals).

Induction	In Vitro										
In vivo	NI					+DOX				+BMP4	
	Clone	GFAP+	Tuj1+	MBP+		GFAP+	Tuj1+	MBP+		GFAP+	Tuj1+	MBP+
NI	C1	69.9	29.6	0.5		67.0	29.8	3.2		83.8	14.5	1.7
	C2	67.2	29.6	3.2		67.1	30.6	2.3		84.5	12.8	2.7
	C3	67.3	30.7	2		60.2	38.3	1.5		88.7	8.4	2.9
	C4	66.5	32.5	1.0		59.8	38.1	1.9		85.8	11.3	2.9
	C5	65.9	30.7	3.4		67.9	31.1	1.0		88.6	9.4	1.9
	Mean±SD	**67.4±1.4**	**30.6±1.0**	**2.0±1.1**		**64.4±3.6**	**33.6±3.8**	**2.0±0.7**		**86.3±2.0**	**11.3±3.8**	**2.4±0.7**
+8	C6	55.4	37.3	7.3		51.0	38.7	10.3		87.1	11.2	1.7
	C7	54.8	37.1	8.1		50.6	39.9	9.5		90.2	8.5	1.2
	C8	56.4	36.9	6.6		51.6	41.0	7.4		83.4	14.7	1.9
	C9	51.9	33.6	14.5		43.5	41.6	14.9		90.3	8.2	1.5
	C10	57.0	37.7	5.2		51.6	40.2	8.3		89.5	8.3	2.2
	Mean±SD	**55.0±2.0**	**36.5±1.7**	**8.3±3.6**		**49.7±3.4**	**40.2±1.1**	**10.0±2.9**		**87.1±2.9**	**10.7±2.8**	**2.1±0.4**
p value, Students t		**$4.0x10^{-6}$**	**$5.4x10^{-6}$**	**0.006**		**0.008**	**0.0015**	**0.005**		**0.6**	**0.5**	**0.05**
		NI vs +8				**+8 vs +8 +DOX**				**NI + BMP4 vs +8+BMP4**		

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