Identification and Characterization of Neural Progenitor Cells in the Central Nervous System Using the Transcription Factor SOX2

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Abstract

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(Under the direction of Larysa Pevny, Ph.D.)

The embryonic and adult central nervous systems (CNS) harbor heterogeneous populations of proliferating neural progenitor cells which are capable of generating both neurons and glia *in vivo* and *in vitro*. These populations serve to generate all neural cell types throughout development as well as maintain neural cell populations during periods of cellular turnover or injury. However, the cellular and molecular mechanisms which regulate the cell-fate decisions of these distinct progenitor populations are unclear. Moreover, the ability to identify neural progenitor populations *in vivo* is hindered by a lack of defined molecular markers which are capable of specifically recognizing these cells. Thus additional tools are necessary for the continued analysis of neural progenitors *in vivo*.

The HMG-BOX transcription factor SOX2 is expressed in a majority of spatially and temporally distinct neural progenitor populations within the developing and adult CNS. SOX2 has been demonstrated to maintain the proliferative and differentiation capacity of neural progenitor cells in the spinal cord and retina and is important for proper neuronal differentiation and cortical development in mice. However, SOX2 has not been fully characterized in molecularly distinct neural progenitor populations in the CNS nor has its function been addressed in neural progenitor cells that appear during later stages of neural development.

In this dissertation I generate and characterize the $SOX2^{EGFP}$ mouse line which allows for the prospective identification of SOX2-positive neural progenitor cells in the developing and adult CNS *in vivo*. I next demonstrate that distinct populations of neural progenitor cells can be prospectively isolated from the CNS based upon their intracellular concentrations of SOX2. Lastly, I demonstrate that SOX2 function is necessary for the proper maintenance of radial glial cells in the dorsal telencephalon as loss of SOX2 results in a decrease in the number of proliferating radial glia and intermediate progenitors, as well as a reduction in their self-renewal capacity. Collectively these results demonstrate that SOX2 (via the $SOX2^{EGFP}$ mouse line) can efficiently identify neural progenitor populations within the CNS and, more importantly, that SOX2 function is critical for the proper maintenance of neural progenitor populations in the developing CNS.

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Preface

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List of Abbreviations

ANOVA- Analysis of Variance

AVE-Anterior Visceral Endoderm

BAC- Bacteria Artificial Chromosome

BF- Bright field

bFGF- basic Fibroblast Growth Factor

bHLH- basic Helix-Loop-Helix

β-Gal- β-galactosidase

BLBP- Brain Lipid Binding Protein

BMP-Bone Morphogenic Protein

BRDU- Bromo-deoxyuridine

BSA- Bovine Serum Albumin

ChIP-Chromatin Immuno-Precipitation

CNS- Central Nervous System

CRE- Cre-recombinase

DIV- Days *In vitro*

DM- Dissociation Medium

DMEM- Dulbecco's Modified Essential Medium

DNA- Deoxyribonucleic Acid

DPC- Days Post Coitus

DT- Diphtheria Toxin

DT- Dorsal Telencephalon

EDTA- Ethylenediaminetetraacetic Acid

EGF- Epidermal Growth Factor

EGFP-Enhance Green Fluorescent Protein

EGFR-Epidermal Growth Factor Receptor

ES-Embryonic Stem

ES-Enzyme Solution

FACS- Fluorescence Activated Cell Sorting

FBS- Fetal Bovine Serum

FGF-Fibroblast Growth Factor

FGFR- Fibroblast Growth Factor Receptor

FITC- Fluorescein Isothiocyanate

GFAP- Glial Fibrillary Acidic Protein

GLAST- Glutamate Astrocyte Specific Transporter

hGFAP- human Glial Acidic Protein

HI- Heavy Inhibitor

HMG- High Mobility Group

ICM- Inner Cell Mass

IP- Intermediate Progenitor

iPS- induced Pluripotent Stem

KB- Kilobase

LI- Light Inhibitor

LV- Lateral Ventricle

MEMFA- MOPS-EDTA-MgSO4 Formaldehyde

miRNA- Micro Ribonucleic Acid

mRNA- Messenger Ribonucleic Acid

NCAM-Neural Cell Adhesion Molecule

NEO-Neomycin

NEP- Neuroepithelial

NSA- Neurosphere Assay

NSC- Neural Stem Cell

NT-Neural Tube

OB- Olfactory Bulb

ORF- Open Reading Frame

PBS- Phosphate Buffered Saline

PCR- Polymerase Chain Reaction

PFA- Paraformaldehyde

PGK- Phosphoglycerate Kinase

PSA-NCAM- Polysialated Neural Cell Adhesion Molecule

qRTPCR- Quantitative Reverse Transcription Polymerase Chain Reaction

RG- Radial Glia

RMS- Rostral Migratory Stream

RTPCR- Reverse Transcription Polymerase Chain Reaction

SC- Spinal Cord

SGZ- Subgranular Zone

SVZ- Subventricular Zone

TGFβ-Transforming Growth Factor β

VZ- Ventricular Zone

Chapter One

Introduction

The elaborate architecture of the vertebrate central nervous system (CNS), including the neocortex and its underlying cellular networks, is established through the precise temporal and spatial integration of multiple neuronal and glial (collectively termed “neural”) cell subtypes. These neural cell types are distinct in regard to their molecular and cellular properties, and each performs highly specialized functions within the CNS (Guillemot 2007). Remarkably, all of these diverse cell populations are ultimately derived from a single, homogeneous population of progenitor cells that becomes neurally-specified (i.e. produces only neural cell types) during early neurogenesis (Gotz and Barde 2005; Hevner 2006; Malatesta et al. 2008). As development proceeds, these early “neural progenitor cells” continuously divide, establishing a cellular hierarchy in which successive generations of distinct neural progenitor cells become increasingly more specialized; a process culminating in the production of multiple neural progenitor populations, each capable of generating unique neuronal or glial cell subtypes (Fig 1.1) (Temple 2001; Farkas and Huttner 2008). This process drives the rapid cellular expansion of the developing CNS, however it also requires tight regulation as any delay or premature onset in neural cell production can result in the improper integration of cell subtypes into the developing neural network (Golestaneh et al. 2006; Chakrabarti et al. 2007). Similarly, either an abundance or deficiency in an individual neural cell population can change the dynamics of neural processing. Therefore, additional insight into the molecular and cellular characteristics that govern these neural progenitor fate decisions is necessary to better understand the function of neural progenitor populations in CNS development.

One of the molecular characteristics shared by a majority of neural progenitor cells in the developing and adult CNS is the expression of the HMG-Box transcription factor SOX2. SOX2 function has been shown to properly maintain neural progenitor populations in regions throughout the CNS including the neural tube and developing

retina (Pevny and Placzek 2005; Wegner and Stolt 2005; Guth and Wegner 2008; Chew and Gallo 2009). However, neither SOX2 expression nor function has been thoroughly characterized in distinct neural progenitor populations within the developing neocortex, leaving several questions unanswered. For instance, in which neural progenitor populations of the developing and adult neocortex is SOX2 expressed? Is this expression consistent between all neural progenitor cell types? Is SOX2 function necessary in these cells and, if so, does it serve a similar role to that observed in other CNS neural progenitor populations? The focus of this dissertation is to therefore address these questions by analyzing the expression and function of SOX2 in neural progenitor populations in the developing neocortex using mouse genetic models.

This introduction will first address the defining cellular characteristics of neural progenitor cells that are currently used to distinguish these cells from other cell types found in the nervous system. The second section will then present the current understanding regarding SOX2 expression and function in neural progenitor cells within the embryonic and adult CNS. Lastly, the third section will introduce the various neural progenitor cell populations that have been identified during the development of the mammalian neocortex.

1.1 Defining Neural Progenitor Cells

In order to understand the mechanisms regulating neural progenitor cells, it is first necessary to define these heterogeneous populations of cells. The phrase "progenitor cell" and the highly related term "stem cell" are commonly used throughout the scientific literature; however, their usage is often inconsistent (Temple 2001). It is therefore critical to distinguish between these two cell classifications and clarify their usage throughout this dissertation. To this effect, two hallmarks of stem and progenitor cells, *differentiation* and *self-renewal*, must be precisely defined.

Differentiation

In all somatic tissues, including the nervous system, stem and progenitor cells are considered *undifferentiated* in that they remain mitotic and have not yet committed to becoming a mature, post-mitotic cell type. The process of *differentiation*, therefore, is the process by which an undifferentiated cell generates one or many mature, post-mitotic ("differentiated") cell types (Smith 2006). The range of differentiated cell types an individual stem or progenitor cell can produce is referred to as *potency* and it can vary greatly among different progenitor populations. Potency can be divided into multiple categories including: *totipotent* (the ability to generate all of the cell types of an organism), *pluripotent* (the ability to generate all of the cell types of a given tissue), *multipotent* (the ability to generate many, but not all, cell types of a given tissue), *oligopotent* (the ability to generate only a few cell types of a given tissue type), or *unipotent* (the ability to generate only one cell type) (Temple 2001; Smith 2006). For use throughout this dissertation, the term "stem cell" will describe a totipotent cell that is capable of generating an entire organism, such as a cell from the blastocyst inner cell

mass (ICM) or an embryonic stem (ES) cell (See section 1.2) (Evans and Kaufman 1981; Martin 1981). Therefore, a "neural stem cell" will define an undifferentiated cell that has become neurally-specified *in vivo* but retains the capacity to generate all somatic cell-types of an organism *in vivo* or *in vitro.* It is important to note, however, that based upon this definition, such a cell has yet to be identified *in vivo*. In contrast, a "progenitor cell" has a more restricted potency, ranging from pluripotent (giving rise to an entire tissue type) to unipotent (one cell type). Thus, in the context of this dissertation, the term "neural progenitor cell" will broadly incorporate all undifferentiated cells that exclusively generate neural cell types *in vivo* and *in vitro (Temple 2001)*.

Self-Renewal

The second hallmark of stem and progenitor cells is *self-renewal*. Self-renewal is the ability of an undifferentiated cell to generate, through multiple rounds of cell division, undifferentiated daughter cells with equivalent differentiation potential as the original cell (Smith 2006). This process requires that cells maintain both their proliferative capacity as well as their progenitor "competence" (i.e. the ability to respond to intra- and extra-cellular cues of a given tissue) (Smith 2006). "Stem cells" are defined as having unlimited self-renewal and therefore can be maintained indefinitely. "Progenitor cells" on the other hand, have limited self-renewal capacity.

The ability to track the self-renewal potential of a cell *in vivo* throughout development is difficult. Therefore, the self-renewal capacity of stem and progenitor cells is often characterized *in vitro* where the process can be more easily observed. In the nervous system, the self-renewal of neural stem and progenitor cells is often conducted using the "neurosphere assay" in which undifferentiated cells are cultured in the presence of growth factors (Fibroblast Growth Factor (FGF) and Epidermal Growth

Factor (EGF)) at extremely low density in non-adherent plates. These cells are then allowed to proliferate, forming free-floating clonal aggregates called “neurospheres” (Fig 1.2) (Reynolds et al. 1992; Reynolds and Weiss 1992; Tropepe et al. 1999; Coles-Takabe et al. 2008; Hutton and Pevny 2008). The low density conditions of the assay are designed to produce one neurosphere per undifferentiated cell (termed *clonal*). Single cells from an individual primary neurosphere can then be isolated and cultured to test for the formation of new, secondary neurospheres (Fig 1.2). The successive passaging of neurospheres is then used to demonstrate self-renewal, with unlimited self-renewal defined as the ability to passage neurospheres indefinitely. In addition, neurospheres can also be induced to differentiate, testing the potency of cells to generate neurons and glia *in vitro* (Fig 1.2).

Summary- The Usage of “Neural Stem” and “Neural Progenitor”

In the context of this dissertation, both neural stem cells and neural progenitor cells are considered to be neural-specified, undifferentiated cells. In addition, neural stem cells will be defined as having the capacity for unlimited self-renewal as well as the ability to produce all of the other somatic cell types of the developing embryo (totipotent), either *in vitro* or following ectopic transplantation. In contrast, neural progenitor cells will be defined as having a restricted self-renewal potential and the capacity to generate neural cell types exclusively. To date, it is unclear whether a neural-specified totipotent cell, capable of being maintained throughout ontogeny (i.e. neural stem cell), exists *in vivo*. Therefore, the term “neural progenitor cell” will be used exclusively to describe all undifferentiated cells from the nervous system that are capable of generating neural cell types, regardless of self-renewal potential.

1.2 SOX2 Structure, Expression and Function in Neural Progenitor Cells of the CNS

Characterizing the cellular and molecular mechanisms that regulate neural progenitor cell-fate decisions is important for understanding CNS development. However, these analyses have been hindered by an inability to conclusively identify neural progenitor cells *in vivo*. This is due, in part, to a limited number of identified molecular markers which can universally label neural progenitor cells. Therefore, the identification and characterization of additional neural progenitor-specific markers is important for the continued analysis of neural progenitor cell regulation.

The identification of SOX2, an HMG-Box transcription factor, as a universal neural progenitor marker in the CNS has provided valuable insight into the mechanisms which regulate neural progenitor cells. SOX2 is expressed in all neurogenic regions in the developing and adult CNS and multiple studies have demonstrated that its function is essential for maintaining neural progenitor identity (Pevny and Placzek 2005; Wegner and Stolt 2005; Guth and Wegner 2008). For instance, in the chick neural tube and mouse retina, SOX2 function is essential for maintaining the proliferative and neural differentiation capacities of neural progenitor cells (Bylund et al. 2003; Graham et al. 2003; Taranova et al. 2006). In addition, hypomorphic mutations in SOX2 result in cerebral cortex defects in both adult mice and humans (Ferri et al. 2004; Taranova et al. 2006; Cavallaro et al. 2008). Furthermore, SOX2 expression and function are evolutionarily conserved. Similar to mice and humans, loss of the *SoxB1* genes *SoxNeuro* and *Dichaete* in *Drosophila* result in neural hypoplasia, while hypomorphic mutations in *Dichaete* also lead to midline glial defects (Russell et al. 1996; Soriano and Russell 1998; Sanchez-Soriano and Russell 2000). Thus, SOX2 function is required in multiple neural progenitor populations throughout the CNS of different species.

However, the expression of SOX2 in molecularly and cellularly distinct neural progenitor populations within the developing mammalian brain has yet to be thoroughly investigated. Moreover, it is unclear whether SOX2 functions in the same capacity in cortical progenitor cells as it does in other neural progenitor populations within the CNS. Therefore, the characterization of SOX2 expression and function in cortical neural progenitor cell populations is a necessary step in discerning the global mechanisms which regulate cell-fate decisions of neural progenitor cells in the developing and adult CNS.

The HMG-Box Superfamily of Transcription Factors

SOX2 is a member of the SOX subfamily of HMG-Box transcription factors. The HMG-Box is a 79-amino acid DNA-binding motif consisting of two units, termed A and B, which together encode a structure containing three alpha helices that bind to the minor groove of DNA (Einck and Bustin 1985; Bustin et al. 1990; Weir et al. 1993; Love et al. 1995). The HMG-Box was originally identified in a subset of High Mobility Group (HMG) proteins, appropriately named for their small size and fast migration in polyacrylamide gels (Dailey and Basilico 2001). The HMG-Box family is divided into two subclasses. The first, called the HMGb/UBF family or "classical" HMG-box family, contains three groups (HMG1, HMG2, and UBF) which have similar sequence homology and DNA-binding characteristics (Einck and Bustin 1985; Jantzen et al. 1999). In animals, the HMGb/UBF family is ubiquitously expressed throughout embryogenesis (Calogero et al. 1999) and family members have also been identified in other kingdoms including plants, protists, and fungi, suggesting that this superfamily is derived from a common ancestor (Laudet et al. 1993). Structurally, HMGb/UBF proteins have been shown to each contain multiple HMG-box motifs and bind DNA in a sequence-independent manner as well as

recognize abnormal DNA structures such as four-way junctions (Grosschedl et al. 1994). Furthermore, their ability to bind to the minor groove of DNA results in a sharp bend in helical structure (Murphy et al. 1999). This, in conjunction with the observations that they do not possess any transcriptional trans-activation/repression activity, suggests that HMGb/UBF proteins serve architectural roles in regulating transcription such as assisting in cofactor binding or chromatin remodeling (Bustin et al. 1990; Grosschedl et al. 1994; Jantzen et al. 1999).

The second HMG-Box family, the SOX/TCF/MATA family, includes the lymphoid T-cell transcription factors TCF and Lef1 (Travis et al. 1991; Waterman et al. 1991), the fungal mating type protein (Mat1-2) (Kjaerulff et al. 1997), and the SRY-box (SOX) family of transcription factors (Soullier et al. 1999). Unlike HMGb/UBF family members, SOX/TCF/Mata family proteins contain only one HMG-box and bind DNA in a sequence-specific manner (see below) (Soullier et al. 1999). Furthermore, in contrast to HMGb/UBF family members which are ubiquitously expressed throughout the embryo, SOX/TCF/MATA family members are restricted in their temporal and spatial tissue expression (Waterman et al. 1991; Dailey and Basilico 2001; Pevny and Placzek 2005; Wegner and Stolt 2005; Guth and Wegner 2008). The SOX family in particular shows high tissue specificity suggesting its members perform distinct functions throughout development (Wegner and Stolt 2005; Kamachi et al. 2009)

The SOX Family of HMG-Box Transcription Factors

SOX family members were first identified based upon their sequence similarity to the HMG-Box binding domain of the mammalian testis-determining factor SRY (Sex-determining Region on the Y chromosome) and all members share at least 50% sequence homology to the SRY HMG-box (Gubbay et al. 1990). In addition, the SOX

HMG-Box is sequence-specific, binding the consensus DNA sequence (A/T)(A/T)CAA(A/T) in the minor groove of DNA (Waterman et al. 1991; Dailey and Basilico 2001; Pevny and Placzek 2005; Wegner and Stolt 2005; Guth and Wegner 2008). SOX proteins are further divided into subfamilies based upon their degree of sequence similarity, with greater than 90% sequence homology observed within the HMG-Box of subfamily members (Bowles et al. 2000). Currently in vertebrates, there are over 20 individual SOX proteins divided into 8 subfamilies (A-H) with some sub-families further subdivided based upon similarities outside of the HMG domain (Waterman et al. 1991; Dong et al. 2004; Wegner and Stolt 2005; Guth and Wegner 2008). In contrast to other HMG-Box members, such as the HMGb/UBF family and MATA family, SOX proteins have only been identified in metazoans and its members are highly tissue and stage specific, with a number of SOX members identified to be expressed in the developing and adult nervous systems (Table 1.1) (Dailey and Basilico 2001; Schepers et al. 2002; Guth and Wegner 2008).

In the mouse, SOX proteins are subdivided into eight groups, SoxA to SoxH, with SRY as the lone SoxA member. SOX2 is a member of the SOXB1 subgroup (along with SOX1 and SOX3), which in addition to the SOXB2 subgroup (SOX14 and SOX21), comprise the SOXB group (Fig 1.4). SOXB genes are found across multiple Metazoan species including: *Drosophila*, sea urchins, urochordates, amphioxus, zebrafish and *Xenopus,* chick, and primates, including humans (Dong et al. 2004; Pevny and Placzek 2005; Guth and Wegner 2008; Chew and Gallo 2009). In addition, many functions of individual SOXB genes are conserved across these species (Wegner and Stolt 2005; Guth and Wegner 2008; Kamachi et al. 2009).

Mechanisms of SOX2 Function

SOX factors regulate their downstream targets through multiple mechanisms; including chromatin modification, post-translational modification, and protein-protein interactions (Dong et al. 2004; Pevny and Placzek 2005; Guth and Wegner 2008; Chew and Gallo 2009; Kamachi et al. 2009). In addition, SOX proteins have also been shown to act as classical transcription factors, with their carboxyl-termini capable of acting as either activator or repressor (Uchikawa et al. 1999; Dong et al. 2004; Pevny and Placzek 2005; Chew and Gallo 2009; Kamachi et al. 2009). The diverse functions of SOX proteins, as well as their broad and overlapping temporal and spatial expression patterns, suggest that SOX factors work in conjunction with tissue-specific co-factors to regulate the expression of their target genes (reviewed by (Guth and Wegner 2008). For example, SOX2 is expressed in multiple cell lineages and in each tissue type interacts with unique factors to regulate the expression of downstream targets. In ES cells, SOX2 has been shown to directly interact with the POU transcription factor OCT3/4 to regulate Fibroblast Growth Factor (Fgf4) expression and thus maintain cell pluripotency (Yuan et al. 1995). Furthermore, SOX2 and OCT3/4 regulate genes that are expressed in and involved in maintaining ES cell potency including Nanog, Lefty1, Fbx15, and Utf1 (Nishimoto et al. 1999; Kuroda et al. 2005; Nakatake et al. 2006). In contrast, in the developing lens, SOX2 directly interacts with PAX6 to bind and activate the expression of *δ1-Crystallin* (Kamachi et al. 2001; Kamachi et al. 2009) and in the vertebrate nervous system, SOX2 interacts with BRN2, a POU factor, to regulate *Nestin* gene expression in neuroepithelial (NEP) cells (Tanaka et al. 2004; Kamachi et al. 2009). However, much is still unknown concerning the mechanisms of SOX2 function, especially in the context of its ability to maintain neural progenitor identity in the CNS.

Role of SOXB1 Factors in Defining Neural Competence

One function of SOX2 and its SOXB1 family members in neural development is in the process of specifying the neural competence (i.e. the ability to respond to neural inducing cues) of a progenitor cell. The entire mammalian embryo, including the nervous system, can trace its origins to a small population of undifferentiated totipotent cells in the ICM of the early blastocyst. These cells, and their *in vitro* derivatives ES cells, are totipotent; both are capable of generating all three germ layers *in vitro* as well as in teratocarcinomas that form when injected into immune-compromised mice (Evans and Kaufman 1981; Martin 1981). Moreover, when both ICM and ES cells are injected into E3.5 blastocysts, they contribute to both somatic and germ line cells in chimera mice (Gardner and Rossant 1979; Bradley et al. 1984; Gardner et al. 1985), a characteristic which has become instrumental in the ability to generate genetically engineered mouse lines. SOX2, in addition to SOX3, is expressed in both ICM and ES cells and is required to maintain the pluripotency of these cells through an interaction with OCT3/4 to regulate the expression of other factors such as NANOG and FGF4 (Collignon et al. 1996; Ambrosetti et al. 1997; Wood and Episkopou 1999; Avilion et al. 2003; Orkin 2005; Rodda et al. 2005; Masui et al. 2007). The importance of SOX2 in the ICM is demonstrated by the peri-implantation lethality of SOX2 deficient embryos; a phenotype resulting from the inability of ICM cells to transition into epiblast cells (Avilion et al. 2003; Masui et al. 2007). During normal developmental conditions, however, epiblast cells will continue to maintain SOX2 and SOX3 expression. During gastrulation, the epiblast cells will then give rise to the three embryonic germ layers: endoderm, mesoderm, and ectoderm (Collignon et al. 1996; Wood and Episkopou 1999; Tam and Loebel 2007; Rossant and Tam 2009). It is from a subpopulation of ectodermal cells that the CNS is ultimately derived (Beddington 1981; Beddington 1982; Beddington 1983; Tam 1989).

During the specification of germ layers, both SOX2 and SOX3 become confined to cells of the ectoderm, including those that will constitute the prospective neuroectoderm (Collignon et al. 1996; Wood and Episkopou 1999). In the developing mouse embryo, this restriction coincides with the onset of SOX1 expression in these cells (Pevny et al. 1998). As evidence for an evolutionarily conserved role of the *SoxB1* factor, the *Drosophila* orthologs of *Sox2* and *SoxB1*, *Dichaete* (or *Fish-hook*) and *SoxNeuro* respectively, show similar confined expression patterns in the developing fly (Nambu and Nambu 1996; Russell et al. 1996; Cremazy et al. 2000). Ultimately, all three SOXB1 factors are then expressed in all subsequent CNS neural progenitor populations throughout ontogeny (Sasai 2001; Sasai 2001).

Functional studies suggest that SOXB1 proteins are essential for the establishment of the neural lineage. For instance, in *Xenopus* embryos, the inhibition of SOXB1 function results in a lack of neural tissue formation and differentiation through the attenuation of bone morphogenic protein (BMP) signaling (Mizuseki et al. 1998). Conversely, overexpression of SOXB1 factors, in conjunction with FGF signaling in naïve ectodermal cells in *Xenopus,* initiates neural differentiation (Mizuseki et al. 1998). Similarly, in ES cells, the constitutive expression of SOX2 induces cells towards a neural fate when released from self-renewal conditions (Zhao et al. 2004). Moreover, the precise temporal and spatial restriction of the *SoxB1* genes to the neural ectoderm suggests that they are regulated by neural inducing signals (Pevny and Placzek 2005). For instance, overexpression of the neural inducer CHORDIN upregulates SOX2 while overexpression of the antagonistic target of CHORDIN, Bone Morphogenic Protein (BMP), suppresses SOX2 expression in the *Xenopus* embryo (Mizuseki et al. 1998). Similarly, *Dpp* and *Sog*, the fly counterparts of BMP4 and CHORDIN, regulate the expression of *SoxNeuro* (Cremazy et al. 2000; Buescher et al. 2002). Lastly, direct evidence for the regulation of SOX2 expression by factors that regulate neural induction

stems from a series of elegant studies in the chick embryo showing that FGFs and WNTs can regulate a *Sox2* enhancer element and activate its expression to initiate neural plate development (Takemoto et al. 2006). These results suggest that SOXB1 factors, including SOX2, are necessary in specifying the identity of early neural progenitor cells in the CNS. However, it is unclear how SOX2 functions, and with what cofactors, to maintain the neural identity of these cells once they become specified. Therefore, it is necessary to determine whether SOX2 functions in maintaining the neural identity of neural progenitor populations after neural induction.

Role of SOXB1 Factors in Maintaining the Identity and Differentiation Capacity of Neural Progenitor Cells of the CNS

Individual members of the SOX transcription factor family play essential roles not only in the acquisition of neural fate but in the maintenance and differentiation of neural progenitor cells of the CNS (Pevny and Placzek 2005; Wegner and Stolt 2005; Guth and Wegner 2008; Chew and Gallo 2009). The SOXB1 proteins mark a common transcriptional state shared by diverse populations of neural progenitors throughout the CNS during development and in the adult. The expression of *SoxB1* genes directly correlates first with the commitment of cells to a neural fate (Pevny and Placzek 2005). Next, after neural induction, all three genes are co-expressed in proliferating neural progenitor cells along the entire antero-posterior axis of the developing vertebrate CNS and are maintained in neurogenic regions of the postnatal and adult CNS. Furthermore, SOXB1 factors mark proliferating neural progenitors in derivatives of the CNS including the neural retina, the olfactory epithelium and the inner ear (Kiernan et al. 2005; Donner et al. 2006; Okubo et al. 2006; Taranova et al. 2006). The importance of SOXB1 factors in the nervous system has been highlighted by both results from mis-expression and dominant interfering studies as well as genetic analyses (Pevny and Placzek 2005).

Gain of function and dominant interference experiments in *Xenopus* and chick embryos as well as mouse cell lines have shown that SOXB1 signaling plays an essential role in the maintenance of neural progenitor identity (Mizuseki et al. 1998; Pevny et al. 1998; Bylund et al. 2003; Graham et al. 2003; Taranova et al. 2006). These data provide evidence that inhibition of SOXB1 signaling in neural progenitor cells results in their premature delamination from the ventricular zone, their exit from the cell cycle and the onset of their neuronal differentiation. Conversely, constitutive expression of SOX2 results in the maintenance of progenitor characteristics (Bylund et al. 2003; Graham et al. 2003). These experiments also provide evidence that SOXB1 factors function by antagonizing the actions of proneural genes (Buescher et al. 2002; Overton et al. 2002; Bylund et al. 2003). Specifically, studies in the chick embryo have shown that the ability of proneural genes to promote neuronal differentiation inversely correlates with the level of SOXB1 expression (Wegner and Stolt 2005). However, pleiotropic effects from the dominant-negative interference of SOX2 function in these studies cannot be ruled out.

The requirement of SOXB1 factors for the maintenance of neural progenitor identity has also been confirmed by genetic studies in a number of species. Analyses of conditional and hypomorphic mutations of *SoxB1* genes in *Drosophila*, zebrafish, mouse and humans have not only verified the absolute requirement of SOXB1 factors but have revealed a dosage-dependent role for them in the maintenance of neural progenitor identity (Fantes et al. 2003; Ferri et al. 2004; Taranova et al. 2006; Cavallaro et al. 2008; Miyagi et al. 2008). The identification of hypomorphic mutations and the generation of compound mutations in the *Drosophila Dichaete* and *SoxNeuro* genes, as well as the generation of conditional, hypomorphic and compound mutations in mouse *SoxB1* genes, permit the assessment of the function of SOXB1 factors in the CNS. In the fly, *SoxNeuro/Dichaete* double mutant embryos show severe neural hypoplasia throughout the CNS, as well as dramatic loss of Achaete expressing proneural clusters (Overton et

al. 2002). These data suggest that members of the *Drosophila SoxB1* subfamily act upstream and in parallel to genes of the *Achaete-Scute* complex. Furthermore, analysis of a *Sox2* hypomorphic allele (Dr11) and directed transgene expression has demonstrated that Dichaete function is necessary for the correct development of midline glia of the CNS (Sanchez-Soriano and Russell 2000). In the mouse, analysis of the tissue specific conditional ablation of SOX2 in neural progenitor cells, dependent on the Cre-loxP system, has provided the first genetic evidence for the requirement for SOX2 in the maintenance of neural progenitor cell identity (Taranova et al. 2006). Specifically, in retinal progenitor cells, where SOX2 is the only SOXB1 member to be expressed, conditional ablation of *Sox2* results in a loss of competence to both proliferate and terminally differentiate. In contrast, in *Sox2* hypomorphic/null mice, a reduction of SOX2 expression causes variable microphthalmia as a result of aberrant neural progenitor differentiation in the retina. In *Sox2* hypomorphic mutant retinas the decrease in levels of SOX2 expression directly correlates with a decrease in the levels of NOTCH1 and its direct downstream effector, HES-5, expression. Moreover, and consistent with the observations made in the fly embryo, in mouse neural retinal progenitor cells that express decreased levels of SOX2 due to germline *Sox2* hypomorphic mutations, the expression of proneural genes such as MATH5 and NEUROD1 are prematurely upregulated (Taranova et al. 2006). In the cortex, where all three SOXB1 members are expressed, recent evidence has demonstrated that the consequences of both SOX2 deletion and hypomorphic SOX2 expression are much more subtle. Loss or reduction of SOX2 at early neural tube stages results in decreased neuron production, enlarged ventricles, as well as behavioral defects, but the overall organization and development of the CNS is intact (Sanchez-Soriano and Russell 2000; Ferri et al. 2004; Cavallaro et al. 2008; Miyagi et al. 2008).

In addition to demonstrating the necessity of SOX2 function in the regulation of neural progenitor cells in the CNS, the hypomorphic mutations in SOX2 also demonstrate that SOX2 function in these cells is dosage-sensitive. Indeed, reductions in the intracellular concentration of SOX2 to below 50% of endogenous levels are sufficient to generate the neural defects observed in the brain and retina (Ferri et al. 2004; Taranova et al. 2006; Cavallaro et al. 2008). However, it remains to be determined whether intracellular concentrations of SOX2 regulate neural progenitor populations under normal physiological conditions.

These studies collectively demonstrate that SOX2 is expressed in neural progenitor populations throughout CNS development. Furthermore, SOX2 functions not only to specify these cells to adopt a neural fate, but also to maintain their capacity to proliferate and properly differentiate. However, SOX2 expression has yet to be thoroughly characterized in distinct populations of neural progenitor cells within the developing and adult CNS. Furthermore, the ablation of SOX2 early in neural development has precluded any analysis of SOX2 function in these late-stage, lineage-restricted neural progenitor populations. Therefore, this dissertation will characterize SOX2 expression in distinct populations of neural progenitor cells in the developing and adult CNS, as well as investigate the function of SOX2 in these cells, utilizing newly developed genetic tools. Furthermore, the studies herein will focus upon the neural progenitor cells of the mouse forebrain, specifically in the neocortex and its embryonic precursor, the developing dorsal telencephalon.

1.3 Neural Progenitor Cells in the Development of the Murine Neocortex

Throughout neural development, populations of neural progenitor cells become spatially and temporally specified to produce distinct neuronal and glial subtypes. These neural progenitor cell populations are derived from a small population of epiblast cells which become specified to generate the neuroepithelial (NEP) cells of the neural plate and neural tube (Fig 1.4A). These NEP cells then give rise to populations of radial glial (RG) cells, including a distinct RG subtype in the developing dorsal telencephalon. Dorsal telencephalic RG cells produce a majority of the projection neurons in the neocortex, either directly or through a secondary neural progenitor called an intermediate progenitor cell (Fig 1.4B). Ultimately, most RG cells will then become postmitotic glial cells in the adult neocortex. However, a subpopulation of RG cells is maintained in the adult subventricular zone (SVZ) where they continue to generate interneurons destined for the olfactory bulb (OB) (Fig 1.4C). Collectively, these different neural progenitor populations display distinct capacities for self-renewal and multipotential differentiation *in vivo* and *in vitro*, as well as unique molecular expression patterns. However, one of the unifying features of these cells is the expression of the HMG-Box transcription factor SOX2. In this section I will introduce the various neural progenitor populations that are involved in the development of the neocortex as well as address what is currently known about SOX2 in each population.

Neural Progenitor Cells of the Early Embryonic Nervous System

Neuroepithelial (NEP) Cells

During the process of gastrulation (between E6.5 and E7.5 in the mouse), beginning at the posterior portion of the embryo called the primitive streak, epiblast cells will segregate into three distinct germ layers: ectoderm, mesoderm, and endoderm. The endoderm gives rise to cells of the gut, liver, and pancreas while the mesoderm generates most of the circulatory system, reproductive system, muscle and bone. Cells of the ectoderm, however, will generate the nervous system and epidermis (skin) (Arnold and Robertson 2009).

The specification of neural identity in the ectoderm occurs in a process called neural induction (age E7.5), in which a subset of ectodermal cells receives extracellular signals from the node at the anterior portion of the primitive streak to adopt a neural fate (Beddington 1994). Simultaneously, signals such as Cerberus 1 (CER1) and Lefty1/2 emanating from the anterior visceral endoderm (AVE) specify the anterior region of the ectoderm to form neural ectoderm (neurectoderm) by inhibiting secreted Transforming Growth Factor β (TGFβ) family members NODAL and BMP (Thomas and Beddington 1996; Kimura et al. 2001; Arnold and Robertson 2009). Fate mapping studies demonstrate that cells isolated from the anterior regions of the epiblast primarily generate the neurectoderm, however they are also capable of generating mesodermal derivatives when ectopically grafted, suggesting they maintain pluripotency (Beddington 1981; Beddington 1982; Beddington 1983; Tam 1989; Lawson et al. 1991; Quinlan et al. 1995). Upon neural induction, these ectodermal cells thicken into a columnar epithelium called the neural plate which then invaginates and closes, forming the neural tube. The most anterior regions of the neural tube continue to expand to form the brain while the

more posterior regions of the neural tube will generate the spinal cord (Wilson and Houart 2004; Rossant and Tam 2009).

The single layer of cells which makes up the neural plate (and later the neural tube) is referred to as a pseudo-stratified epithelium (or neuroepithelium) and is comprised of NEP cells (Fig 1.4A). Morphologically, NEP cells have a columnar appearance and extend the length of the neuroepithelium, contacting both the apical surface (in contact with the ventricular lumen) as well as the pial surface, which gives them a "radial" appearance (Boulder-Committee 1970). The epithelial characteristics of NEP cells include a highly polarized apical-basal axis in which transmembrane proteins, as well as tight and adherens junctions, are concentrated at the apical end of the cell (Aaku-Saraste et al. 1996; Corbeil et al. 1999). During the cell-cycle, the nuclei of NEP cells migrate along the entire apical-basal axis in a process called interkinetic nuclear migration. During S-phase, nuclei reside in the basal portion of the cell but migrate to the apical side during G2 phase where they undergo mitosis before returning to the basal region during G1 phase (Takahashi et al. 1993). This nuclear migration gives the neuroepithelium a pseudo-stratified appearance.

As NEP cells are the first neural specified descendents of epiblast cells, they are generally assumed to have the capacity to generate all neural tissue types and are therefore referred to as pluripotent neural progenitor cells. *In vivo*, NEP cells have been observed to initially undergo successive rounds of symmetric division in which one NEP cell produces two equal daughter cells (Noctor et al. 2001; Haubensak et al. 2004; Miyata et al. 2004), serving to expand the neural progenitor pool in the neural plate/neural tube. This initial wave of expansion is followed by the onset of neurogenesis, in which NEP cells begin to directly generate neurons, this time by asymmetrically dividing to form an additional NEP cell as well as a nascent neuron, which then migrates towards the preplate (Haubensak et al. 2004; Miyata et al. 2004;

Noctor et al. 2004). In addition, *in vitro* isolation studies have demonstrated that NEP cells isolated from the E8.5 anterior neural plate and either cultured at low density or retrovirally labeled, are capable of differentiating into all three neural cell types as well as maintaining limited self-renewal capacity (Williams and Price 1995; Qian et al. 2000; Tropepe et al. 2001). However, at E9.5 isolated cells were shown to already be restricted to either neuronal or glial fates, suggesting that NEP cells are functionally heterogeneous (McCarthy et al. 2001).

Molecularly, NEP cells can be distinguished from epiblast cells by the expression of the intermediate filament protein Nestin, a widely used neural progenitor marker, in addition to its posttranslational modifications labeled with the antibodies RC1 and RC2 (Frederiksen and McKay 1988; Misson et al. 1988; Edwards et al. 1990; Malatesta et al. 2008). Furthermore, mRNA transcripts of brain lipid binding protein (BLBP) have also been observed in NEP cells, although antigenic labeling of BLBP protein is not detected until later stages (Anthony et al. 2004). NEP cells have also been observed to become dependent upon Notch signaling as loss of Notch targets such as Hes1, 3, and 5 results in the inability of NEP cells to differentiate into radial glial cells (Hatakeyama et al. 2004).

As in epiblast cells, SOX2 and SOX3 continue to be expressed in NEP cells. However, coincident with neural induction, the last SOXB1 member, SOX1 also becomes expressed (Pevny et al. 1998; Wood and Episkopou 1999). At this stage, SOX2 continues to be functionally important. For instance, hypomorphic mice containing one SOX2-null allele (in which the SOX2 coding sequence was replaced with a β-geo reporter cassette) and one hypomorphic SOX2 allele (generated from the deletion of a neural cell-specific enhancer of SOX2) display a reduction in SOX2 expression in the telencephalon and several associated neural defects (Zappone et al. 2000; Ferri et al. 2004). These defects included circling behavior, epileptic spike

recordings, neurodegeneration, reduction in cortical size, ventricular enlargement, and impaired neural progenitor proliferation and differentiation (Ferri et al. 2004). In another series of experiments, the conditional ablation of SOX2 in NEP cells using a Nestin-Cre driven excision also resulted in enlarged ventricles as well as a reduction in the neurosphere forming potential of cortical progenitors and embryonic lethality (Miyagi et al. 2008). These results suggest that SOX2 has a unique role in the maintenance of NEP cells and in their ability to generate mature neurons. Furthermore, it suggests that these functions cannot be completely compensated for by its highly related SOXB1 family members SOX1 and SOX3. However, it should be noted that these studies ablate or reduce SOX2 expression in neural plate/tube stages of embryogenesis (at the onset of NEP formation), but only analyze the effects later in development into adulthood. Thus, the effects observed may not address the function of SOX2 in maintaining NEP cells, but rather its function in specifying neural competence of these cells. Therefore, in order to address whether SOX2 functions in the maintenance of neural progenitor populations, it is important to utilize genetic tools to direct SOX2 ablation at precise temporal stages to specific neural progenitor cell types (as described in Chapter 4).

Neural Progenitor Populations in the Dorsal Telencephalon

During anteroposterior patterning of the developing neural tube, the most anterior portion becomes specified to generate the two hemispheres of the telencephalon, or cerebrum. In turn, the telencephalon is further divided into the dorsal and ventral telencephalic regions. The dorsal telencephalon, or pallium, will then generate the cerebral cortex of the adult brain (also called the neocortex) while the ventral telencephalon, or subpallium, will generate the basal ganglia (Koop et al. 1986; Puelles 2001; Wilson and Houart 2004). Phylogenetically, the neocortex is the most recently

developed region of the brain and in mammals is involved in many higher order processes such as memory, language, perception, and consciousness (Campbell 2003; Wilson and Houart 2004). These complex processes require neocortical neurons to establish intricate networks of connections between themselves as well as project connections to neurons of other cortical regions. Therefore, a vast majority of neurons that populate the neocortex are projection neurons (Hevner 2006).

Projection neurons (or pyramidal neurons) found in the dorsal telencephalon are generated from neural progenitor cells located within the dorsal telencephalon (Anderson et al. 1997; Tan et al. 1998). The two predominant classes of neural progenitor populations that have been characterized in the developing dorsal telencephalon appear at approximately E10.5 in the mouse (Boulder-Committee 1970; Hartfuss et al. 2001; Hevner 2006; Guillemot 2007). The first, radial glial (RG) cells, are direct descendents of NEP cells and exhibit multipotent and self-renewal capabilities (Hartfuss et al. 2001; Haubensak et al. 2004; Malatesta et al. 2008). The second neural progenitor population, intermediate progenitor (IP) cells, is directly generated by RG cells but has limited self-renewal capacity, if any, and is strictly neurogenic (Gotz and Barde 2005; Pontious et al. 2008; Kowalczyk et al. 2009). However, both progenitor populations can generate projection neurons.

Radial Glial Cells

At the onset of neurogenesis (E10-E11 in the mouse), NEP cells begin to undergo a glial transition to form RG cells (Fig. 1.4B) (Feng et al. 1994; Malatesta et al. 2000; Alvarez-Buylla et al. 2001; Hartfuss et al. 2001; Anthony et al. 2004; Haubensak et al. 2004; Gotz and Barde 2005; Basak and Taylor 2007; Farkas and Huttner 2008; Malatesta et al. 2008). During this period, epithelial characteristics, such as tight

junctions, are lost, as is the apical-basal polarity of some plasma membrane proteins such as hemagglutinin and the viral envelope G protein (Aaku-Saraste et al. 1996; Aaku-Saraste et al. 1997). However, RG cells continue to maintain contact with both the apical and basal surfaces of the neuroepithelium. Furthermore, RG cells also maintain interkinetic nuclear migration, although the presence of newly generated neurons in the basal neuroepithelium limits their nuclei migration to the apical neuroepithelium, a region referred to as the ventricular zone (VZ) (Boulder-Committee 1970). It is in the VZ that most mitotic events are subsequently observed.

In addition to the loss of epithelial characteristics, RG cells adopt glial features starting between E10-E11. These features include the expression of genes characteristic of astrocytes including brain-lipid-binding-protein (BLBP), glutamate astrocyte specific transporter (GLAST), vimentin, Tenascin-C, S100β, and in primates, glial fibrillary acidic protein (GFAP); although in mice the human GFAP (hGFAP) promoter is active in RG cells (Brenner et al. 1994; Feng et al. 1994; Shibata et al. 1997; Mori et al. 2005; Casper and McCarthy 2006). Interestingly, while BLBP protein is observed at approximately E11, its mRNA is found to be expressed at E10 in NEP cells, suggesting that it is an early indicator of the NEP to RG transition (Hartfuss et al. 2001; Anthony et al. 2004; Gotz and Barde 2005). In addition to molecular changes, RG cells can also be characterized by the presence of glycogen granules, which are a glial characteristics (Fig 1.6) (Choi 1981).

The glial properties of RG cells originally led researchers to conclude that they serve as scaffolding on which newly generated neurons can migrate towards the pial surface (Boulder-Committee 1970; Rakic 1971). However, analysis of isolated radial glial cells using mice expressing green fluorescent protein (GFP) under the hGFAP promoter was the first direct demonstration that these cells were capable of generating neurons (Malatesta et al. 2000). Subsequent lineage tracing studies using BLBP- and

GLAST-promoter driven Cre as well as retroviral labeling confirmed that RG cells are capable of generating neurons as well as astrocytes (Malatesta et al. 2000; Hartfuss et al. 2001; Noctor et al. 2001; Malatesta et al. 2003; Anthony et al. 2004).

Although many RG molecular and cellular characteristics are ubiquitous within the CNS, including the expression of BLBP and GLAST, regional differences in RG cells are observed in the developing telencephalon. In particular, RG cells in the dorsal telencephalon of E13.5 mice have been shown to express the hGFAP promoter whereas RG cells in the ventral telencephalon do not (Malatesta et al. 2000; Malatesta et al. 2003). In addition, dorsal telencephalic RG cells express the transcription factor PAX6 and can directly generate neurons, which rarely occurs in ventral telencephalic RG cells (Malatesta et al. 2000; Heins et al. 2002; Malatesta et al. 2003; Anthony et al. 2004).

Recent studies in the mouse have suggested that subpopulations of RG cells exist in the developing dorsal telencephalon (E10-E17). It has been demonstrated that all RG cells are mitotic, incorporating the S-phase marker bromo-deoxyuridine (BrDU) after 12 hours of exposure (Hartfuss et al. 2001; Magavi and Macklis 2008). However, labeling studies using retrovirally labeled cells observed neurogenic, gliogenic, and multipotential clusters after multiple days in culture (Walsh and Cepko 1988; Grove et al. 1993; Reid et al. 1995; Noctor et al. 2001). In addition, studies using genetic labeling demonstrated that many RG cells are lineage restricted, generating only neuronal or glial progeny, although multipotential RG cells were also observed (Luskin et al. 1988; Walsh and Cepko 1988; Malatesta et al. 2000; Hartfuss et al. 2001; Malatesta et al. 2003; Anthony et al. 2004; Miyata et al. 2004; Noctor et al. 2004). Interestingly, *in vivo* lineage studies have suggested that this restriction may occur at the onset of neurogenesis as neural progenitors in the mouse neural tube that were retrovirally labeled at E9.5 were able to give rise to neuronal and glial restricted clones in the adult (McCarthy et al. 2001). It has also been demonstrated in time-lapse cultures of cortical cells isolated

from embryonic and postnatal mice that as neurogenesis proceeds, radial glial cells transition from neurogenic to gliogenic, producing primarily astrocytes and oligodendrocytes at early postnatal stages (Qian et al. 2000). These results suggest that a majority of RG cells are not multipotent *in vivo* and therefore heterogeneous populations of RG cells exist in the dorsal telencephalon.

To understand the cellular diversity of RG cells within the dorsal telencephalon, molecular characterizations of RG cells have been conducted. RG cells share common expression of molecular markers with NEP cells including Nestin, RC1, RC2, as well as the expression of all three SOXB1 members (Frederiksen and McKay 1988; Misson et al. 1988; Edwards et al. 1990; Wood and Episkopou 1999; Avilion et al. 2003). In addition, RG cells, similar to NEP cells, are dependent upon Notch-signaling. Gain of functions studies have shown that RG cells transfected with the constitutively active form of Notch1 were induced to form molecularly and cellularly distinct RG cells and inhibit neuronal differentiation (Gaiano et al. 2000; Hitoshi et al. 2002; Yoon et al. 2004). Furthermore, RG cells can be isolated based upon high levels of enhanced green fluorescent protein (EGFP) when its expression is driven by Hes5 , a downstream transcription factor of Notch1 signaling, as well as high levels of CBF1-driven EGFP, a downstream Notch1 effector (Basak and Taylor 2007; Mizutani et al. 2007). However, Notch signaling in RG cells is also heterogeneous, as RG cells negative for Hes5- and CBF1-EGFP are also found, although only the former were demonstrated to be able to generate neurospheres (Basak and Taylor 2007; Mizutani et al. 2007). As mentioned previously, PAX6 is also expressed in dorsal telencephalic RG cells and is important in their maintenance. Loss of PAX6 results in a reduction in RG cells and postmitotic neurons in the dorsal telencephalon as well as premature differentiation of RG cells to IP cells (Gotz et al. 1998; Heins et al. 2002; Haubst et al. 2004). Furthermore, the level at which PAX6 is expressed in RG cells is also important. Although PAX6 is necessary to

maintain RG cells, high levels of PAX6 are able to induce RG cells to adopt an IP fate (Sansom et al. 2009). These studies demonstrate that multiple molecular factors and signaling pathways are involved in the regulation of cell-fate decisions in RG cells.

As mentioned above, the expression of SOX2, as well as SOX1 and SOX3, continues to be maintained in RG cells (Cavallaro et al. 2008). However, it has yet to be determined whether the function of SOX2 in these cells is similar to its role in NEP cells since the *in vivo* functional studies to date have ablated SOX2 early in NEP cells (Ferri et al. 2004; Cavallaro et al. 2008; Miyagi et al. 2008). Radial glial cells are maintained in the dorsal telencephalon through much of neural development which, in conjunction with previous observations demonstrating the importance of SOX2 in maintaining other neural progenitor populations, suggests that SOX2 may play an important role in maintaining the neural progenitor identity of radial glial cells.

Intermediate Progenitor Cells

In addition to RG cells, a second population of neural progenitor cells has been identified in the dorsal telencephalon, called intermediate progenitor (IP) cells. IP cells appear at the onset of neurogenesis and were originally identified as a mitotic progenitor population distinct from RG cells based upon their rounded shape and lack of interkinetic nuclear migration (Fig. 1.5B) (Boulder-Committee 1970; Smart 1973; Haubensak et al. 2004; Miyata et al. 2004; Noctor et al. 2004). In addition, IP cells are located at the basal edge of the apical VZ and are, therefore, also referred to as “basal progenitors”. IP cells reside in the SVZ, which can be distinguished from the VZ at approximately E13 in the mouse. Live-imaging and lineage tracing studies have demonstrated that IP cells are the product of asymmetrically dividing RG cells which typically produce one self-

renewing RG daughter cell and one IP daughter cell. This IP cell then migrates from the VZ into the SVZ where it divides symmetrically to produce two differentiated neurons that migrate into the cortical plate where they ultimately develop into projection neurons (Haubensak et al. 2004; Miyata et al. 2004; Noctor et al. 2004). Thus, IP cells are generally regarded as unipotential progenitors with no self-renewal capacity. However, instances of symmetrically dividing IP cells resulting in two IP cells, and ultimately 4 neurons, have been observed suggesting some IP cells may maintain limited self-renewal potential (Noctor et al. 2004; Kowalczyk et al. 2009). Furthermore, IP cells with unipolar and multipolar processes have been distinguished suggesting that IP populations may also be heterogeneous (Kawaguchi et al. 2008; Kowalczyk et al. 2009). Collectively, these observations demonstrate that IP cells rapidly expand the neuronal population of the dorsal telencephalon through symmetric division during the peak stages of neurogenesis (E12-E18). Furthermore, IP cells have been shown to generate a majority of projection neurons in the neocortex (Haubensak et al. 2004; Miyata et al. 2004).

Molecularly, IP cells have been well characterized. During RG transition into IP cells, IP cells upregulate the proneural basic helix-loop-helix gene Neurogenin2 (Ngn2) in direct response to high levels of PAX6 (Scardigli et al. 2003; Holm et al. 2007; Sansom et al. 2009), which is subsequently downregulated in IP cells (Englund et al. 2005). Ngn2 has been shown to regulate the migration of newly generated IP cells into the SVZ as Ngn2-deficient mice show an accumulation of bromo-deoxyuridine (BrDU)-positive dividing cells in the SVZ (Britz et al. 2006). Ngn2, in turn, directly upregulates the T-domain transcription factor TBR2 which exclusively identifies IP cells (Bulfone et al. 1999; Kimura et al. 1999; Englund et al. 2005; Ochiai et al. 2009). TBR2 is necessary in the maintenance of IP cells as mice with conditionally ablated TBR2 display decreased cortical thickness, a reduction in the number of SVZ IP cells, and a reduction

in the number of cortical neurons (Arnold et al. 2008; Sessa et al. 2008). Conversely, TBR2 misexpression is sufficient to induce IP cell specification in RG cells (Sessa et al. 2008). In addition, IP cells express CUX2, which serves to limit the proliferation of IP cells in the SVZ (Zimmer et al. 2004; Cubelos et al. 2008). The molecular regulation of CUX2 in IP cells, however, remains unclear. Lastly, as IP cells differentiate into postmitotic neurons, they downregulate TBR2 and CUX2 concomitant with the upregulation of TBR1 and CUX1, which specify upper-layer cortical neuron populations (Hevner et al. 2001; Nieto et al. 2004).

These data demonstrate that IP cells have a restricted capacity for self-renewal and are highly limited in their differentiation potential *in vivo*. In contrast, few studies to date have examined the behavior of IP cells *in vitro.* Despite the existence of a TBR2-EGFP transgenic mouse line (Kwon and Hadjantonakis 2007), it has yet to be demonstrated that isolated TBR2-positive IP cells are capable of forming neurospheres in culture or are able to differentiate into neuronal and/or glial populations. Therefore, it remains to be determined whether IP cells maintain the capacity for multipotential differentiation and self-renewal *in vitro*.

SOX2 has been shown to be expressed in the SVZ of the dorsal telencephalon (D'Amour and Gage 2003; Ellis et al. 2004; Ferri et al. 2004; Bani-Yaghoub et al. 2006) but its relative expression compared to IP cell markers is unclear. Furthermore, it remains to be determined the extent to which SOX2 functionally regulates the limited proliferative and self-renewal potential of IP cells or whether SOX2 downregulation in IP cells is necessary for their ability to differentiate. Therefore, further characterization of SOX2 expression and function specifically in IP cells is necessary.

Neural Progenitor Cells in the Adult Neocortex

At the end of neurogenesis, RG cells begin to lose their bipolar morphology and migrate toward the cortical plate where they differentiate into astrocytes (Voigt 1989; Mission et al. 1991; Noctor et al. 2008). However, a small population of RG cells are maintained into adulthood in multiple regions including the SVZ of the neocortex, the subgranular zone (SGZ) of the hippocampus, and the spinal cord (Gil-Perotin et al. 2009; Suh et al. 2009). Retroviral labeling of postnatal (P0) RG cells shows that these cells are capable of generating olfactory bulb interneurons, cortical oligodendrocytes, and parenchymal astrocytes (Merkle et al. 2004). In the neocortical SVZ, RG cells also generate the four potential neural progenitor populations that have been currently identified including the ependymal cells lining the lateral ventricles as well as three molecularly characterized populations called Type A, Type B, and Type C cells (Doetsch et al. 1999; Johansson et al. 1999). The characterization of adult neural progenitor populations is still in its infancy; therefore, the true identity and function of these cells is not fully understood. Furthermore, it remains unclear whether the mechanisms that regulate these neural progenitor populations are similar to the mechanisms utilized by embryonic neural progenitor populations. However, SOX2 has been demonstrated to be expressed in the neurogenic regions of the adult CNS and may therefore provide a means with which to identify and characterize these neural progenitor populations (D'Amour and Gage 2003; Ellis et al. 2004; Ferri et al. 2004) .

Subventricular Zone (SVZ) Progenitor Cells

By early postnatal stages in the rodent dorsal telenecephalon, neurogenesis has declined and a majority of RG cells have begun to differentiate into mature glial cells (Schmechel and Rakic 1979). However, a small population of mitotically active RG cells

continues to be maintained in the SVZ of the postnatal and adult neocortex (Reynolds and Weiss 1992; Richards et al. 1992; Merkle et al. 2004; Ventura and Goldman 2007). In contrast to embryonic RG cells, these adult neural progenitor cells do not generate cortical cells, but are responsible for the production of olfactory bulb granule and periglomerular interneurons which migrate anteriorly along the rostral migratory stream (RMS) to the OB where they incorporate into the granule and glomerular layers (De Marchis et al. 2007). Four classes of cells have been characterized in the adult SVZ: Types A, B, and C, and ependymal cells. Type B cells have been suggested to serve as the self-renewing neural progenitor population in the SVZ (Doetsch et al. 1999). Type B cells are slowly dividing and, similar to RG cells, express GFAP, GLAST, VIMENTIN, NESTIN, and contain glycogen granules (Doetsch and Alvarez-Buylla 1996; Jankovski and Sotelo 1996; Doetsch et al. 1997; Peretto et al. 1997; Bolteus and Bordey 2004). In addition, although most Type B cells reside in the SVZ, some cells have also been observed to come into contact with the ventricular surface where they extend a single cilium into the ventricular lumen (Doetsch et al. 1997).

Next, Type B cells give rise to a population of rapidly dividing cells called Type C cells. Clusters of Type C cells are found intermittently within the SVZ and are thus also known as transit-amplifying progenitor cells. Molecularly, Type C cells can be identified by the expression of transcription factors Mash1 and Dlx2, but in contrast to Type B cells, they downregulate GFAP expression (Doetsch et al. 1997; Parras et al. 2004). Type B cells will ultimately generate a third cell type, Type A cells, which serve as committed neuroblasts that migrate through glial tubes along the RMS and differentiate into OB interneurons, although mitotic Type A cells have also been observed (Doetsch et al. 1997). Similar to Type C cells, Type A cells also express Dlx2, but in addition express DCX and polysialated neural cell adhesion molecule (PSA-NCAM) (Doetsch et al. 1997; Parras et al. 2004). The last cell type found in the SVZ is the ependymal cell.

Ependymal cells are specialized, multiciliated epithelial cells which line the luminal surface of the ventricles and have been shown to be mitotic (Boulder-Committee 1970; Doetsch et al. 1997; Parras et al. 2004). Interestingly, ependymal cells are also derived from RG cells and continue to express the neural progenitor marker Nestin, suggesting they may also have neural progenitor capacity (Alonso 1999; Johansson et al. 1999).

The capacity of either Type B cells or ependymal cells to act as a neural progenitor population in the adult SVZ remains controversial. Several lines of evidence suggest that the Type B cells are the progenitor population in the SVZ as they directly give rise to both Type A and Type C cells. For example, after acute pharmaceutical ablation of all mitotic Type A and C cells in the SVZ with Ara-C, regeneration of both populations is observed after several days, likely by a slowly dividing Type B cells which were not affected by the drug (Doetsch et al. 1999). In addition, Type B cells transfected with an EGFP plasmid driven by the hGFAP promoter were shown to generate Type C cells (Doetsch et al. 1999). In contrast, other studies have also suggested that ependymal cells have the capacity to act as neural progenitor cells (Johansson et al. 1999; Gleason et al. 2008; Carlen et al. 2009; Moreno-Manzano et al. 2009). Recently, it was demonstrated that under normal physiological conditions, ependymal cells do not serve as neural progenitor cells. However, during periods of stroke, ependymal cells can self-renew and are able to generate both neuroblasts and astrocytes (Gleason et al. 2008; Carlen et al. 2009; Moreno-Manzano et al. 2009). Furthermore, ependymal cells maintain their self-renewal and multipotent differentiation capacity through Notch1 signaling (Carlen et al. 2009). These results suggest that both ependymal cells and Type B cells serve as neural progenitor populations in the adult SVZ *in vivo*.

Interestingly, all four cell types in the SVZ have been shown to express SOX2 which, given the role of SOX2 in maintaining neural progenitor populations throughout

the CNS, suggests that they may each retain limited progenitor characteristics (D'Amour and Gage 2003; Ellis et al. 2004). However, it remains to be determined what relation, if any, these different progenitor populations have to each other and whether SOX2 functions in the same capacity within each. Therefore, further studies utilizing SOX2 as a marker to analyze these cells may provide insight into their capacity to serve as neural progenitor cells.

1.4 Hypothesis

SOX2 has been demonstrated to be important in maintaining neural progenitor identity in defined regions of the CNS, specifically in the developing retina and spinal cord. However, its expression has not been clearly established nor its function analyzed in the cellular and molecularly distinct neural progenitor populations residing in the developing dorsal telencephalon. **Therefore, my hypothesis is that through the analyses of SOX2 expression and function, distinct neural progenitor populations can be identified in the developing dorsal telencephalon and furthermore, that SOX2 functions to maintain the proliferative, self-renewal, and differentiation capacities of these cells *in vivo* and *in vitro*.** To test this hypothesis, I have examined three main questions:

1) Can SOX2 expression be used to identify embryonic and adult neural progenitor populations *in vivo*? In this chapter I describe and characterize the $SOX2^{EGFP}$ mouse line in which the expression of enhanced green fluorescent protein (EGFP) recapitulates endogenous SOX2 expression in neural progenitor populations throughout ontogeny.

2) Can the SOX2^{EGFP} mouse line be used to prospectively identify and isolate distinct populations of neural progenitor cells? In this chapter, I demonstrate that radial glial cells, intermediate progenitor cells, and differentiated neurons can be prospectively isolated based upon their differential levels of EGFP and SOX2 expression using Fluorescence Automated Cell Sorting (FACS).

3) Is SOX2 necessary in the proper development of dorsal telencephalic neural progenitor populations? In this chapter I ablate SOX2 expression specifically in radial glial cells of the dorsal telencephalon using the hGFAP^{Cre} mouse line and find that loss of SOX2 in these cells results in a decrease in the number of radial glial cells and intermediate progenitor cells, as well as a thinning of the dorsal telencephalon and increased embryonic lethality.

Overall, my results are the first demonstrate that SOX2 is differentially expressed in distinct populations of the developing dorsal telencephalon and this expression can be used to prospectively isolate and identify these cells. Furthermore, I show that SOX2 is necessary to properly maintain populations of proliferating radial glia and intermediate progenitor cells in the developing dorsal telencephalon. These data support previous reports that SOX2 is necessary for the proper development of neural progenitor populations in other CNS regions and that SOX2 function cannot be completely compensated for by other SOXB1 transcription factors.

Table 1.1

Family		Member	Function
B	1	1	Maintains neural progenitor characteristics in mouse ES and P19 cells. Regulates migration of mouse telencephalic neurons. Required for differentiation of neurons in mouse ventral striatum.
		2	Maintenance of SOX2 expression biases Xenopus animal cap cells and mouse ES cells towards neural fate. Maintains neural progenitor characteristics in ES cells, chick. spinal cord, mouse retinal and rat oligodendrocyte progenitor cells. Required for neuronal differentiation in anterior thalamus, dorsal striatum and septum. Required for maintenance of chick neural crest progenitor identity.
		3	Maintains neural progenitor characteristics in chick spinal cord. Required in a subset of mouse hypothalamic neurons that regulate the hormonal output of the anterior pituitary.
	2	14	Promotes neuronal differentiation, cell cycle exit, delamination of chick neural progenitors.
		21	Promotes neuronal differentiation, cell cycle exit, delamination of chick neural progenitors.
C		4	Promotes activation of differentiated neuronal markers in chick embryonic spinal cord.
		11	Promote activation of differentiated neuronal markers in chick embryonic spinal cord. Regulates of neuronal survival and neurite outgrowth of chick neurons.
D		5	Represses specification and terminal differentiation and influences mouse oligodendrocyte migration patterns.
		6	Represses specification and terminal differentiation and influences mouse oligodendrocyte migration patterns.
E		8	Regulates *Xenopus* and chick neural crest cell migration.
		9	Required for neural crest induction in *Xenopus*, chick and mouse embryos. Promotes chick oligodendrocyte differentiation.
		10	Maintenance and survival of neural crest progenitors in *Xenopus* and mouse embryos. Induction and survival of glial lineages in *Xenopus* and mouse embryos. Promotes oligodendrocyte differentiation.

Table 1.1. SOX Factor Functions in Nervous System Development. Table of SOX members grouped by family and subgroup. Also listed are the identified function(s) of each Sox gene within the central and peripheral nervous systems.

Figure 1.1

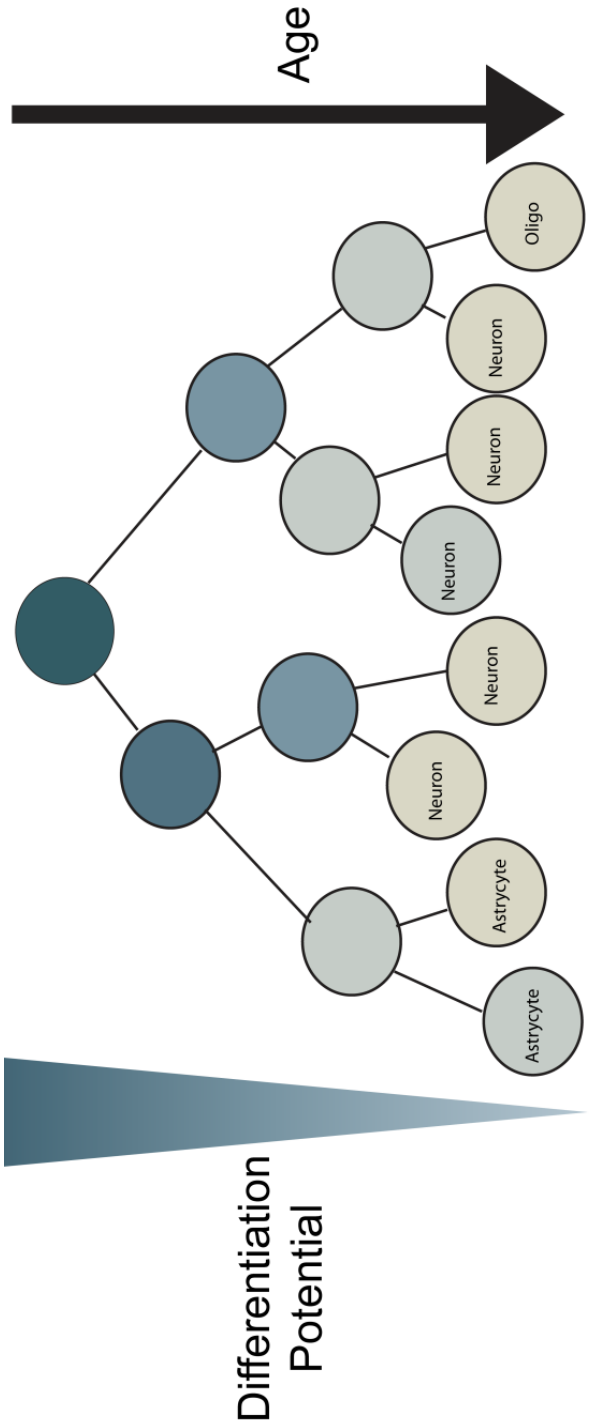
Age
Oligo
Neuron
Neuron
Neuron
Neuron
Neuron
Astrycyte
Astrycyte
Differentiation
Potential

Figure 1.1 Stem/Progenitor Cell Hierarchy. Illustration demonstrating that as a totipotent progenitor cell divides throughout development, its potency becomes more restricted in its subsequent progeny after each successive division. Differentiation potential is indicated by the darkness of the circle. Abbreviation: Oligo, oligodendrocyte

Figure 1.2

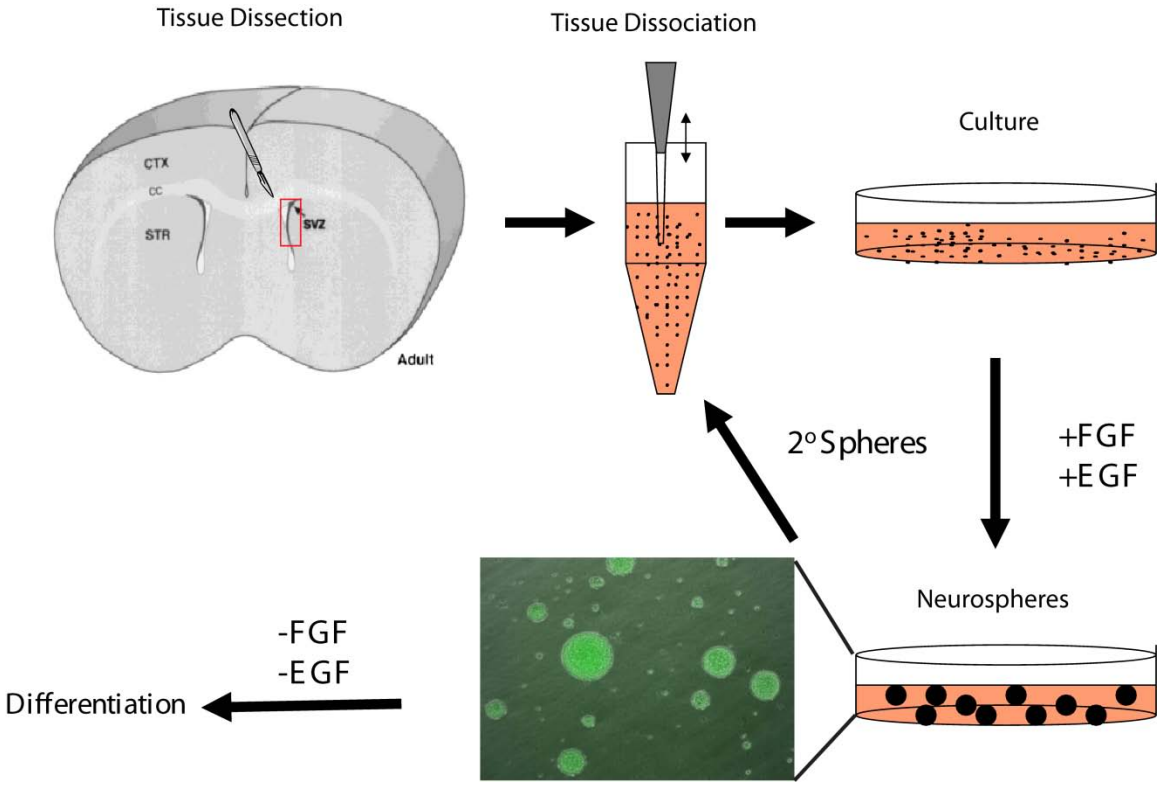
Tissue Dissection
Tissue Dissociation
CTX
CC
STR
SVZ
Adult
Culture
2° Spheres
+FGF
+EGF
Neurospheres
-FGF
-EGF
Differentiation

Figure 1.2 The Neurosphere Assay. To generate neurospheres, a region of interest is dissected from the tissue and enzymatically dissociated into a single cell suspension. These cells are then plated at low density in non-adherent dishes in the presence of growth factors and allowed to proliferate, forming neurospheres. Neurospheres can then either be dissociated and re-plated to generate secondary neurospheres, or induced to differentiate by the withdrawal of growth factors.

Figure 1.3

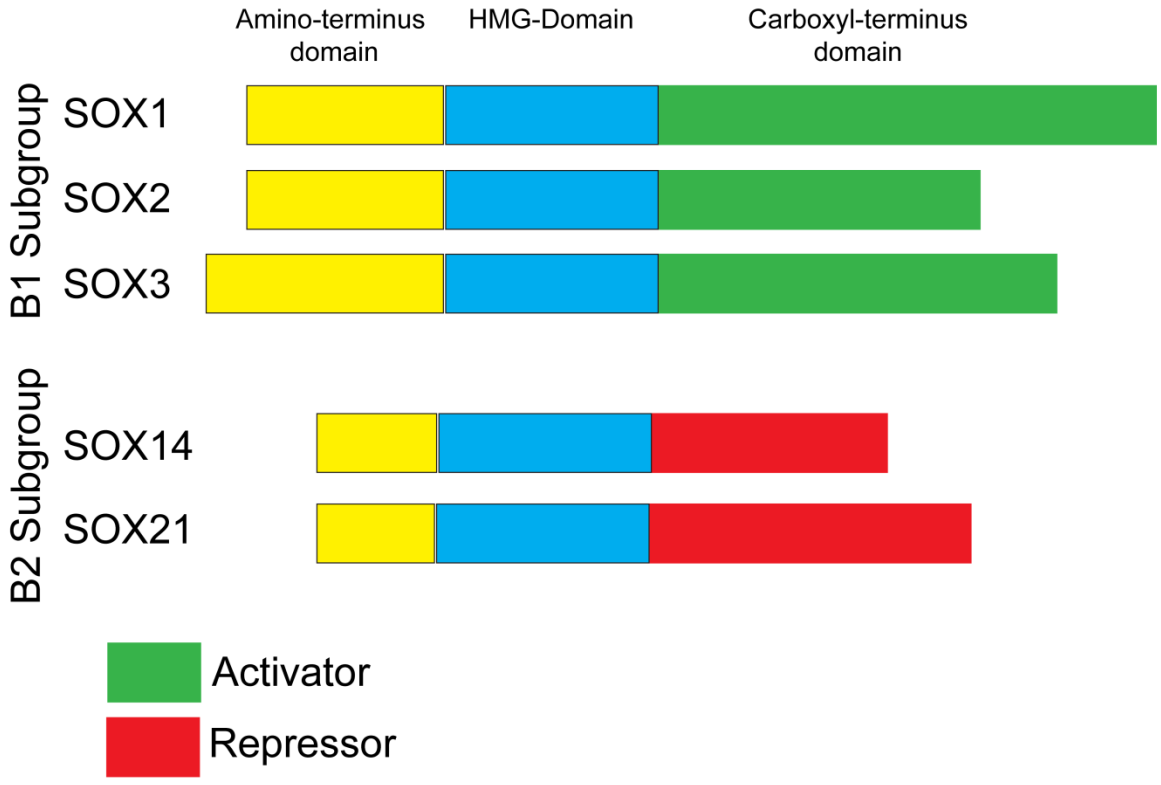
Amino-terminus domain
HMG-Domain
Carboxyl-terminus domain
B1 Subgroup
SOX1
SOX2
SOX3
B2 Subgroup
SOX14
SOX21
Activator
Repressor

Figure 1.3. Structure of SOXB Factors. All SoxB members contain an N-terminus domain (yellow) and HMG-BOX DNA-binding domain (blue). However, the C-terminus domain is different between SOXB1 and SOXB2 member. The C-terminus of SOXB1 members serves as a trans-activating domain (green) whereas the C-terminus of SOXB2 members functions as a trans-repressor domain (red).

Figure 1.4

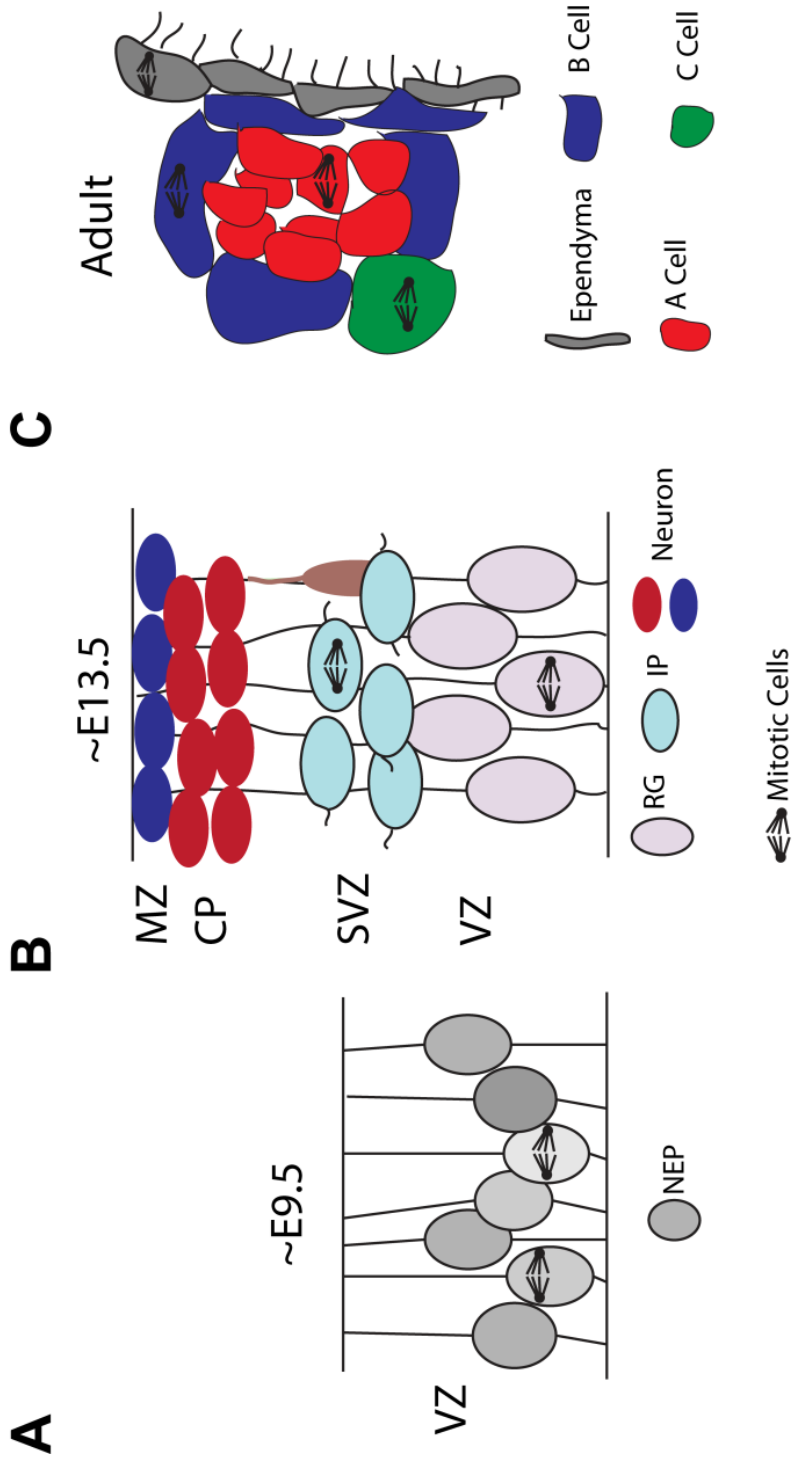
A
~E9.5
VZ
NEP
B
~E13.5
MZ
CP
SVZ
VZ
Neuron
IP
RG
Mitotic Cells
C
Adult
B Cell
C Cell
Ependyma
A Cell

Figure 1.4 Cortical Architecture During CNS Development. (A): At early stages of neural development (~E9.5), the developing CNS consists of a pseudo-stratified sheet of neuroepithelial (NEP) cells which extend the entire width of the neuroepithelium. **(B):** At later stages of neural development, NEP cells have been replaced by Radial Glial (RG) cells which also extend the entire width of the developing dorsal telencephalon, although their soma are restricted to the ventricular zone (VZ). In addition, RG cells also produce Intermediate Progenitor (IP) cells which reside basally in the subventricular zone (SVZ). Both RG and IP cells are capable of generating nascent neurons which migrate along the RG processes to the cortical plate (CP). **(C):** In the adult SVZ, ependymal cells lining the lateral ventricles are capable of proliferating and undergoing neurogenesis. In addition, glial-like Type B cells can also proliferate and generate neurons, usually through an intermediate progenitor-type cell (Type C cell) which then forms a migrating neuroblast (Type A cell). Abbreviations: VZ, ventricular zone; SVZ, subventricular zone; CP, cortical plate; MZ, mantle zone; NEP, neuroepithelial; RG, radial glia; IP, intermediate progenitor.

Chapter Two

The Generation and Characterization of a SOX2-EGFP Mouse Line for the Analysis of Neural Progenitor Populations *In Vivo*

Summary of Chapter

The developing and adult mammalian central nervous system (CNS) contains multiple populations of proliferating neural progenitor cells that are capable of generating neurons and glia *in vivo* and *in vitro* (Gage and Verma 2003; Gil-Perotin et al. 2009). These progenitor populations serve diverse functions throughout ontogeny including: regulating the production of distinct neural cell types in developing CNS tissues, maintaining specific neural populations in regions of high cellular turnover, and upregulating neuronal or glial cell production in response to injury (Farkas and Huttner 2008). However, the ability to identify defined populations of neural progenitor cells *in vivo* has been difficult due to a limited number of molecular markers which recognize these cells. Moreover, many of the markers that have currently been identified, including NESTIN, MUSASHI, NOTCH and Neuronal Cell Adhesion Molecule (NCAM), are not specific for neural progenitor cells (Hockfield and McKay 1985; Lendahl et al. 1990; Weinmaster et al. 1991; Sakakibara et al. 1996; Sakakibara and Okano 1997). This, in turn, has hindered attempts to elucidate the cellular and molecular mechanisms which regulate the cell-fate decisions of neural progenitor cells. This problem is further compounded by the current dependence upon retrospective *in vitro* assays, such as the neurosphere assay, to accurately determine whether a cell exhibits progenitor capacity. Therefore, the development of tools and protocols to prospectively identify, isolate, and characterize distinct neural progenitor cell populations from the CNS is essential to advance the understanding of both the cellular and molecular mechanisms that regulate neural progenitor cell identity and differentiation potential.

The HMG-Box transcription factor SOX2 has also been identified as a neural progenitor cell marker. SOX2 is expressed in a majority of embryonic and adult neural progenitor cells throughout the CNS (Collignon et al. 1996; Wood and Episkopou 1999;

Avilion et al. 2003; D'Amour and Gage 2003; Ellis et al. 2004) and functions to retain the progenitor identity of these cells, serving to maintain both their proliferative and neural differentiation capacities before its expression is downregulated upon neural differentiation (Bylund et al. 2003; Graham et al. 2003; Ferri et al. 2004; Brazel et al. 2005; Miyagi et al. 2006; Taranova et al. 2006; Cavallaro et al. 2008). These properties therefore suggest that SOX2 expression can serve as a valuable marker with which to identify neural progenitor populations *in vivo*. However, as a transcription factor, SOX2 is localized to the nucleus, and therefore its use for immunocytochemistry requires chemical fixation and cell permeablization; process which then preclude the use of labeled cells for *in vitro* culture (O'Leary et al. 2009). Thus, less evasive labeling techniques are required to utilize SOX2 expression in the prospective isolation of viable neural progenitor cells.

In this chapter I detail the tools and techniques that were generated in the lab to address the limitations in prospectively isolating neural progenitor populations. The first section describes the generation and characterization of the $SOX2^{EGFP}$ mouse line. While much of this work was a collaborative effort and published as such, I was responsible for the characterization of SOX2-EGFP expression in the developing and adult CNS of $SOX2^{EGFP}$ mice. The second section of this chapter consists of the modified protocol which I developed in order to increase the efficiency of neural progenitor cell isolation from $SOX2^{EGFP}$ mice that was necessary for the experiments discussed in Chapter 3.

Generation and Characterization of the $SOX2^{EGFP}$ Mouse Line

To overcome the limitations in identifying and isolating neural progenitor cells from the CNS, our laboratory generated a transgenic mouse line ($SOX2^{EGFP}$) in which

the endogenous Sox2 regulatory regions drive the expression of Enhanced Green Fluorescent Protein (SOX2-EGFP). Fluorescent protein expression has been extensively used as a cellular marker for a wide range of applications including fluorescent microscopy, laser dissection, flow cytometery, and fluorescence automated cell sorting (FACS) (Galbraith et al. 1999; Nowotschin et al. 2009). Furthermore, the EGFP variant is stable in that it does not degrade quickly and importantly, unlike other fluorescent protein variants, is non-toxic to cells *in vivo* (Chalfie et al. 1994; Galbraith et al. 1999). This is essential for *in vivo* models in which labeled cells need to be identified and tracked for extensive periods of time as well as *in vitro* models in which cells must be maintained for multiple passages.

To generate the $SOX2^{EGFP}$ mouse line we utilized a genetic "knock-in" approach. This technique ensures that the expression of an inserted transgene faithfully recapitulates the endogenous SOX2 expression due to the maintenance of undisturbed regulatory elements within the SOX2 locus (Nagy et al. 2003). In methods detailed in Section One of this chapter, a targeting vector was generated containing an EGFP-loxP-neomycin-loxP cassette flanked by a 12 kb genomic region 5' to the *Sox2* coding region and a 2.5 kb genomic region 3' to the *Sox2* coding region. The SOX2-EGFP targeting construct was then knocked-in to the endogenous *Sox2* locus by homologous combination into mouse ES cells. Homologously-recombined, EGFP-positive ES clones were identified and injected into E3.5 blastocysts and transplanted into pseudo-pregnant female mice to generate germline chimeras. One mouse line, named the $SOX2^{EGFP}$ line, expresses EGFP in all neurogenic of the CNS throughout ontogeny. In addition, a second mouse line was generated ($SOX2^{Random}$) in which only a subset of neural progenitor populations in the CNS expresses EGFP (Ellis et al. 2004). Here we demonstrate that the $SOX2^{EGFP}$ line not only provides a novel tool for identifying and isolating neural progenitor populations from the embryonic and adult central nervous

system *in vivo*, but also for characterizing SOX2 expression profiles in these cells as well.

Several key findings resulted from these analyses. First, this work demonstrates that SOX2-EGFP expression faithfully recapitulates endogenous SOX2 expression in the embryonic and adult CNS *in vivo*. This finding is important as it demonstrates that the SOX2-EGFP targeting construct is precisely regulated throughout the CNS similar to the endogenous Sox2 gene. Second, I show that SOX2-EGFP expression is co-localized with markers of distinct neural progenitor populations within the CNS, including Nestin (all progenitors), CD24 (Ependyma), GFAP (Type C,), and PSA-NCAM (Type A) in the adult SVZ. Lastly, I demonstrate that all self-renewing, multipotential neurospheres generated from distinct CNS regions, during different stages of development, express SOX2-EGFP, thus establishing the $SOX2^{EGFP}$ mouse line as a valuable tool in the analysis of neural progenitor cell populations. Subsequently, the $SOX2^{EGFP}$ mouse line has been utilized in the characterization of progenitor cell populations outside of the CNS including the tongue, inner ear, and lung (Okubo et al. 2006; Que et al. 2007; Dabdoub et al. 2008)

The ability to analyze viable neural progenitor cell populations *in vitro* requires a method in which to identify and prospectively isolate these cells from CNS tissue. The process of fluorescence-automated cell Sorting (FACS) is ideal for this analysis as it allows for the precise selection of individual cells based upon fluorescent labeling of molecular markers (Galbraith et al. 1999; Maric and Barker 2004). However, current technical limitations only allow for the fluorescent labeling of cell-surface antigens on living cells as the labeling of nuclear-localized proteins, such as transcription factors, requires the chemical fixation of proteins and degradation of the cell membrane; a process resulting in cell lethality (Ibrahim and van den Engh 2007). Few cell-surface antigens have been identified in neural progenitor cells from the CNS, in contrast to the

hematopoietic system in which many have been identified (Maric and Barker 2004; Challen et al. 2009). Furthermore, the two most prominent neural progenitor cell-surface antigens, CD133 and CD34, have been shown to be expressed in a cell-cycle dependent manner and are thus not constitutively expressed in neural progenitor populations (Sun et al. 2009). Therefore, the $SOX2^{EGFP}$ mouse line, in which SOX2-EGFP is endogenously expressed in living cells, overcomes the dependency on cell-surface antigens for FACS and as well as provides a perpetual marker for identifying neural progenitor cell populations *in vivo*.

The generation and analysis of the $SOX2^{EGFP}$ mouse line demonstrates the importance of this valuable tool for the characterization of neural progenitor populations within the CNS (Ellis et al. 2004). Our work shows that SOX2-EGFP expression is expressed in multiple progenitor populations, including ES, ICM, epiblast, and neural progenitor cells, and that this expression faithfully recapitulates endogenous SOX2 expression in these cells. In addition, we demonstrate that SOX2-EGFP is expressed in spatially and temporally distinct neural progenitor populations within the CNS. Lastly, we demonstrate that SOX2-EGFP cells can be cultured *in vitro* to generate neurospheres, and that all neurospheres generated from the $SOX2^{EGFP}$ mouse line, regardless of age or tissue of origin, express SOX2-EGFP. Thus, the $SOX2^{EGFP}$ mouse line identifies a majority of the neural progenitor populations within the CNS and provides a tool in which we can prospectively identify and isolate living neural progenitor populations for analysis.

Development of Methods to Efficiently Isolate and Culture Neural Progenitor Cells

Section Two of this chapter describes a neurosphere assay protocol which was developed to more efficiently analyze the gene expression profiles and *in vitro* characteristics of neural progenitor cell populations from the CNS (Hutton and Pevny

2008). To date, the most efficient method to identify a neural progenitor cell has been through retrospective analysis using *in vitro* culturing assays. Methods such as the neurosphere assay have led to a better understanding of the unique mechanisms which regulate neural progenitor cells from the CNS, however they do not allow for the analysis of neural progenitors in their *in vivo* environment (Reynolds et al. 1992; Reynolds and Weiss 1992). Furthermore, the ability to analyze the molecular and cellular properties of isolated neural progenitor populations, as well as their self-renewal and differentiation capacities *in vitro,* requires a highly efficient method to retrieve large numbers of viable cells from a given tissue. This is especially important in regions where neural progenitor populations are sparse, such as in the rodent adult CNS (<0.1% of cells) (Reynolds and Weiss 1992). The generation of neurospheres requires the dissociation of tissue into single cells and although traditional methods using mechanical or Trypsin-mediated dissociation are effective in this process, they also result in high cell mortality rates in our hands. This, in turn, precludes the ability to accurately analyze certain neurosphere characteristics such as the percentage of neurosphere-forming cells within a cell population. Therefore, to properly isolate and characterize neural progenitor populations isolated from the $SOX2^{EGFP}$ mouse line, it was necessary to develop protocols that efficiently dissociate tissue but also minimize stress to the cells so that they are able to survive during subsequent time-intensive processes such as FACS. A previously published protocol for the *ex vivo* culture of brain slices has utilized the enzyme Papain in place of Trypsin for tissue dissociation (Polleux and Ghosh 2002). The modification of current neurosphere and FACS protocols to utilize this technique has resulted in a method which increases the survival rate of neural progenitor cells after dissociation compared to other neurosphere protocols (Hutton and Pevny 2008). In turn, this allows for a more accurate and thorough characterization of neural progenitor cells *in vitro*.

Conclusion

Collectively, the results presented within this chapter are the first to demonstrate that SOX2 is expressed in distinct classes of neural progenitor cells in the embryonic and adult CNS, including the putative neural progenitor populations identified in the adult SVZ, hippocampus, and spinal cord. Furthermore, we show that SOX2-EGFP expression in the $SOX2^{EGFP}$ mouse line is able to faithfully recapitulate endogenous SOX2 expression in all regions of the CNS. Importantly, this characteristic then allows for the prospective identification, isolation and analysis of distinct populations of living neural progenitor cells from the CNS (Discussed in Chapter 3). In addition, to compliment the use of the $SOX2^{EGFP}$ mouse line in neural progenitor analysis, the development of protocols for the efficient isolation and culture of these cells is important in order to perform the *in vitro* analyses necessary to characterize the self-renewal and differentiation capacities of these cells. Here I have developed modified protocols which increase the number of viable cells that can be recovered after undergoing the processes of tissue dissociation, FACS analysis, and neurosphere culture. Ultimately, both of these tools were necessary for the characterization of SOX2 expression within distinct neural progenitor cells types, as will be discussed in Chapter 3.

SOX2 a persistent marker for multipotential neural stem cells derived from ES cells, the embryo or the adult.

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Abstract

Multipotent neural stem cells (NSCs) are present throughout the development of the central nervous system, persist into adulthood in defined locations, and can be derived from more primitive embryonic stem cells. We show that SOX2, an HMG box transcription factor, is expressed in multipotent neural stem cells at all stages of mouse ontogeny. We have generated transgenic mice expressing green fluorescent protein (EGFP) under the control of the endogenous locus regulatory regions of the *Sox2* gene to prospectively identify neural stem/progenitor cells *in vivo* and *in vitro*. Fluorescent cells co-express SOX2 protein, and EGFP fluorescence is detected in proliferating neural progenitor cells of the entire anterior-posterior axis of the CNS from neural plate stages to adulthood. SOX2-EGFP cells can form neurospheres that can be passaged repeatedly and can differentiate into neurons, astrocytes and oligodendrocytes. Moreover, prospective clonal analysis of SOX2-EGFP positive cells shows that all neurospheres whether isolated from the embryonic CNS or the adult CNS, express SOX2-EGFP. In contrast, the pattern of SOX2-EGFP expression using randomly integrated *Sox2* promoter/reporter construct differs and neurospheres are heterogeneous for EGFP expression. These studies demonstrate that SOX2 may meet the requirements of a universal neural stem cell marker and provides a means to identify cells which fulfill the basic criteria of a stem cell: self-renewal and multipotent differentiation.

Introduction

Neural stem cells are self-renewing multipotent progenitors that give rise to neurons, astrocytes, and oligodendrocytes in the central nervous system (CNS). Recent studies demonstrate that multipotent neural stem cells, with the capacity for at least limited self-renewal, are present throughout development of the nervous system. Initially they can be found in the cells of the neural plate, and then later along the entire antero-posterior axis of the ventricular zone (VZ) of embryonic CNS. In certain locations they persist into adulthood (reviewed in (Barres 1999; Momma et al. 2000). Neural stem cells can also be derived from more primitive embryonic stem cells (Gage 1998), however, the relationship between "stem cell" populations at different stages of ontogeny and different rostro-caudal and dorso-ventral locations remains unclear.

Neural stem cells isolated from embryonic and adult CNS are defined by common properties. First, cells isolated from the embryonic VZ and subventricular zone (SVZ), cells surrounding the adult lateral ventricle (LV) and subgranular zone (SGZ) of the dentate gyrus in the hippocampus, along with cells from the central canal of the adult spinal cord all share the ability to form neurospheres, the ability to self-renew, and the ability to differentiate into neurons, astrocytes and oligodendrocytes *in vitro* (Gritti et al. 1996; Johe et al. 1996; Shihabuddin et al. 1997). Second, both embryonic and adult neural stem cells of the CNS can differentiate appropriately after transplantation into a new host (Brustle and McKay 1995; Campbell et al. 1995; Fishell 1995; Vicario-Abejon et al. 1995). For example, adult hippocampal stem cells can give rise to specific and region appropriate cell types not only in the hippocampus but also in the olfactory bulb (OB), cerebellum and retina (Gage et al. 1995; Suhonen et al. 1996; Takahashi et al. 1998). Stem cells derived from the human embryonic nervous system and expanded *in vitro* by oncogenic immortalization exhibit a similarly broad developmental potential

when transplanted *in vivo* (Flax et al. 1998). Stem cells isolated at different developmental stages do share expression of some universal molecular markers, these include among others, Nestin, Musashi, Notch, B-FABP and NCAM. The expression of the majority of these markers is activated during the initial phases of neural induction (Hockfield and McKay 1985; Frederiksen and McKay 1988; Lendahl et al. 1990; Weinmaster et al. 1991; Sakakibara et al. 1996; Sakakibara and Okano 1997; Johansson et al. 1999; Johansson et al. 1999) and is then maintained in stem cell populations throughout ontogeny. However, these stem cells are also regionally specified, express unique molecular markers and respond differently to growth factors. Consistent with this regional restriction of cell fate *in vivo*, a number of studies have demonstrated the importance of cell autonomous mechanisms in maintaining identity of neuroepithelial cells *in vitro*. It has now been clearly shown that positional markers, which define the rostrocaudal and dorso-ventral identity of stem cells, persist over multiple generations *in vitro* (Nakagawa et al. 1996; Zappone et al. 2000; Hitoshi et al. 2002*)*. For example, neural stem cell colonies derived from embryonic (E) 14.5 cortex and spinal cord differentially express regional marker genes along the anteroposterior axis (Zappone et al. 2000) expression that persists for at least forty generations. In addition, uncultured neural progenitor cells from the cortical VZ of middle-staged ferret embryos can generate neurons in stage-appropriate layers when transplanted to older but not younger hosts, suggesting that they are more restricted in the subtypes of cells they generate (Desai and McConnell 2000). Also, when SVZ progenitors that normally only generate interneurons in the OB are transplanted to the embryonic nervous system they do not give rise to the long projection neurons which are normally generated from endogenous progenitor cells (Lim et al. 1997).

Thus, to date it remains unclear whether there exists a generic neural stem cell, as found in the hematopoietic system. It appears that the CNS consists of heterogenic

stem cells which, although restricted in their potency, all retain the ability to self-renew, to differentiate into neurons and glia, and to express a set of universal markers.

To understand exactly what characteristics define a neural stem cell it is first necessary to elucidate the lineage relationship between the various types of stem cells and how they contribute to the formation and maintenance of the central nervous system. To achieve this certain methodologies need to be developed for the prospective isolation of neural stem cells from defined regions of the CNS during defined developmental stages.

SOX2, an HMG-box transcription factor, is expressed throughout mouse embryogenesis in neural progenitors of the CNS (Collignon et al. 1996; Wood and Episkopou 1999; Zappone et al. 2000). We have been able to isolate neural progenitor cells from embryonic stem cells based on SOX2 expression (SOX selection) (Li et al. 1998). To determine in detail which progenitor populations of the CNS express SOX2, in both the embryo and in the adult, we generated transgenic mice by replacing the SOX2 open reading frame (ORF) with that of enhanced green fluorescent protein (EGFP) at the *Sox2* locus by homologous recombination (SOX2-EGFP) as well as random integration of the targeting construct (SOX2-RANDOM). In the SOX2-EGFP mouse line, EGFP fluorescence recapitulates endogenous SOX2 expression and is restricted to proliferating neural progenitor cells in the CNS from neural plate stages and throughout embryogenesis. Expression of SOX2-EGFP is detected in neurogenic and non-neurogenic regions in the post-natal and adult CNS, including the dentate gyrus of the hippocampus, the SVZ, the ependymal layer surrounding the LV, and the ependyma of the central canal. Moreover, through prospective clonal analysis of SOX2-EGFP positive cells, we demonstrate that multipotential stem cells isolated from embryonic stem cells, the embryonic CNS, and the adult CNS all express SOX2-EGFP. These data provide evidence that SOX2 serves as a universal stem cell marker and can be used to isolate,

characterize, and manipulate neural stem cells by prospective analysis. In contrast, EGFP expression in transgenic mice generated by the random integration of the SOX2-EGFP targeting vector is displayed in only a subset of endogenous SOX2 expression, specifically, it is restricted to the dorsal telencephalon. In the adult SOX2-RANDOM line, EGFP is found in neurogenic regions of the SVZ and hippocampus, but is excluded from the ependymal zone. Moreover, only subsets of neurospheres isolated from the adult LV of these mice are EGFP positive, thus allowing the direct comparison of distinct adult neural stem cell populations.

Results

Generation of the SOX2-EGFP Targeted Mice and SOX2-specific EGFP expression in embryonic stem cell-derived neural progenitor cells.

We have previously shown that SOX2 is restricted to neural progenitors during *in vitro* differentiation of embryonic stem cells and is excluded from postmitotic neurons (Rex et al. 1997; Li et al. 1998; Graham et al. 2003). A SOX2 targeting vector, illustrated in Figure 2.1A, was constructed in which the entire ORF and 1 KB of 3' untranslated region were replaced with an EGFP loxP neomycin loxP cassette. The targeting vector was introduced into ES cells and two independently and homologously targeted ES cell clones were used to generate mouse strains that carried the mutant SOX2-EGFP allele. SOX2-EGFP +/- animals were both viable and fertile (Figure 2.1B).

To examine whether SOX2-EGFP fluorescence recapitulates the SOX2 expression profile we differentiated SOX2-EGFP targeted embryonic stem cells into neuroepithelial derivatives. Following selection for neural progenitors under defined culture conditions (Okabe et al. 1996) EGFP fluorescence, like endogenous SOX2, was observed in clusters of cells with neuroepithelial morphology (Figure 2.1 C-K), these cells were immunoreactive for neural progenitor markers such as SOX1 (Figure 2.1 F-H) and Nestin (Figure 2.1 I-K). Subsequent replating on poly-lysine/laminin coated dishes and incubation in B-27 supplemented Neurobasal medium resulted in differentiation of progenitor cells into β-tubulin-type III + neurons, GFAP+ astrocytes and O4+ oligodendrocytes (Figure 2.1 L-N).

SOX2-EGFP expression is restricted to proliferating neural progenitors during the development of the embryonic CNS.

During early stages of the formation of the nervous system EGFP is detected throughout the neuroepithelial cells of the neural plate and early neural tube in SOX2EGFP/+ embryos. At early gastrulation EGFP is expressed throughout the anterodistal ectoderm of SOX2EGFP/+ embryos, which at this stage corresponds to the neural plate (Figure 2.2 A and 2 B). EGFP is then expressed along the entire anteroposterior axis of the developing nervous system (10.0 *dpc* mouse embryo, Figure 2.2 C and D). After neural tube closure, neuroepithelial cells begin to differentiate into defined classes of neurons at specific dorsoventral positions within the spinal cord (Altman and Bayer 1984). In the early neural tube, proliferating progenitors are organized into a pseudostratified epithelium into which the processes of these cells extend from the inner to outer surface. At later stages the neural tube becomes progressively thicker and can be divided into different zones of proliferation. The proliferating CNS progenitors are largely restricted to the inner ventricular zone (VZ), migrating toward the outer layer after completing their final mitosis. The expression ofSOX2-EGFP is downregulated in a stereotypic ventral to dorsal progression coincident with the restriction of cell proliferation within the developing spinal cord. At early stages (9.5 dpc) EGFP is expressed throughout the cells of the neural tube (Figure 2.2 E). Expression is then precisely downregulated in a ventral to dorsal progression in the regions of neural tube differentiation. For example, EGFP expression is first downregulated in the ventral horns, the region where the first neurons differentiate (10.5 dpc; Figure 2.2 F). Eventually the expression of EGFP (14.5 dpc; Figure 2.2 G) is restricted to the thin VZ surrounding the lumen of the embryonic spinal cord. Overall, the expression of EGFP directly mirrors expression of SOX2 (Figure 2.2 H-K).

EGFP-SOX2 expression marks zones of proliferative neuroepithelial cells throughout the embryonic axis, including the ventricular (Figure 2.3 A-C) and subventricular zone (SVZ) of the cortex and the ventricular cells of the midbrain (Figure 2.3 D-F), hindbrain and spinal cord (Figure 2.3 G-I) as well as proliferative progenitors of the neural retina (Figure 2.3 J-L) and olfactory epithelium (data not shown). The regulation of EGFP expression reflects the post-mitotic downregulation of SOX2 expression, as is evident by the generally mutually exclusive expression of EGFP and β-tubulin-type III (Figure 2.3 C, F, I, L). These data indicate that SOX2-EGFP fluorescence directly correlates with SOX2 expression and both are mutually exclusive of terminal differentiation markers at these stages of development.

SOX2-EGFP expression defines ongoing neurogenesis in post-natal and adult CNS.

Although the vast majority of cells in the mammalian nervous system appear during the embryonic and early postnatal period, new neurons are continuously added in certain regions of the adult brain (Altman and Bayer 1984). The SVZ of the LV (Garcia-Verdugo et al. 1998) and the dentate gyrus of the hippocampus (Gage 1998) are two brain regions with active adult neurogenesis. These neurons are thought to derive from a population of neural stem cells. In the postnatal and adult brain, as illustrated on schematic diagram in figure 2.4 A (taken from (Johansson et al. 1999), the cells of the SVZ, located in association with the LVs of the brain, give rise to immature proliferating neurons that migrate along the rostral migratory stream (RMS) to the olfactory bulb (OB). Once these neurons reach the OB they differentiate and integrate as interneurons.

To date the expression of SOX2 has not been determined in the post-natal or adult CNS. Thus, to characterize the expression of SOX2-EGFP in the neonate and adult EGFP expression was assessed in the SVZ, RMS and OB. The migration of neural

progenitor cells from the LV to the OB is marked by EGFP fluorescence in SOX2-EGFP/+ neonates (Figure 2.4 B). In the adult SOX2-EGFP was expressed in a thin layer surrounding the LV (Figure 2.4 C) that included the SVZ, this EGFP signal extends along the RMS to the OB.

SVZ cell types can be distinguished by their morphological and immunocytochemical characteristics (Doetsch et al. 1999). Interestingly, all ependymal and SVZ cell types express Nestin (Lendahl et al. 1990). Adjacent to the SVZ is the layer of multiciliated ependymal cells that express markers such as CD24 and S100. The migratory neuroblasts (type A cells) of the RMS express a polysialated form of neuronal cell adhesion molecule (PSA-NCAM). Type B cells, the slowly dividing SVZ astrocytes, contain intermediate filament bundles with Glial Fibrillary Acidic Protein (GFAP) - a marker of mature astrocytes as well as the cell surface antigen SSEA-1 (Capela and Temple 2002). Type C cells have recently been shown to express Epidermal Growth Factor Receptor (EGFR) and *Dlx2* (Doetsch et al. 2002). To determine in more detail whether SVZ and RMS cells express SOX2 we used a combination of GFP fluorescence and immunohistochemistry (See Table 2.1). In the adult brain SOX2-EGFP is expressed in proliferating cells of the SVZ as marked by Ki67 expression (Figure 2.4 D-G), ependymal cells surrounding the LV co-express markers such as CD24 (Figure 2.4 H-K) and S100 (data not shown) as well as scattered cells in the SVZ which co-express GFAP (Figure 2.4 L-O), SSEA-1 (data not shown) and EGFR (Figure 2.4 P-S).

In addition to the germinal zone of the SVZ, continued neurogenesis is known to occur in the adult hippocampus: cell proliferation leading to neurogenesis has been described in the dentate gyrus granular layer (Altman and Das 1965; Kuhn et al. 1996). Progenitor cells are found along a thin strip of cells, referred to as the subgranular zone (SGZ), located between the hilar region and the granule cell layer (Gage 1998). Furthermore, progenitor cells can be identified by co-expression of EGFP-SOX2 with

PSA-NCAM, partial co-expression with GFAP, and mutually exclusive expression with NeuN. Consistent with EGFP-SOX2 marking neurogenesis in the adult brain, EGFP fluorescence is mostly confined to the SGZ of the adult hippocampus (Figure 2.5A-I) and co-localizes with PSA-NCAM and GFAP but is mutually exclusive of NeuN expression.

It has also been reported that multipotential cells can be isolated from non-neurogenic regions of the adult mammalian CNS such as the spinal cord (Weiss et al. 1996; Shihabuddin et al. 1997; Johansson et al. 1999; Johansson et al. 1999; Shihabuddin et al. 2000). Recent studies have suggested that the ependymal cells lining the central canal of the postnatal spinal cord possess certain properties of neural stem cells (Johansson et al. 1999). These cells are reported to surround the central canal of the adult spinal cord. Consistent with this we have found SOX2-EGFP positive cells surrounding the ventricle of the adult spinal cord. SOX2-EGFP expression is detected in the ependymal layer of the central canal of the spinal cord where it is coexpressed with PSA-NCAM (Figure 2.5 M-O) but is mutually exclusive of GFAP (Figure 2.5 J-L) and β-tubulin-type III (Figure 2.5 P-R). Taken together these expression data illustrate that SOX2-EGFP fluorescence is localized to regions of the embryonic and adult CNS that have been directly correlated with neurogenic regions of the CNS.

The SOX2-EGFP mouse line analyzed in this study was generated by replacing the open reading frame of *Sox2* with an EGFP expression cassette by homologous recombination, thus all of the endogenous regulatory domains of the *Sox2* gene remain intact. This is in contrast to previous mouse lines generated by random integration of SOX2 promoter regions (Zappone et al. 2000) where the marker expression recapitulates only part of the endogenous SOX2 expression pattern even within restricted regional domains. To directly compare and develop a potential tool to distinguish between CNS stem cell populations we generated mouse lines by random integration of *Sox2* targeting vector (see Materials and Methods) in which only a subset of endogenous SOX2

expression is recapitulated (Figure 2.6 A and B). In the adult brain, EGFP expression is detected in the SVZ, RMS and SGZ of the hippocampus. However, whereas SOX2-EGFP is expressed both in ependymal and SVZ cells (Figure 2.4 and Figure 2.6 C-E, and Figure 2.6 I-K) in mouse line (transgenic-C11) EGFP expression is detected in the RMS and a subset of the SVZ, but is excluded from ependymal cells (see Figure 2.6 F-H, and Figure 2.6 L-N).

Multipotential stem cells isolated from both embryonic and adult CNS are derived from SOX2-EGFP positive cells.

The previous *in vivo* expression analysis demonstrates that SOX2-EGFP is expressed in proliferating neural progenitors throughout the entire axis of the CNS and throughout ontogeny. We therefore used this mouse strain to determine whether SOX2-EGFP can be considered a universal neural stem cell marker, specifically whether all or only a subset of self-renewing and multipotential (i.e. gives rise to neurons, oligodendrocytes and astrocytes) neural stem cell populations of the developing and mature CNS express SOX2-EGFP. To date the most widely accepted method of defining a neural stem cell is by cloning cells *in vitro* and showing that a single cell can self renew and give rise to multiple phenotypes (Gage 1998). In the presence of mitogens such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), dissociated neural cells proliferate and form floating multicellular structures termed neurospheres (Reynolds and Weiss 1996; Nakamura et al. 2000). A proportion of the cells (Morshead et al. 1998) in a neurosphere are clonally derived from a single CNS stem cell/progenitor and are thought to possess the characteristics of CNS stem cells i.e., they have self-renewing activity and are multipotent (Reynolds and Weiss 1996; Nakamura et al. 2000). Using the neurosphere assay we examined whether the EGFP-SOX2 positive cell

population contained neural stem cells that were multipotent and had the capacity to self-renew. Disaggregated cells from the embryonic (cortex and spinal cord (E10.5 and E14.5)) (Figure 2.7 A and B) and the adult nervous system (SVZ) (Figure 2.7 H) were cultured in serum-free medium supplemented with bFGF and EGF. At E10.5 most of the spheres formed from E10.5 neuroepithelial cells coexpressed SOX2-EGFP (Figure 2.7 C) and Nestin (Figure 2.7 D). By 7 days, highly fluorescent spheres had formed (Figure 2.7 E-F and I-J). We next examined the lineage potential of the sphere-initiating cells. For this purpose we transferred spheres formed in theses cultures to poly-lysine/laminin coated dishes and cultured as monolayers for 14d, after which the cell sheets were stained with antibodies against neuronal (β-tubulin-type III) and the general glial marker (GFAP) (Figure 2.7 G and K). After examination, all colonies that were mulitpotential were derived from SOX2-EGFP positive cells (Table 2.2). Finally, to examine the self-renewal capacity of the SOX2-EGFP individual primary spheres were transferred to separate wells and then dissociated into single cells. These single-sphere derived cells were then cultured and assessed for secondary sphere formation. After 7 days, spheres showing EGFP fluorescence similar to the primary spheres were generated. These results further indicate that the sphere initiating cells were both self-renewing and multipotent. The above *in vivo* expression and *in vitro* clonogenic analysis demonstrates that SOX2-EGFP serves as a universal stem cell marker and provides a means by which we can prospectively identify CNS progenitors *in vivo* and select live CNS progenitor cells.

SOX2-RANDOM distinguishes two stem cell populations of the adult brain.

As described above, in transgenic mice generated by random integration of the SOX2-EGFP targeting vector, EGFP fluorescence recapitulates only a subset of

endogenous SOX2 expression. Specifically, in the adult SVZ it is excluded from the ependymal cell layer but found in the adjacent SVZ and RMS (Figure 2.6). The subset of endogenous SOX2 expression reflected by EGFP in SOX2-RANDOM mouse line led us to address what percentage of neurospheres isolated from the SOX2-RANDOM mouse line would be EGFP positive and how this correlates with the *in vivo* identity of stem cells of the SVZ. Using identical conditions to those described above, dissociated SVZ cells from adult SOX-RANDOM mice were cultured in serum-free medium supplemented with FGF and EGF. In contrast to SOX2-EGFP mice, where all multipotent neurospheres generated were EGFP positive (Figure 2.7 I-J), neurospheres from the SOX2RANDOM mouse line were both EGFP positive and negative (Figure 2.7 L-M). The majority of EGFP+ neurospheres expressed GFAP, indicating that they may be derived from type B cells in the subventricular cells (Figure 2.7 P-R). What is more, both EGFP positive and negative spheres from the SOX2-RANDOM mouse line were multipotent, differentiating into neurons and glia. Moreover, both EGFP+ and EGFP- cells (Figure 2.7 N-O) formed multipotent secondary spheres (data not shown). These results indicate that in addition to the type B astrocytic cell located in the SVZ of the LV, there are distinct populations of cells that display stem cell characteristics and retain the capacity to give rise to multipotent neurospheres.

Discussion

To develop an *in vivo* system for analyzing neurogenesis, we generated transgenic mice expressing green fluorescent protein (EGFP) under the control of the regulatory regions of the *Sox2* gene. We show here that SOX2-EGFP remains expressed in proliferating neural progenitor cells throughout vertebrate embryogenesis along the entire rostrocaudal axis including proliferating cells in the mouse adult CNS, specifically in neurogenic regions such as the SVZ of the LV, the SGZ of the hippocampus, and the ependyma of the adult central canal. Moreover, prospective analysis of SOX2-EGFP+ CNS cells indicates that all multipotent neurospheres isolated from the SOX2-EGFP mouse line originate from a SOX2 expressing cell. These results contrast with our and previously published reports utilizing *Sox2/βgal* reporter mice where *Sox2* driven GFP/βgal expression appeared to label a subset of stem cells (Zappone et al. 2000). Of the three lines utilizing the *Sox2* promoter driving βgal or GFP that were integrated randomly in the genome none of the expression patterns matched that of the endogenous gene (see results and (Zappone et al. 2000). The present results provide strong evidence that SOX2, a SOXB1-HMG protein, acts as a universal neural stem cell marker and provides a means to identify cells which fulfill the basic criteria of a neural precursor cell: self-renewal and multipotential differentiation.

The present results show that SOX2 is expressed in proliferating progenitor cells throughout embryogenesis and, to our knowledge, provides the first line of evidence that SOX2 is expressed in neurogenic regions of the adult CNS (Figure 2.3, 2.4, 2.5). Taken together these expression data confirm previous results that have suggested that SOX2 represents a conserved pan-neural molecular marker (Uwanogho et al. 1995; Penzel et al. 1997; Rex et al. 1997; Streit et al. 1997; Mizuseki et al. 1998; Wood and Episkopou 1999; Cremazy et al. 2000; Hardcastle and Papalopulu 2000). SOX2 expression is

present in the neural plate as soon as it can be demarcated from the ectoderm and expression is seen in all proliferating cells. Expression is maintained in culture and clonal analysis confirms that individual SOX2-expressing cells are multipotent self-renewing cells. At later stages of development expression is limited to the proliferating VZ and labeled cells do not express markers of differentiation. Thus, the SOX2-expressing population includes all stem cells present in the brain at all stages of development along the entire rostrocaudal axis. This expression is maintained in culture over multiple passages allowing the use of SOX2-EGFP expression to prospectively identify and follow neural stem cells. We cannot at this stage say that all SOX2-expressing cells in the adult are stem cells due to technical limitations of the neurosphere assay, though we have not observed any restricted clones in any of our experiments.

The inability to prospectively sort and localize stem cells at different stages has been a major issue in neural stem cell biology. Several techniques of negative and positive selection have been reported (reviewed in (Pevny and Rao 2003)) but no single method has been proven to both localize stem cell populations *in vivo* and follow stem cells *in vitro.* For example, negative selection cannot serve to localize cells *in vivo* and some of the candidate negative selection markers may be expressed by subsets of stem cells. CD24 selection for example cannot be used as a negative marker in tissue as it labels a subset of SOX2-expressing ependymal cells. The two positive selection strategies reported are of limited utility in localizing stem cell populations. AC133 expression is seen on astrocytes in human tissue (Majka et al. 2000; Uchida et al. 2000; D'Arena et al. 2002) and the antibody does not cross react with the rodent epitope. RT-PCR indicates that Prominin (AC133 homologue) is expressed by differentiated cells as well (Cai et al. 2002). SSEA1 labels only a subset of stem cells (unpublished results) and also labels differentiated cells in sections (Capela and Temple 2002). Non specific labeling strategies such as Hoechst and Rhodhamine dye uptake (Quesenberry et al.

1999; Hulspas and Quesenberry 2000) cannot be used to localize stem cells *in vivo* or in slice cultures (unpublished results) making it difficult to identify the cell of origin for the neurosphere forming cell, to purify stem cells in large numbers, or prospectively follow them *in vivo*. The transgenic mice that we have developed provide an opportunity to bridge the gap between *in vitro* and *in vivo* results. The persistence of SOX2-EGFP expressing cells in zones of ongoing neurogenesis and the generation of multipotent neurospheres *in vitro* suggest that SOX2 expression can be used to localize stem cells *in vivo* and directly select purified stem cell populations for assessment.

While our data clearly indicate that SOX2 is expressed by all multipotent stem cells at all stages of development it is clear that the population of SOX2 expressing cells, although similar overall in its multipotency and ability to self-renew, is a heterogeneous population. The *in vivo* double labeling studies and the localization of SOX2 expressing cells clearly indicate that in the adult SOX2-EGFP expressing cells are present in distinct locales, express different markers, and likely have varying differentiation capability (Figure 2.4 and 2.5). The present results are consistent with previous reports of such heterogeneity and confirm that this heterogeneity is maintained *in vitro* (see above and (Zappone et al. 2000; Hitoshi et al. 2002)) (reviewed by (Pevny and Rao 2003)). Our experiments comparing multipotent neurospheres derived from the adult LV of SOX2-RANDOM mouse line, in which EGFP reflects only a subset of SOX2 expression, clearly show the heterogeneity of the neurosphere population, and predict that neurospheres isolated from neonatal tissue and later stages of development, are likely to be a heterogeneous population whose properties may differ depending on the region from which the cells are isolated.

The ability to distinguish between neurosphere forming populations using different transgenic strains offers a simple and reliable way to quantitate the degree of heterogeneity, determine the cell of origin and resolve the controversy on the localization

of stem cells in the adult. For example one hypothesis suggests that adult neural stem cells are located in the ependymal layer of the LV of the adult forebrain (Johansson et al. 1999), however, different groups have proposed other possibilities. In the adult, neurosphere forming stem cells may be localized to the type B GFAP+ astrocytic cells in the SVZ or the type C GFAP–cell in the SVZ (reviewed in (Garcia-Verdugo et al. 1998; Alvarez-Buylla et al. 2002) or may represent a distinct population that has not been clearly defined (discussed in (Capela and Temple 2002)).

Our data also provide an explanation for some of these differing results. SOX2 expression is seen in both populations of cells and comparison of neurosphere formation from different transgenic lines confirms that the adult CNS cells that can generate multipotent neurospheres derive from distinct populations *in vivo*. Specifically, only a subset of multipotent neurospheres derived from SOX2-RANDOM transgenic mice are EGFP positive, these neurospheres appear to arise from a GFAP positive population in the SVZ. This mouse line provides a tool by which to distinguish between molecularly distinct stem cell populations.

The ability to identify the stem cell pool *in vivo* and follow it *in vitro* with standard double labeling experiments may provide clues to positional and functional heterogeneity of other proposed stem cell populations. Indeed, preliminary results have suggested such heterogeneity than can be distinguished based on the coexpression of SOX2-EGFP with selected cell surface markers. For example SOX2 is expressed by ependymal cells, as well as cells in the RMS. Double labeling with polysialated NCAM - variously described as being specific to a neuronal progenitor (Mayer-Proschel et al. 1997), expressed by glial progenitors or by stem cells (Keirstead et al. 1999) shows that polysialated NCAM (Marmur et al. 1998) and *Sox2* are co-expressed and in cells in the RMS and in the subventricular region in the forebrain and spinal cord.

SOX genes comprise a large family of different members and play important conserved roles in cell fate specification (reviewed by (Pevny and Lovell-Badge 1997) (Wegner 1999)). There is now increasing evidence that SOX factors may play a global role in maintaining progenitor/stem cell fates in a variety of tissues including the nervous system. Members of the *SOX* gene family are expressed in a variety of embryonic and adult tissues where their expression, and in some cases function, is associated with the specification and/or maintenance of progenitor identity (Pevny and Lovell-Badge 1997; Wegner 1999; Bowles et al. 2000). The expression of SOX2 in all neural stem cell populations supports the likelihood of common generic molecular mechanisms shared by neural stem cells throughout ontogeny. Recent data has provided compelling evidence that members of the SOXB1 family are both sufficient and essential to maintain characteristics that define neural progenitor identity, specifically their proliferative capacity and inhibition of neuronal differentiation (Graham et al. 2003). Specifically, inhibition of SOX2 signaling results in delamination from the VZ, a general loss of pan-neural and regional progenitor markers, and the onset of expression of early neuronal differentiation markers suggesting that constitutive expression of SOX2 inhibits differentiation and promotes precursor cell characteristics. In ES cells SOX2, POU homeodomain proteins (Oct3/4), FGFs (FGF4) and REX-1 interact to maintain the undifferentiated state of ES cells (Dailey et al. 1994). It is intriguing to note that NSC specific POU homeodomain factors, FGF and FGFR, have been described. While speculative, it is possible that a functionally conserved group of gene families evolved to participate in basic stem cell functions, including stem cell self-renewal and maintenance of multipotency and that this overlapping set of gene products may represent a molecular signature of stem cells. Perturbing SOX2 expression or monitoring alteration in EGFP expression when candidate genes are modulated will allow us to dissect out the molecular interaction between interacting regulators of the stem cell state.

Overall our results indicate that SOX2 promoter EGFP transgenic mice can be utilized to visualize regions of neurogenesis throughout ontogeny and provides a means by which to prospectively identify, isolate, and characterize neural progenitor cells at defined developmental stages *in vivo* and *in vitro*. Furthermore, these data indicate that SOX2 signaling defines a transcriptional mechanism shared amongst stage and regionally heterogeneous neural stem cell populations, and that these mice can be used to dissect out the stem cell signature of NSCs. Our future experiments are directed at determining how many classes of adult stem cells are present and determining the functional consequence of the loss of SOX2 expression at different stages of development.

Materials and methods

Generation of SOX2-EGF targeted and SOX2-RANDOM mice.

Mouse *Sox2* genomic clones were isolated from a 129/Sv BAC genomic library. A fragment of floxed PGKneo cassette (Zhang et al. 2001) was obtained by ClaI digestion, followed by fill-in reaction and then BamHI digestion. This fragment was subcloned into pBluescriptKSII that was digested with XbaI, followed by fill-in reaction and then BamHI digestion to give pKSfneo. A 2.5 kb SalI fragment of Sox2 genomic DNA 3' to the coding region was cloned into pKSfneo to obtain pKSfneo2.5Sal. A Not-PmeI fragment containing EGFP (Clontech), ß-actin intron, and rabbit ß-globin polyA sequence (Niwa et. al. 1991) was ligated with NotI-PmeI fragment of pKSfneo2.5Sal pKSIGfneo2.5Sal. A 12.5kb NotI fragment of Sox2 genomic sequence 5' to the coding region was subcloned into NotI site of pKSIGfneo2.5Sal to obtain the targeting vector. The orientation of the NotI fragment was confirmed with restriction mapping. Two independent homologously recombined clones were injected into C57Bl/6J blastocysts to generate chimeric mice that transmitted the mutant allele through the germline. To generate SOX2-RANDOM mouse lines, five random integrant clones were injected into C57Bl/6J blastocysts and three clones generated chimeric mice that transmitted the mutant allele through the germline.

Immunohistochemistry.

Embryos were fixed at room temperature for 1 hour in MEMFA (Li et al. 1998) cryoprotected with 30% sucrose in PBS, and cryosectioned. Frozen sections were incubated overnight at 4°C with primary antibodies. Adult mice were perfused with 4% paraformaldehyde (PFA) and the brains were post-fixed for 2 hours. 15um sections were cut on a Cryostat, blocked in 1% goat serum in PBX/0.1% Triton-X 100 and incubated at

4°C overnight with anti-nestin (mouse IgG, 1:500: Developmental Studies Hybridoma Bank), anti-GFP (mouse IgG, 1:500; Chemicon), anti-β-tubulin III (mouse IgG, 1:1000; Covance), anti-PSA-NCAM (mouse IgM, 1:1000; G Rougon, Univ. of Marseilles), anti-GFAP (mouse, 1:500; Boehringer Mannheim), anti-S100 (mouse IgG, 1:500 DAKO), mEGF-R (1:50; Upstate Biotechnology), mCD24 (heat stable antigen) (mouse IgG, 1:1000), O4 (mouse IgM, 1:2000; Chemicon) Secondary antibodies Cy3-anti-mouse, Cy3-anti-rabbit, FITC-anti-mouse (Sigma) were used to visualize immuno-staining. Primary antibody was omitted resulted in no immunostaining for all antibodies except SOX2 where some staining was observed in the parenchyma of the adult spinal cord.

Neurosphere Preparation, Differentiation and Immunostaining.

For neurosphere cultures disaggregated cells were treated as described previously (Reynolds and Weiss 1996; Kawaguchi et al. 2001; Shimazaki et al. 2001). Secondary sphere formation: the number of spheres was counted approximately 14 days later. After mechanical dissociation of each sphere into single cells, each pool of cells derived from single spheres was cultured again for secondary sphere formation. For differentiation assays, spheres at 10-14 days were in vitro plated onto poly-D-lysine/laminin coated chamber slides and cultured for another 5-7 days in DMEM/F12 containing 1% fetal bovine serum (FBS). Cells were observed under an inverted fluorescent microscope (LEICA) equipped with a digital camera (SPOT2). Neurospheres were prepared from the SVZ of the lateral wall of the LV of adult mice, passaged, differentiated, and immunostained as described (Doetsch et al. 1999).

Table 2.1

Marker	LV (Ependyma)	LV (SVZ) A	B	C	RMS	Hippocampus (SGZ)	Spinal Cord (Ependyma)
EGFP (targeted)	+	+	+	+	+	+	+
EGFP (random)	-	+	+	nd	+	+	-
SOX2	+	+	+	+	+	+	+
Nestin	+	+	+	+	+	+	+
GFAP(mono)	-	-	-	+	+	+	-
PSA-NCAM	+	+	-	-	+	+	+
CD24 (HSA)	+	+	-	-	-	-	+
S100	+	-	-	-	+/-	-	+
EGFR	-	nd	+	+	+	-	-
SSEA1	-	nd	+	+	nd	nd	nd

Table 2.1. Comparison of EGFP expression with markers of distinct adult neural cell populations

Table 2.2

Mouse Strain	Age and Region	Number of Positive Multipotent Primary Spheres
SOX2-EGFP homologous recombinant	10.5 dpc neural tube	58/58
SOX2-EGFP " "	14.0 dpc cortex	64/64
SOX2-EGFP " "	14.0 spinal cord	40/40
SOX2-EGFP " "	Adult SVZ	39/39
SOX2-RANDOM " "	Adult SVZ	7/20

Table 2.2. Number of multipotent primary neurospheres derived from the embryonic and adult CNS of SOX2-EGFP transgenic mice.

Figure 2.1

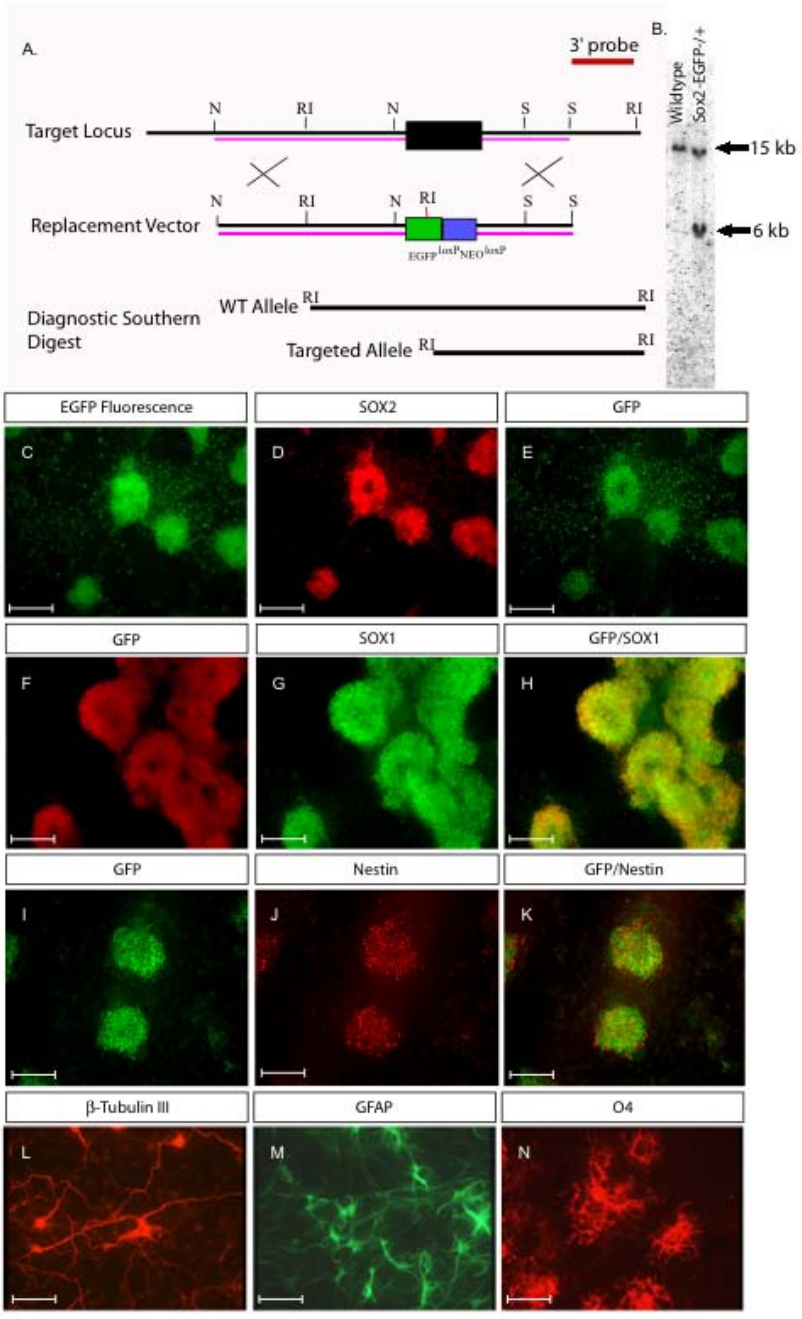

A.
3' probe
Target Locus
N
RI
N
S
S
RI
Replacement Vector
N
RI
N
RI
S
S
EGFP loxP NEO loxP
Diagnostic Southern Digest
WT Allele
RI
RI
Targeted Allele
RI
RI
B.
Wildtype
Sox2-EGFP-/+
15 kb
6 kb
EGFP Fluorescence
SOX2
GFP
C
D
E
GFP
SOX1
GFP/SOX1
F
G
H
GFP
Nestin
GFP/Nestin
I
J
K
β-Tubulin III
GFAP
O4
L
M
N

Figure 2.1. **Strategy applied to generate SOX2-EGFP targeted mice.** A. The genomic structure of the wild-type *Sox2* locus (top) and the structure of the targeting vector (bottom). The targeting vector contains 12kb of genomic sequence 5' and 2.5kb 3' to the *Sox2* coding region. The entire ORF and 1 kb of 3' untranslated region were replaced with an EGFP loxP neomycin loxP cassette. The neomycin (neo) was used for positive selection in ES cells. The probe used for Southern hybridization is shown (P), as are the sizes of the predicted DNA fragments obtained after EcoRI digestion of wildtype and mutant alleles. B. Southern blot analysis of EcoRI-digested genomic DNA from wildtype and SOX2-EGFP+/- animals. C-K. EGFP fluorescence (C) and SOX2 antibody (D) and GFP antibody (E, F, I) immunostaining images of rosettes of neural progenitors, which express SOX1 (G and H) and Nestin (J and K), derived from SOX2-EGFP embryonic stem cells plated in N2B27+bFGF. L-N. β-tubulin-type III (L), GFAP (M) and O4 (N) immunostaining of neurons, astrocytes and oligodendrocytes, respectively, generated 10-14d after replating monolayer progenitors in N2B27 (plus 1% serum for oligodendrocytes). Scale Bars: C-N = 100 μm.

Figure 2.2

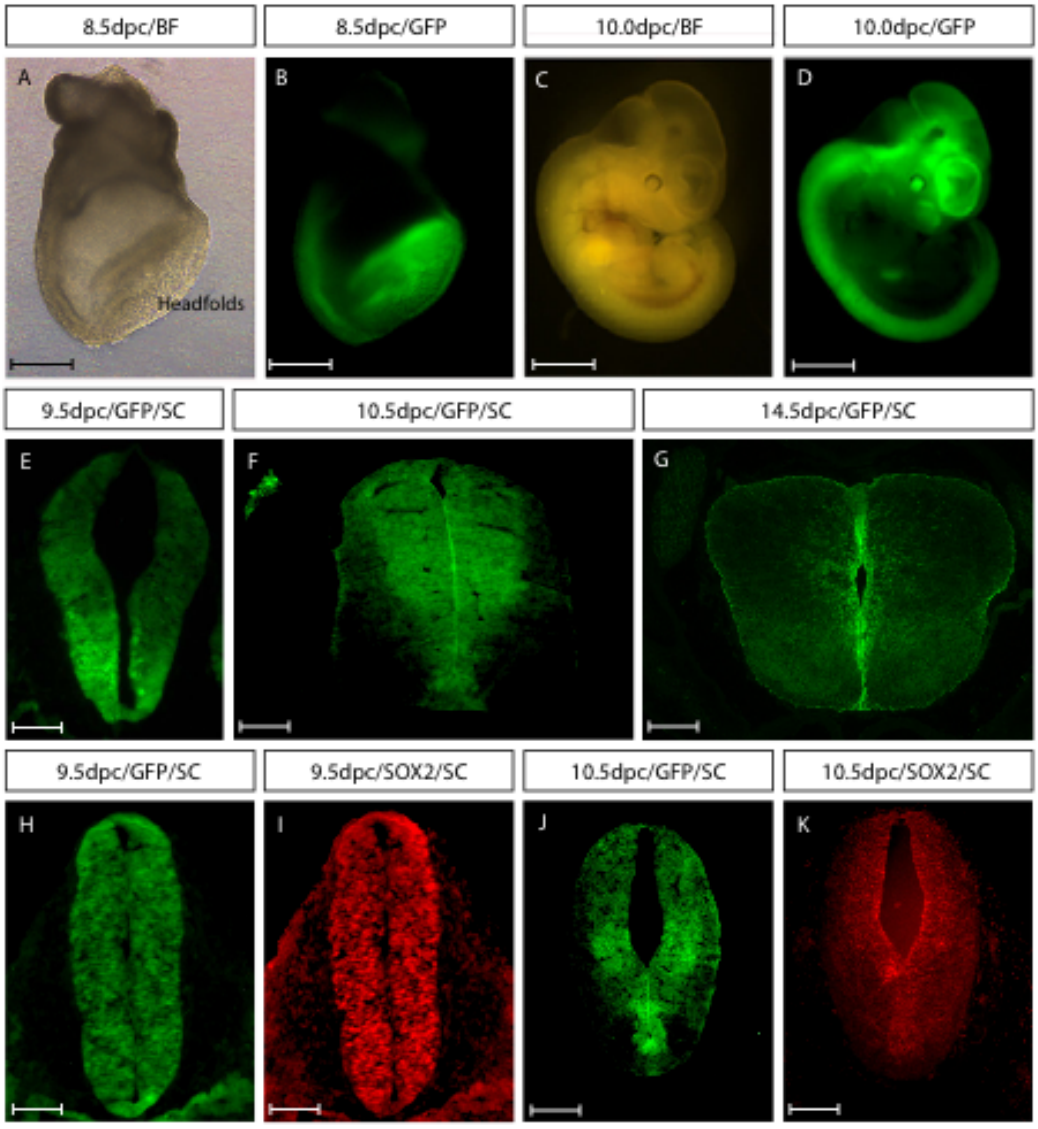

8.5dpc/BF
8.5dpc/GFP
10.0dpc/BF
10.0dpc/GFP
A
B
C
D
Headfolds
9.5dpc/GFP/SC
10.5dpc/GFP/SC
14.5dpc/GFP/SC
E
F
G
9.5dpc/GFP/SC
9.5dpc/SOX2/SC
10.5dpc/GFP/SC
10.5dpc/SOX2/SC
H
I
J
K

Figure 2.2. SOX2-EGFP expression is restricted to proliferating neural progenitors during the development of the embryonic CNS. (A-B). Bright Field (BF) image and EGFP expression in an embryonic day 8.5 (E8.5) mouse embryo. EGFP is expressed throughout the anterior/distal ectoderm that at this stage corresponds to the neural plate. (C-D). EGFP expression in an embryonic day 10.0 mouse embryo. EGFP is expressed throughout the neuroepithelium along the entire anteroposterior axis. (E-G). Localization of EGFP in transverse thoracic sections through the spinal cord (SC) of mouse embryos (9.5 dpc – 14.5dpc). (E). At 9.5 dpc EGFP is expressed throughout the majority of cells in the neural tube. (F). At 10.5 dpc EGFP fluorescence is restricted to the medial VZ and excluded from ventro-lateral regions of the neural tube. (G). By 14.5 dpc EGFP fluorescence is restricted to a small population of cells around the lumen of the neural tube. (H-K) EGFP expression recapitulates endogenous SOX2 expression during the development of the mouse spinal cord. Scale bars: A & B = 75 µm, C-K = 100 µm.

Figure 2.3

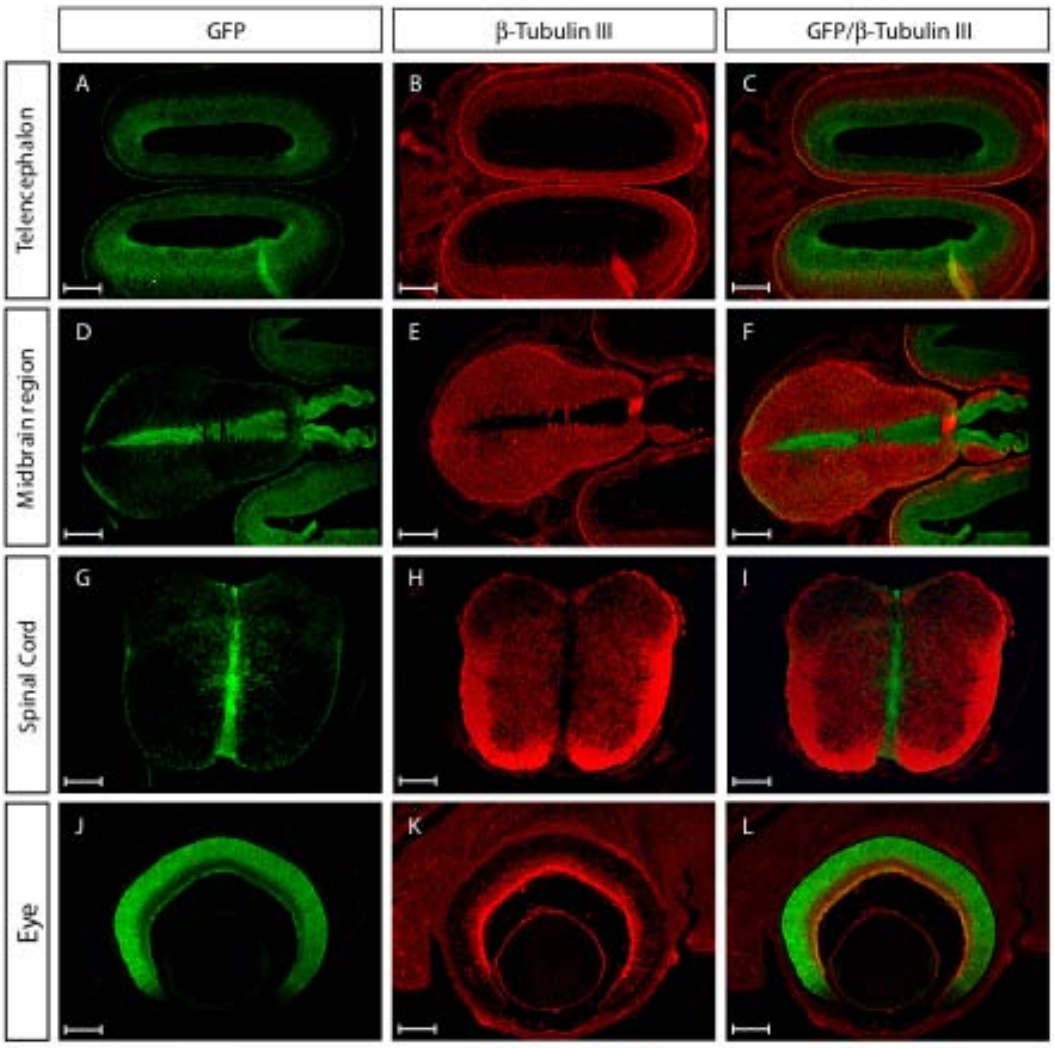

GFP
β-Tubulin III
GFP/β-Tubulin III
Telencephalon
Midbrain region
Spinal Cord
Eye
A
B
C
D
E
F
G
H
I
J
K
L

Figure 2.3. EGFP expression is mutually exclusive of post-mitotic markers. (A-I). Localization of EGFP expression in transverse sections through the telencephalon, mesencephalon and spinal cord of E 13.5 SOX2EGFP embryos. EGFP (green) expression is mutually exclusive of marker of differentiated post-mitotic neurons marked by β-tubulin-type III (red). (J-L). EGFP expression is detected in the proliferating neural retinal progenitors but excluded from β-tubulin-type III positive neurons. Scale bars: A-L = 100 μm.

Figure 2.4

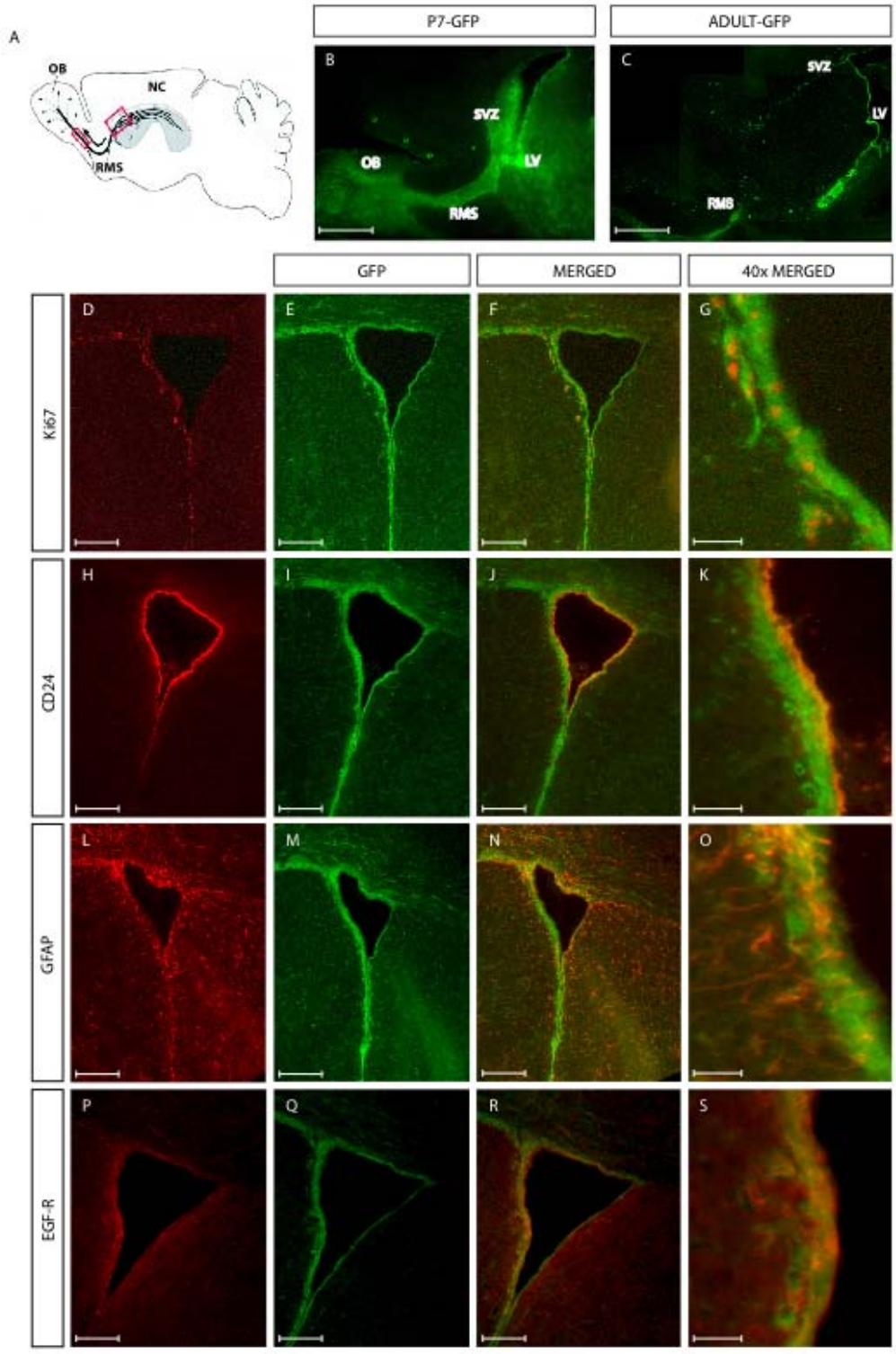

A
OB
NC
RMS
P7-GFP
ADULT-GFP
B
SVZ
LV
OB
RMS
C
SVZ
LV
RMB
GFP
MERGED
40x MERGED
Ki67
CD24
GFAP
EGF-R
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S

Figure 2.4. Overview of EGFP expression in neonatal and adult brain and immunohistochemical characterization of EGFP-expressing cells in the lateral ventricle. (A). Schematic diagram of neurogenic regions of adult mammalian brain. (B). P7 brain. Strong EGFP fluorescence is observed in the SVZ, RMS and OB. (C). Adult brain (6 months after birth). EGFP fluorescence is observed in the SVZ and RMS. (D-S) Superimposition of EGFP signal and immunoreactivity for marker proteins (red) (D-G) SOX2-EGFP is expressed by dividing cells expressing Ki67. (H-K) SOX2-EGFP is expressed in CD24 positive ependymal cells (L-O) SOX2-EGFP is expressed in a subset of GFAP positive cells of the SVZ. (P-S) SOX2-EGFP is expressed by EGF-R positive cells in the SVZ. Scale bars: B & C = 200 μm, D-F, H-J, L-N, P-R = 100 μm, G, K, O & S = 50 μm.

Figure 2.5

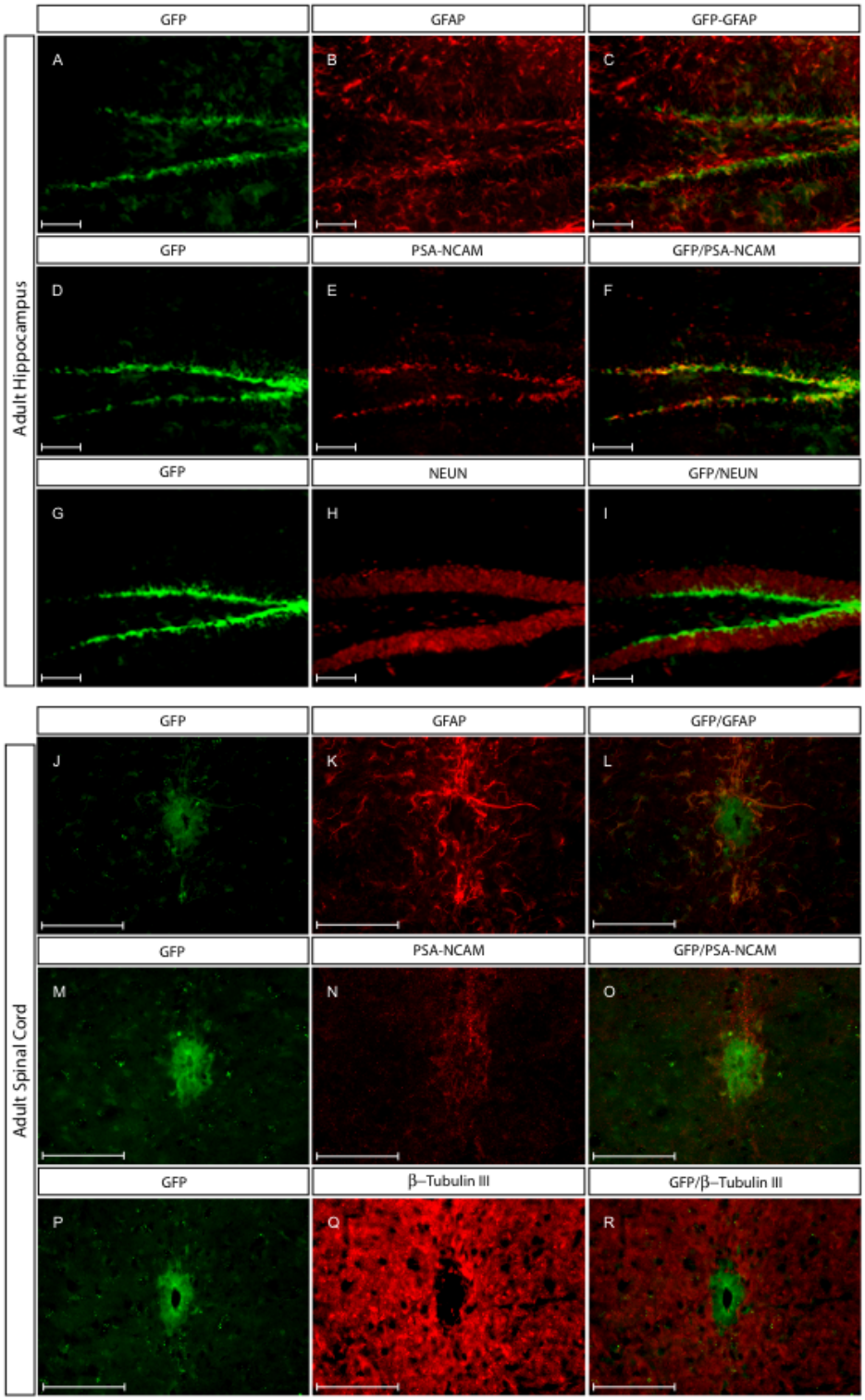
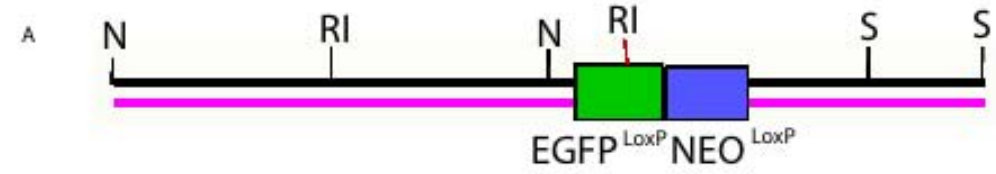

Adult Hippocampus
GFP
GFAP
GFP-GFAP
A
B
C
GFP
PSA-NCAM
GFP/PSA-NCAM
D
E
F
GFP
NEUN
GFP/NEUN
G
H
I
Adult Spinal Cord
GFP
GFAP
GFP/GFAP
J
K
L
GFP
PSA-NCAM
GFP/PSA-NCAM
M
N
O
GFP
β–Tubulin III
GFP/β–Tubulin III
P
Q
R

Figure 2.5. SOX2-EGFP marks neurogenic regions of the adult hippocampus and spinal cord. Localization of EGFP in transverse sections through the adult (> 6 weeks) SOX2-EGFP mice. EGFP expression is restricted to the SGZ of the dentate gyrus where (A) EGFP is coexpressed with GFAP (B-C) and PSA-NCAM (D-F) but is mutually exclusive of markers of terminally differentiated neurons, NeuN (G-I). EGFP expression is restricted to the ependymal layer of the central canal (J, M, P). Immunostaining of the periventricular region with anti GFAP (J-L), anti-PSA-NCAM (M-O) and anti-β-tubulin-type III (P-R). Scale bars: A-R = 100 μm.

Figure 2.6



B

# of independent integrations	Embryonic telencephalon	Adult forebrain excluding the ependyma	Ectopic expression
3	3	3	0

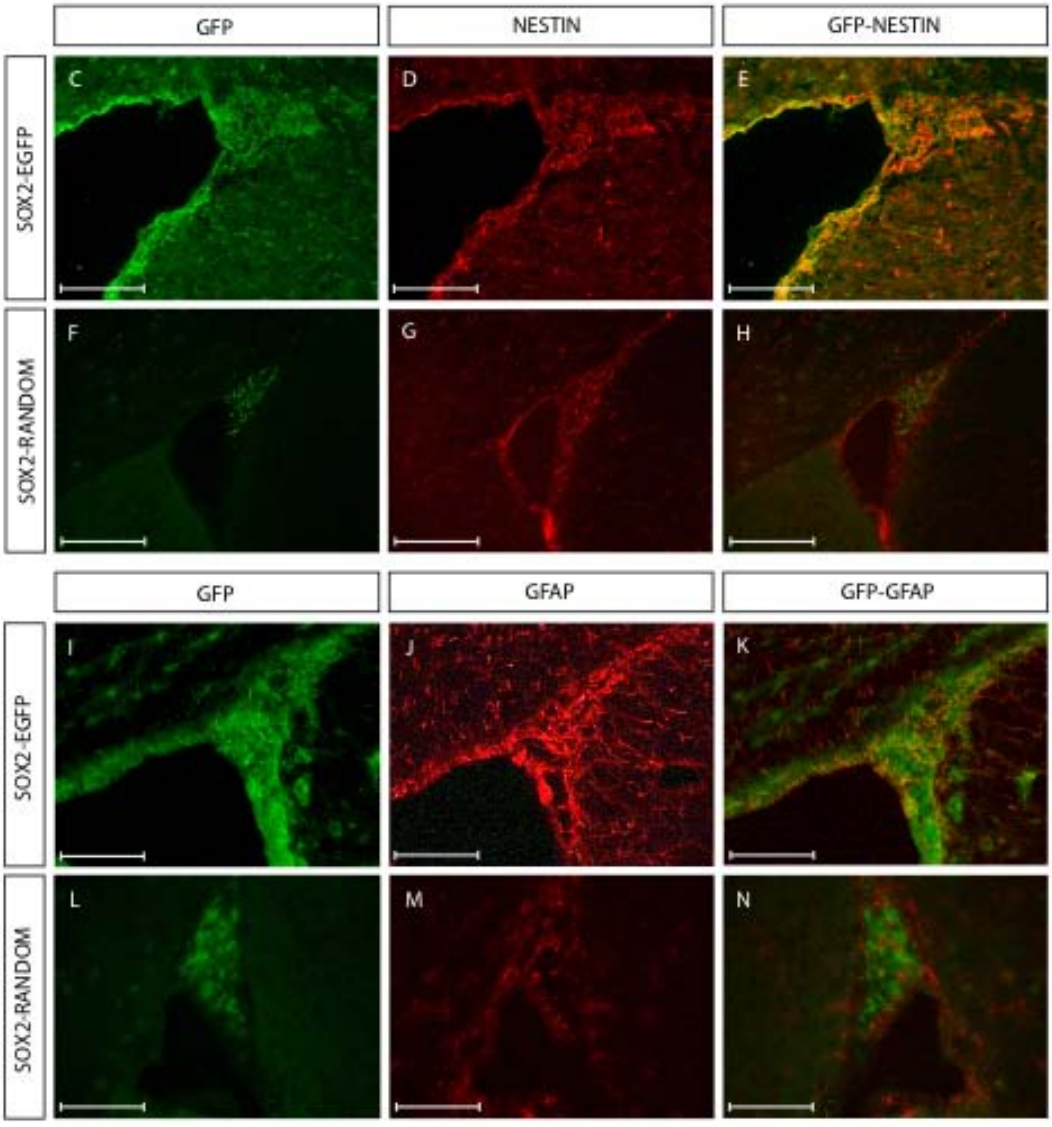


Figure 2.6. In SOX2-RANDOM mouse line EGFP expression marks a subset of SOX2 expressing cells. Localization of EGFP fluorescence in coronal section of the SVZ in SOX2-EGFP and SOX2-RANDOM adult mice (>6 weeks) immunostained for Nestin (red) and GFAP (red). (A) Schematic diagram of DNA construct injected to generate SOX2-RANDOM mouse lines. (B) Summary of mouse strains generated with *Sox2* targeting vector. (C-E) In SOX2EGFP adult mice EGFP fluorescence colocalizes with Nestin expression both in the ependymal and SVZ zone. (F-H) In SOX2RANDOM adult mice EGFP fluorescence is excluded from the Nestin (red) positive ependymal zone but is co-localized with Nestin in the SVZ region surrounding the LV. (I-N) EGFP fluorescence in both SOX2-EGFP and SOX2-RANDOM is co-expressed with GFAP in the SVZ region. Scale bars: C-N = 100 μm.

Figure 2.7

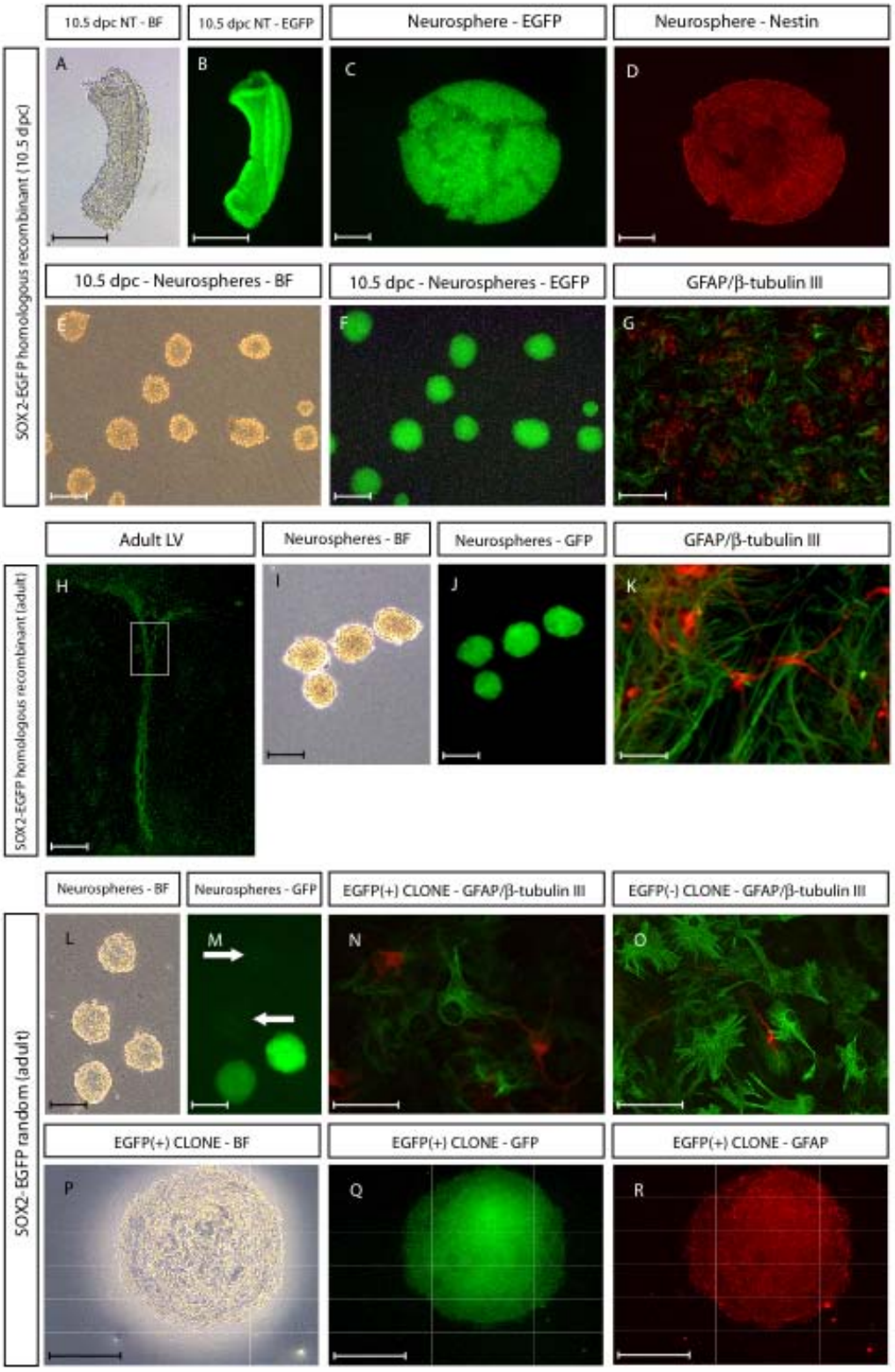

SOX2-EGFP homologous recombinant (10.5 dpc)
10.5 dpc NT - BF
10.5 dpc NT - EGFP
Neurosphere - EGFP
Neurosphere - Nestin
A
B
C
D
10.5 dpc - Neurospheres - BF
10.5 dpc - Neurospheres - EGFP
GFAP/β-tubulin III
E
F
G
SOX2-EGFP homologous recombinant (adult)
Adult LV
Neurospheres - BF
Neurospheres - GFP
GFAP/β-tubulin III
H
I
J
K
SOX2- EGFP random (adult)
Neurospheres - BF
Neurospheres - GFP
EGFP(+) CLONE - GFAP/β-tubulin III
EGFP(-) CLONE - GFAP/β-tubulin III
L
M
N
O
EGFP(+) CLONE - BF
EGFP(+) CLONE - GFP
EGFP(+) CLONE - GFAP
P
Q
R

Figure 2.7. SOX2-EGFP universally marks multipotential neurosphere forming cells in the embryonic and adult nervous system. (A-B) Isolated neuroepithelium from 10.5 dpc SOX2-EGFP mouse embryos (A) Bright field (B) EGFP fluorescence. The majority of EGFP positive neurospheres from E10.5 EGFP+ neuroepithelial cells (C) are NESTIN positive (D). All neurospheres isolated from 10.5 dpc neuroepithelium are EGFP positive (E-bright field, F-EGFP) and are multipotent (G - neurons (β-tubulin-type III - red) and glial cells (GFAP-green)) Neurospheres derived from adult anterior LV SVZ of SOX2-EGFP mouse (H) are EGFP positive ((I, J), (I) bright field – (J) EGFP fluorescence). K. EGFP positive neurosphere are multipotent and give rise to neurons (β-tubulin-type III - red) and glial cells (GFAP-green). SOX2-RANDOM distinguishes two-neurosphere-forming populations of the adult SVZ. (L) Bright field photograph of neurospheres derived from SOX2-RANDOM SVZ cells. (M) Fluorescent image of the same field in (L) showing that some of the neurospheres generated are EGFP positive and some negative (arrow). The majority of EGFP-positive neurospheres from SOX2-RANDOM express GFAP (N-P). Both GFP positive and GFP negative SVZ cells generate neurospheres that can be passaged to make secondary neurospheres and are multipotent generating neurons (red) and glial (green) cells (Q, R). Scale bars: A, B, G, H, K = 100 μm, E, F, I, J, L, M = 200 μm, N & O = 50 mm, P-R = 1000 μm.

The Isolation, Culture and Differentiation of Progenitor Cells from the Central Nervous System

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Introduction

The ability to prospectively identify and characterize neural progenitor cells *in vivo* has been difficult due to a lack of cell-surface markers specific for these cell types. A widely used *in vitro* culture method, known as the Neurosphere Assay (NSA), has provided a means to retrospectively identify neural progenitor cells as well as to determine both their self-renewal capacity and their ability to generate the three primary cell types of the nervous system; neurons, astrocytes and oligodendrocytes. Today, coupled with the establishment of multiple transgenic mouse strains expressing fluorescent markers and advances in cell isolation techniques such as Fluorescent-Activated Cell Sorting (FACS), the NSA provides a powerful system to prospectively elucidate neural progenitor characteristics and functions. Here we describe methods for the isolation, culture, and differentiation of neural progenitors from the developing mouse and adult cortex.

RELATED INFORMATION

Protocol is adapted from Polleux and Ghosh (2002).

Materials

Reagents

Bovine Serum Albumin (BSA) (Sigma A3294), 1% in sterile 1X Dulbecco's Phosphate Buffered Saline (PBS)

Sterile filter and dilute to 0.1% BSA with 1X PBS.

<recXXXX>Dissociation medium (DM)

Epidermal Growth Factor (EGF) and basic Fibroblast Growth Factor (bFGF) (Gibco 13247051 and 13256029)

Add 1 ml of 0.1% BSA solution to 10 µg bFGF or 100 µg EGF to prepare solutions of 10 ng/µl and 100 ng/µl respectively. Freeze at -20°C in 50-µl aliquots.

Mice-Embryonic or Adult

<recXXXX>Enzyme solution (ES)

Prepare immediately before use.

<recXXXX>Heavy inhibitory solution (HI)

Prepare immediately before use.

Laminin (Sigma L2020), stock solution of 1 mg/ml in 0.1-ml aliquots. Store at -20°C.

Just before use, dilute 0.1 ml of laminin stock solution in 5 ml of sterile 1X PBS supplemented with Ca++ and Mg++ (Sigma D8662; a 1:50 dilution); see Step 13.

<recXXXX>Light inhibitory solution (LI)

Prepare immediately before use.

<recXXXX>Neurobasal medium

<recXXXX>NEP basal medium

NEP basal medium containing 2% heat-inactivated horse serum (Invitrogen 16050130, 30 minute inactivation at 60°C) is also required; see Step 16.

In addition, prepare NEP complete medium by adding 10 µl of EGF (100 ng/µl) and 10 µl of bFGF (10 ng/µl) to 10 ml of NEP basal medium; see Step 10.

Dulbecco's Phosphate Buffered Saline (PBS), 1X sterile (Sigma D8537)

Paraformaldehyde (PFA-Sigma P6148), 4% in 1X PBS

Poly-D-lysine (Sigma P7280), reconstitute in sterile ddH20 for a stock solution of 1 mg/ml in 1.0-ml aliquots

Just before use, dilute the stock solution 1:50 in sterile ddH_2O; see Step 12.

Trypsin-EDTA (Sigma T3924) (optional; see Step 18)

Equipment

Polystyrene Dishes, 6- or 10-cm, non-treated (Falcon 351007 and 351029)

Polystyrene 96-well plates, Low Cell Binding, Flat Bottom (Corning 3474)

Forceps, #5 pointed, sterilized (Fine Science Tools 11252-30)

Microsurgical knife (5mm) (MSP Surgical 7516) (optional, see Step 1.i)

Micro-spring scissors (8.5cm), sterilized (Fine Science Tools 15009-08)

Razor blade, sterile

Slides, 8-well chamber (Nunc 177402)

Transfer pipettes, disposable (Fisher 13-711-9D)

Tubes, 15- and 50-ml conical, sterile

Tubes, 1.5ml microcentrifuge tubes, sterile

Water bath set to 37°C

Hemacytometer

Methods
Dissection

1. Carefully remove the brain from the skull of an embryonic or adult mouse and place in a clean dish containing ice-cold 1X PBS. To dissect early embryo tissue (<E16), follow Step 1.i. To collect tissue from a late-stage embryo (>E16) or adult, follow Step 1.ii.

i. Separate the two hemispheres of the brain. Carefully separate the region of interest (e.g. dorsal telencephalon) using a microsurgical knife (Fig 2.8). Next, using fine-tipped forceps and a microsurgical knife, carefully remove the meninges from the tissue.

Meninges must be removed from the tissue, since they will not digest efficiently in Enzyme solution.

ii. Using a razor blade, cut a coronal slice of the brain containing the region of interest (Lateral Ventricle, Hippocampus, etc.) (Fig. 2.9a). Carefully remove the tissue of interest using the forceps and microsurgical knife (Fig. 2.9b).

2. Using a sterile transfer pipette, carefully transfer the tissue to a non-treated polystyrene dish containing cold 1X PBS. To speed up enzymatic digestion, cut the tissue into smaller pieces using the micro-spring scissors.

3. Using a sterile transfer pipette, transfer the tissue to a 15-ml conical tube containing 10 ml of Enzyme solution. Minimize the amount of PBS transferred with the sample. Incubate at 37°C for 20 minutes, carefully mixing approximately every 5 minutes. Do not vortex.

4. Add another 10 ml of Enzyme solution. Incubate for 20 minutes at 37°C, mixing occasionally.

Incubation times may vary. The tissue is ready when it achieves a thick, viscous consistency.

5. In a sterile hood, carefully remove the Enzyme solution using a pipettor, leaving the tissue at the bottom of the tube.

6. Add 4.5 ml of LI solution to the tube. Carefully flick the tube, remove the solution, and repeat with another 4.5 ml of LI solution.

Caution-The tissue will go into solution easily and should not be mixed with a pipette.

7. Remove the LI solution, leaving the tissue at the bottom of the tube, and add 6 ml of HI solution. Incubate for 2 minutes at 37°C. Gently remove the HI solution.

8. Add 5 ml of NEP basal medium, flick the tube, and remove the medium.

9. Add 0.5ml -1.0 ml of NEP Complete medium and triturate 10 to 20 times, until the tissue pieces are dissociated.

More medium may be required, depending upon the amount of tissue used.

10. Count the cells using a hemacytometer and add the appropriate number to a non-treated polystyrene dish containing NEP complete medium (with FGF and EGF).

Cell density should be $1x10^6$ cells per 6-cm plate, or $2x10^6$ cells per 10-cm plate. However, the plating density will vary between different age points and different brain regions. See Discussion.

11. Incubate cells in a humidified 37°C incubator (+5% CO_2). Monitor the dishes daily for neurosphere formation (Fig 2.10a).

Adult lateral ventricle neurospheres take approximately 1 week to form, while embryonic neurospheres are observed after a few days. Once spheres have formed, replace medium every three days by transferring spheres to a 15-ml conical tube and letting them settle to the bottom by gravity at 37°C. Centrifugation is not recommended. After the spheres have settled, remove the medium and replace with fresh NEP complete medium. Transfer spheres to a fresh dish.

Slide Coating

12. Add 0.2 ml of diluted Poly-D-lysine to each well of an 8-well chamber slide. Incubate for 1 hour at room temperature.

13. Wash wells three times with sterile ddH_2O. Add 0.2 ml of diluted laminin to each well and incubate overnight at 4°C.

Slides should be stored at 4°C until use and wrapped in parafilm to minimize evaporation.

14. Before use, remove laminin and wash wells once with Neurobasal medium.

Do not remove the laminin from the wells until immediately before use.

Differentiation

15. Add 100 µl of NEP complete medium to each well of a poly-D-lysine/laminin coated 8-well chamber slide (prepared as described in Steps 12-14). Transfer one sphere using a P200 pipettor to each well and incubate overnight at 37°C.

16. After 24 hours, ensure spheres have attached to the slide (Fig. 2.10B), then carefully remove medium. Add 200 µl of NEP basal medium containing 2% heat-inactivated horse serum.

17. Culture for 2-3 days, changing medium daily. For fixation, remove medium, rinse once with 1x PBS, and fix with 4% PFA in 1X PBS for 1 hour at 4°C for immunohistochemical analysis Fig. 2.10C).

Secondary Neurospheres

18. Using a P20 or P200 pipettor, mechanically dissociate a single neurosphere in a 1.5-ml microcentrifuge tube.

If this is difficult, incubate spheres in trypsin for five minutes and mechanically dissociate.

19. Centrifuge the cells at 3000 rpm for 5 minutes and resuspend in NEP complete medium. Cells can be plated individually in 96-well, low cell binding plates or multiple spheres can be bulk passaged as described in Step 10.

Discussion

The development of a cell culture system designed to isolate and propagate putative stem cells from neural tissue (Reynolds et al. 1992; Reynolds and Weiss 1992)

has greatly advanced our understanding of these cell populations. This technique has not only provided a means to monitor the physical characteristics of these cells but has also enhanced our ability to manipulate the genetic and epigenetic factors that regulate both their capacity to self-renew as well as differentiate into the three defined cell types of the nervous system; neurons, astrocytes, and oligodendrocytes. In the presence of EGF and/or bFGF, these cell populations can be reliably expanded and maintained in the form of neurospheres and, upon removal of these growth factors, can efficiently generate the three major CNS cell types.

Today, advances in mouse genetic manipulation as well as the development of more powerful analytical technologies (e.g. Fluorescent-automated Cell Sorting) has resulted in multiple modified protocols of the neurosphere assay. For instance, the utilization of mouse lines containing multiple targeted mutations typically generates only a small fraction of animals carrying the desired genotype. Given that the total percentage of neural stem cells can be quite small (<0.1% in adults (Reynolds and Weiss 1992)), it is therefore often important to maximize the yield of neural stem cells from these animals. We have found that the often used trypsin-based enzymatic dissociation can be deleterious to cells, resulting in a high percentage of cell death after isolation. Here we provide an alternative protocol utilizing the enzyme papain which, in our hands, is more efficient and less destructive in dissociating neural tissue and therefore provides a greater yield of viable stem cells. It should be noted however that we have found that culture conditions, including enzymatic incubation times and plating densities, vary between different age points as well as between different neural stem cell populations and therefore may need to be determined on an individual basis.

Although historically utilized as a means to retrospectively identify neural progenitors, today the Neurosphere Assay can be used in the prospective isolation and propagation of neural progenitors. The generation of mouse strains expressing

fluorescent proteins under the control of neural progenitor specific markers (Nestin-EGFP (Kawaguchi et al. 2001); Sox2-EGFP (Ellis et al. 2004); hGFAP-EGFP (Zhuo et al. 1997)) coupled with the ability to efficiently isolate these cell types using FACS has led to the ability to culture highly enriched populations of neural progenitor cells, and using the NSA, examine the proliferative, self-renewal, and multipotential capacities of these cells *in vitro*.

Recipes

Dissociation medium

Reagent	Amount to add	Final concentration
1 M Na_2SO_4	20.44 ml	98 mM
0.5 M K_2SO_4	15 ml	30 mM
1 M $MgCl_2$	1.45 ml	5.8 mM
100 mM $CaCl_2$	0.63 ml	0.25 mM
1M HEPES (pH 7.4)	250 µl	1 mM
1M Glucose	5 ml	20 mM
Phenol Red (0.5%)	0.5 ml	0.001%
0.1 N NaOH	~0.1 ml	0.03 mN
Add above volumes to a sterile, dedicated 250-ml tissue culture bottle. Adjust volume to 250 ml with sterile ddH_20. Sterile filter using a 0.2-µm bottle filter. Store solutions at 4°C (Na_2SO_4 and K_2SO_4 solutions should be stored at room temperature to avoid formation of precipitate). Use Dissociation medium within 2 weeks. pH should be approximately 7.4.		

Enzyme solution

Reagent	Amount to add
<recXXXX>Dissociation medium	20 ml
Cysteine (Sigma C1276)	6.4 mg
Papain (Roche 108014)	200 µl
Combine above reagents and incubate for 15 minutes in a 37°C water bath to dissolve. Mix and adjust to pH ~7.4 with 0.1 N NaOH (this usually requires about 3 drops of 0.1 N NaOH). The pH can be monitored by the color of the solution: pink is too basic and yellow is too acidic. Filter through a 0.2-µm syringe filter. Warm the solution in a water bath at 37°C before use.	

Heavy inhibitory solution

Reagent	Amount to add
<recXXXX>Dissociation medium	6 ml
BSA (Sigma A3294)	60 mg
Trypsin inhibitor (Sigma T6522)	60 mg
Warm Dissociation medium to 37°C, add BSA and trypsin inhibitor and mix. Incubate at 37°C for approximately 1 hour to completely dissolve. Adjust to pH ~7.4 with 0.1 N NaOH to about 7.4 (this usually requires about 3 drops of 0.1 N NaOH). The pH can be monitored by the color of the solution: pink is too basic and yellow is too acidic. Filter through a 0.2-µm syringe filter. Warm the solution in a water bath at 37°C before use.	

Light inhibitory solution

Reagent	Amount to add
<recXXXX>Dissociation medium	9 ml
<recXXXX>Heavy inhibitory solution	1 ml
Warm Dissociation medium to 37°C and add the Heavy inhibitory solution. Filter through a 0.2-µm syringe filter. Warm the solution in a water bath at 37°C before use.	

Neurobasal medium

To a 500-ml bottle of Neurobasal medium (Invitrogen 21103049), add one 5-ml aliquot of 100X Penicillin-Streptomycin (Gibco 15160122) and one 5-ml aliquot of 100X L-glutamine (Gibco 250300810). Mix well.

NEP basal medium

In a 50-ml conical tube, combine 48.5 ml of Neurobasal medium with a 1-ml aliquot of B27 supplement (Gibco 17504044) and a 0.5-ml aliquot of N2 supplement (Gibco 17502048). Mix well.

Figure 2.8

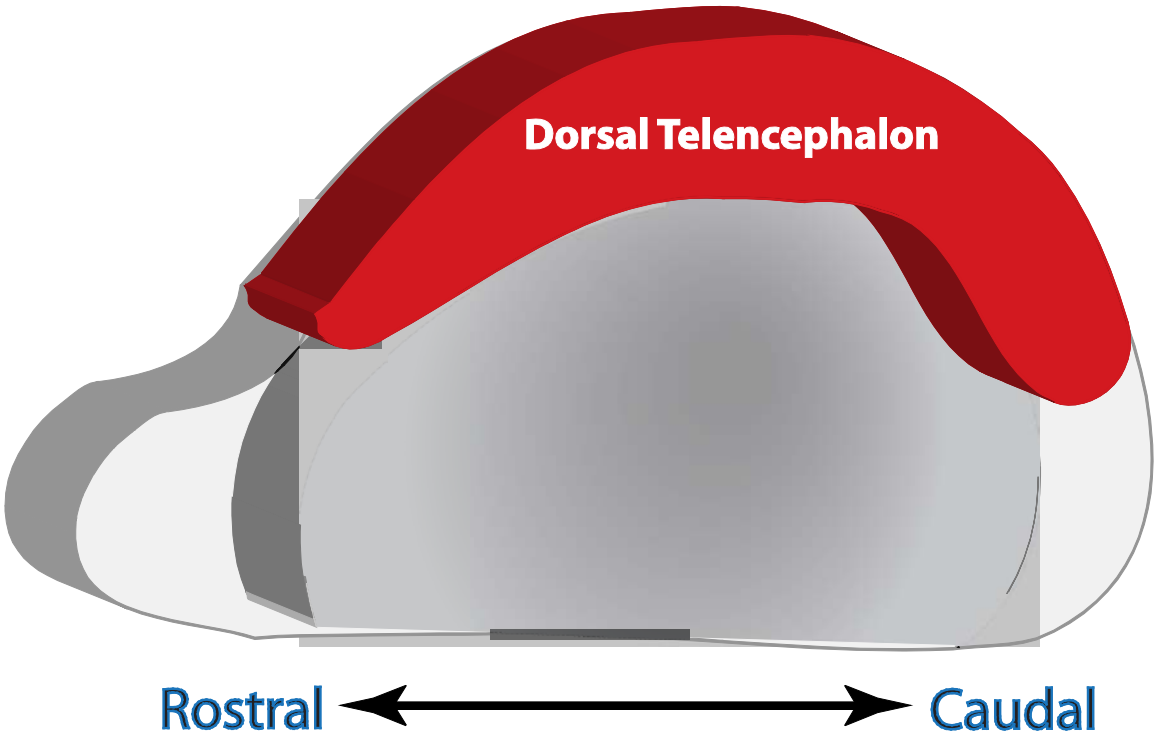


Fig 2.8. Location of the Dorsal Telencephalon

A sagital view of a single cerebral hemisphere from an E12.5 mouse embryo demonstrating the location of the dorsal telencephalon.

Figure 2.9

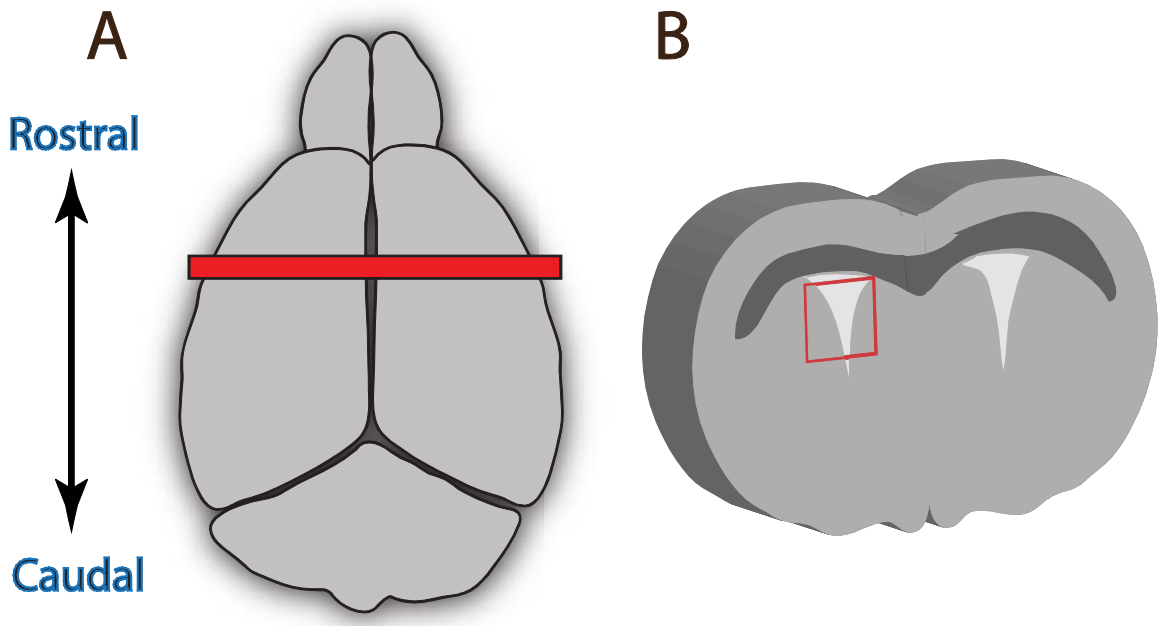


Fig 2.9. Schematic of Adult Brain

A). Dorsal view of the adult mouse brain. The red bar indicates the location of the cut to isolate periventricular tissue. B) The resulting cross-section of tissue with lateral ventricles exposed. Periventricular tissue should be dissected where indicated by the red box.

Figure 2.10

Fig 2.10 Differentiation of Neurospheres.

A). Neurospheres derived from E12.5 mouse dorsal telencephalon after 6 days in culture. B). Neurosphere attachment to a poly-D-lysine/laminin coated slide; 1 day after plating. C). β-tubulinIII (red) and Glial Fibrillary Acidic Protein (GFAP; green) labeling of neurosphere derived neurons and astrocytes, respectively.

Chapter Three

SOX2 Levels Define Distinct Cortical Progenitor Cell Populations

Abstract

The *in vivo* identification and direct isolation of neural progenitor populations from the developing CNS has been difficult due to a limited repertoire of cell-surface markers. Previous studies have demonstrated that the HMG-Box transcription factor SOX2 is commonly expressed in all neural progenitor cell populations throughout the developing and adult central nervous system (CNS). Furthermore, SOX2 has been shown to function in a dosage-dependent manner in the maintenance of neural progenitor identity. Here, using cellular and molecular analyses, we demonstrate that isolated dorsal ventricular cells expressing high, intermediate and low levels of SOX2-EGFP distinguish between radial glia, intermediate progenitors, and differentiated neurons respectively. Collectively, we show that distinct neural progenitor populations from the developing dorsal telencephalon can be prospectively isolated based upon their differential levels of SOX2 expression.

Introduction

In regions throughout the developing mammalian central nervous system (CNS) heterogeneous populations of neural progenitor cells, each with distinct molecular and cellular characteristics, have been identified. In the dorsal telencephalon, two such progenitor populations have been characterized. The first population, located primarily in the ventricular zone (VZ), consists of self-renewing, multipotent radial glial (RG) cells that have the capacity to generate both neurons and glia *in vivo* (Malatesta et al. 2000; Noctor et al. 2001; Malatesta et al. 2003; Anthony et al. 2004). RG cells, in turn, generate a second neural progenitor cell population, intermediate progenitor (IP) cells (or basal progenitor cells) which, in contrast to RG cells, reside in the subventricular zone (SVZ), are exclusively neurogenic, and have limited self-renewal capacity (Haubensak et al. 2004; Miyata et al. 2004; Noctor et al. 2004). Interestingly, subtypes of both RG and IP populations have also been observed in the dorsal telencephalon, including unipotential RG cells, which are exclusively neurogenic or gliogenic, as well as IP subpopulations with distinct morphological characteristics (Kawaguchi et al. 2008; Kowalczyk et al. 2009). Thus, the developing dorsal telencephalon harbors a diverse mixture of distinct cellularly-defined neural progenitor cell populations.

A number of transcription factor networks have been identified which regulate progenitor cell diversity (Guillemot 2007). In the dorsal telencephalon, the interplay of several key transcription factors defines and regulates the “radial glial-intermediate progenitor-neuron” transition (Englund et al. 2005). One transcription factor, the paired-domain protein PAX6, is highly expressed in RG cells and functions to maintain their progenitor identity as evidenced by a decrease in the number of both RG cells and postmitotic neurons in PAX6 deficient mice, a phenotype most likely due to the premature differentiation of RG cells into IP cells (Gotz et al. 1998; Heins et al. 2002; Haubst et al. 2004). During their normal transition into IP cells, RG cells downregulate

PAX6 concomitant with the upregulation of, among other genes, the proneural basic helix-loop-helix transcription factor Neurogenin 2 (NGN2) and the T-Box transcription factor 2 (TBR2), which are necessary for neuronal and IP fate specification, respectively (Bulfone et al. 1999; Kimura et al. 1999; Miyata et al. 2004; Englund et al. 2005; Arnold et al. 2008; Sessa et al. 2008). These genes, in turn, are downregulated upon neuronal differentiation coincident with the upregulation of neuronal subtype-specific genes such as T-Box transcription factor 1 (TBR1) (Hevner et al. 2001; Englund et al. 2005). Thus, the temporal and spatial expression of these key transcription factors is important in regulating neural progenitor cell fate in the dorsal telencephalon.

Recent evidence suggests that the specification of distinct progenitor cell identity depends not only upon the expression or absence of certain transcription factors, but also upon the levels at which these transcription factors are expressed. For example, in the dorsal telencephalon the expression of PAX6 has been shown to be important in maintaining the progenitor identity of RG cells as well as promoting their neuronal differentiation (Bishop et al. 2000; Stoykova et al. 2000; Toresson et al. 2000; Yun et al. 2001; Estivill-Torrus et al. 2002; Heins et al. 2002; Quinn et al. 2007). This paradox was recently attributed to a dosage-sensitive function of PAX6 where increased concentrations of PAX6 are important in driving RG cells to an IP cell fate, through the activation of NGN2, whereas lower concentrations of PAX6 serve to maintain the progenitor identity of RG cells (Scardigli et al. 2003; Sansom et al. 2009). In addition, RG and IP cells express high and low levels of the transcription factor HES5, respectively, and can also be distinguished based upon high and low NOTCH1 signaling activity (Basak and Taylor 2007; Mizutani et al. 2007). Furthermore, examples of a dosage-dependent function of transcription factors are also observed in progenitor populations from other tissue systems. For instance, in the hematopoietic system, varying levels of transcription factors such as E2A, RUNX1 (AML1) and GATA-1 have

been shown to be important in both the maintenance and fate determination of hematopoietic stem and progenitor cells and, moreover, these variations can be used to prospectively isolate distinct populations of hematopoietic progenitors (Suzuki et al. 2003; Lorsbach et al. 2004; Sun and Downing 2004). Therefore, the concentration, as well as the spatial-temporal expression, of key transcription factors in the developing nervous system may similarly be important in progenitor maintenance and fate determination. In addition, due to a current lack of suitable cell-surface markers that can be used to identify and isolate neural progenitor populations, these variations in transcription factor levels may also provide a valuable method to selectively isolate distinct progenitor populations from the nervous system.

Here we show that one such dosage-dependent transcription factor is SOX2, a SOXB1 HMG-box transcription factor that is expressed in all neural progenitors throughout the developing and adult CNS (Uwanogho et al. 1995; Collignon et al. 1996; Uchikawa et al. 1999; Wood and Episkopou 1999; Zappone et al. 2000; D'Amour and Gage 2003; Ellis et al. 2004; Bani-Yaghoub et al. 2006). SOX2 is initially expressed in cells of the inner cell mass (ICM) (Collignon et al. 1996; Wood and Episkopou 1999) and its ablation at this stage results in the loss of proliferating epiblast cells and subsequent peri-implantation lethality of the embryo (Avilion et al. 2003). In order to circumvent the early lethality of SOX2-deficient mice and investigate the role of SOX2 in neural progenitor populations, misexpression and dominant-negative studies were originally conducted in the embryonic chick neural tube. These studies demonstrated that the overexpression of SOX2 was sufficient to maintain cells in a neural progenitor state while dominant-negative SOX2 induced cell cycle exit and precocious neuronal differentiation (Bylund et al. 2003; Graham et al. 2003). However, *in vivo* hypomorphic SOX2 mutations in mice have suggested that the intracellular concentrations of SOX2 in these cells also play an important role. In the cortex, hypomorphic levels of SOX2 result

in decreases in both progenitor proliferation and neuronal production (Ferri et al. 2004; Cavallaro et al. 2008), while in the retina, aberrant neuronal differentiation is observed (Taranova et al. 2006). In addition, several hereditary eye and hippocampal defects have also been attributed to hypomorphic SOX2 mutations in humans (Fantes et al. 2003; Hagstrom et al. 2005; Ragge et al. 2005; Sisodiya et al. 2006; Bakrania et al. 2007). Further evidence supporting a dose-dependent role of SOX2 in the dorsal telencephalon comes from immunocytochemical studies demonstrating a gradient of SOX2 expression in the cortex (Bani-Yaghoub et al. 2006) as well as *in vitro* studies in which cells that express high levels of SOX2 demonstrate greater neurosphere-forming potential as well as increased expression of "stem cell" genes such as Notch1 and Nestin (D'Amour and Gage 2003; Ellis et al. 2004). Collectively, these studies suggest that intracellular concentrations of SOX2 are important for proper neural development. However, it remains to be determined whether endogenous variations in SOX2 expression occur between distinct neural progenitor cell populations such as RG and IP cells. To address this, adequate tools are needed to prospectively isolate distinct progenitor populations based upon different levels of SOX2 expression.

Here we characterize SOX2 expression in RG and IP cells and demonstrate that not only is SOX2 differentially expressed between these two populations, it also uniquely marks a select population of neural progenitor cells in the VZ undergoing direct neuronal differentiation. Furthermore, we prospectively isolate RG cells, IP cells, and differentiated neurons from the developing dorsal telencephalon based upon the levels of SOX2 expression using a "knock-in" $SOX2^{EGFP}$ mouse line (Ellis et al. 2004). These results suggest that utilizing SOX2 expression levels, in conjunction with the expression of other dorsal telencephalic progenitor markers such as PAX6 and TBR2, is an efficient method to identify and isolate distinct populations of neural progenitor cells from the dorsal telencephalon.

Results

Detailed Analysis of SOX2 Expression in Neural Progenitor Populations of the Dorsal Telencephalon

Here we address whether SOX2 levels vary between distinct neural progenitor populations. We first compared SOX2 expression in relation to neural progenitor markers in the E17.5 dorsal telencephalon using immunocytochemistry. SOX2 is highly expressed in the VZ and at lower levels in the SVZ (Fig 3.1A). In comparison, the RG marker PAX6 is similarly expressed in the VZ and SVZ (Fig 3.1B) and double-labeling revealed that PAX6 and SOX2 are largely coexpressed in the VZ (Fig 3.1C), demonstrating that RG cells express high levels of SOX2. Interestingly, populations of PAX6-negative cells expressing high levels of SOX2 were also observed in the SVZ (Fig 3.1C arrows), suggesting the existence of multiple neural progenitor populations within the dorsal telencephalon. Next, we compared SOX2 and PAX6 expression with that of the IP cell marker TBR2. TBR2-positive IP cells predominantly reside in the SVZ (Fig 3.1E) and express little or no SOX2 (Fig 3.1F), although a subset of TBR2-positive cells expressing high levels of SOX2 are found scattered throughout the VZ/SVZ boundary (Fig 3.1F, arrowhead) and, based on their location, are presumed to be newly generated IP cells migrating out of the VZ. Consistent with previously published reports, we also observed scattered TBR2-positive cells expressing high levels of PAX6 in the VZ (Fig 3.1G-I) (Englund et al. 2005). In addition, a majority of TBR2-positive cells maintain a low level of PAX6 expression (Fig 3.1I), demonstrating that PAX6 continues to be expressed in IP cells, albeit at low levels. These data suggest that SOX2 and PAX6 are maintained in RG cells at high levels, but SOX2 is downregulated in IP cells coincident with the upregulation of TBR2, whereas PAX6 continues to be maintained at lower levels in TBR2-positive IP cells. Furthermore, the identification of a SOX2-positive, PAX6-

negative population suggests that a molecularly distinct neural progenitor cell population is maintained in the SVZ in addition to IP cells.

Lastly, we examined SOX2 in relation to the immature neuronal marker β-Tubulin III. SOX2-positive cells were mutually exclusive of β-Tubulin III-positive cells in superficial layers of the dorsal telencephalon (Zappone et al. 2000; Ellis et al. 2004), however both SOX2 and β-Tubulin III were found to be coexpressed in sporadic, newly generated neurons in the VZ (Fig 3.1J, asterisks). In contrast, we did not observe any PAX6-positive or TBR2-positive cells co expressing β-Tubulin III (Fig. 3.1K-L), nor did these β-Tubulin III-positive cells coexpressed the GABAergic marker Calbindin, demonstrating that they are not interneurons migrating from the ventral telencephalon (data not shown). These data suggest that a population of SOX2-positive neural progenitor cells in the E17.5 dorsal telencephalon is capable of generating β-Tubulin III-positive neurons independent of the PAX6-TBR2 lineage.

Distinct Neural Progenitor Populations can be Prospectively Isolated Based Upon SOX2 Expression Levels

Our immunocytochemistry results suggest that SOX2 is differentially expressed between RG and IP populations. To more accurately ascertain whether the level of SOX2 expression can be used to identify distinct neural progenitors, we isolated populations of neocortical progenitor cells based upon their level of SOX2 expression and analyzed the gene expression profile as well as the self-renewal and differentiation capacity of each sorted population. To this end, we utilized the $SOX2^{EGFP}$ mouse line in which an enhanced green fluorescent protein (EGFP) expression cassette is inserted into the SOX2 locus using homologous recombination, faithfully recapitulating endogenous SOX2 expression in the CNS (Ellis et al. 2004). In the $SOX2^{EGFP/+}$ dorsal telencephalon, SOX2 is expressed in the VZ and SVZ of the lateral ventricles (Fig. 3.2A,C-D). EGFP is similarly expressed in the VZ and SVZ, but is also observed in the

more superficial layers, a difference we attribute to the increased stability of the EGFP protein (Fig. 3.2B,C-D) (Cubitt et al. 1995). However, all cells which express endogenous SOX2 protein also express EGFP (Fig. 3.2B-D), thus allowing for the prospective isolation of cells based upon SOX2-EGFP expression levels.

We first compared EGFP-intensity profiles of $SOX2^{EGFP/+}$ dorsal telencephalic cells isolated from early (E12.5) and late (E17.5) periods of neurogenesis using flow cytometry. At E12.5, the dorsal telencephalon consists primarily of RG cells located within the proliferative VZ (Malatesta et al. 2000; Hartfuss et al. 2001; Malatesta et al. 2003). However, by E17.5, a majority of the dorsal telencephalon is comprised of post-mitotic neurons, although populations of RG cells and IP cells continue to be maintained in the VZ and SVZ (Malatesta et al. 2000; Hartfuss et al. 2001; Malatesta et al. 2003). In both E12.5 and E17.5 $SOX2^{EGFP/+}$ mice, EGFP-positive cells can be distinguished from $SOX2^{+/+}$:EGFP-negative littermate controls using flow cytometry (Fig 3.2E). At E12.5, dorsal telencephalic progenitor cells express high levels of EGFP (871.3±31.44 fluorescent units) and display a normal distribution curve (Fig 3.2E, green). In contrast, the E17.5 cells, on average, express lower levels of EGFP compared with E12.5 cells (306.8±26.24 fluorescent units)(Fig 3.1E,red). However the E17.5 intensity profile is skewed, suggesting that, even though most E17.5 cells express low levels of EGFP, a small population of cells is maintained that expresses EGFP levels equivalent to those levels observed in E12.5 embryos (Fig 3.2E, red). These results suggest that cells expressing high levels of SOX2 (as indicated by EGFP expression) are found in the dorsal telencephalon during both early (~E12.5) and late (~E17.5) stages of neurogenesis whereas cells expressing low levels of SOX2 are only observed at later stages. Based on these data, we hypothesized that the E17.5 dorsal telencephalon contains multiple progenitor populations (including RG and IP cells) which express

distinct levels of SOX2, and moreover, these populations can be prospectively separated based upon their levels of SOX2-EGFP expression.

Neurosphere Size, Self-Renewal, and Multipotency Correlate with High Levels of SOX2 Expression

To isolate distinct neural progenitor cell populations, we focused our analyses on the E17.5 dorsal telencephalon. Fluorescence Automated Cell Sorting (FACS) was utilized to isolate and separate the E17.5 EGFP-positive cells into three subpopulations based upon their EGFP-intensity levels: $EGFP^{High}$, $EGFP^{Int}$, and $EGFP^{Low}$. Post-sort analyses confirmed that each subpopulation was distinct in regard to its intensity of EGFP fluorescence (Fig 3.3A) and analyses of *Sox2* and *Egfp* transcript levels utilizing quantitative reverse transcription polymerase chain reaction (qRT-PCR) demonstrate that both *Sox2* and *Egfp* transcript levels correspond to their respective EGFP fluorescent intensity levels (Fig 3.3B).

The stem cell capacity of each subpopulation was then analyzed using the *in vitro* neurosphere assay. Neurospheres generated from $EGFP^{High}$, $EGFP^{Int}$, and $EGFP^{Low}$ cells were tested for their proliferative and self-renewal capabilities, as well as the ability to undergo multipotent differentiation.

First, to test the capacity of $EGFP^{High}$, $EGFP^{Int}$, and $EGFP^{Low}$ populations to generate neurospheres, freshly sorted cells from each subpopulation were plated at clonal density (282.9 cells/cm^2) and cultured for six days (Coles-Takabe et al. 2008). All three subpopulations generated EGFP-positive neurospheres (Fig 3.3C), however $EGFP^{Low}$ cells produced statistically fewer neurospheres compared to $EGFP^{High}$ and $EGFP^{Int}$ subpopulations (Fig 3.3D). In contrast, EGFP-negative cells were unable to produce neurospheres (data not shown). Moreover, $EGFP^{High}$ cells produced larger neurospheres compared to $EGFP^{Int}$ and $EGFP^{Low}$ populations, suggesting a difference in neurosphere growth rate (Fig 3.3C,E). To directly examine neurosphere growth,

neurospheres were isolated from bulk cultures at 6 days *in vitro* (DIV) and individually maintained in 96-well plates to prevent aggregation. The diameter of each individual neurosphere was then measured every two days. At 6 DIV, $EGFP^{High}$ cells produced significantly larger neurospheres than both $EGFP^{Int}$ and $EGFP^{Low}$ cells and these differences were maintained throughout the culture period up to 10 DIV (Fig 3.3E). To better gauge the neurosphere size variation within each subpopulation, neurospheres were classified into one of three categories based upon their diameter at 6 DIV. The proportion of small-diameter neurospheres (<100µm) was significantly greater in $EGFP^{Low}$ cultures compared to both $EGFP^{High}$ and $EGFP^{Int}$ subpopulations (Fig 3.3F). In contrast, intermediate sized neurospheres (100-200µm) were more prevalent in the $EGFP^{High}$ and $EGFP^{Int}$ subpopulations (Fig 3.3F). Moreover, the $EGFP^{High}$ cultures also contained a significant percentage of large-diameter spheres (>200µm) compared to both $EGFP^{Int}$ and $EGFP^{Low}$ populations (Fig 3.3F). These data suggest that cells with high levels of SOX2-EGFP have an increased neurosphere forming potential and growth rate.

Next, to analyze self-renewal potential, individual primary neurospheres from $EGFP^{High}$, $EGFP^{Int}$, and $EGFP^{Low}$ cultures were dissociated and allowed to form secondary, followed by tertiary, neurospheres (Coles-Takabe et al. 2008). $EGFP^{High}$ primary neurospheres generated significantly more secondary and tertiary neurospheres then both $EGFP^{Int}$ and $EGFP^{Low}$ primary neurospheres (Fig 3.4A). Moreover, $EGFP^{Low}$ primary neurospheres produced very few secondary neurospheres and were unable to generate any tertiary neurospheres (Fig 3.4A), suggesting they have a more restricted capacity for self-renewal. These data indicate that high levels of SOX2-EGFP expression are directly correlated with an increased capacity for self-renewal.

Lastly, to test the multipotent differentiation capacity of the $EGFP^{High}$, $EGFP^{Int}$, and $EGFP^{Low}$ subpopulations, primary neurospheres from each subpopulation were

induced to differentiate after 10 DIV. Multipotential neurospheres, capable of generating both β-Tubulin III-positive neurons and GFAP-positive astrocytes, were observed in each subpopulation (Fig. 3.4B). However, $EGFP^{High}$ cells produced more multipotential neurospheres than $EGFP^{Int}$ or $EGFP^{Low}$ cells (Fig 3.4D). In addition, unipotent neurospheres, generating only β-Tubulin III-positive neurons, were identified in all three populations (Fig 3.4C). However, $EGFP^{Low}$ cells generated more unipotent neurospheres compared to both $EGFP^{Int}$ and $EGFP^{High}$ cells (Fig 3.4D). Interestingly, we found that the multipotent capacity of a neurosphere was directly correlated with its size, regardless of sorting population (Fig 3.4D). At 10 DIV, neurospheres with small diameters (<150μm) only generated neurons, while larger neurospheres (>150μm) generated both neurons and glia. We did not observe any glial-specific neurospheres using these culture conditions.

Gene Expression Profiles of $EGFP^{High}$, $EGFP^{Int}$, and $EGFP^{Low}$ sorted populations

The results from our *in vitro* studies demonstrate that the $EGFP^{High}$, $EGFP^{Int}$, and $EGFP^{Low}$ sorted populations each have distinct proliferative, self-renewal, and multipotent differentiation properties. The low neurosphere forming potential of the $EGFP^{Low}$ cells shows that this population consists primarily of post-mitotic neurons while the significant differences in neurosphere size and self-renewal capacity between the $EGFP^{High}$ and $EGFP^{Int}$ subpopulations indicate that these two subpopulations of cells contain distinct neural progenitor populations as well.

We next addressed whether $EGFP^{High}$, $EGFP^{Int}$, and $EGFP^{Low}$ cells could be molecularly distinguished by utilizing qRT-PCR to determine transcript levels of ubiquitously expressed and neural cell-type specific genes. As mentioned previously, EGFP fluorescent intensity is a faithful indicator of Sox2 and Egfp transcript levels (Fig 3.3B). Sox3, a SOXB1 homologue which is coexpressed with Sox2 in neocortical

progenitor cells, showed a similarly dynamic regulation as *Sox3* levels directly correlate with Sox2 levels (Fig 3.5A). Moreover, Notch1, a known effector of SOX2 which is highly expressed in neural progenitor cells, was high in both $EGFP^{High}$ and $EGFP^{Int}$ populations, but low in $EGFP^{Low}$ cells (Fig 3.5A). These data suggest that both the $EGFP^{High}$ and $EGFP^{Int}$ subpopulations are enriched for neural progenitor cells compared to $EGFP^{Low}$ cells.

Our data demonstrate that the $EGFP^{High}$ subpopulation generates more self-renewing, multipotential, large-diameter neurospheres than $EGFP^{Int}$ and $EGFP^{Low}$ cells, suggesting that the $EGFP^{High}$ population consists primarily of stem-cell like radial glial cells (Mizutani et al. 2007). We confirmed this by Blbp expression and find that $EGFP^{High}$ cells express higher levels of Blbp transcript than both $EGFP^{Int}$ and $EGFP^{Low}$ populations (Fig 3.5B) (Hartfuss et al. 2001). In contrast, Pax6 transcript levels are also high in $EGFP^{High}$ cells; however its levels are significantly higher in $EGFP^{Int}$ cells (Fig 3.5B). Given that Pax6 is widely used as a RG marker, this result was unexpected. However, these results are supported by recent findings that lower levels of Pax6 maintain the progenitor identity of RG cells while higher Pax6 levels promote IP formation (Sansom et al. 2009). Thus, our data demonstrate that $EGFP^{High}$ populations are enriched for RG progenitor cell markers and suggest that the $EGFP^{Int}$ populations may contain IP cells.

$EGFP^{Int}$ cells generate smaller neurospheres with limited self-renewal capacity, similar to IP cells (Mizutani et al. 2007). To address whether $EGFP^{Int}$ cells are enriched for IP cell molecular markers, transcript levels for IP genes Ngn2 and Tbr2 (Miyata et al. 2004; Englund et al. 2005), as well as the neuronal-fate marker Tis-21 (Iacopetti et al. 1999) were analyzed. Tbr2 and Ngn2 levels were significantly higher in the $EGFP^{Int}$ subpopulation compared to $EGFP^{High}$ cells (Fig 3.5B) while $EGFP^{Low}$ cells, in contrast, expressed low levels of both transcripts. Similarly, in $EGFP^{Int}$ cells, Tis-21 was more than 2-fold higher than $EGFP^{High}$ cells (Fig 3.5B). However, consistent with Tis-21

expression in cells undergoing a neuronal transition, higher levels of Tis-21 are also observed in EGFP^{Low} cells compared to EGFP^{High} cells (Iacopetti et al. 1999). Thus, these results show that cells expressing intermediate levels of SOX2-EGFP are enriched for IP cell markers.

Lastly, the low neurosphere forming potential of EGFP^{Low} cells suggests that these cells have lost the capacity to proliferate and may consist largely of postmitotic neurons. We therefore examined the expression of two neuronal genes, β-Tubulin III, which labels immature cells, and Tbr1, which marks mature neuronal populations (Fig 3.5C). Indeed, both β-Tubulin III and Tbr1 transcript levels were higher in EGFP^{Low} cells compared to EGFP^{High} and EGFP^{Int} populations and were slightly higher in EGFP^{Int} cells compared to EGFP^{High} cells, suggesting that some cells from the EGFP^{Int} sort have undergone neuronal differentiation. Interestingly, detectable levels of β-Tubulin III transcript are observed in EGFP^{High} cells, whereas Tbr1 transcript is not, suggesting that a small population of EGFP^{High} cells may also be generating immature neurons. Overall, these results support our hypothesis that cells expressing low levels of SOX2 and EGFP are enriched for post-mitotic neurons.

Discussion

In comparison to other mammalian progenitor populations, neural progenitor cells remain largely uncharacterized. This is due, in part, to a lack of cell-surface markers available to identify and isolate distinct neural progenitor populations from the nervous system. One marker, the surface antigen CD133 (Prominin1), has been used extensively in the isolation and analysis of neural progenitor cells from human (Uchida et al. 2000) and mouse nervous system tissue (Lee et al. 2005; Corti et al. 2007). In addition, another cell-surface marker, CD15 (SSEA-1, LeX) has also been used to isolate neural progenitor cells from the mouse (Capela and Temple 2002) and has been

shown to be expressed by a subset of radial glial cells (Mai et al. 1998). However, neither CD133 nor CD15 expression is restricted to the nervous system as both are expressed in progenitor cells from other tissues as well (Solter and Knowles 1978; Fox et al. 1981; Gomperts et al. 1994; Jiang et al. 2002; Mizrak et al. 2008). Moreover, it was recently reported that clonogenic, tripotent cells lacking CD133 and CD15 can be isolated from neural stem cell cultures (Sun et al. 2009). Thus, the isolation of neural progenitor cells based upon the expression of CD133 and CD15 is not sufficient and additional methods of identifying and isolating neural progenitor cells are necessary.

To date, SOX2 is found to be expressed in all stem/progenitor populations throughout the central nervous system, as well as other non-neural populations such as the stomach, lung, and tongue (Ishii et al. 1998; Pevny and Placzek 2005; Okubo et al. 2006; Que et al. 2007). In the CNS, all neurosphere forming cells express SOX2 (Ellis et al. 2004) suggesting that, unlike CD133 and CD15, SOX2 is constitutively expressed in neural progenitor cells. The recent generation of mouse lines expressing the EGFP reporter protein under the control of the *Sox2* promoter has therefore provided a valuable tool in the characterization of neural progenitor cells (D'Amour and Gage 2003; Ellis et al. 2004). Previously, D'Amour and Gage (D'Amour and Gage 2003) isolated distinct populations of cells based upon SOX2 expression levels utilizing a transgenic mouse line in which a SOX2-EGFP transgene was used to drive EGFP expression in a subset of SOX2-expressing cells in the developing telencephalon. Similar to the results we report here, the authors observed differential levels of SOX2 expression in the telencephalon and found that SOX2-EGFP-positive isolated cells had a greater propensity to generate neurospheres. However, the restriction of SOX2-EGFP expression to the telencephalon and the observation of SOX2-EGFP-negative neurospheres derived from this region suggests that not all neural progenitor populations are marked by SOX2-EGFP in this mouse line. In contrast, in this study we utilize a

$SOX2^{EGFP}$ mouse line in which the SOX2 open reading frame has been directly replaced, using homologous recombination at the *Sox2* locus, with an EGFP expression cassette and demonstrate that all neurosphere generating cells from the developing dorsal telencephalon express SOX2. Furthermore, we demonstrate the power of this tool in the characterization of neural progenitor populations by isolating and enriching for subtypes of neocortical cells based upon their level of SOX2 expression.

Our results demonstrate that radial glial cells express high intracellular levels of SOX2. Isolated cells expressing high levels of SOX2 and EGFP ($EGFP^{High}$) produced a greater number of large-diameter, multipotential neurospheres compared with $EGFP^{Int}$ and $EGFP^{Low}$ cells. Furthermore, these cells were enriched for radial glial markers such as BLBP and neural progenitor markers such as SOX3 and Notch1. *In situ*, SOX2 was also found to be highly expressed in radial glial cell bodies in the VZ and was largely coexpressed with PAX6 in these cells. Interestingly, in the SVZ, SOX2-positive;PAX6-negative cells were also observed. It is possible that these cells are radial glia cells downregulating PAX6 expression as they transition into intermediate progenitor cells. However, this scenario seems unlikely as we also noted that a majority of TBR2-positive intermediate progenitor cells maintain low levels of PAX6 expression. Moreover, SOX2 was not detectable in most TBR2-positive cells. Another possibility that we propose is that these SOX2-positive;PAX6-negative cells are in fact a subset of radial glial cells that do not proceed through the radial glial-intermediate progenitor lineage but undergo direct neurogenesis. This is supported by two of our results. First, we identified a number of SOX2-positive;β-Tubulin III-positive cells located in the SVZ but no β-Tubulin III-positive cells coexpressing either PAX6 or TBR2, the later in line with previous reports (Englund et al. 2005). Second, our quantitative RT-PCR analysis of $EGFP^{High}$ cells revealed detectable levels of β-Tubulin III transcript but not of the more specific neuronal marker Tbr1, which is expressed by mature projection neurons. Our results therefore support

the later, a direct-neurogenesis scenario in which SOX2-positive;PAX6-negative radial glial cells undergo neurogenesis to produce β-Tubulin III-positive neurons. It remains to be determined, however, whether these neural progenitor cells initially express PAX6 only to downregulate its expression prior to neuronal differentiation or are in fact a small population of radial glial cells that do not express PAX6, which has been previously reported (Gotz et al. 1998).

In addition to prospectively isolating radial glial populations, we are also able to utilize SOX2 expression levels to distinguish between intermediate progenitor cells and differentiated neurons. $EGFP^{Int}$ cells express intermediate levels of EGFP and SOX2 and are highly enriched for the bHLH proneural gene Ngn2, the intermediate progenitor marker Tbr2, and the neuronal-fate marker Tis-21. However, although these cells can generate neurospheres, they are smaller and less likely to be multipotent compared to $EGFP^{High}$ neurospheres. These observations are inline with data reported in previous analyses of isolated radial glial/intermediate progenitor populations (Mizutani et al. 2007) and correspond to our *in situ* data in which most TBR2-positive intermediate progenitors express very low levels of SOX2 protein. In contrast, $EGFP^{Low}$ cells rarely generate neurospheres and are enriched for neuronal markers β-Tubulin III and TBR1, demonstrating that these cells are differentiated neurons.

Conclusion

The identification and characterization of neural progenitor populations *in vivo* has been hindered due to a limited number of available cell-specific markers. Our results demonstrate that one such marker, the transcription factor SOX2, is differentially expressed between radial glia, intermediate progenitors, and differentiated neurons within the dorsal telencephalon. Furthermore, utilizing the $SOX2^{EGFP}$ mouse line (Ellis et al. 2004), we are able to prospectively isolate and enrich for these populations based

upon SOX2-EGFP intensity levels, thus providing a powerful tool for the isolation of distinct, viable neural progenitor populations from the CNS.

Materials and Methods

Animal Breeding

All animals were used and maintained in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of North Carolina-Chapel Hill. The generation of the $SOX2^{EGFP}$ mouse line has been described previously (Ellis et al. 2004). $SOX2^{EGFP}$/+ litters were generated by crossing $SOX2^{EGFP}$/+ male mice with C57Bl6 females (Jackson Laboratory). Pregnant females were then euthanized and the embryos harvested at E12.5 and E17.5 (plug date was recorded as E0.5).

Tissue Dissociation, Fluorescence-Activated Cell Sorting (FACS), and Neurosphere Assay

Tissue dissociation and neurosphere generation were conducted using published protocols (Hutton and Pevny 2008). In short, the dorsal telencephalon was dissected from E17.5 $SOX2^{EGFP/+}$ and $SOX2^{+/+}$ embryos and incubated in Papain (Roche) followed by treatments with Trypsin Inhibitor (Sigma) and a final wash with Neurobasal Medium (Gibco). The tissue was then mechanically dissociated into a single-cell suspension in Neurobasal Medium containing B27 and N2 supplements (Gibco) and 10ng/ml bFGF and EGF (Invitrogen).

Fluorescent analysis and cell sorting were conducted at the University of North Carolina Flow Cytometry Facility (http://flowcytometry.med.unc.edu) using a MoFlo flow cytometer (Beckman-Coulter) and Summit v4.3 software (Dako). Freshly dissociated cells were maintained in supplemented Neurobasal medium and kept on ice. EGFP-positive cells were sorted into three subpopulations based upon the level of their fluorescent intensity. Immediately after sorting, cell density was calculated using a hemacytometer.

To generate neurospheres, freshly isolated cells were seeded at a clonal density of 2000 cells/6cm dish (283 cells/cm^2) (Coles-Takabe et al. 2008; Hutton and Pevny 2008). After six days in culture, the number of neurospheres per dish was counted. Individual neurospheres were then isolated in single wells of 96-well plates and their diameter measured every two days using Image Pro Express Software. To generate secondary and tertiary neurospheres, individual neurospheres were mechanically dissociated into single cell suspensions and then plated again at clonal density. For differentiation analysis, individual neurospheres were plated in 8-well chamber slides (Nunc) coated with Poly-D-Lysine and Laminin and allowed to attach for 24 hours, after which the medium was replaced with Neurobasal medium (+2% horse serum) lacking basic Fibroblast Growth Factor (bFGF) and Epidermal Growth Factor (EGF). Neurospheres were then cultured for 1 week under these conditions at which time they were fixed with 4% Paraformaldehyde (PFA) for 30 min at room temperature and stained using the immunocytochemistry procedures below.

Quantitative RT-PCR

The remaining isolated cells not used for the Neurosphere Assay were utilized for gene transcript analysis. Total RNA was isolated from cell pellets using Trizol Reagent (Invitrogen) and quantitated using an ND1000 spectrophotometer (Nanodrop). cDNA was then generated from 50µg of total RNA using a Superscript First Strand Synthesis Kit (Invitrogen). Quantitative real-time PCR reactions were run on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems) using a SYBR Green labeling kit (Applied Biosystems). All samples were run in triplicate and normalized to GAPDH expression. The following primer sequences were used: **β-Tubulin III-F:** 5'-tcacgcagcagatgttcgat-3', **β-Tubulin III-R:** 5'-gtggcgcgggtcaca-3'; **BLBP-F:** 5'-cgcaacctggaagctgaca-3', **BLBP-R:** 5'-gcccagagctttcatgtactca-3'; **EGFP-F:** 5'-gccacaagttcagcgtgtcc-3', **EGFP-R:** 5'-

gcttctcgttggggtctttgc-3'; **Ngn2-F:** 5'-cggcgtcatcctccaact-3', **Ngn2-R:** 5'ggctagcgggcgataaagt-3'; **Notch1-F:** 5'-ggatcacatggaccgattgc-3', **Notch1-R:** 5'-atccaaaagccgcacgatat-3'; **PAX6-F:** 5'-caggccctggttggtatcc-3', **PAX6-R:** 5'-ggtgttctctccccctcctt-3'; **SOX2-F:** 5'-cgcggcggaaaacca-3', **SOX2-R:** 5'-cctccgggaagcgtgtact-3'; **SOX3-F:** 5'-tgcggtgcacatgaagga-3', **SOX3-R:** 5'-tgagcagcgtcttggtcttg-3'; **Tis21-F:** 5'-cattacaaacaccactggtttccag-3', **Tis21-R:** 5'-gctggctgagtccaatctggctg-3'; **TBR1-F:** 5'-ctcgctctttcacttgaccc-3', **TBR1-R:** 5'-actcgactcgcctaggaaca-3'; **TBR2-F:** 5'-tgaatgaaccttccaagactcaga-3', **TBR2-R:** 5'-ggcttgaggcaaagtgttgaca-3'; **GAPDH-F:** 5'-tgtgtccgtcgtggatctga-3', **GAPDH-R:** 5'-cctgcttcaccaccttcttga-3'.

Immunocytochemistry

Mouse embryos were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight at 4°C. After fixation, the tissue was then sequentially treated to a 10%, 20%, and 30% sucrose gradient and finally embedded in OCT medium (Tissue-Tek). 12µm coronal sections were made using a cryostat and collected on Superfrost Plus coated glass slides (VWR). Slides were blocked for 1 hour at room temperature with 10% goat serum/1% Trition X-100 in PBS. All primary and secondary antibodies were diluted in 5% goat serum/0.1% Triton X-100 in PBS. Primary antibodies used are: SOX2 (1:500 Millipore; 1:100 R&D Systems), PAX6 (1:100 Developmental Studies Hybridoma Bank), TBR2 (1:500 AbCam), and β-Tubulin III (TUJ1, 1:1000 Covance). Goat secondary antibodies used for the detection of primary antibodies were: anti-rabbit Alexa 488 or 546 (1:1000 Invitrogen), anti-mouse (IgG1 and IgG2A) Alexa 488 or 546 (1:1000 Invitrogen). Fluorescent images were obtained using a Leica Microsystems (Wetzlar, Germany) DM-IRB inverted fluorescent microscope and a QImaging (Surrey,

BC, Canada) Retiga-SRV camera using Image Pro Express software (Media Cybernetics).

Statistical Analysis

All data were statistically analyzed using Prism 3.0 software (GraphPad). Samples were analyzed using One-Way ANOVA and post-hoc analysis was conducted using Tukey's range test.

Figure 3.1

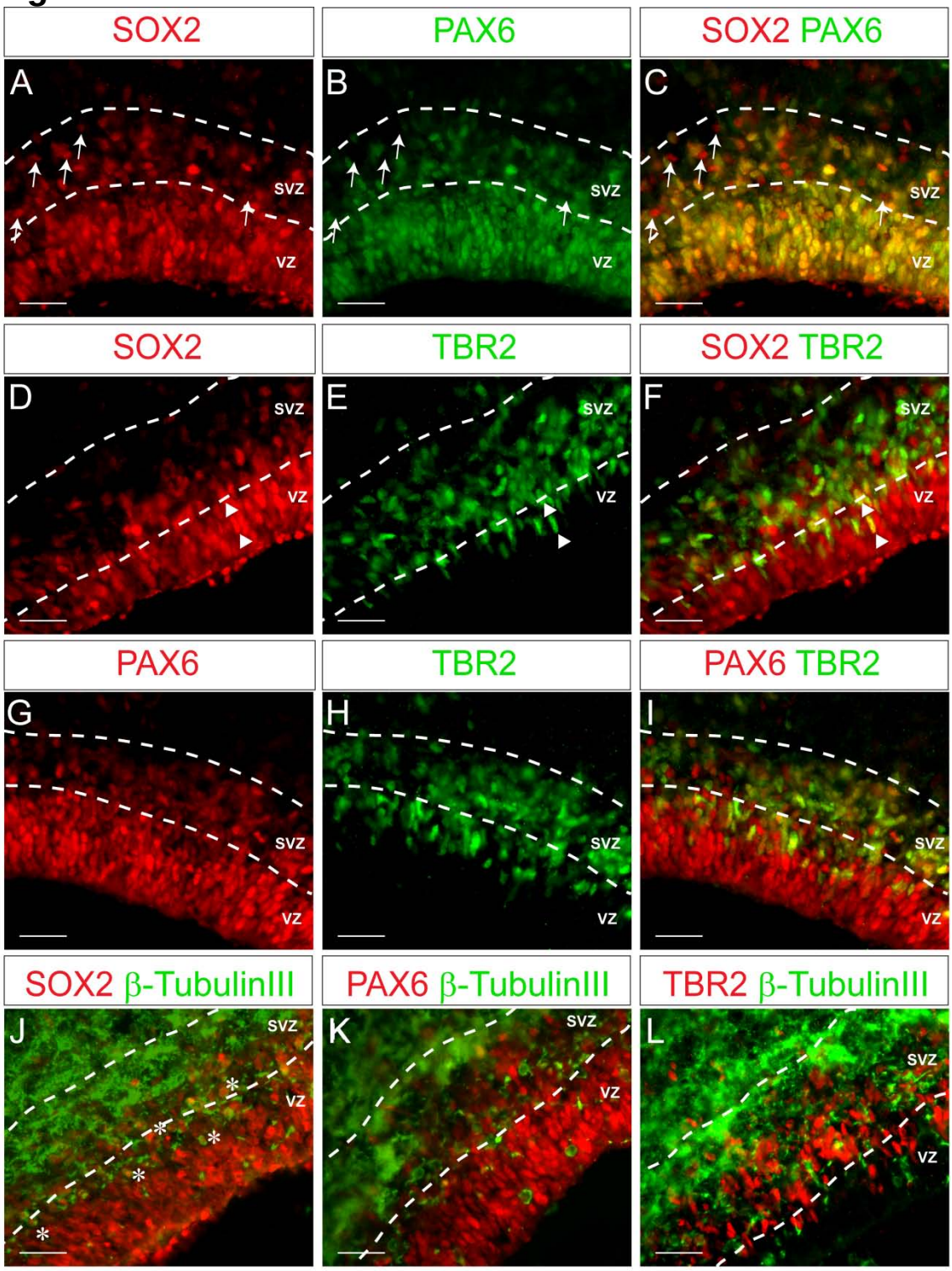

SOX2
PAX6
SOX2 PAX6
A
B
C
SVZ
VZ
SOX2
TBR2
SOX2 TBR2
D
E
F
PAX6
TBR2
PAX6 TBR2
G
H
I
SOX2 β-TubulinIII
PAX6 β-TubulinIII
TBR2 β-TubulinIII
J
K
L

Figure 3.1. Immunocytochemical characterization of SOX2-positive cells in the dorsal telencephalon. E17.5 coronal sections of wildtype mice were analyzed for coexpression of dorsal telencephalon cell-type markers. **(A-C):** SOX2 **(A)** and PAX6 **(B)** are expressed heterogeneously in the VZ and SVZ . SOX2 is coexpressed in all PAX6-positive radial glial cells in the VZ **(C)** but scattered SOX2-positive; PAX6-negative cells are also observed in the SVZ **(C,arrows). (D-F):** SOX2 is weakly expressed in the SVZ **(D)** where the intermediate progenitor marker TBR2 is highly expressed **(E).** Faint levels of SOX2 can be observed coexpressing with TBR2 in the SVZ **(F)** while intermittent cells coexpressing high levels of SOX2 and TBR2 are observed in the VZ/SVZ boundary **(F,arrowheads). (G-I):** PAX6 **(G)** and TBR2 **(H)** show weak coexpression in the SVZ **(I). (J):** SOX2 expression is largely exclusive of β-Tubulin III except for scattered cells in the VZ/SVZ boundary coexpressing both markers **(J, asterisks).** Both PAX6 **(K)** and TBR2 **(L)** cells do not coexpress β-Tubulin III. Scale Bar=50μm. Abbreviations: VZ, ventricular zone; SVZ, subventricular zone.

Figure 3.2

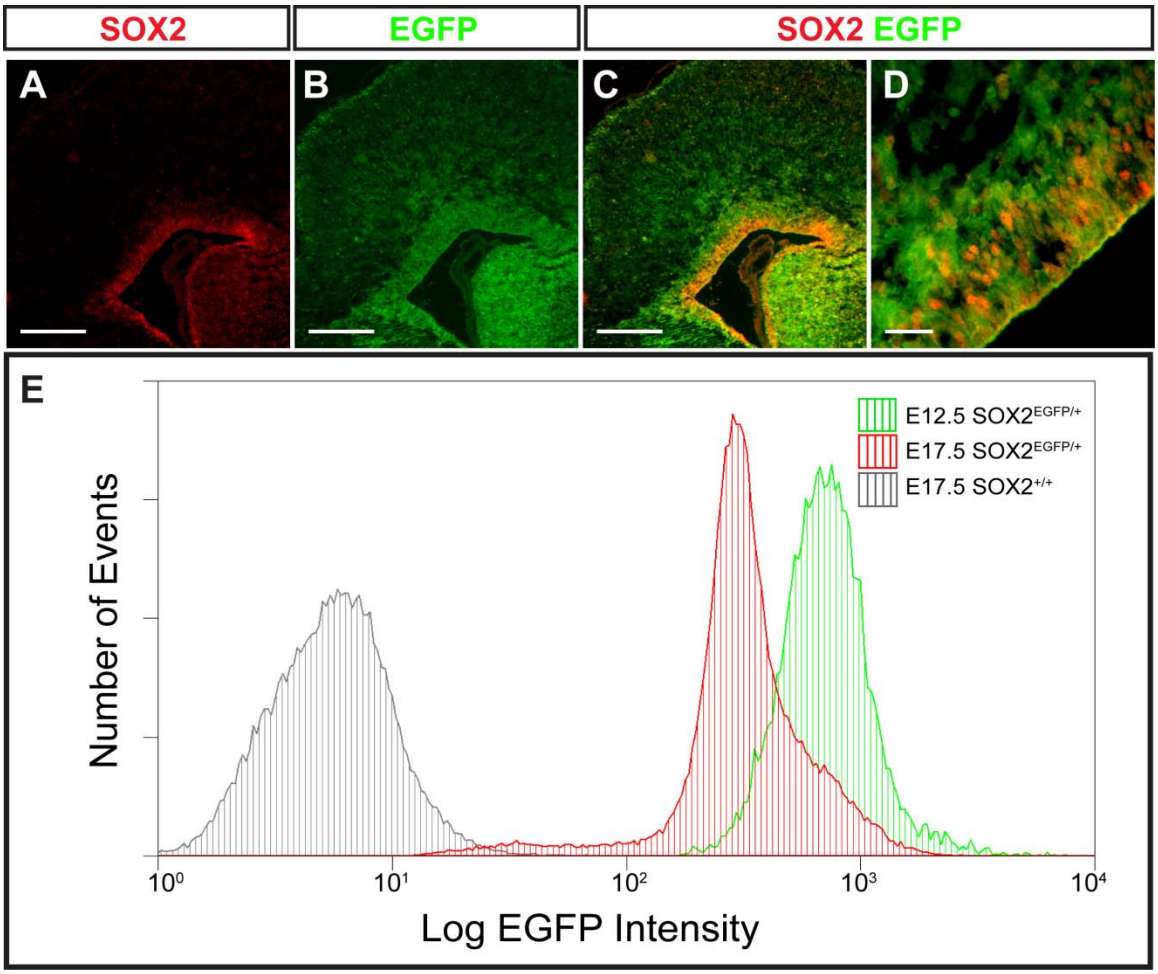

SOX2
EGFP
SOX2 EGFP
A
B
C
D
E
E12.5 SOX2EGFP/+
E17.5 SOX2EGFP/+
E17.5 SOX2+/+
Number of Events
10⁰
10¹
10²
10³
10⁴
Log EGFP Intensity

Figure 3.2. SOX2 expression in the developing dorsal telencephalon. Coronal sections of E17.5 $SOX2^{EGFP/+}$ mice labeled with an anti-SOX2 antibody **(A)** and expressing EGFP driven by the endogenous SOX2 promoter **(B)**. Cells coexpressing SOX2 and EGFP are located within the VZ and SVZ of the dorsal telencephalon **(C-D)**. Dissociated cells from E12.5 and E17.5 $SOX2^{EGFP/+}$ dorsal telencephalon were analyzed for EGFP-intensity using flow cytometry **(E)**. E12.5 cells express higher mean levels of EGFP **(E; green)** compared to E17.5 cells **(E; red)** and can be distinguished from $SOX2^{+/+}$, EGFP-negative cells **(E; grey)**. However, some E17.5 cells continue to express high EGFP-levels equal to E12.5 cells as indicated by the overlap in the two profiles. Abbreviations: EGFP, enhanced green fluorescent protein; VZ, ventricular zone; SVZ, subventricular zone. Scale Bars: (A-C)=250µm; (D)=20µm.

Figure 3.3

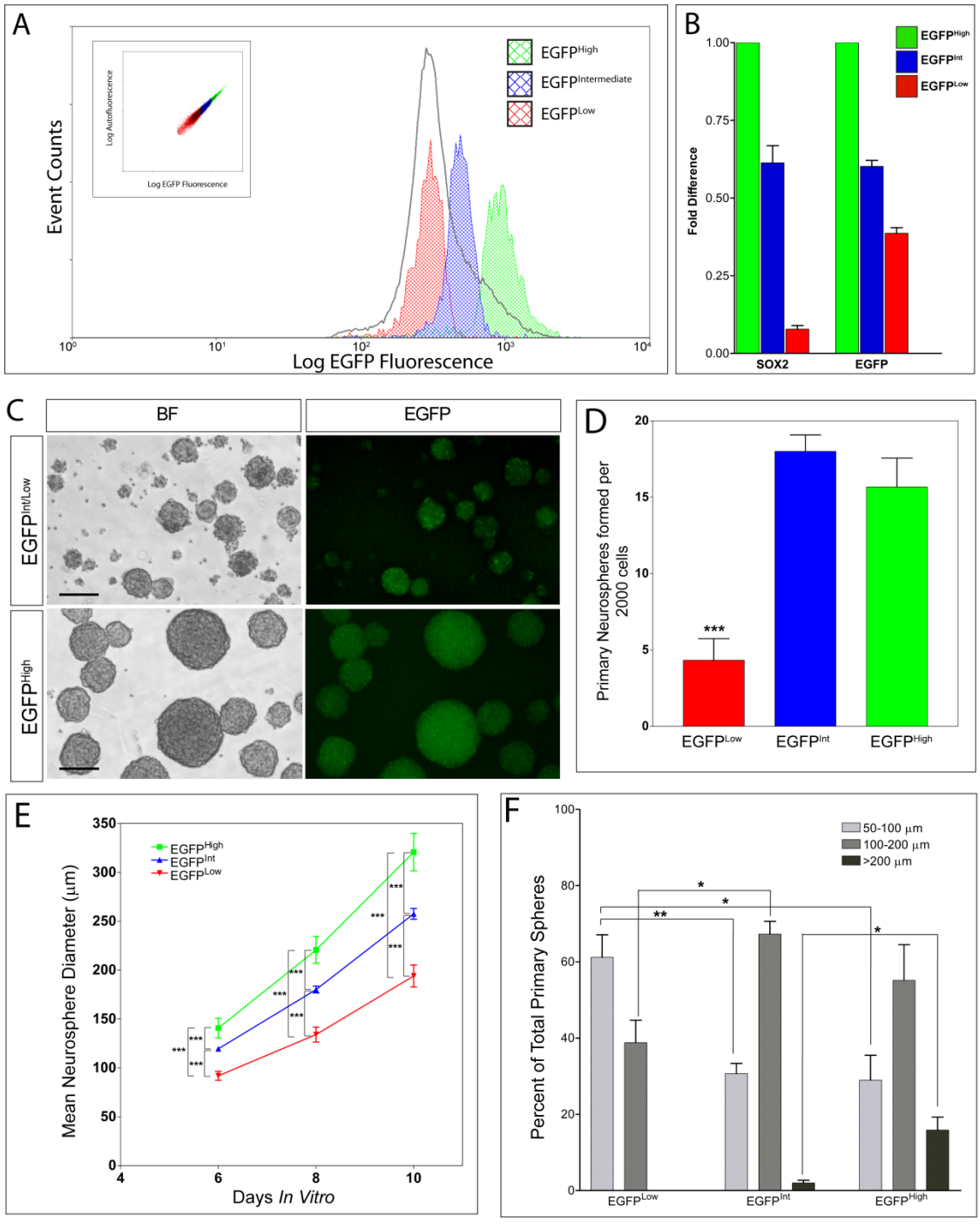
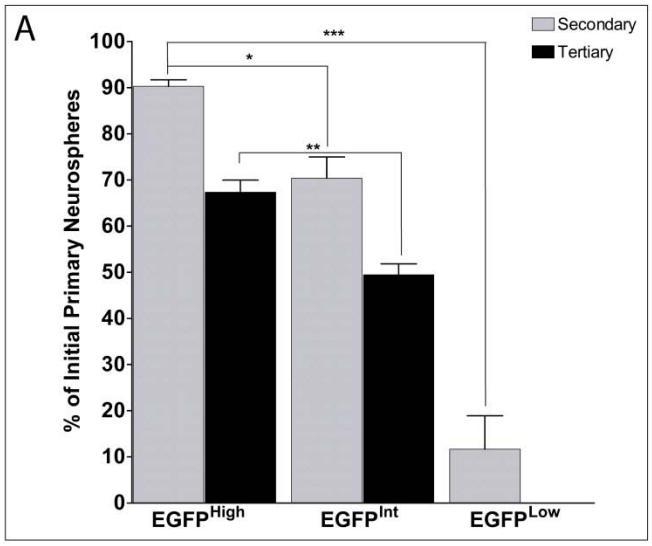
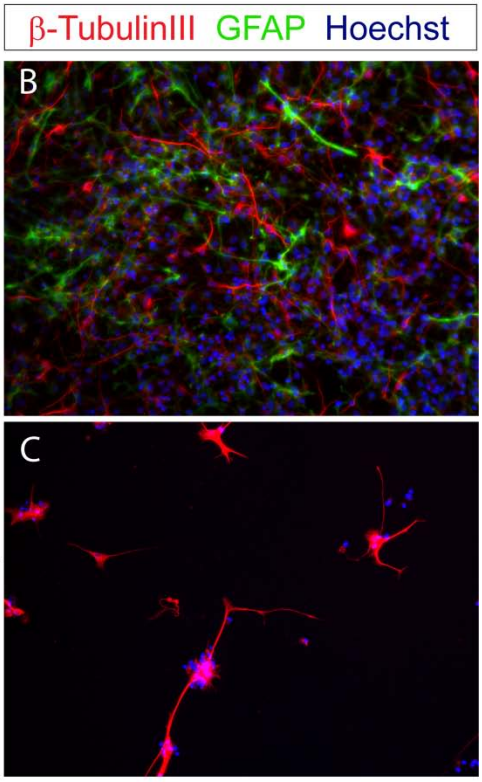

A
Event Counts
Log EGFP Fluorescence
Log Autofluorescence
EGFP^High
EGFP^Intermediate
EGFP^Low
B
Fold Difference
SOX2
EGFP
C
BF
EGFP
EGFP^Int/Low
EGFP^High
D
Primary Neurospheres formed per 2000 cells
***
E
Mean Neurosphere Diameter (μm)
Days In Vitro
F
Percent of Total Primary Spheres
50-100 μm
100-200 μm
>200 μm

Figure 3.3. *In vitro* analyses of cells isolated based upon SOX2-EGFP intensity levels. Cells from E17.5 dorsal telencephalon were separated into three subpopulations based upon their level of SOX2-EGFP expression: $EGFP^{High}$, $EGFP^{Int}$, and $EGFP^{Low}$ (n=4 for each sort)**(A)**. SOX2 and EGFP mRNA transcript levels from $EGFP^{High}$, $EGFP^{Int}$ and $EGFP^{Low}$ sorted cells (n=3) **(B)**. While each subpopulation was able to generate free-floating, EGFP-positive neurospheres, $EGFP^{High}$ neurospheres were larger than $EGFP^{Int}$ and $EGFP^{Low}$ neurospheres **(C)**. At 6 days *in vitro* (DIV), $EGFP^{Low}$ cells produced fewer neurospheres than $EGFP^{High}$ and $EGFP^{Int}$ cells **(D)**. Furthermore, the mean neurosphere diameter was larger for $EGFP^{High}$ cells, followed by $EGFP^{Int}$ and $EGFP^{Low}$ **(Cand E)**. Neurospheres were then cultured individually for four additional days and their diameters measured every 2 days. At 8 and 10 DIV, these size differences were maintained **(E)**. To compare the size of neurospheres within the subpopulations, neurospheres were categorized based upon size: small (100µm), medium (100-200µm) and large (>200µm) **(F)**. $EGFP^{Low}$ cells produced predominantly small neurospheres while $EGFP^{High}$ and $EGFP^{Int}$ cells generated predominantly medium sized neurospheres. However, $EGFP^{High}$ cells also produced a significant number of large neurospheres as well. Scale bars= 75µm. P Values: $p<0.05$ (*), $p<0.01$(**), $p<0.001$(***).

Figure 3.4





D

	EGFP^{High}	EGFP^{Int}	EGFP^{Low}
Percentage of Large Primary Neurospheres (>150μm)	89.16 ± 2.627	86.51 ± 1.73	62.34 ± 6.633
Percentage of Multipotential Neurospheres	87.5 ± 7.217	79.17 ± 4.167	54.17 ± 4.167

Figure 3.4. Analyses of self-renewal and multipotency. **(A):** To test for self-renewal capacity, dissociated $EGFP^{High}$, $EGFP^{Int}$ and $EGFP^{Low}$ primary neurospheres (n=16 neurospheres/condition) were cultured to form secondary and tertiary neurospheres. Secondary neurospheres were generated from all three subpopulations, but a significantly greater percentage of $EGFP^{High}$ primary neurospheres were capable of generating secondary neurospheres, followed by $EGFP^{Int}$ and $EGFP^{Low}$ primary neurospheres. Similarly, a significantly greater percentage of $EGFP^{High}$ secondary neurospheres were capable of generating tertiary neurospheres. $EGFP^{Low}$ secondary neurospheres, however, were unable to produce tertiary neurospheres, suggesting they were unable to self-renew. **(B-C):** Primary neurospheres from $EGFP^{High}$, $EGFP^{Int}$, and $EGFP^{Low}$ subpopulations were plated on coated slides at 12 DIV and cultured in the presence of 2% horse serum for one week to induce differentiation. Both multipotential neurospheres, labeled with both β-Tubulin III and GFAP **(B)** and neuronal-specific (β-Tubulin III only) **(C)** neurospheres were identified in all three subpopulations. **(D):** $EGFP^{High}$ and $EGFP^{Int}$ neurospheres were predominantly multipotential, whereas $EGFP^{Low}$ neurospheres had an increased percentage of unipotent, neuronal-specific neurospheres. Multipotential capacity of neurospheres is directly correlated with neurosphere size, with neurospheres less than 150μm exclusively identified as unipotential. Thus, the percentage of large neurospheres (>150μm) produced from each subpopulation was similar to the percentage of multipotential neurospheres identified from each subpopulation. Abbreviations: EGFP, Enhance Green Fluorescent Protein; GFAP, Glial Fibrillary Acidic Protein. P Values: $p<0.05$ (*), $p<0.01$(**), $p<0.001$(***).

Figure 3.5

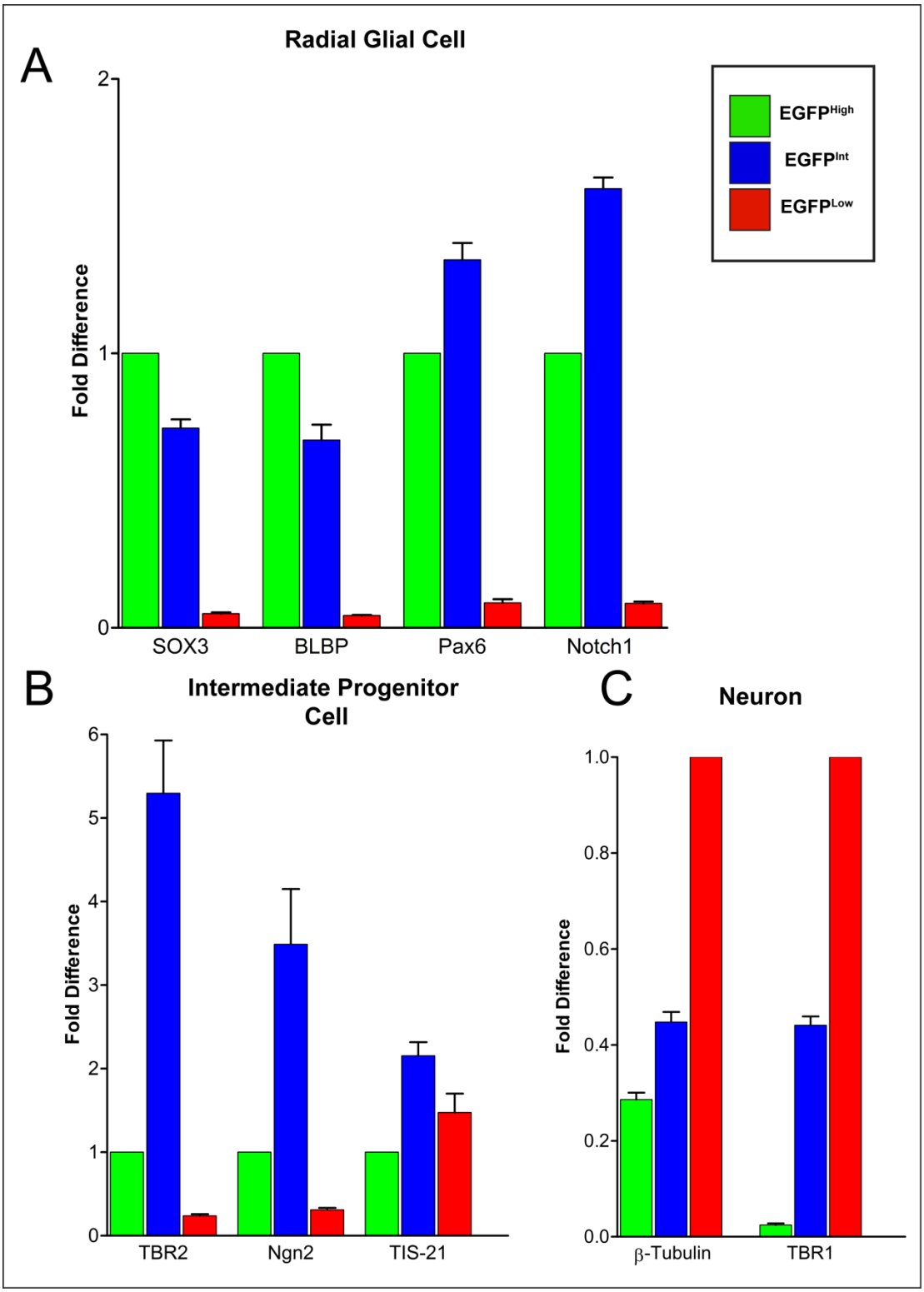

A
Radial Glial Cell
EGFP^High
EGFP^Int
EGFP^Low
Fold Difference
2
1
0
SOX3
BLBP
Pax6
Notch1
B
Intermediate Progenitor Cell
Fold Difference
6
5
4
3
2
1
0
TBR2
Ngn2
TIS-21
C
Neuron
Fold Difference
1.0
0.8
0.6
0.4
0.2
0.0
β-Tubulin
TBR1

Figure 3.5. Quantitative RT-PCR analyses of $EGFP^{High}$, $EGFP^{Int}$, and $EGFP^{Low}$ cells. After isolation using FACS, gene expression was analyzed from each subpopulation using qRT-PCR. **(A):** $EGFP^{High}$ cells express high levels of neural progenitor markers SOX3 and NOTCH1 and radial glial markers BLBP and PAX6. $EGFP^{Int}$ cells also express high levels of NOTCH1 and PAX6 however both SOX3 and BLBP are reduced. $EGFP^{Low}$ cells express low levels of SOX3, NOTCH1, BLBP, and PAX6. **(B)** TBR2 and NGN2, markers for intermediate progenitor cells, were highly expressed in $EGFP^{Int}$ cells compared to $EGFP^{High}$ and $EGFP^{Low}$ populations. TIS-21, a marker for neuronal-fated cells, is also highly expressed in $EGFP^{Int}$ cells but also in $EGFP^{Low}$ cells. **(C)** Neuronal markers β-Tubulin III and TBR1 are highly expressed in $EGFP^{Low}$ cells, suggesting they are post-mitotic neurons. Abbreviation: EGFP, Enhanced Green Fluorescent Protein; BLBP, Brain Lipid Binding Protein; NG2, Neurogenin 2; FACS, Fluorescent-automated cell sorting. N=4 litters.

Chapter Four

SOX2 Function is Necessary for the Proper Maintenance of Radial Glial Cells in the Dorsal Telencephalon

Introduction

SOX2, a member of the SOXB1 family of transcription factors, has been shown to play a fundamental role in the maintenance of a variety of stem/progenitor cell populations throughout embryonic development into adulthood (Guth and Wegner 2008; Chew and Gallo 2009). In mammals, SOX2 is expressed in early embryonic epiblast cells and its expression is maintained in a majority of neural progenitor cells of the central nervous system (CNS) including neuroepithelial (NEP) cells, radial glia, and intermediate progenitor cells (as shown in Chapter 3) (Collignon et al. 1996; Wood and Episkopou 1999; Avilion et al. 2003). However, the functional role of SOX2 in specific subsets of neural progenitor cell populations remains unclear. Studies in the developing chick spinal cord have demonstrated that inhibiting SOX2 function in neural progenitors results in cell cycle exit and restricted precocious neuronal differentiation, whereas constitutive expression of SOX2 inhibits differentiation and maintains progenitor characteristics (Bylund et al. 2003; Graham et al. 2003). However, these studies were performed using dominant-interfering constructs in which an Engrailed repressor domain was fused to the Sox2 or SOX3 DNA-binding domain and, therefore, have the potential to bind and disrupt downstream targets of other SOX factors expressed in the CNS. This is further supported by the observation that SOX14, a SOXB2 family member, can mimic the effects of SOX2-Engrailed and that either SOX1 or SOX3 can rescue these effects. Recently, genetic approaches have been used to address SOX2 function while minimizing the pleiotropic effects of misexpression and dominant-interference studies.

The genetic assessment of SOX2 function in the nervous system has proven difficult, due in part to the peri-implantation lethality of Sox2 homozygous knockout mice (Avilion et al. 2003). In addition, in many regions of the CNS SOX2 is coexpressed with its highly related SOXB1 homologues SOX1 and SOX3, which have been suggested to

partially compensate for the loss of SOX2 function in the nervous system (Collignon et al. 1996; Wood and Episkopou 1999). To circumvent this, investigations have focused on the developing retina, a region where SOX2 is exclusively expressed (Collignon et al. 1996; Wood and Episkopou 1999). Mice with hypomorphic mutations in SOX2 show aberrant neuronal differentiation in the retina while conditional ablation of SOX2 in retinal progenitor cells results in a loss of proliferating cells, suggesting that SOX2 is important in maintaining the proliferative capacity of neural progenitors in the eye (Taranova et al. 2006). Investigations into the function of SOX2 in the cortex are less clear. SOX2 hypomorphic mice display a decrease in neurogenesis and progenitor proliferation in the adult cortex, however the overall architecture of the cortex remains intact (Ferri et al. 2004; Cavallaro et al. 2008). Recently, SOX2 expression was conditionally ablated in neural progenitors using a conditional Sox2-knockout mouse line crossed to a Nestin-Cre mouse line (Miyagi et al. 2008). The resulting Nestin^{Cre}; SOX2 conditional-null homozygous mice display ventricular enlargement, reduced neurosphere forming potential, and embryonic lethality (Miyagi et al. 2008). These studies further show that SOX2 loss had no effect on the ability of neural progenitor cells to generate neurons and glia or in their ability to self renew *in vitro* (Miyagi et al. 2008). It is important to note, however, that both the conditional ablation and hypomorphic mutation of SOX2 in these studies occur at early neural tube stages, and therefore the effects of SOX2 loss on the specification of early neural progenitor cells cannot be disregarded. In addition, these investigations do not address the temporal dependence of SOX2 in distinct neural progenitor populations generated after neural induction, such as those in the developing cortex or the adult. Therefore, it remains to be determined whether SOX2 functions to maintain neural progenitor capacity throughout development, and if so, whether this function is essential or can be functionally compensated for by other SOXB1 factors.

Multiple populations of neural progenitor cells have been identified in regions throughout the developing and adult CNS. In the developing dorsal telencephalon, the precursor to the adult neocortex, two classes of neural progenitor cells have been identified: radial glial cells and intermediate (basal) progenitor cells. Together, these two cell types produce a majority of the glutamatergic projection neurons (also called pyramidal or excitatory neurons), as well as astrocytes and oligodendrocytes, found in the adult neocortex (Hartfuss et al. 2001; Malatesta et al. 2003; Haubensak et al. 2004; Casper and McCarthy 2006; Hevner 2006; Pontious et al. 2008). Radial glial cells are originally derived from the NEP cells of the neural plate and neural tube and were traditionally believed to act as glial scaffolding for newly generated migrating neurons (Rakic 1971). However, recent findings demonstrate that radial glial cells act as the predominant neural progenitor population in the dorsal telencephalon since they generate both neuronal and glial populations *in vivo* (Malatesta et al. 2000; Hartfuss et al. 2001; Haubensak et al. 2004). Furthermore, radial glial cells also give rise to the second neural progenitor population found in the dorsal telencephalon, the intermediate progenitor cells, which, in contrast to their multipotential precursors, exclusively differentiate into pyramidal neurons (Haubensak et al. 2004; Pontious et al. 2008; Kowalczyk et al. 2009).

Radial glial cells and intermediate progenitor cells can be distinguished by their cellular and molecular characteristics (Gotz and Huttner 2005). Cellularly, radial glial cells project long processes that extend the entire apical-basal axis of the dorsal telencephalon neuroepithelium (Hevner 2006; Malatesta et al. 2008). Furthermore, radial glia soma are confined to the apical, or ventricular, lumen region of the neuroepithelium called the ventricular zone (VZ) (Boulder-Committee 1970). In contrast, intermediate progenitor cells are spherical in shape, extend relatively short processes, and reside in a region basal to the VZ called the subventricular zone (SVZ) (Boulder-

Committee 1970). Molecularly, radial glial cells have been shown to express neural progenitor and astroglial markers including NESTIN, glutamate astrocyte specific transporter (GLAST), brain lipid binding protein (BLBP), PAX6, and in rodents activate the human promoter of the glial fibrillary acidic protein (hGFAP) (Casper and McCarthy 2006; Hevner 2006; Malatesta et al. 2008). Intermediate progenitor cells, on the other hand, express the proneural basic helix-loop-helix transcription factor Neurogenin2 (NGN2), as well as the T-Box transcription factor TBR2 and the *Cut* homeodomain transcription factor CUX2 (Bulfone et al. 1999; Kimura et al. 1999; Cai et al. 2000; Nieto et al. 2004; Zimmer et al. 2004; Ochiai et al. 2009). Both radial glial cells and intermediate progenitor cells, however, express SOX2; although it is unclear if SOX2 is necessary to maintain the proliferative and differentiation capacity of these cell populations as it does in other neural progenitor populations in the CNS.

In this chapter we conditionally ablate SOX2 expression in radial glial cells of the mouse developing dorsal telencephalon using a conditional SOX2-knockout line ($^{SOX2Cond/+}$). To target radial glial cells, we utilized a mouse line in which the expression of Cre-recombinase is driven by the hGFAP promoter. The hGFAP promoter has been demonstrated to be active in radial glial populations of the telencephalon at E13.5 and lineage tracing studies using both GFP and LacZ reporter mouse lines show that hGFAP driven CRE recombination occurs in pyramidal neurons and glia in the developing dorsal telencephalon; however its expression in ventral telencephalic neurons remains inconclusive (Brenner et al. 1994; Malatesta et al. 2000; Malatesta et al. 2003; Casper and McCarthy 2006). The resulting $hGFAP^{Cre}$;$SOX2^{Cond/Cond}$ mice demonstrate a loss of SOX2 in dorsal telencephalic neural progenitor cells and, as a result, a decrease in the number of proliferating radial glial cells and intermediate progenitor cells. Interestingly, we find that SOX2-deficient cells from the dorsal telencephalon are capable of generating neurospheres in numbers comparable to SOX2-positive littermate controls;

however these neurospheres are significantly smaller in size. Although the SOX2-mutant neurospheres maintain their ability to generate neurons and glia, they show a significantly diminished self-renewal potential. These analyses demonstrate that radial glial cells are dependent upon SOX2 to maintain their proliferative and self-renewal capacity *in vivo* and that this function is not fully compensated for by other SOXB1 members.

Results

Efficient Expression and Recombination of hGFAP^{Cre} in Radial Glia of the Dorsal Telencephalon

A 2.2kb fragment of the human promoter of Glial Fibrillary Acidic Protein (hGFAP) has been shown to drive expression of heterologous proteins such as LacZ or GFP in radial glial populations in the developing dorsal telencephalon (Brenner et al. 1994; Malatesta et al. 2000; Ellis et al. 2004). Lineage studies utilizing hGFAP-driven EGFP and LacZ expression have demonstrated that hGFAP-positive radial glia cells located within the developing dorsal telencephalon serve as precursors to a majority of the pyramidal projection neurons that are ultimately generated in this region, as well as to parenchymal astrocytes and oligodendrocytes that appear later in development (Brenner et al. 1994; Casper and McCarthy 2006). Therefore, to directly target radial glial populations for SOX2 ablation, we utilized a transgenic mouse line in which the expression of Cre-Recombinase (Cre) is driven by the hGFAP promoter (hGFAP^{Cre}) (Casper and McCarthy 2006).

In the mouse, radial glia populations emerge in the neuroepithelium at approximately E9.5 (Pinto and Gotz 2007). However, the onset of expression for the hGFAP promoter has been shown to occur at E13.5 (Brenner et al. 1994). To determine the spatial and temporal expression of hGFAP-driven Cre, we first analyzed coronal brain sections of E12.5 hGFAP^{Cre}-positive mice. At E12.5, CRE protein cannot be detected in the developing cortex using anti-CRE antibodies (Data not shown). However, by E15.5, CRE protein can be readily detected in the ventricular zone (VZ) of the developing dorsal telencephalon (Fig 4.1A-C). In addition, to test the recombination efficiency of the CRE enzyme, hGFAP^{Cre} mice were crossed to the Rosa26-LacZ reporter mouse strain (Rosa26$^{LacZ\text{-}Rep}$) in which a cassette containing the β-galactosidase

gene (LacZ), preceded by a stop sequence flanked by two loxP sites, is inserted into the ubiquitously expressed Rosa26 locus (Friedrich and Soriano 1993; Mao et al. 1999). In the presence of CRE, the loxP sites undergo recombination, excising the stop sequence and allowing for the expression of β-galactosidase (β-gal) which can then be detected using the substrate X-gal (Nagy 2000). In E15.5 hGFAP^{Cre};Rosa26$^{LacZ\text{-}Rep}$ embryos, β-gal is expressed throughout the VZ of the dorsal telencephalon, corresponding to the observed Cre expression (Fig 4.1D). In addition, β-gal is also observed distally in the cortical plate which supports previous observations that nascent neurons derived from radial glial cells in the dorsal telencephalon migrate toward the cortical plate (Fig 4.1D) (Malatesta et al. 2000; Hartfuss et al. 2001; Malatesta et al. 2003; Haubensak et al. 2004; Casper and McCarthy 2006). These results demonstrate that hGFAP^{Cre} expression and Cre-mediated recombination efficiently occur in dorsal telencephalic radial glial cells.

SOX2 Loss in the Dorsal Telencephalon Results in Reduced Cortical Thickness and Increased Embryonic Lethality

To temporally and spatially ablate SOX2 expression in radial glial cells, hGFAP^{Cre} mice were crossed to a mouse line carrying a conditional Sox2 allele in which the SOX2 open reading frame (ORF) is flanked by two loxP sequences (Sox2$^{Cond/+}$) (Taranova et al. 2006). At E12.5, before the onset of hGFAP-Cre expression, SOX2 is expressed in the dorsal telencephalon in both hGFAP^{Cre};Sox2$^{Cond/Cond}$ homozygous mice (Fig 4.2A) and hGFAP^{Cre};Sox2$^{Cond/+}$ heterozygous littermates (Fig 4.2B). However, at E14.5 there is a reduction in the number of cells expressing SOX2 in the VZ of hGFAP^{Cre};Sox2$^{Cond/Cond}$ mice (Fig 4.2C) compared to hGFAP^{Cre};Sox2$^{Cond/+}$ controls (Fig 4.2D). By E15.5, SOX2 expression is completely ablated in the dorsal telencephalon of

hGFAP^{Cre};Sox2$^{Cond/Cond}$ embryos (Fig 4.2E) but continues to be maintained throughout the VZ in hGFAP^{Cre};Sox2$^{Cond/+}$ mice (Fig 4.2F). Similarly, at E17.5 SOX2 expression cannot be detected in the hGFAP^{Cre};Sox2$^{Cond/Cond}$ dorsal telencephalon (Fig 4.2G) but is maintained in hGFAP^{Cre};Sox2$^{Cond/+}$ controls (Fig 4.2H). Throughout these embryonic stages, SOX2 expression is maintained in the ventral telencephalon of both hGFAP^{Cre};Sox2$^{Cond/Cond}$ (Fig 4.2A,C,E,G) and hGFAP^{Cre};Sox2$^{Cond/+}$ mice (Fig 4.2B,D,F,H), consistent with the lack of Cre expression in this region (Fig 4.1A-D). Moreover, no overt differences were observed in neuronal production using the pan-neuronal marker β-Tubulin III, which was expressed abundantly in the dorsal telencephalon of both hGFAP^{Cre};Sox2$^{Cond/Cond}$ and hGFAP^{Cre};Sox2$^{Cond/+}$ embryos (Fig 4.2 I-P). Furthermore, SOX2 expression was mutually exclusive of β-Tubulin III expression in all embryos analyzed (Fig 4.2Q-X).

Coincident with loss of SOX2 expression, we also observe a decrease in the number of hGFAP^{Cre};Sox2$^{Cond/Cond}$ embryos obtained after E12.5 (approximately 50% of the expected Mendelian level)(Fig 4.3, green). In contrast, the number of hGFAP^{Cre};Sox2$^{Cond/+}$ control embryos that are obtained are near expected Mendelian levels, demonstrating that this observation is genotype specific (Fig 4.3, red). Furthermore, although rare, we were able to obtain viable hGFAP^{Cre};Sox2$^{Cond/Cond}$ postnatal pups (Fig 4.3). Many of these animals exhibited unilateral, stressed-induced circling behavior suggesting they harbor neurological defects (Data not shown) and most die within 60 days of birth (Fig 4.3).

In addition to increased embryonic lethality of hGFAP^{Cre};Sox2$^{Cond/Cond}$ animals, we also observe a reduction in the cortical thickness in hGFAP^{Cre};Sox2$^{Cond/Cond}$ embryos . Quantitative analysis of E15.5 embryos (n=4) demonstrates that there is a statistically significant reduction in the thickness of the dorsal telencephalon in hGFAP^{Cre};Sox2$^{Cond/Cond}$ embryos (Fig 4.4A) in contrast to hGFAP^{Cre};Sox2$^{Cond/+}$ littermate

controls (Fig 4.4 B,C $p<0.05$), with no difference observed in the thickness of the ventricular zone between these groups (Fig 4.4C). However, the inability to detect any differences in β-Tubulin III expression between wildtype and mutant embryos suggests that further analysis is necessary to determine if differences in other neuronal markers are observed.

The Loss of SOX2 in the Dorsal Telencephalon Results in a Decrease in the Number of Proliferating Radial Glial Cells and Intermediate Progenitor Cells

The reduced cortical thickness in $hGFAP^{Cre};Sox2^{Cond/Cond}$ mice suggests that loss of SOX2 expression in the dorsal telencephalon may impair proper cortical development. Therefore, we assessed the progenitor characteristic of SOX2 mutant cells by examining the expression of radial glial and intermediate progenitor cell markers as well as their proliferative capacity using BrDU. SOX1, a SOXB1 homologue of SOX2, is coexpressed with SOX2 in neural progenitor cells including radial glia throughout the nervous system and its high sequence similarity to SOX2 suggests that it may be able to compensate for SOX2 loss (Collignon et al. 1996; Wood and Episkopou 1999; Avilion et al. 2003). At E15.5, SOX1 expression is maintained in radial glial cells throughout the VZ and SVZ of the dorsal telencephalon in $hGFAP^{Cre};Sox2^{Cond/Cond}$ and $hGFAP^{Cre};Sox2^{Cond/+}$ embryos (Fig 4.5A-B), suggesting that the loss of SOX2 expression does not directly affect SOX1 expression.

Next, we addressed whether radial glial populations are affected by the loss of SOX2 using the radial glial marker PAX6 (Gotz et al. 1998). PAX6 is expressed in both $hGFAP^{Cre};Sox2^{Cond/+}$ (Fig 4.5C) and $hGFAP^{Cre};Sox2^{Cond/Cond}$ embryos at E15.5 (Fig 4.5D). However, the number of PAX6-positive cells was significantly reduced in $hGFAP^{Cre};Sox2^{Cond/Cond}$ embryos, suggesting that there was a decrease in the number of radial glial cells in the dorsal telencephalon of these embryos (Fig 4.5I, $p<0.01$). Since

radial glial cells give rise to the intermediate progenitor population which resides in the SVZ, we hypothesized that the reduction in radial glial cells in hGFAP^{Cre};Sox2$^{Cond/Cond}$ animals would also decrease the number of intermediate progenitor cells as well. We observed a significant reduction in the number of TBR2 positive cells in the SVZ of hGFAP^{Cre};Sox2$^{Cond/Cond}$ embryos compared to hGFAP^{Cre};Sox2$^{Cond/+}$ controls (Fig 4.5E-F,J, $p<0.01$). Together, these data suggest that the loss of SOX2 in radial glial cells may affect their ability to proliferate, resulting in fewer radial glial cells, and as a consequence, fewer intermediate progenitor cells. Therefore, to determine whether there is a reduction in proliferating cells in the hGFAP^{Cre};Sox2$^{Cond/Cond}$ dorsal telencephalon, we labeled cells with the S-phase marker bromo-deoxyuridine (BrDU). The hGFAP^{Cre};Sox2$^{Cond/Cond}$ embryos show a significant reduction in the number of BrDU-positive cells in the dorsal telencephalon compared to hGFAP^{Cre};Sox2$^{Cond/+}$ controls (Fig 4.5G-H, K, $p<0.05$). These results collectively demonstrate that the loss of SOX2 results in a reduction in proliferating radial glial and intermediate progenitor cell populations in the dorsal telencephalon which subsequently leads to a thinning of the cortex. It remains to be determined if this phenotype is the result of a decrease in neuronal production or defects in neuron maturation or morphology.

SOX2-deficient Neural Progenitor Cells Have a Reduced Proliferative and Self-renewal Capacity and are able to Generate Multipotential Neurospheres.

To test the self-renewal capacity and differentiation potency of SOX2-deficient cells, the dorsal telencephalons of E15.5 hGFAP^{Cre};Sox2$^{Cond/Cond}$ and hGFAP^{Cre};Sox2$^{Cond/+}$ embryos were dissociated and cultured at clonal density *in vitro* to form free-floating colonies called neurospheres (Coles-Takabe et al. 2008; Hutton and Pevny 2008). Interestingly, both hGFAP^{Cre};Sox2$^{Cond/Cond}$ and hGFAP^{Cre};Sox2$^{Cond/+}$ cells

were able to generate equal numbers of neurospheres in culture (Fig 4.6A-B,C). However, the neurospheres generated from hGFAP^{Cre};Sox2$^{Cond/Cond}$ embryos were significantly smaller than those generated from hGFAP^{Cre};Sox2$^{Cond/+}$ embryos (Fig 4.6A-B, D; $p<0.001$). To verify that the neurospheres generated from hGFAP^{Cre};Sox2$^{Cond/Cond}$ mice were derived from SOX2-negative cells, neurospheres from both hGFAP^{Cre};Sox2$^{Cond/Cond}$ and hGFAP^{Cre};Sox2$^{Cond/+}$ embryos were allowed to attach to adherent slides and stained with SOX2 antibody. The hGFAP^{Cre};Sox2$^{Cond/Cond}$ neurospheres were negative for SOX2 expression (Fig 4.7A-C) while hGFAP^{Cre};Sox2$^{Cond/+}$ neurospheres continue to express SOX2 (Fig 4.7D-F). These results suggest that SOX2-deficient dorsal telencephalic neural progenitor cells are capable of generating neurospheres *in vitro*, albeit at a reduced size compared to control littermates.

To test the self-renewal capacity of SOX2-deficient neural progenitor cells, individual primary neurospheres were dissociated into single cells and re-cultured at clonal density to form secondary neurospheres. Both hGFAP^{Cre};Sox2$^{Cond/Cond}$ (Fig 4.8A) and hGFAP^{Cre};Sox2$^{Cond/+}$ (Fig 4.8B) primary neurospheres were able to generate secondary neurospheres, however significantly fewer hGFAP^{Cre};Sox2$^{Cond/Cond}$ secondary neurospheres were generated compared to hGFAP^{Cre};Sox2$^{Cond/+}$ cells (Fig 4.8C; $p<0.001$), demonstrating that SOX2-deficient primary neurospheres have a reduced ability to self-renew. Furthermore, similar to the results obtained from the primary neurosphere cultures, hGFAP^{Cre};Sox2$^{Cond/Cond}$ secondary neurospheres were significantly smaller than hGFAP^{Cre};Sox2$^{Cond/+}$ neurospheres (Fig 4.8D; $p<0.001$). These data suggest that SOX2-deficient neural progenitor cells have a decreased capacity for self-renewal *in vitro* compared to littermate controls.

Lastly, to test the ability of SOX2-deficient neurospheres to generate neurons and glia (multipotency), primary neurospheres from hGFAP^{Cre};Sox2$^{Cond/Cond}$ and

hGFAP^{Cre};Sox2$^{Cond/+}$ embryos were transferred to laminin coated slides and induced to differentiate. Both hGFAP^{Cre};Sox2$^{Cond/+}$ (Fig 4.8E-G) and hGFAP^{Cre};Sox2$^{Cond/Cond}$ (Fig 4.8H-J) neurospheres were capable of differentiating into both β-Tubulin III-positive neurons and GFAP-positive astrocytes *in vitro*. These results suggest that Sox2-deficient neurospheres are capable of multipotential differentiation.

Collectively, we demonstrate that the loss of SOX2 in radial glial cells significantly reduces the number of proliferating radial glial cells and intermediate progenitor cells in the developing dorsal telencephalon. This reduction in the number of neural progenitors results in a thinning of the developing cortex. In addition, SOX2-deficient cells are still capable of generating multipotential neurospheres *in vitro*; however they are smaller and have a decreased ability to self-renew.

Discussion

In vivo investigations into the role of SOX2 in neural progenitor cells in the mammalian cortex have been difficult due to early embryonic lethality of SOX2-null mice (Avilion et al. 2003). To circumvent this obstacle, we have utilized a mouse line in which SOX2 can be conditionally ablated in a temporally defined and spatially restricted manner (Sox2^{Cond}) (Taranova et al. 2006). By crossing the Sox2^{Cond} mouse line with the hGFAP^{Cre} mouse line, we specifically addressed the role of SOX2 in radial glial cells of the dorsal telencephalon. The ablation of SOX2 in embryonic radial glial cells results in a decrease in the number of proliferating radial glial cells and in their direct descendents, intermediate progenitor cells. Furthermore, the loss of SOX2 also results in a reduction in cortical thickness and, in conjunction with the *in vitro* analyses (neurosphere assay), suggest that SOX2-deficient cells have decreased proliferative and self-renewal

capacities. These results are in concert with previous findings that demonstrate that loss of SOX2 in early mouse retinal progenitor cells leads to a hypocellular eye phenotype (Taranova et al. 2006), as well as to a decrease in proliferating cortical cells when SOX2 is ablated at neural plate stages (Miyagi et al. 2008). Moreover, interfering with SOX2 function, using dominant-negative constructs in chick neural tube, also results in neural progenitor cells prematurely exiting the cell cycle (Bylund et al. 2003; Graham et al. 2003). These data suggest that SOX2 is important in maintaining the proliferative and differentiation capacity of neural progenitor cells throughout the developing the CNS.

The results of the *in vitro* examination of hGFAP^{Cre};SOX2-deficient radial glial cells are in contrast with observations made using the Nestin-Cre mouse line to ablate SOX2 (Miyagi et al. 2008). Primarily, we demonstrate that SOX2-deficient cells are equally as capable at generating primary neurospheres as SOX2-positive cells but are severely inhibited in generating secondary neurospheres. Miyagi et al. (2008) showed a decrease in primary neurospheres generated by SOX2-deficient cells but no reduction in secondary neurosphere formation. There are several possibilities to explain these discrepancies. First, the two SOX2 conditional knockout mouse lines used in these studies were independently generated. Therefore, it is unclear whether the efficiency of SOX2 excision is equal between the two. Second, we utilize the hGFAP^{Cre} mouse strain to drive CRE which is expressed at approximately E13.5, a stage after many neural progenitor populations, including radial glial cells, have already become specified (Malatesta et al. 2000; Casper and McCarthy 2006). Thus, we are ablating SOX2 function in established radial glial populations and their derivatives. In contrast, Miyagi et al. (2008) utilize a Nestin-driven Cre-recombinase mouse line to direct SOX2 ablation. The onset of Nestin expression occurs at about E7.5 (Dahlstrand et al. 1995; Kawaguchi et al. 2001) and it is expressed in all NEP cells of the neural plate

(approximately E9.5), which will collectively go on to generate the entire CNS (Frederiksen and McKay 1988). Therefore, in these mice, SOX2 expression is lost in all NEP cells at the very onset of neural development which, in turn, may adversely affect the specification and development of all subsequent neural progenitor populations (which may also account for their inability to obtain viable postnatal mice). Therefore, the *in vitro* differences observed between the two studies may be an effect of molecular changes occurring in neural progenitor cells during early or late stages of development that influence their fate specification and commitment. Lastly, the discrepancies observed between the two *in vitro* studies may not be due to cell intrinsic mechanisms, but rather to differences in the culture conditions that were used, which in turn may overtly affect the behavior of neural progenitor cells *in vitro* (Deleyrolle and Reynolds 2009). Further studies are necessary using different Cre mouse strains to help clarify these observations.

Our results are the first to demonstrate that SOX2 expression is necessary for the proper maintenance of specified neural progenitor populations at mid to late stages of neural development. The loss of SOX2 in dorsal radial glial cells reduces the number of neural progenitor cells within the VZ and SVZ and adversely effects the proper development of the dorsal telencephalon, resulting in a thinning of the neocortex. In addition, these results show that SOX1 and SOX3 cannot completely functionally compensate for SOX2 in these cells, confirming the importance of SOX2 in neural progenitor populations of the CNS.

Materials and Methods

Animal Breeding and Husbandry

All animals were used and maintained in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of North Carolina-Chapel Hill. The generation of the $Sox2^{Cond}$ mouse line has been published previously (Taranova et al. 2006). The $hGFAP^{Cre}$ mouse line was generously provided by Ken McCarthy (Univ. of North Carolina-Chapel Hill) and has been described previously (Casper and McCarthy 2006). The $hGFAP^{Cre}$;$Sox2^{Cond}$ mouse line was produced by mating $hGFAP^{Cre}$ mice with $SOX2^{Cond/+}$ mice; subsequent generations were maintained on a Sox2 heterozygous background ($hGFAP^{Cre}$;$Sox2^{Cond/+}$). To produce $hGFAP^{Cre}$;$Sox2^{Cond/Cond}$ mutants, $hGFAP^{Cre}$;$Sox2^{Cond/+}$ males and females were crossed. For proliferating analysis, pregnant females were injected intraperitoneally with BrDU (100mg/kg body mass) 2 hours before being euthanized for embryo harvest. The morning of the plug date was recorded as E0.5.

Tissue Dissociation and Neurosphere Assay

The generation of neurospheres was conducted using previously published protocols (Hutton and Pevny 2008). The dorsal telencephalon was dissected from $hGFAP^{Cre}$;$Sox2^{Cond/Cond}$ and $hGFAP^{Cre}$;$Sox2^{Cond/+}$ embryonic brains (n=4 for each genotype) and incubated in Papain (Roche) followed by successive treatments with Trypsin Inhibitor (Sigma) and a final wash in Neurobasal medium (Gibco). The tissue was then mechanically dissociated using a pipettor into a single-cell suspension in Neurobasal medium containing B27 and N2 supplements (Gibco) and 10ng/ml basic Fibroblast Growth Factor (bFGF) and Epidermal Growth Factor (EGF) (Invitrogen). Dissociated cells were then seeded at clonal density of 2000 cells/6cm dish (283 cells/cm^2) (Coles-Takabe et al. 2008) in non-adherent plates (Nunc). After five days in

culture, the number of neurospheres was counted per plate and the diameter of individual neurospheres measured using ImagePro Express Software (MediaCybernetics). To generate secondary neurospheres, individual primary neurospheres were mechanically dissociated into a single cell suspension and the cells plated again at clonal density. For differentiation analysis, individual neurospheres were plated in 8-well chambers slides (Nunc) coated with Poly-D-Lysine and Laminin in Neurobasal medium containing bFGF and EGF. After 24 hours, the media was removed and replaced with Neurobasal medium (+ 2% heat-inactivated horse serum). Cells were cultured for one week under these conditions after which they were fixed with 4% paraformaldehyde (PFA) for 30 minutes at room temperature and stained using the immunocytochemistry procedures below.

Immunocytochemistry

Dissected mouse embryos were fixed in 4% PFA overnight at 4°C. After fixation, tissue was subjected to a sucrose gradient (10%, 20%, 30%) and frozen in OCT medium (Tissue-Tek). Frozen 12µm coronal sections were taken using a cryostat on Superfrost Plus slides (VWR). For staining, slides were blocked for one hour at room temperature with 10% Goat Serum/1% Triton-100 in Phosphate Buffered Saline (PBS). Primary and secondary antibodies were diluted in 5% goat serum/0.1% Triton-100 in PBS. Slides were treated with primary antibodies overnight at 4°C and secondary antibodies for 1 hour at room temperature. Primary antibodies used are: SOX2 (1:3000 Millipore; 1:100 R&D Systems), PAX6 (1:100 Developmental Studies Hybridoma Bank), TBR2 (1:500 AbCam), β-Tubulin III (TUJ1, 1:1000 Covance), Bromo-Deoxyuridine (BrDU, 1:500, BD Biosciences), Glial Fibrillary Acidic Protein (GFAP, 1:500, Dako), Cre-recombinase (1:2000 Novagen), and SOX1 (1:1000 (Pevny et al. 1998)). Secondary antibodies used

were: anti-rabbit Alexa 488 or 546 (1:1000 Invitrogen) and anti-mouse (IgG1 and IgG2A) Alexa 488 and 546 (1:1000 Invitrogen). All slides were counterstained with Hoechst 33258 (Invitrogen). Fluorescent images were taken on a Leica Microsystems DM-IRB inverted fluorescent microscope (Wetzlar, Germany) and a QImaging Retiga-SRV camera (Surrey, BC, Canada) using ImagePro Express software (MediaCybernetics).

Hematoxylin-Eosin Staining

Frozen coronal sections were incubated in PBS for 30 minutes at room temperature to remove excess OCT. Slides were then incubated in Harris' Hematoxylin Solution (Sigma) for 5 minutes and then washed with dH20. Next, slides were dipped twice in acid alcohol (1% HCl in 70% EtOH) rinsed with copious amounts of tap water, followed by dH20. Slides were then dehydrated using 95% ethanol, placed in alcoholic Eosin Y Solution (Sigma) for 30 seconds, then rinsed with two steps of 95% and then 100% ethanol.

Measuring Cortical Thickness and Cell Density

To measure cortical thickness, 12µm coronal slices were measured from the ventricular surface to the pial surface using the measurement tool in the ImagePro Express Software at 20x magnification. The mean of three measurements was recorded per 12µm slice with three to four consecutive slices measured from each embryo (n=4/genotype).

For cell count comparisons, 12µm coronal sections were stained for cell-specific antibodies and counterstained with Hoechst 33258 to label nuclei. Pictures were taken at 20x magnification and a 300µm x 300 µm box was drawn over the dorsal telencephalon using Photoshop software. Cells double-labeled for Hoechst and

antibody were then counted. Four consecutive slices were measured for each embryo (n=3/genotype).

Statistical Analysis

All data were analyzed using Prism 3.0 software (GraphPad). Samples were analyzed using a two-tailed T-test.

Figure 4.1

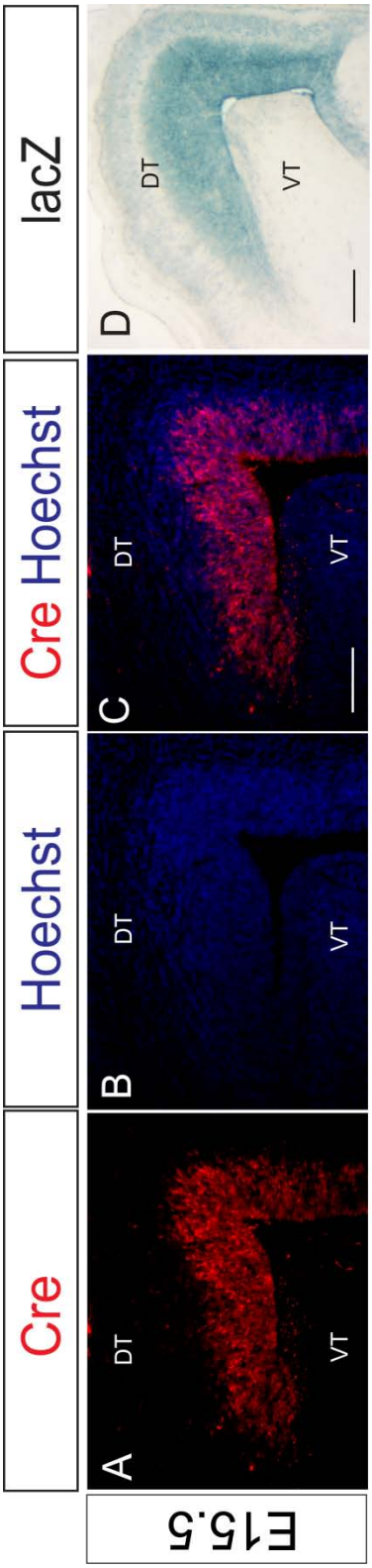


Figure 4.1. hGFAP driven Cre-recombinase expression in the E15.5 dorsal telencephalon. E15.5 coronal sections of hGFAP^{Cre} embryos labeled with anti-Cre antibody (A,C) and counterstained with Hoechst 33258 to label nuclei (B,C). hGFAP^{Cre};Rosa26$^{LacZ\text{-}Rep}$ embryo treated with X-Gal to identify cells in which Cre-mediated recombination occurs (D). Cre is expressed in the DT but not VT (A,C-D). Abbreviations: hGFAP, human Glial Fibrillary Acidic Protein; DT, dorsal telencephalon; VT, ventral telencephalon. Scale Bars=200μm.

Figure 4.2

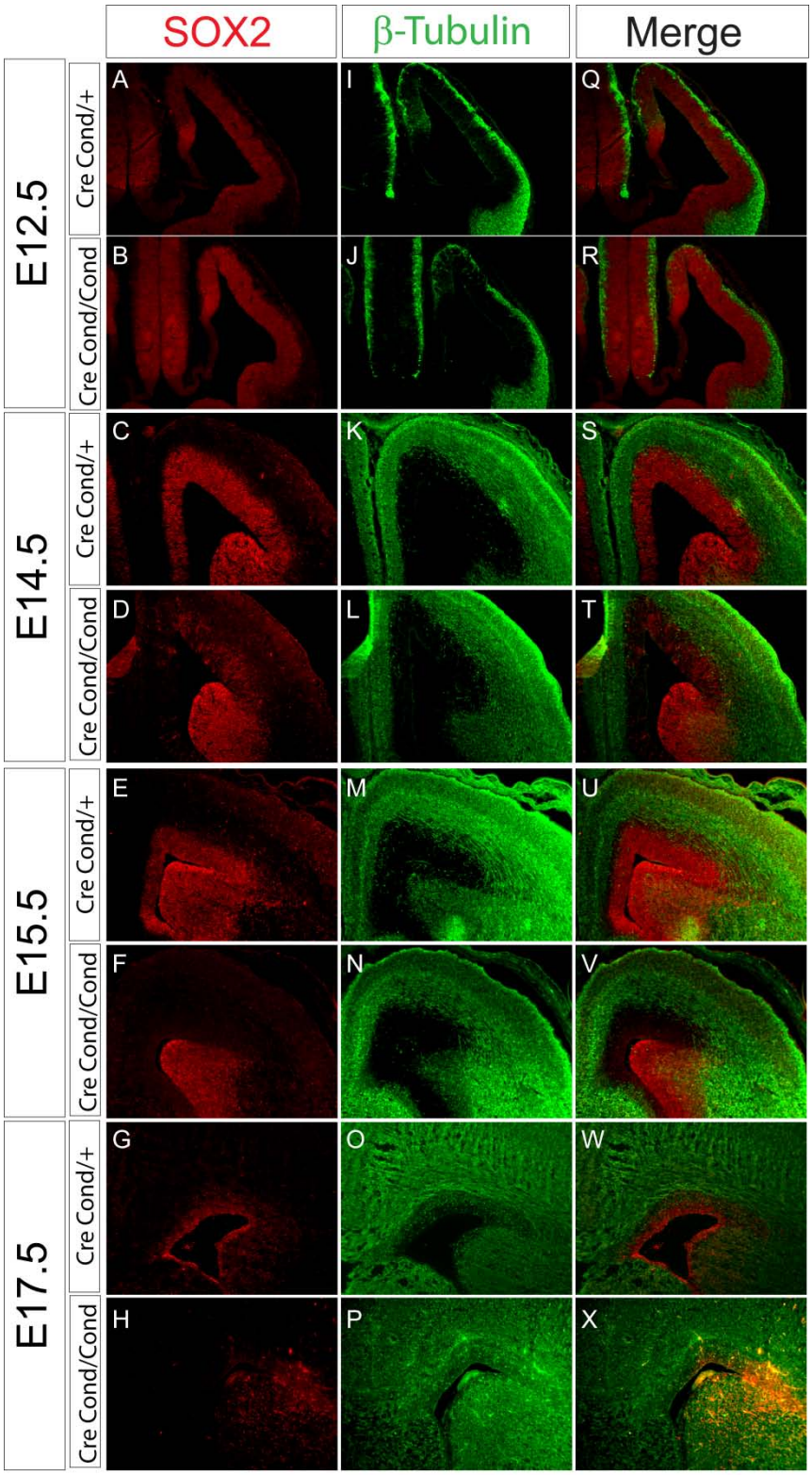

SOX2
β-Tubulin
Merge
E12.5
E14.5
E15.5
E17.5
Cre Cond/+
Cre Cond/Cond
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X

Figure 4.2. **Developmental time course of SOX2 expression in hGFAP^{Cre};SOX2$^{Cond/+}$ and hGFAP^{Cre};SOX2$^{Cond/Cond}$ mice.** 5X images of SOX2 **(A-H, Q-X)** and β-Tubulin III **(I-P, Q-X)** antibody staining in hGFAP^{Cre};SOX2$^{Cond/+}$ and hGFAP^{Cre};Sox2$^{Cond/Cond}$ embryos at E12.5, E14.5, E15.5 and E17.5. SOX2 is expressed in both hGFAP^{Cre};SOX2$^{Cond/+}$ and hGFAP^{Cre};Sox2$^{Cond/Cond}$ embryos **(A and B)** at E12.5. In hGFAP^{Cre};Sox2$^{Cond/Cond}$ embryos, a reduction in the number of SOX2-positive cells found in the DT occurs at E14.5 **(D)** while a complete loss of SOX2-positive cells is seen by E15.5 **(F)** into E17.5 **(H)**. In contrast, SOX2 is maintained in the DT of hGFAP^{Cre};SOX2$^{Cond/+}$ embryos throughout development **(A,C,E,G).** β-Tubulin III is expressed in both hGFAP^{Cre};SOX2$^{Cond/+}$ and hGFAP^{Cre};Sox2$^{Cond/Cond}$ embryos **(I-P, Q-X)** and is mutually exclusive of SOX2 expression **(Q-X).** Abbreviations: DT, Dorsal Telencephalon.

Figure 4.3

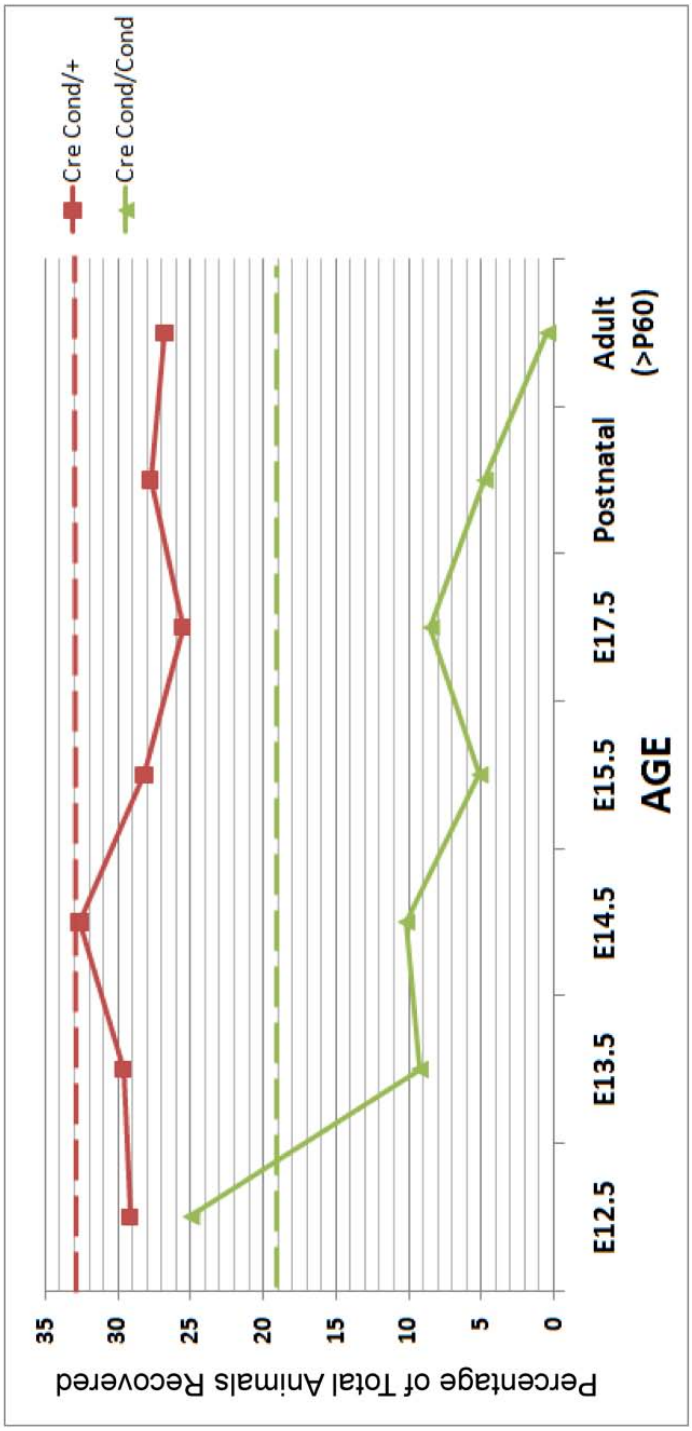

Cre Cond/+
Cre Cond/Cond
Percentage of Total Animals Recovered
35
30
25
20
15
10
5
0
E12.5
E13.5
E14.5
E15.5
E17.5
Postnatal
Adult (>P60)
AGE

Figure 4.3 Distribution of hGFAP^{Cre};SOX2$^{Cond/+}$ and hGFAP^{Cre};Sox2$^{Cond/Cond}$ genotypes obtained throughout development. The percentage of hGFAP^{Cre};SOX2$^{Cond/+}$ embryos **(Red solid line)** was near the expected Mendelian distribution **(Red dotted line)**. In contrast, hGFAP^{Cre};Sox2$^{Cond/Cond}$ embryos **(Green solid line)** were obtained at a much lower percentage than is predicted by the Mendelian distribution **(Green dotted line)** suggesting embryonic lethality occurs after E12.5.

Figure 4.4

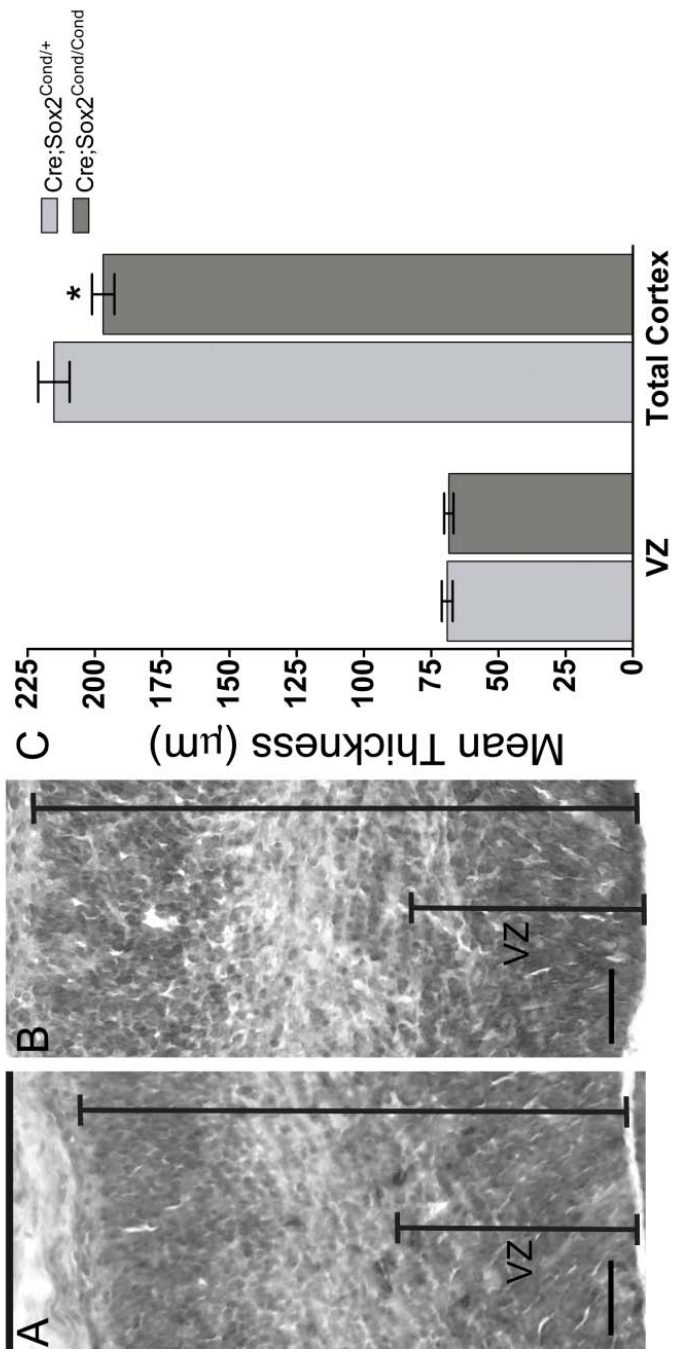

A
VZ
B
VZ
C
Mean Thickness (μm)
225
200
175
150
125
100
75
50
25
0
VZ
Total Cortex
*
Cre;Sox2Cond/+
Cre;Sox2Cond/Cond

Figure 4.4. Reduction in Cortical Thickness in hGFAP^{Cre};Sox2$^{Cond/Cond}$ embryos. Hematoxylin-Eosin stained sections of E15.5 dorsal telencephalon of hGFAP^{Cre};Sox2$^{Cond/Cond}$ **(A)** and hGFAP^{Cre};SOX2$^{Cond/+}$ **(B)** embryos. There is a statistically significant reduction in the cortical thickness of hGFAP^{Cre};Sox2$^{Cond/Cond}$ embryos compared to hGFAP^{Cre};SOX2$^{Cond/+}$ controls **(C)** but no difference was seen in the thickness of the VZ **(A-C).** Scale Bars=50µm. (*) =$p<0.05$.

Figure 4.5

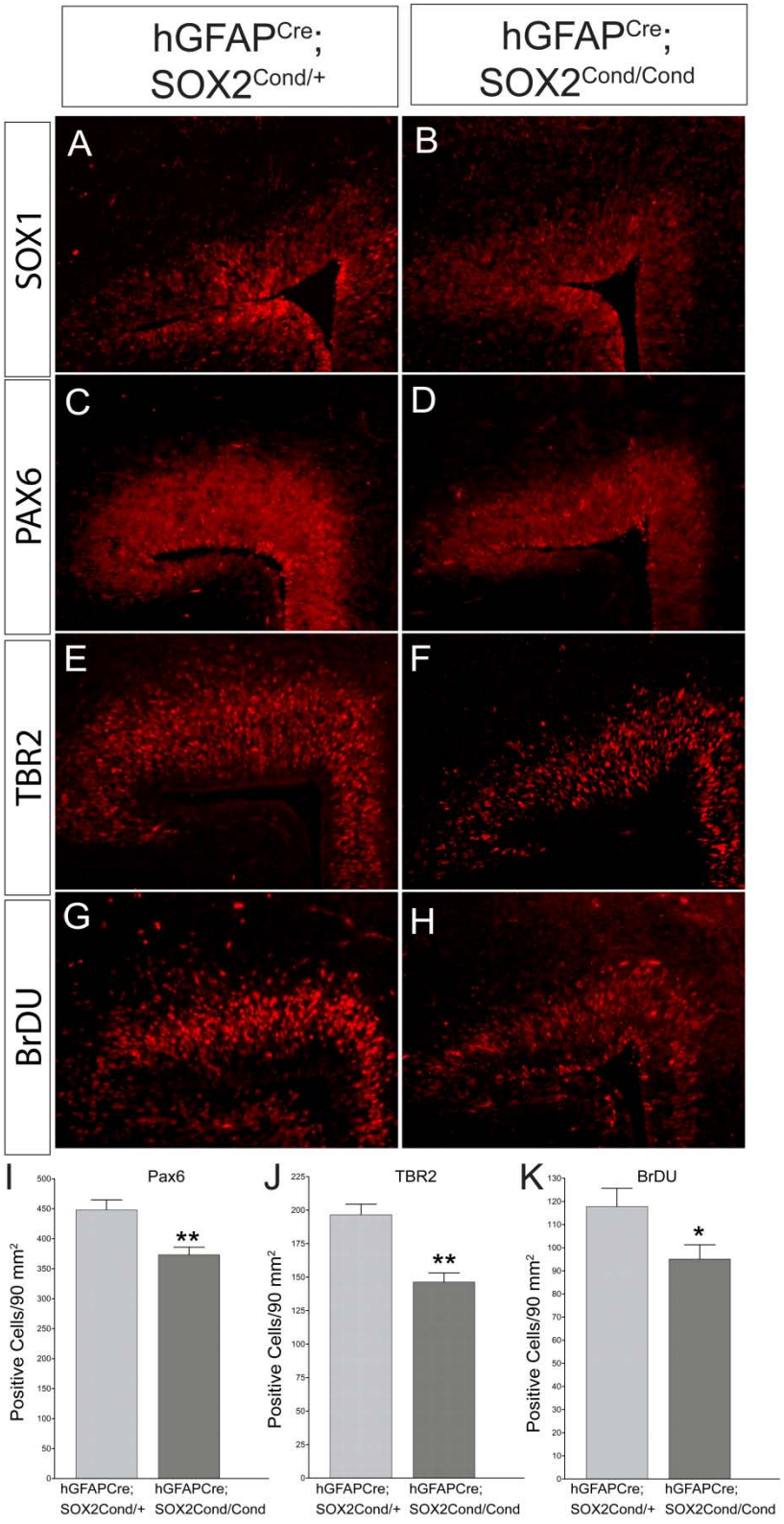

hGFAP^Cre; SOX2^Cond/+
hGFAP^Cre; SOX2^Cond/Cond
SOX1
PAX6
TBR2
BrDU
A
B
C
D
E
F
G
H
I
Pax6
Positive Cells/90 mm²
**
hGFAPCre; SOX2Cond/+
hGFAPCre; SOX2Cond/Cond
J
TBR2
Positive Cells/90 mm²
**
hGFAPCre; SOX2Cond/+
hGFAPCre; SOX2Cond/Cond
K
BrDU
Positive Cells/90 mm²
*
hGFAPCre; SOX2Cond/+
hGFAPCre; SOX2Cond/Cond

Figure 4.5 Expression of Dorsal Telencephalic Cell-specific Markers in E15.5 hGFAP^{Cre};SOX2$^{Cond/+}$ and hGFAP^{Cre};Sox2$^{Cond/Cond}$ embryos. The neural progenitor marker SOX1 is expressed in the DT of both hGFAP^{Cre};SOX2$^{Cond/+}$ **(A)** and hGFAP^{Cre};Sox2$^{Cond/Cond}$ **(B)** embryos. The radial glial marker PAX6 is expressed in the DT of hGFAP^{Cre};SOX2$^{Cond/+}$ mice **(C)** but reduced in hGFAP^{Cre};Sox2$^{Cond/Cond}$ embryos **(D, I)**. The intermediate progenitor marker TBR2 is also maintained in the DT of hGFAP^{Cre};SOX2$^{Cond/+}$ embryos **(E)** and reduced in hGFAP^{Cre};Sox2$^{Cond/Cond}$ embryos **(F, J).** Proliferating cells labeled by BrDU incorporation in the DT of hGFAP^{Cre};SOX2$^{Cond/+}$ **(G)** and hGFAP^{Cre};Sox2$^{Cond/Cond}$ **(H)** animals. There is a reduced number of BrDU-positive cells in hGFAP^{Cre};Sox2$^{Cond/Cond}$ embryos **(K)**. Abbreviations: DT, Dorsal Telencephalon. P values: (*)=$p<0.05$; (**)=$p<0.01$.

Figure 4.6

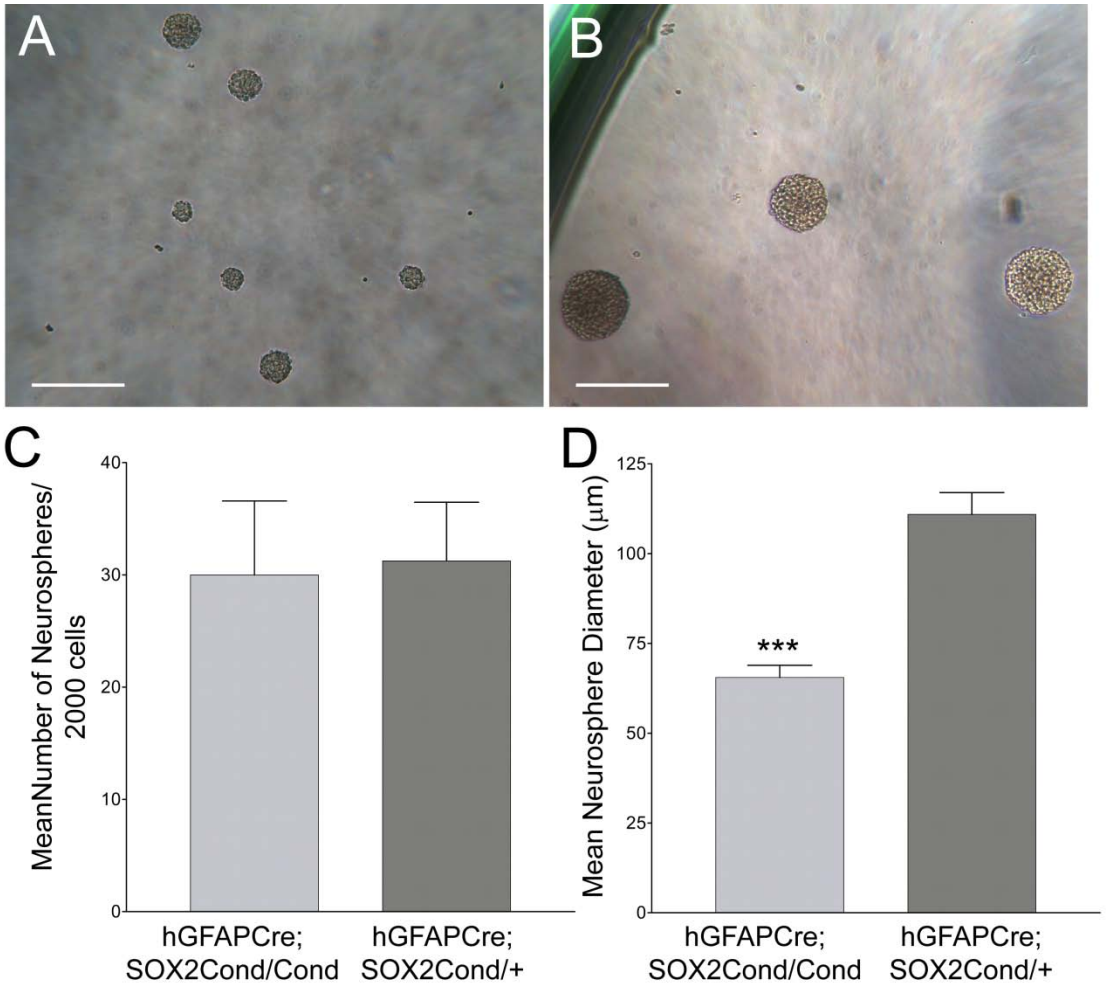

A
B
C
MeanNumber of Neurospheres/ 2000 cells
40
30
20
10
0
hGFAPCre; SOX2Cond/Cond
hGFAPCre; SOX2Cond/+
D
Mean Neurosphere Diameter (μm)
125
100
75
50
25
0
***
hGFAPCre; SOX2Cond/Cond
hGFAPCre; SOX2Cond/+

Figure 4.6 Primary Neurosphere Formation. Cells isolated from the DT of hGFAP^{Cre};Sox2$^{Cond/Cond}$ mice are able to generate primary neurospheres **(A,C)** at the same frequency as hGFAP^{Cre};SOX2$^{Cond/+}$ isolated cells **(B,C).** However, hGFAP^{Cre};Sox2$^{Cond/Cond}$ primary neurospheres have a smaller diameter **(A,D)** than the hGFAP^{Cre};SOX2$^{Cond/+}$ controls **(B,D)** suggesting there are proliferative differences between the two genotypes. Abbreviation: DT, dorsal telencephalon. (*)=$p<0.001$. Scale Bar= 250µm

Figure 4.7

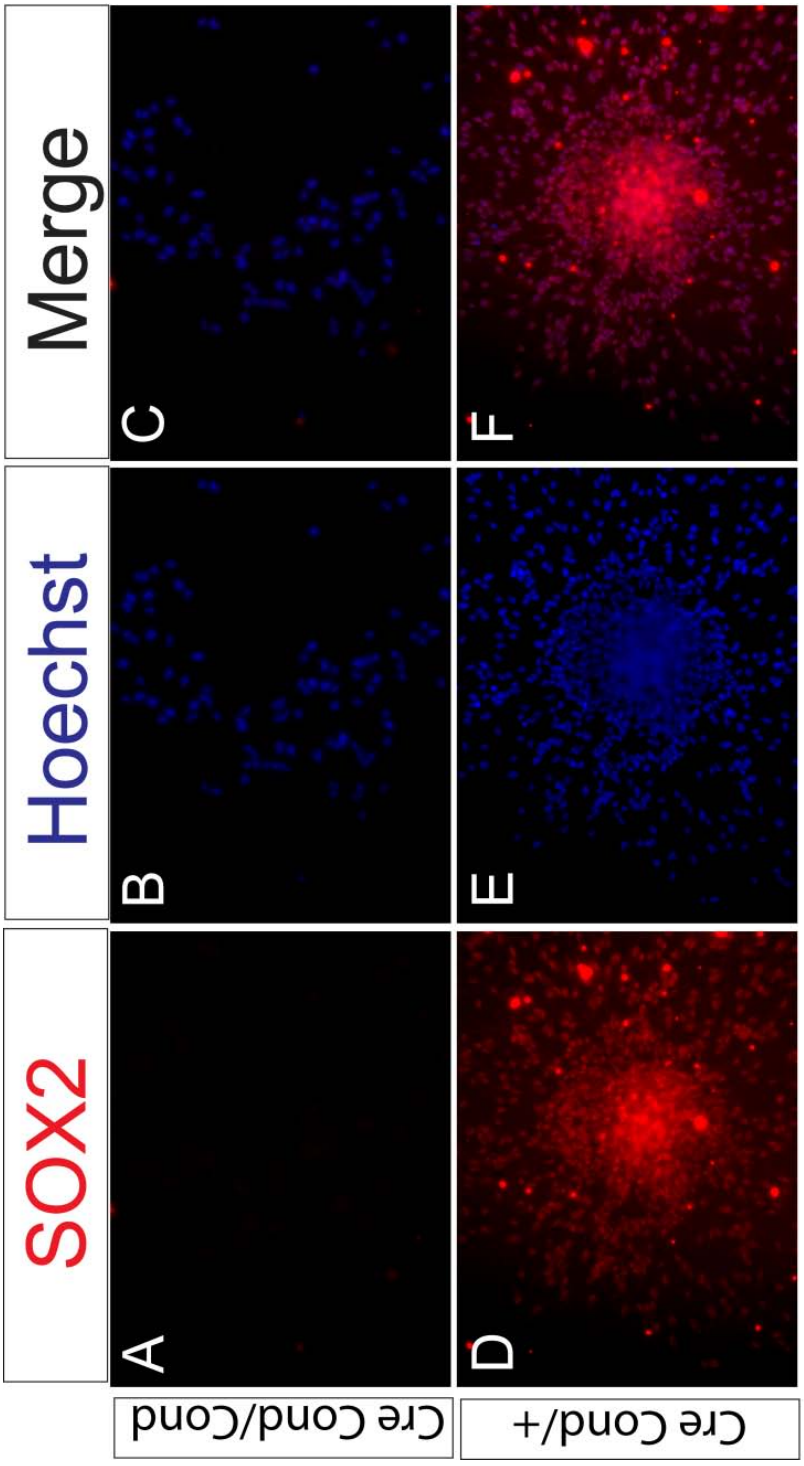


Figure 4.7. Primary neurospheres generated from hGFAP^{Cre};Sox2$^{Cond/Cond}$ DT cells do not express SOX2. Primary neurospheres generated from cells isolated from the DT of hGFAP^{Cre};SOX2$^{Cond/+}$ embryos express SOX2 **(A,C)** and counterstained with Hoechst 33258 to label nuclei **(B,C).** Primary neurospheres generated from the DT of hGFAP^{Cre};Sox2$^{Cond/Cond}$ embryos do not express SOX2 **(D,F)** but are labeled with Hoechst 33258 **(E-F)**, demonstrating that they are generated from SOX2-deficient cells. Abbreviations: DT, Dorsal Telencephalon.

Figure 4.8

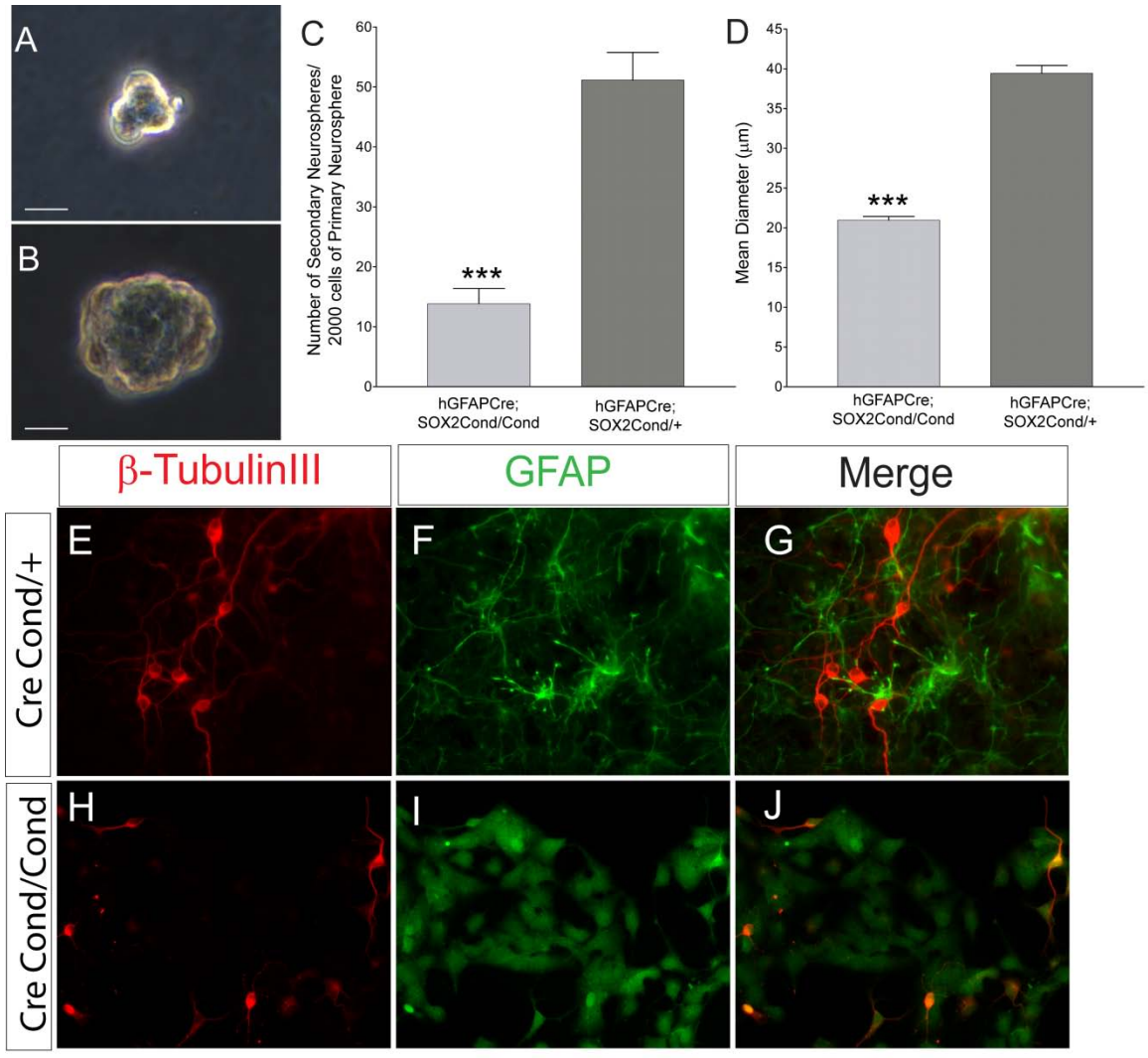

A
B
C
Number of Secondary Neurospheres/ 2000 cells of Primary Neurosphere
60
50
40
30
20
10
0
***
hGFAPCre; SOX2Cond/Cond
hGFAPCre; SOX2Cond/+
D
Mean Diameter (μm)
45
40
35
30
25
20
15
10
5
0
***
hGFAPCre; SOX2Cond/Cond
hGFAPCre; SOX2Cond/+
β-TubulinIII
GFAP
Merge
Cre Cond/+
Cre Cond/Cond
E
F
G
H
I
J

Figure 4.8 Self-renewal and multipotency of primary neurospheres. Upon primary neurosphere dissociation, both hGFAP^{Cre};Sox2$^{Cond/Cond}$ **(A)** and hGFAP^{Cre};SOX2$^{Cond/+}$ **(B)** embryos are able to generate secondary neurospheres. However, hGFAP^{Cre};Sox2$^{Cond/Cond}$ embryos generate fewer secondary neurospheres than hGFAP^{Cre};SOX2$^{Cond/+}$ controls **(C)** and are smaller in diameter than hGFAP^{Cre};SOX2$^{Cond/+}$ secondary neurospheres **(D).** Primary neurospheres were allowed to differentiate to generate neurons and glia. The hGFAP^{Cre};SOX2$^{Cond/+}$ neurospheres were able to generate β-Tubulin III-positive neurons **(A, C)** and GFAP-positive astrocytes **(B,C)**. The hGFAP^{Cre};Sox2$^{Cond/Cond}$ neurospheres could also generate β-Tubulin III-positive neurons **(H,J)** and GFAP-positive astrocytes **(I,J)**, however the two markers were often coexpressed in single cells **(J)**. Abbreviations: GFAP, Glial Fibrillary Acidic Protein. Scale markers=20μm. (***)=p<0.001.

Chapter Five

Discussion

Summary of Findings

Chapter Two

- SOX2-EGFP expression faithfully recapitulates endogenous SOX2 expression in the $SOX2^{EGFP}$ mouse line.
- SOX2 (and SOX2-EGFP) labels ES cells, embryonic neural progenitor cells and distinct populations of adult neural progenitor cells in the CNS.
- $SOX2^{Random}$ mice express SOX2-EGFP in a subset of neural progenitor cells in the CNS, suggesting the existence of heterogeneous populations of neural progenitor cells which differentially regulate SOX2 expression.
- All neurospheres generated from $SOX2^{EGFP}$ mice, regardless of tissue region or developmental stage, express SOX2-EGFP.
- Modified protocols for the dissociation, isolation, and culture of neural progenitor cells from the CNS result in an increase in cell viability for subsequent analyses.

Chapter Three

- SOX2 is expressed in PAX6-positive radial glial cells and in a subset of TBR2-positive intermediate progenitor cells in the developing dorsal telencephalon.
- Levels of SOX2-EGFP expression can be distinguished in $SOX2^{EGFP}$ mice using flow cytometry.
- Utilizing the $SOX2^{EGFP}$ mouse line, radial glia, intermediate progenitors, and differentiated neurons can be prospectively isolated using FACS from the developing dorsal telencephalon based upon high, intermediate, or low intracellular levels of SOX2 expression, respectively.
- SOX2 is co-expressed with a small population of β-Tubulin III-positive cells in the VZ of the dorsal telencephalon, independent of PAX6 or TBR2 expression,

suggesting that a small population of neural progenitor cells undergo direct neuronal differentiation

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- hGFAP^{Cre} mice express Cre-Recombinase in the dorsal telencephalon beginning at E13.5.
- SOX2 expression is lost in the dorsal telencephalon of hGFAP^{Cre};SOX2$^{Cond/Cond}$ mice beginning at E13.5 and is completely ablated by E15.5.
- hGFAP^{Cre};SOX2$^{Cond/Cond}$ embryos have a reduction in the number of PAX6-positive radial glial cells and TBR2-positive intermediate progenitor cells in the dorsal telencephalon at E15.5.
- hGFAP^{Cre};SOX2$^{Cond/Cond}$ embryos have a reduction in the number of BrDU labeled cells in the dorsal telencephalon at E15.5.
- The dorsal telencephalon is thinner in hGFAP^{Cre};SOX2$^{Cond/Cond}$ compared to hGFAP^{Cre};SOX2$^{Cond/+}$ controls.
- SOX2-deficient cells are able to generate multipotential neurospheres, however these neurospheres are smaller and have limited self-renewal capacity compared to controls.
- hGFAP^{Cre};SOX2$^{Cond/Cond}$ embryos display increased embryonic lethality. Postnatal animals exhibit stress-induced, unilateral circling and die within 60 days.

Brief Overview of Findings

The results presented in this dissertation are the first to collectively demonstrate: that SOX2 is expressed in distinct neural progenitor populations throughout the developing and adult CNS, that distinct progenitor populations in the dorsal telencephalon can be prospectively identified and isolated based upon their intracellular concentrations of SOX2, and that SOX2 function is necessary to maintain the proliferative and self-renewal capacity of established radial glial populations in the developing dorsal telencephalon.

This dissertation was designed to address the hypothesis **that through the analyses of SOX2 expression and function, distinct neural progenitor populations can be prospectively identified in the developing dorsal telencephalon and furthermore, that SOX2 functions to maintain the proliferative, self-renewal, and differentiation capacities of these cells *in vivo* and *in vitro*.**. The rationale supporting this hypothesis is that SOX2 has been shown to maintain progenitor cell identity and function in neural progenitor populations in the chick spinal cord and mouse developing retina (Bylund et al. 2003; Graham et al. 2003; Taranova et al. 2006). Moreover, SOX2 is also expressed in the developing and adult mammalian brain, although it is unclear in what cell types it is expressed and what function it serves in these cells (D'Amour and Gage 2003; Ellis et al. 2004; Ferri et al. 2004; Bani-Yaghoub et al. 2006). Hypomorphic expression and conditional ablation of SOX2 at neural tube stages in the mouse result in neural defects including enlarged ventricles, aberrant neuronal differentiation, and decreases in neuronal production (Ferri et al. 2004; Cavallaro et al. 2008; Miyagi et al. 2008). However, the temporal and spatial dependence upon SOX2 function at later stages of neural development is not known. In this dissertation, I have tested this hypothesis by first generating and characterizing the expression of SOX2 in a $SOX2^{EGFP}$ mouse line to identify in which neural progenitor

populations in the developing and adult CNS express SOX2 (Ellis et al. 2004). After establishing that SOX2-EGFP is expressed in distinct neural progenitor cells throughout the CNS, I next demonstrate that differential levels of SOX2 expression can be used to prospectively identify and isolate distinct progenitor classes from the dorsal telencephalon. Lastly I address the function SOX2 in neural progenitors during mid to late stages of neural development (>E13.5), specifically by genetically ablating SOX2 in radial glial progenitor cells in the developing dorsal telencephalon. Here I find that loss of SOX2 results in a reduction in the number of progenitor populations (radial glia and intermediate progenitors) within the dorsal telencephalon due to decreases in the proliferative and self-renewal capacity of these cells.

In Chapter Two I address whether SOX2 expression can be used to identify neural progenitor populations in both the embryonic and adult CNS. By characterizing the expression pattern of the $SOX2^{EGFP}$ mouse line generated in the lab, I demonstrate that SOX2-EGFP expression faithfully recapitulates endogenous SOX2 expression (Ellis et al. 2004). Furthermore, I show that SOX2-EGFP is found in molecularly distinct neural progenitor populations in embryonic and adult neurogenic regions. These results further demonstrate that all neurospheres generated from $SOX2^{EGFP}$ mice express SOX2-EGFP, confirming the use of SOX2 as a general neural progenitor marker with which neural progenitor cells can be prospectively identified. In addition, I present protocols that more efficiently isolate SOX2-EGFP positive neural progenitor populations from tissue for use in *in vitro* analyses.

In Chapter Three I show that using the $SOX2^{EGFP}$ mouse line, populations of radial glial cells, intermediate progenitor cells, or differentiated neurons can be isolated and enriched based upon their expression of high, intermediate or low levels of SOX2-EGFP, respectively. These results are the first to demonstrate that SOX2 is differentially expressed between distinct neural progenitor populations in the dorsal telencephalon. In

addition, immunocytochemical analyses identify unique populations of SOX2 positive cells undergoing direct neuronal differentiation that do not coexpressed either PAX6 or TBR2, suggesting that these cells do not follow the canonical radial glial-intermediate progenitor cell differentiation pathway.

Lastly, in Chapter Four I genetically dissect the role of SOX2 in radial glial cells of the developing dorsal telencephalon by conditionally ablating SOX2 in $hGFAP^{Cre};Sox2^{COND/COND}$ embryos. As a consequence of SOX2 ablation, the number of proliferating radial glia and their progeny, intermediate progenitor cells, is decreased, resulting in a thinning of the developing cortex. SOX2-deficient cells also demonstrate decreased proliferative and self-renewal capacity *in vitro*. Collectively these results demonstrate that SOX2 function is necessary to maintain populations of radial glial cell in the dorsal telencephalon.

Discussion of Major Findings and Future Directions

SOX2-EGFP Identifies Distinct Populations of Neural Progenitor Cells in the Embryonic and Adult Central Nervous System.

In chapter two I demonstrate that an EGFP reporter "knocked-in" to the SOX2 locus can faithfully and efficiently recapitulate the expression of SOX2 protein in cells throughout the CNS. Moreover, these results are the first to demonstrate that SOX2 is expressed in distinct populations of neural progenitor cells, such as those identified in the adult SVZ, hippocampus, and spinal cord. However, one of the most important results from this chapter is the finding that all neurospheres generated from $SOX2^{EGFP}$ mice, regardless of region or developmental stage, express EGFP. This establishes that SOX2 is expressed in all neurosphere-forming neural progenitor cells and also demonstrates that EGFP faithfully reports SOX2 expression. In turn, this supports the

use of SOX2 expression as a tool for prospectively identifying neural progenitor cells *in vivo*.

The establishment of the $SOX2^{EGFP}$ mouse line as a tool to identify neural progenitor cells suggests that additional, more advanced genetic tools can be generated utilizing SOX2 expression. For instance, one disadvantage of the $SOX2^{EGFP}$ line is that EGFP is ubiquitously expressed in all SOX2-positive cells throughout ontogeny. While this is useful in identifying progenitor cells, it is not conducive for lineage analyses of distinct populations. Therefore, the generation of SOX2 inducible reporter mouse lines, in which the expression of a fluorescent protein can be conditionally activated by a tissue specific Cre line, can allow for the temporal and spatial labeling of distinct neural progenitor cells (Nagy 2000). Similarly, the development of an inducible SOX2-Cre mouse line, in which Cre expression can be temporally and spatially activated in the presence of the estrogen receptor ligand Tamoxifen, can be useful in targeting other genes of interest for Cre-mediated recombination in distinct neural progenitor populations in the CNS (Bockamp et al. 2002).

One specific question that can be addressed using additional SOX2 genetic tools is the identity and lineage contribution of neural progenitor populations in the adult SVZ. The results in Chapter 2 demonstrate that SOX2 is expressed in putative neural progenitor populations in the adult subventricular zone (SVZ) and hippocampal subgranular zone (SGZ) (Ellis et al. 2004). In the SVZ, however, the precise identities of the multipotent neural progenitor populations remain unclear. Multiple studies have indicated that the GFAP-positive Type B astrocytes residing in the SVZ serve as the multipotential neural progenitor population; ultimately generating the other progenitor classes (Type A and Type C cells) as well as the post-mitotic interneurons of the olfactory bulb (Doetsch et al. 1997; Doetsch et al. 1999; Doetsch et al. 1999; Capela and Temple 2002). However, conflicting studies have suggested that the ependymal

cells lining the ventricular lumen are the true neural progenitor cell population (Johansson et al. 1999; Carlen et al. 2009; Moreno-Manzano et al. 2009). Both arguments are supported by strong data, but I believe that our observation that SOX2 is expressed in both populations suggests that each may have neural progenitor capacity. Thus, SOX2 expression in these cells can be used as a tool to identify the neural progenitor population in the SVZ *in vivo*.

To definitively identify which of these populations serve as neural progenitor cells, an *in vivo* loss of function approach is required. To accomplish this, a mouse line will be generated in which the expression of a potent cellular toxin gene (Diphtheria Toxin (DT)) is conditionally driven by the SOX2 promoter exclusively in the presence of Cre-Recombinase ($SOX2^{DTA}$). $SOX2^{DTA}$ mice will then be crossed to either inducible $hGFAP^{CreERT2}$ or $CFTR^{CreERT2}$ mice to target either Type B cells or ependymal cells for ablation, respectively, when administered the drug Tamoxifen. Mice will then be analyzed for reductions in neurogenesis. If neurogenesis is affected by the ablation of one population but not the other, it will suggest that the ablated cell population functions as the neural progenitor population in the SVZ.

SOX2 is Differentially Expressed between Distinct Neural Progenitor Populations

In chapter three I demonstrate that distinct populations of radial glia, intermediate progenitors, and postmitotic neurons can be isolated from $SOX2^{EGFP/+}$ mice based upon their expression of high, intermediate, or low levels of SOX2, respectively. Previous studies have shown that intracellular concentrations of SOX2 are important as mice with hypomorphic mutations in SOX2 expression (below 50% of endogenous levels) present with multiple neurological defects. In neural progenitor cells of the developing retina, a reduction in SOX2 expression results in aberrant neuronal differentiation and hypocellularity (Taranova et al. 2006). Similarly, in *Yellow Submarine* (ysb) mice,

disruptions in specific regulatory elements which direct SOX2 expression in the developing inner ear result in decreased levels of SOX2 and a reduction in the number of sensory inner ear hair cells (Kiernan et al. 2005). In the developing brain, deletion of a neural cell-specific enhancer of SOX2 (in conjunction with a SOX2-null allele) results in reduced levels of SOX2 expression in the telencephalon and subsequent cellular defects including neuron degeneration, increased ventricular size, cortical thinning and impaired neurogenesis in adults (Zappone et al. 2000; Ferri et al. 2004; Cavallaro et al. 2008). Hypomorphic SOX2 mutations in humans have also been observed with patients diagnosed with anophthalmia and mental retardation (Gardner and Rossant 1979; Fantes et al. 2003). Thus, the intracellular concentration of SOX2 is important for the proper maintenance and differentiation of neural progenitor cells in the developing and adult nervous systems. However, it remained unclear whether SOX2 is differentially expressed between distinct neural progenitor populations under normal physiological conditions. The results I present in Chapter Three are the first direct evidence that distinct neural progenitor populations, specifically radial glia and intermediate progenitors, differentially express SOX2 *in vivo*.

Despite the finding that SOX2 is differentially expressed in distinct neural progenitor populations, it is still unclear how SOX2 concentrations may affect cell-fate decisions. Therefore, it is important to identify genes that are differentially expressed in cell populations with distinct levels of SOX2 as well as identify the mechanisms by which SOX2 regulates these genes. First, potential targets of SOX2 modification can be identified utilizing techniques such as DNA microarray to screen for the differential regulation of thousands of genes. Given that the SOX2 loss of function studies presented in chapter four demonstrate a reduction in proliferating cells, it will be important to look at genes involved in cell cycle regulation as well as genes involved in the conversion of radial glia to intermediate progenitor cells such as Ngn2, Tbr2, and

Cux2. After the identification of candidate genes, I believe it is necessary to determine the mechanisms by which SOX2 levels function to regulate these genes. One potential mechanism is through the modification of chromatin structure. The high sequence-specificity of the SOX2 DNA-binding domain, as well as the observation that SOX2 binds the minor groove of DNA resulting in a sharp bend in the helix, suggests that SOX2 functions in chromatin modification (Soullier et al. 1999; Dailey and Basilico 2001). To analyze this is, bisulfite sequencing can be used to determine the extent of DNA methylation of candidate genes identified in the microarray screens (Fraga and Esteller 2002). A second method in which SOX2 may regulate progenitor cell fate decisions is through direct protein-protein interactions. For instance, in the nervous system, SOX2 directly interacts with the transcription factor BRN2 to regulate the expression of NESTIN in neural progenitor cells (Tanaka et al. 2004). Potential shifts in the levels of SOX2 expression can result in less SOX2 protein available to form transcriptional complexes, which in turn may allow other proteins to out-compete SOX2 and either activate or repress gene transcription. Thus, in conjunction with the microarray analysis, potential targets of SOX2 protein-protein interactions can be identified using Chromatin Immunoprecipitation (ChIP) from cell populations differentially expressing SOX2. Collectively, these studies will help to elucidate the mechanisms in which SOX2 regulates and maintains neural progenitor cells in the developing CNS.

The ability to identify neural progenitor populations that express distinct levels of SOX2 can also be applied to cell populations outside of the CNS. Recent studies have demonstrated that a cocktail for four transcription factors (SOX2, OCT4, KLF4, and C-MYC) can be transfected into differentiated fibroblast cells and induce molecular and epigenetic changes that result in the reprogramming of these cells into stem cell-like cells called induced pluripotent stem cells (iPS cells) (Takahashi and Yamanaka 2006; Takahashi et al. 2007; Takahashi et al. 2007; Wernig et al. 2007). In an effort to reduce

the number of reprogramming factors used in this process, further investigations have eliminated the necessity for SOX2 transfection by utilizing cell populations in which high levels of SOX2 are endogenously expressed, such as adult neural progenitor cells (Eminli et al. 2008; Kim et al. 2008). However, reprogramming neural progenitor cells, especially in humans, for therapeutic uses is impractical given the difficulties in acquiring these cells from living patients. Therefore, alternative sources of SOX2-expressing cells are needed. One potential cell population that expresses SOX2 is the dermal papilla cells located in the hair follicle of the epidermis (Driskell et al. 2009). These cells express SOX2-EGFP in $SOX2^{EGFP}$ mice and can therefore also be isolated using FACS (Ellis et al. 2004). Skin cells also provide a more practical cell population from which to generate iPS cells since harvesting skin cells is less intrusive than harvesting neural progenitor cells. However, it remains to be determined whether dermal papilla cells express SOX2 levels comparable to neural progenitor cell populations. Therefore, using the $SOX2^{EGFP}$ mouse line, dissociation protocols, and FACS procedures described in Chapters 2 and 3, it will be important to determine if dermal papilla cells express high levels of SOX2, and if so, whether they are able to generate iPS cells in the same capacity as neural progenitor populations. Given the therapeutic potential of iPS cells (Rolletschek and Wobus 2009; Webb 2009), these experiments may provide insight for future advances in the iPS field.

SOX2 is Necessary to Maintain Radial Glial Populations in the Dorsal Telencephalon

The results presented in Chapter Four are the first to demonstrate that SOX2 plays an important role in maintaining the number of radial glial progenitor cells in the developing dorsal telencephalon. Previous investigations into the function of SOX2 in neural progenitor populations have manipulated SOX2 expression during the onset of

neural development (E9.5) and analyzed the effects at later developmental and adult stages (Ferri et al. 2004; Cavallaro et al. 2008; Miyagi et al. 2008). While these methods demonstrate that loss of or reduction in SOX2 expression in early stages of neural development has phenotypic consequences in the overall development of the nervous system, they do not address the role of SOX2 in neural progenitor populations. Furthermore, it cannot be ruled out that the manipulation of SOX2 at such an early stage does not affect the fate-specification of these early progenitor cells which may, in turn, affect the specification of their progeny. This has been demonstrated in *Xenopus* studies in which reduced SOX2 signaling in ectodermal cells results in the inability of these cells to adopt either a neural or epidermal fate, suggesting that SOX2 signaling is essential in development of early neural ectoderm (Kishi et al. 2000). Therefore, the studies I present in chapter four utilize the $hGFAP^{Cre}$ mouse line to ablate SOX2 in established radial glial populations at E13.5, after they have adopted a radial glial and neural identity (Casper and McCarthy 2006). The loss of SOX2 at this embryonic stage results in a decrease in the number of proliferating radial glial cells, as well as intermediate progenitor cells, and a decrease in cortical thickness. Furthermore, *In vitro* studies demonstrate that SOX2-deficient ($hGFAP^{Cre}$; $SOX2^{Cond/Cond}$) cells proliferate and maintain their multipotency, however their self-renewal capacity is significantly decreased and they proliferate at a slower rate than controls, resulting in smaller neurospheres.

Whether SOX2 functions in regulating cell-cycle remains unclear. Previous studies have demonstrated that dominant-negative inhibition of SOX2 leads to cell-cycle arrest in neural progenitor cells in the chick neural tube (Graham et al. 2003). Furthermore, misexpression of *Xsox3* in conjunction with the transcription factor XBF-1 dose-dependently inhibits the cell-cycle inhibitor $p27^{XIC1}$, which leads to increased proliferation of neuroectoderm (Hardcastle and Papalopulu 2000). Moreover, recently it

was discovered that in ES cells, SOX2 (along with OCT4) regulates the translation of miR-302, a microRNA which represses the transcription of cyclin-D1 (Card et al. 2008). Cyclin D1 is important in the regulation of the G1 phase of the cell cycle and its repression leads to a decrease in the number of cells in G1 and an increase in cells in S-phase, in essence decreasing cell cycle length. This lends credence to the hypothesis that loss of SOX2 in radial glial progenitor cells results in a loss of miR-302, or other similar factor, which in turn increases the length of time a cell spends in the G1 phase of the cell-cycle. Indeed, approximately 70% of all miRNAs identified to date are expressed in the mammalian brain with many implicated in the control of neuronal differentiation (Cao et al. 2006). However, to date their relationship with SOX2 has yet to be investigated

Based upon the above observations and studies, I hypothesize that SOX2 is directly involved in cell-cycle regulation. This can be addressed by analyzing cell-cycle regulation in SOX2-deficient cells. First, to determine whether SOX2-deficient and SOX2-expressing cells differ in the length of their cell-cycle, cells can be exposed to bromodeoxyuridine (BrDU) for an extended time period to label all proliferating cells, followed by a short-term pulse of iododeoxyuridine (IDU) to acutely label only those cells currently in S-phase. The cell-cycle rate can then be calculated based upon the ratio of IDU to BRDU labeled cells (Burns and Kuan 2005). Next, cell-cycle regulators such as Cyclin genes and microRNAs can also be analyzed for differential express between SOX2-postive and SOX2-deficient cells, with SOX2-deficient cells expected to show increased levels of cell-cycle inhibitors. Collectively, these experiments will help to elucidate whether SOX2 acts upon cell-cycle regulators in neural progenitor cells of the CNS.

Functional Redundancy of SOXB1 Transcription Factors in the Developing Dorsal Telencephalon

The three members of the SOXB1 family (Sox1, 2, and 3) share high sequence similarity and are co-expressed in many neural progenitor populations in the developing telencephalon (Collignon et al. 1996; Wood and Episkopou 1999). Moreover, the three SOXB1 members have the capacity to activate the same target genes. For instance, in lens tissue, all three members are able to activate the δ1-crystalin D5 enhancer *in vitro* through interactions with Pax6, whereas other SOX family members cannot (Kamachi et al. 1995; Kamachi et al. 1998; Kamachi et al. 2001). In addition, in *Drosophila* mutants lacking the *SoxNeuro* gene, severe neural defects are observed in regions where *SoxNeuro* is not co-expressed with the other *Drosophila SOXB1* factor, *Dichaete*; whereas in regions of *SoxNeuro* and *Dichaete* coexpression, the phenotypic defects are mild (Overton et al. 2002). Furthermore, in the mouse, homozygous knockouts for either Sox1 or Sox3 show only mild phenotypes, primarily in regions where each is uniquely expressed (the developing lens and pituitary respectively), suggesting that the loss of either SOX1 or SOX3 can be functionally compensated for. However, other studies have demonstrated that these three factors may not be completely functionally redundant. For example, SOX2-null mice die at peri-implantation stages despite the coexpression of SOX3 (Avilion et al. 2003). In addition, conditional ablation and hypomorphic expression of SOX2 at neural tube stages results in neural defects even when SOX1 and SOX3 are both expressed in the effected neural progenitor populations (Ferri et al. 2004; Cavallaro et al. 2008; Miyagi et al. 2008). Moreover, the results presented in Chapter 4 also demonstrate that the loss of SOX2 in radial glial cells produces phenotypic defects despite the maintenance of SOX1 and SOX3 expression. Given the overall importance of SOX2 in maintaining neural progenitor capacity in ES cells and neural progenitor cells, it can be suggested that the otherwise normal

development of these various SOX2 mutant lines demonstrates at least partial functional redundancy by SOX1 and SOX3. However, this phenomenon has yet to be genetically analyzed.

The question of whether SOXB1 factors are functionally redundant can be addressed in two ways. The first method is to genetically ablate either two or all three SOXB1 factors in neural progenitor populations. Two-factor ablation can be accomplished by generating $SOX2^{Cond/Cond}$ mice on either a SOX1- or SOX3-null background. $SOX1^{-/-}$, $SOX3^{-/-}$, and $SOX2^{Cond/Cond}$ mice are all viable, although it remains unclear whether double homozygotes are viable as well. If double homozygotes are viable, ablation of SOX2 at later stages via a tissue-specific Cre would thus leave only SOX1 (or SOX3) to compensate for the loss of the other two factors. Any additional phenotypic defects observed beyond what is seen in Cre;$SOX2^{Cond/Cond}$ mutants would suggest that functional redundancy between the three SOXB1 factors is limited. In contrast, the generation of a triple mutant (Cre;$SOX1^{-/-}$, $SOX3^{-/-}$,$SOX2^{Cond/Cond}$) would be technically difficult but would conclusively address the function of SOXB1 genes in neural development.

A second approach to genetically address functional redundancy is to engineer alleles in which one SOXB1 member can be expressed by the promoter of another, as has been previously demonstrated to investigate SOX1 function in postmitotic cells (Ekonomou et al. 2005). To test functional redundancy, an allele can be generated in which the endogenous SOX2 open reading frame (ORF) is replaced with the SOX1 ORF. This would result in a mouse carrying one SOX2-null allele in which SOX1 is expressed by the endogenous SOX2 promoter ($Sox2^{SOX1/+}$). This mouse can then be crossed to a $Sox2^{Cond/+}$ line to generate $Sox2^{SOX1/Cond}$ embryos. Functional redundancy can then be tested in a region where SOX2 is exclusively expressed, such as in the developing retina. We have previously shown that $SOX2^{EGFP}/^{Cond}$ mice, in which the

EGFP acts as a null allele, are hypomorphic, generating small eye phenotypes (Taranova et al. 2006). Therefore, if ectopic SOX1 expression is capable of compensating for SOX2 loss, than $Sox2^{SOX1/Cond}$ mice would be expected to present with a milder eye phenotype than $SOX2^{EGFP/COND}$ mice. Moreover, the extent of this compensation can be further tested by crossing $Sox2^{SOX1/Cond}$ mice with an eye specific Cre-mouse line to test whether ectopic SOX1 can compensate for complete SOX2 loss in the eye. Similar experiments can be conducted with SOX3 as well.

Conclusion

In this dissertation I have provided the first evidence that intracellular concentrations of SOX2 vary between distinct neural progenitor populations within the developing CNS. I have also demonstrated, utilizing the $SOX2^{EGFP}$ mouse line, that these neural progenitor populations can be prospectively identified and isolated based upon their levels of SOX2 expression. Moreover, I have demonstrated that SOX2 function is necessary for maintaining populations of proliferating radial glial and intermediate progenitor cells in the developing dorsal telencephalon. Ultimately, these findings have elucidated the role of SOX2 in neural progenitor populations within the embryonic CNS and have also established tools for future analyses of SOX2 function in progenitor populations throughout development.

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