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SOX2 expression levels distinguish between neural progenitor populations of the developing dorsal telencephalon

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ABSTRACT

The HMG-Box transcription factor SOX2 is expressed in neural progenitor populations throughout the developing and adult central nervous system and is necessary to maintain their progenitor identity. However, it is unclear whether SOX2 levels are uniformly expressed across all neural progenitor populations. In the developing dorsal telencephalon, two distinct populations of neural progenitors, radial glia and intermediate progenitor cells, are responsible for generating a majority of excitatory neurons found in the adult neocortex. Here we demonstrate, using both cellular and molecular analyses, that SOX2 is differentially expressed between radial glial and intermediate progenitor populations. Moreover, utilizing a $SOX2^{EGFP}$ mouse line, we show that this differential expression can be used to prospectively isolate distinct, viable populations of radial glia and intermediate cells for *in vitro* analysis. Given the limited repertoire of cell-surface markers currently available for neural progenitor cells, this provides an invaluable tool for prospectively identifying and isolating distinct classes of neural progenitor cells from the central nervous system.

Introduction

Heterogeneous populations of neural progenitor cells, each with distinct molecular and cellular characteristics, reside in neurogenic regions throughout the developing mammalian central nervous system (CNS). In the rodent dorsal telencephalon (dTel), two such progenitor populations have been characterized. The first population, located primarily in the ventricular zone (VZ), consists of self-renewing, multipotent radial glial cells (RGCs) that have the capacity to generate both neurons and glia *in vivo* (Anthony et al., 2004; Malatesta et al., 2003, 2000; Noctor et al., 2001). RGCs are also capable of generating a second, transient neural progenitor population of intermediate progenitor cells (IPCs; or basal progenitor cells) which, in contrast to RGCs, 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 RGC and IPC populations have also been observed in the dTel, including unipotential RGCs which are exclusively neurogenic or gliogenic, as well as IPC subpopulations with distinct morphological characteristics (Gal et al., 2006; Kawaguchi et al., 2008; Kowalczyk et al., 2009; Mizutani et al., 2007; Stancik et al., 2010). Thus, the developing dTel harbors a diverse mixture of distinct cellularly-defined neural progenitor cell populations.

The mechanisms which regulate neural progenitor cell diversity include a variety of transcriptional networks (Guillemot, 2007). In the dTel for instance, the interplay of several key transcription factors defines and regulates the "radial glial–intermediate progenitor–neuron" transition (Englund et al., 2005). RGCs express high levels of the paired-domain protein PAX6 which is necessary to properly maintain their radial glial identity (Gotz et al., 1998; Haubst et al., 2004; Heins et al., 2002). IPCs, in contrast, 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 specify neuronal and IPC fates, respectively (Arnold et al., 2008; Bulfone et al., 1999; Englund et al., 2005; Kimura et al., 1999; Miyata et al., 2004; 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)(Englund et al., 2005; Hevner et al., 2001). Thus, the temporal and spatial gradient of expression of these and other key transcription factors is important in regulating neural progenitor cell fate in the dTel.

The SOXB1 transcription factor SOX2 is expressed in neural progenitor cells throughout the developing and adult CNS (Bani-Yaghoub et al., 2006; Brazel et al., 2005; Cavallaro et al., 2008; Collignon et al., 1996; D'Amour and Gage, 2003; Ellis et al., 2004; Favaro et al., 2009; Miyagi et al., 2008; Uchikawa et al., 1999; Uwanogho et al., 1995; Wood and Episkopou, 1999; Zappone et al., 2000). In the chick neural tube, SOX2 expression is sufficient to maintain cells in a neural progenitor state while its loss of function induces cell cycle exit and precocious neuronal differentiation (Bylund et al., 2003; Graham et al., 2003). In mice, *in vivo* hypomorphic

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Sox2 mutations suggest that the intracellular concentrations of SOX2 play an important role in neural progenitor cells. In the cortex, hypomorphic levels of SOX2 result in decreases in both progenitor proliferation and neuronal production (Cavallaro et al., 2008; Favaro et al., 2009; Ferri et al., 2004), while in the retina, aberrant neuronal differentiation is observed (Taranova et al., 2006). Moreover, hereditary eye and hippocampal defects have also been attributed to hypomorphic SOX2 mutations in humans (Bakrania et al., 2007; Fantes et al., 2003; Hagstrom et al., 2005; Ragge et al., 2005; Sisodiya et al., 2006). Further evidence supporting a dose-dependent role of SOX2 in the dTel comes from immunocytochemical studies illustrating a gradient of SOX2 expression in the cortex (Bani-Yaghoub et al., 2006) as well as *in vitro* studies which demonstrate that SOX2-expressing cells are responsible for all neurospheres generated from the telencephalon (D'Amour and Gage, 2003; Ellis et al., 2004) and express high levels of "neural stem cell" genes such as Notch1 and Nestin (D'Amour and Gage, 2003). Thus, these findings collectively suggest that the intracellular concentration of SOX2 plays an important role in the maintenance and differentiation of neural progenitor cells as a whole. However, taking into consideration that multiple, distinct populations of neural progenitor cells reside in the CNS, often within the same region (i.e. dorsal telencephalon), these studies have failed to address an important question—whether SOX2 is differentially expressed between distinct neural progenitor populations *in vivo*.

To directly address this question, first we characterize SOX2 expression in RGC and IPCs in the dTel and show that SOX2 is differentially expressed between these two populations. In addition, we employ a transgenic $SOX2^{EGFP}$ reporter mouse line to illustrate that the prospective isolation of RGCs, IPCs, and differentiated neurons from the developing dTel can be accomplished based upon their differential expression of SOX2. Thus, these results demonstrate that the intracellular concentration of SOX2 varies between distinct classes of neural progenitor cells in the dTel, which in turn can be utilized to efficiently identify and isolate distinct populations of viable neural progenitor cells from the dTel for use in both *in vivo* and *in vitro* investigations.

Materials and methods

Animals

All animals were used and maintained in accordance with guidelines published in the NIH *Guide for the Care and Use of Laboratory Animals* and all protocols were 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 female mice (Jackson Laboratory). Pregnant females were euthanized and the embryos harvested at embryonic day (E)12.5 and E16.5 (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 dTel was dissected from E16.5 $SOX2^{EGFP/+}$ and $SOX2^{+/+}$ embryos and incubated in Papain (Roche) followed by treatments with Trypsin Inhibitor (Sigma) and a final wash with Neurobasal medium (Invitrogen). The tissue was then mechanically dissociated into a single-cell suspension in supplemented Neurobasal medium (Invitrogen) containing B27 (Invitrogen) and N2 supplements (Invitrogen) and 10 ng/ml bFGF and EGF (Invitrogen).

Fluorescent analysis and cell sorting were conducted at the University of North Carolina Flow Cytometry Facility 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. The top tenth percentile for intensity was considered $EGFP^{High}$, 40–60th percentile $EGFP^{Int}$, and bottom tenth percentile $EGFP^{Low}$. 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/6 cm dish (283 cells/cm^2) (Coles-Takabe et al., 2008; Hutton and Pevny, 2008). After 6 days in culture, the number of neurospheres per dish was counted. Individual neurospheres were then isolated in single wells of 96-well, non-adherent plates and their diameter measured every 2 days using Image Pro Express Software (Media Cybernetics). 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 h, 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

Isolated cells not used for the Neurosphere Assay were utilized for gene transcriptome analysis. Total RNA was isolated from cell pellets using Trizol Reagent (Invitrogen) and the concentration was determined using an ND1000 spectrophotometer (Nanodrop). cDNA was then generated from 50 μg 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. Primer sequences are as follows: 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′-cattacaaacaccactggtttc-cag-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′ and GAPDH-R: 5′-cctgcttcac-caccttcttga-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 immersed in a 10%, 20%, and 30% sucrose gradient and finally embedded in OCT medium (Tissue-Tek). 12 μm coronal sections were made using a cryostat and collected on Superfrost Plus coated glass slides (VWR). Slides were blocked for 1 h at room temperature with 10% goat serum/1% Triton 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 (1:2000 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) and anti-mouse (IgG1 and IgG2A) Alexa 488, 546, and 647 (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).

Cell counting

For cell count comparisons, 12 μm coronal sections were stained for cell-specific antibodies and counterstained with Hoechst 33258 to label nuclei. Pictures were taken at 20× magnification and a 300 μm × 300 μm box was drawn over the dorsal telencephalon using Photoshop software. Cells double-labeled for both Hoechst and antibody were then counted. Four consecutive slices were measured for each embryo (n = 4 embryos/genotype).

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.

Results

Sox2 is highly expressed in radial glial cells and at lower levels in intermediate progenitor cells

To molecularly characterize progenitor populations in the dTel, we triple labeled E16.5 coronal sections with SOX2, PAX6, and TBR2. SOX2 is highly expressed in the VZ and at lower levels in the SVZ (Fig. 1A). In comparison, the RGC marker PAX6 (Anthony et al., 2004; Malatesta et al., 2003, 2000; Noctor et al., 2001) shows a similar expression pattern in the VZ and SVZ (Fig. 1B) and a majority of SOX2-positive cells (94.8% ± 3.01) within the VZ co-express PAX6 (Fig. 1C), demonstrating that RGCs express high levels of both transcription factors. Next, we compared SOX2 and PAX6 expression with that of the IPC marker TBR2 (Arnold et al., 2008; Bulfone et al., 1999; Englund et al., 2005; Kimura et al., 1999; Sessa et al., 2008). TBR2-positive IPCs predominantly reside in the SVZ (Fig. 1D) and express low levels of SOX2 (Fig. 1E), although a subset of TBR2-positive cells expressing high levels of SOX2 are found scattered throughout the VZ/SVZ boundary which, based upon their location, we presumed to be newly generated IPCs migrating out of the VZ (Fig. 1E, arrowheads). Similarly, 99% (98.8% ± 0.06%) of TBR2-positive IPCs express PAX6, with SVZ IPCs expressing low levels of PAX6 and scattered VZ IPCs expressing high levels of PAX6 (Fig. 1E and F, arrows), consistent with previously studies (Englund et al., 2005). Interestingly, SOX2 was not expressed in all TBR2-positive; PAX6-positve IPCs (Fig. 1G, red arrows). These data demonstrate that both SOX2 and PAX6 are maintained at high levels in RGCs, but at lower levels in TBR2-positive IPCs. Lastly, most SOX2-positive cells were mutually exclusive of β-Tubulin III-positive neurons throughout the dTel (Ellis et al., 2004; Zappone et al., 2000), although SOX2-positive; β-Tubulin III-positive cells were intermittently found in the VZ and SVZ (Fig. 1H, asterisks).

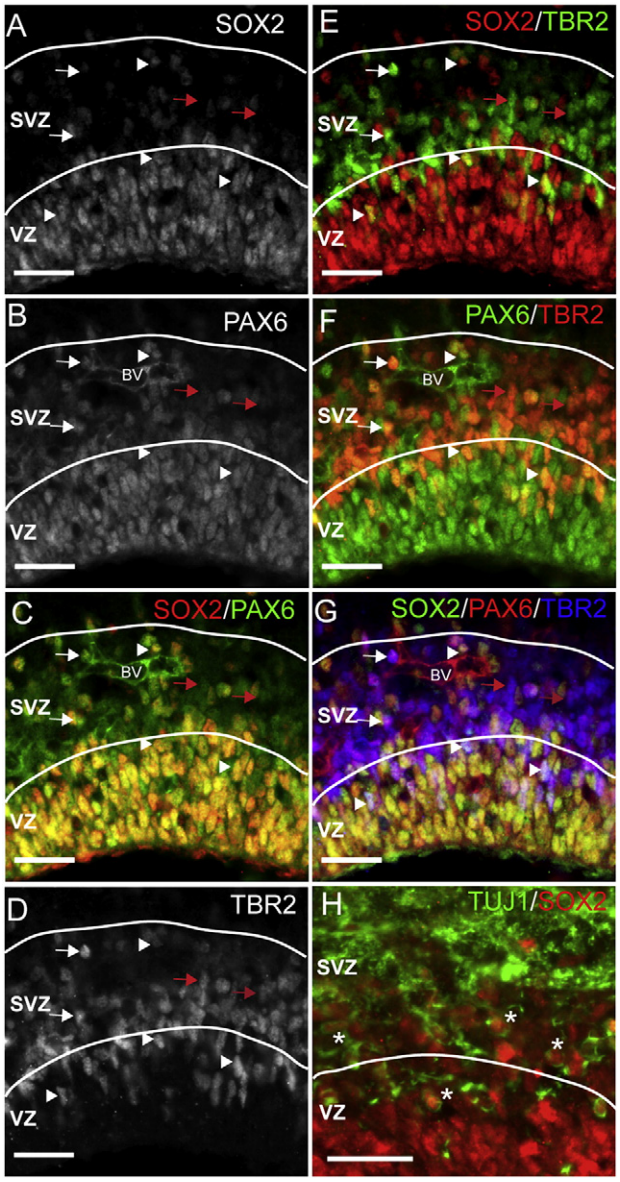

Fig. 1. Immunocytochemical characterization of SOX2-positive cells in the E16.5 dorsal telencephalon. (A–G) Triple-labeling of SOX2, PAX6, and TBR2 in the VZ and SVZ. White arrowheads show cells co-expressing high levels of SOX2 and TBR2. White arrows indicate cells co-expressing all three transcription factors while red arrows label cells that co-express PAX6 and TBR2 but not SOX2. (H) SOX2 expression is largely exclusive of β-Tubulin III expression, although SOX2-positive, β-Tubulin III-positive cells are found intermittently throughout the VZ and SVZ (asterisks). Scale bar = 50 μm (A–G); 25 μm (H). Abbreviations: VZ, ventricular zone; SVZ, subventricular zone and BV, blood vessel.

Distinct neural progenitor populations can be prospectively isolated based upon SOX2 expression levels

Our immunocytochemistry results indicate that SOX2 is differentially expressed between RGC and IPC 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 $SOX2^{EGFP/+}$ dTel, SOX2 is expressed in the VZ and SVZ of the lateral ventricles (Fig. 2A). 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. 2B) (Cubitt et al., 1995). Thus, the coexpression of endogenous SOX2 with EGFP (Fig. 2B, inset) allows for the prospective isolation of progenitor 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 (E16.5) periods of neurogenesis using flow cytometry. We hypothesized that at E12.5, when RGCs are the predominant progenitor populations in the dTel (Hartfuss et al., 2001; Malatesta et al., 2003, 2000), cells would express high EGFP intensity levels. In contrast, at E16.5 when RGCs, IPCs, and post-mitotic neurons constitute the dTel (Hartfuss et al., 2001; Malatesta et al., 2003, 2000), we would observe a shift towards lower EGFP

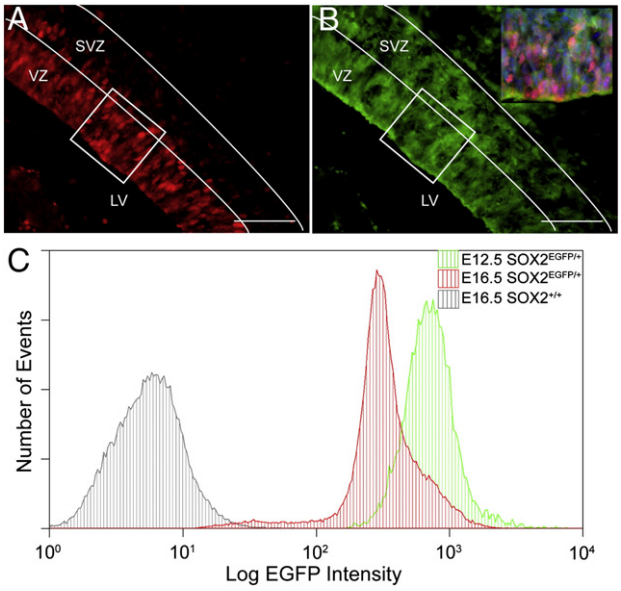

Fig. 2. Expression profile of the SOX2^{EGFP} mouse line using flow cytometry. (A–B) Coronal sections of E16.5 SOX2$^{EGFP/+}$ mice labeled with an anti-SOX2 antibody and expressing endogenous EGFP driven by the SOX2 promoter. (B, inset) The boxed regions of panels A and B show cells that co-express anti-SOX2 and EGFP located within the VZ and SVZ of the dorsal telencephalon. (C) Dissociated cells from E12.5 and E16.5 SOX2$^{EGFP/+}$ dorsal telencephalon were analyzed for EGFP intensity using flow cytometry. E12.5 SOX2$^{EGFP/+}$ intensity profile is in green and E16.5 SOX2$^{EGFP/+}$ intensity profile in red. Cells from SOX2$^{+/+}$ animals (gray) were used as an EGFP-negative control. Abbreviations: EGFP, enhanced green fluorescent protein; VZ, ventricular zone and SVZ, subventricular zone. Scale bars: (A–B) = 250 μm.

intensity levels as SOX2 expression is reduced or lost in IPCs and post-mitotic neurons, respectively. In both E12.5 and E16.5 SOX2$^{EGFP/+}$ mice, EGFP-positive cells can be distinguished from SOX2$^{+/+}$:EGFP-negative littermate controls using flow cytometry (Fig. 2C, red and green vs. gray). 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. 2C, green) consistent with a uniform population of RGCs expressing high levels of SOX2. In contrast, the E16.5 cells, on average, express lower levels of EGFP compared with E12.5 cells (306.8 ± 26.24 fluorescent units) (Fig. 2C, red). However the E16.5 intensity profile is skewed, suggesting that, even though a majority of E16.5 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. 2C, red). These results demonstrate that RGCs expressing high levels of SOX2 (as indicated by EGFP expression) are found in the dTel during both early (~E12.5) and late (~E16.5) stages of neurogenesis whereas IPCs expressing low levels of SOX2 are only observed at later stages. Therefore, we next determined whether 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

Based upon our immunocytochemical and flow cytometry results, we hypothesized that RGCs express higher amounts of SOX2 (and SOX2–EGFP) than IPCs, and therefore this differential expression can be utilized to isolate viable populations of each cell type. Using Fluorescence-Automated Cell Sorting (FACS), we separated E16.5 EGFP-positive cells from SOX2$^{EGFP/+}$ embryos 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. 3A) and analyses of *Sox2* and *Egfp* mRNA transcript levels utilizing quantitative reverse transcription polymerase chain reaction (qRT-PCR) demonstrate that both *Sox2* and *Egfp* mRNA transcript levels correspond to their respective EGFP fluorescent intensity levels (Fig. 3B).

Next, the stem cell capacity of each subpopulation was analyzed using the *in vitro* neurosphere assay (Hutton and Pevny, 2008; Reynolds and Weiss, 1992). To first 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 6 days (Coles-Takabe et al., 2008). All three subpopulations generated EGFP-positive neurospheres (Fig. 3C–D), however EGFP^{Low} cells produced statistically fewer neurospheres compared to EGFP^{High} and EGFP^{Int} subpopulations (Fig. 3C). In contrast, EGFP-negative cells isolated from SOX2^{EGFP} dTel were unable to produce neurospheres (data not shown). Moreover, EGFP^{High} cells produced larger diameter neurospheres compared to EGFP^{Int} and EGFP^{Low} populations, suggesting a difference in neurosphere growth rate (Fig. 3D–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 individual neurospheres was then measured every 2 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. 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 (<100 μm) was significantly greater in EGFP^{Low} cultures compared to both EGFP^{High} and EGFP^{Int} subpopulations (Fig. 3F). In contrast, intermediate sized neurospheres (100–200 μm) were more prevalent in the EGFP^{High} and EGFP^{Int} subpopulations (Fig. 3F). Moreover, the EGFP^{High} cultures also contained a significant percentage of large-diameter spheres (>200 μm) compared to both EGFP^{Int} and EGFP^{Low} populations (Fig. 3F). These data thus demonstrate 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; Hutton and Pevny, 2008). EGFP^{High} primary neurospheres generated significantly more secondary and tertiary neurospheres than both EGFP^{Int} and EGFP^{Low} primary neurospheres (Fig. 4A). Moreover, EGFP^{Low} primary neurospheres produced very few secondary neurospheres and were unable to generate any tertiary neurospheres (Fig. 4A), suggesting they have a more restricted capacity for self-renewal. These data indicate that cells with high levels of SOX2–EGFP expression have 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 with EGFP^{High} cells generating more multipotential neurospheres (89.2 ± 2.63%) compared to either EGFP^{Int} (86.5 ± 1.73%) or EGFP^{Low} (62.3 ± 6.63%) cells (Fig. 4B and D) In addition, unipotent neurospheres, generating only β-Tubulin III-positive neurons, were identified in all three populations (Fig. 4B and E). However, both EGFP^{Int} and EGFP^{Low} cells generated more unipotent neurospheres than EGFP^{High} cells with EGFP^{Low} cells generating the most unipotent neurospheres of all three subpopulations (Fig. 4B). Interestingly, we found that the multipotent capacity of a neurosphere was correlated with its size, regardless of sorting population (Fig. 4B–C). At 10 DIV, neurospheres with small diameters (<150 μm) only generated neurons, while large neurospheres (>150 μm) generated both neurons and glia. We did not observe any glial-specific neurospheres using these culture conditions.

EGFP^{High}, EGFP^{Int}, and EGFP^{Low} populations express molecular markers of radial glia, intermediate progenitors, and neurons, respectively

The results from our *in vitro* studies demonstrate that the EGFP^{High}, EGFP^{Int}, and EGFP^{Low} sorted populations each have distinct proliferative,

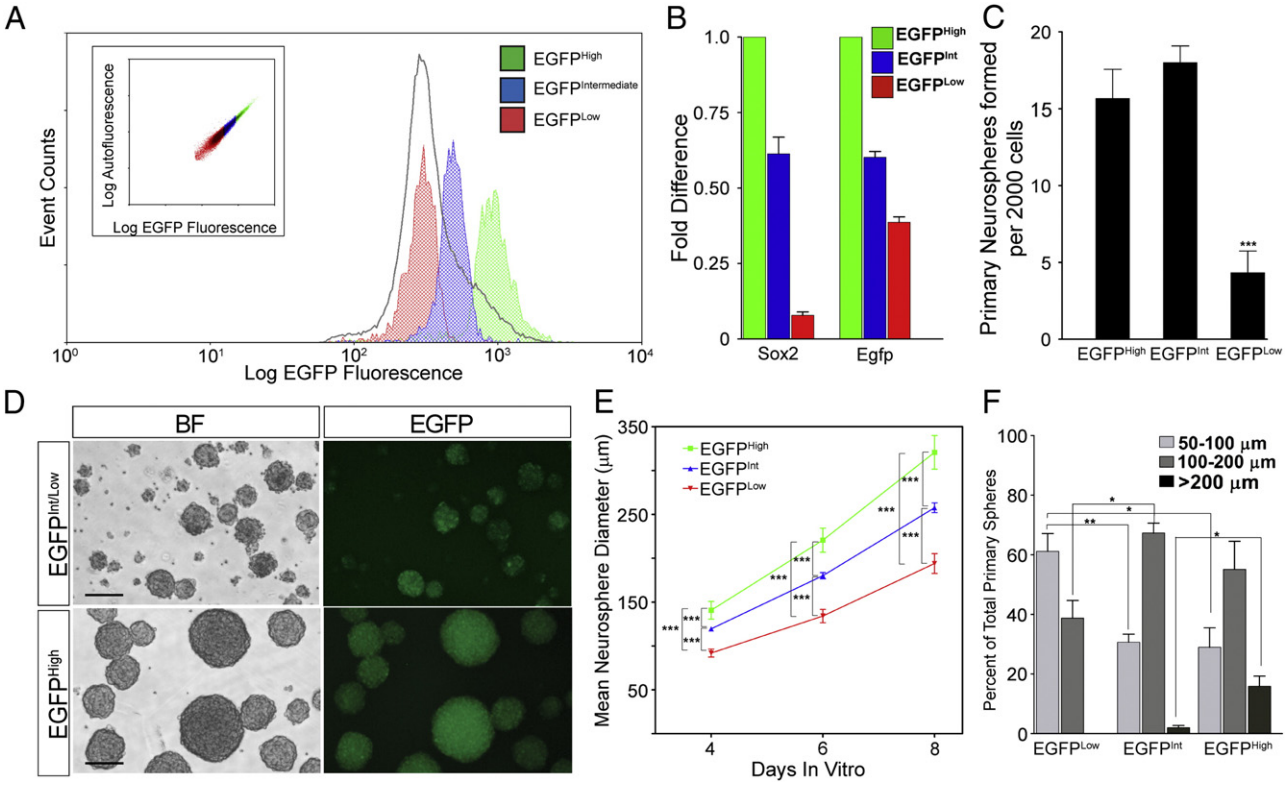

Fig. 3. Generation and analysis of primary neurospheres. *In vitro* analyses of cells isolated based upon SOX2–EGFP intensity levels. (A) Representative EGFP intensity profile of $EGFP^{High}$, $EGFP^{Int}$, and $EGFP^{Low}$ subpopulations isolated from E16.5 dorsal telencephalon (n = 4 for each sort). (B) *Sox2* and *Egfp* mRNA transcript levels from $EGFP^{High}$, $EGFP^{Int}$ and $EGFP^{Low}$ sorted cells (N = 3 separate sorting experiments). Fold difference is relative to $EGFP^{High}$ values for each gene. (C) Number of neurospheres formed per 2×10^3 cells plated from $EGFP^{High}$, $EGFP^{Int}$ and $EGFP^{Low}$ sorted cells. $EGFP^{Low}$ values are significantly different ($p<0.001$(***)). (D) Representative images of $EGFP^{High}$, $EGFP^{Int}$ and $EGFP^{Low}$ primary neurospheres after 6 days in culture. Scale bars = 75 μm. (E) Mean neurosphere diameters over 8 days of culturing. All values were significantly different ($p<0.001$(***)). (F) Percentage of primary neurospheres from each subpopulation after separation into one of three categories based upon diameter: small (100 μm), medium (100–200 μm) and large (>200 μm). P values: $p<0.05$ (*) and $p<0.01$(**).

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. *Sox3*, a SOXB1 homologue which is coexpressed with *Sox2* in neocortical progenitor cells (Collignon et al., 1996; Uwanogho et al., 1995; Wood and Episkopou, 1999), shows a similarly dynamic regulation and its levels directly correlate with those of *Sox2* (Fig. 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. 5A) (Mizutani et al., 2007). These data show that both the $EGFP^{High}$ and $EGFP^{Int}$ subpopulations are enriched for neural progenitor cells compared to $EGFP^{Low}$ cells.

These results 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 of a larger proportion of stem cell like radial glial cells. We confirmed this by analyzing Brain Lipid Binding Protein (*Blbp*) expression, a marker of radial glial (Hartfuss et al., 2001), and find that $EGFP^{High}$ cells express higher levels of *Blbp* transcript than both $EGFP^{Int}$ and $EGFP^{Low}$ populations (Fig. 5A). In contrast, *Pax6* transcript levels are not only high in $EGFP^{High}$ cells but also in $EGFP^{Int}$ cells as well (Fig. 5A). Given that Pax6 is widely used as a RGC marker, this result was unexpected. However, these results are supported by recent findings that lower levels of Pax6 maintain the progenitor identity of RGCs while higher Pax6 levels are necessary to promote IPC formation (Sansom et al., 2009). Thus, our data demonstrate that $EGFP^{High}$ populations are enriched for RGC progenitor cell markers and suggest that the $EGFP^{Int}$ populations may contain IPCs.

$EGFP^{Int}$ cells generate smaller neurospheres with limited self-renewal capacity. To address whether $EGFP^{Int}$ cells are enriched for IPC molecular markers, transcript levels for IPC genes *Tbr2* and *Ngn2* (Englund et al., 2005; Miyata et al., 2004), 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 while $EGFP^{Low}$ cells, in contrast, expressed low levels of both transcripts (Fig. 5B). Similarly, in $EGFP^{Int}$ cells, *Tis-21* was more than 2-fold higher than $EGFP^{High}$ cells (Fig. 5B). 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 IPC markers.

Lastly, the low neurosphere forming potential of $EGFP^{Low}$ cells suggests that these cells have lost the capacity to proliferate and consist largely of post-mitotic neurons. We therefore examined the expression of two neuronal genes, β-Tubulin III, which labels immature neuronal cells, and Tbr1, which marks mature neuronal populations (Fig. 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

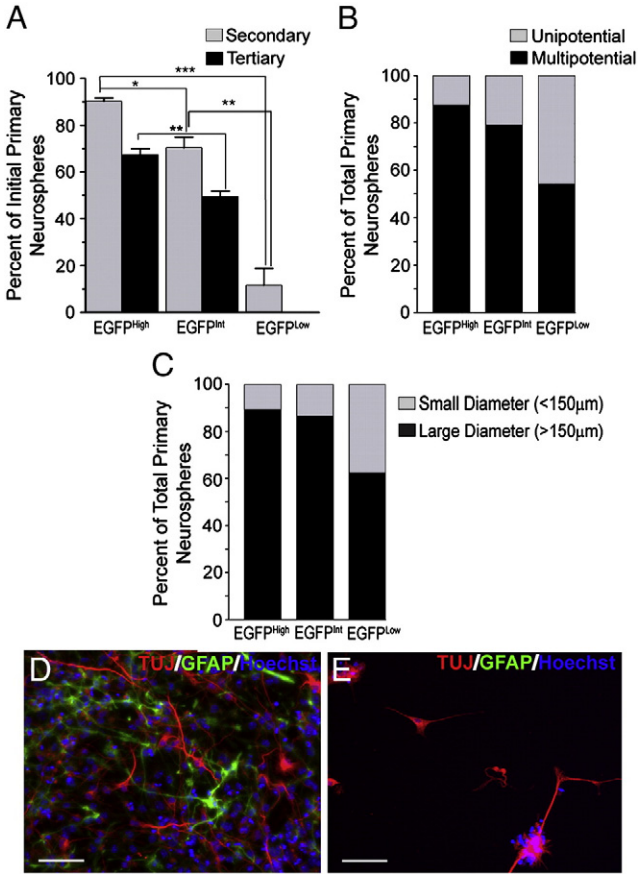

Fig. 4. Functional analyses of neurosphere self-renewal and multipotency. (A) Percentage of EGFP^{High}, EGFP^{Int} and EGFP^{Low} primary neurospheres capable of generating secondary and tertiary neurospheres (n = 16 neurospheres/condition). Significant differences in secondary and tertiary neurosphere generation were observed between all three populations. P values: $p<0.05$ (*), $p<0.01$(**) and $p<0.001$(***). (B) The percentage of primary neurospheres from each subpopulation that produce neurons and glia (multipotential) or neurons only (unipotential). No glial-specific neurospheres were observed. (C) The percentage of primary neurospheres from each subpopulation that contained large-diameter (>150 μm) and small-diameter (<150 μm) neurospheres. (D) Differentiated multipotential neurosphere labeled with β-Tubulin III (neurons), GFAP (astrocytes), and the nuclear marker Hoechst 33258. (E) Example of a differentiated unipotential neurosphere which generates β-Tubulin III-positive neurons only.

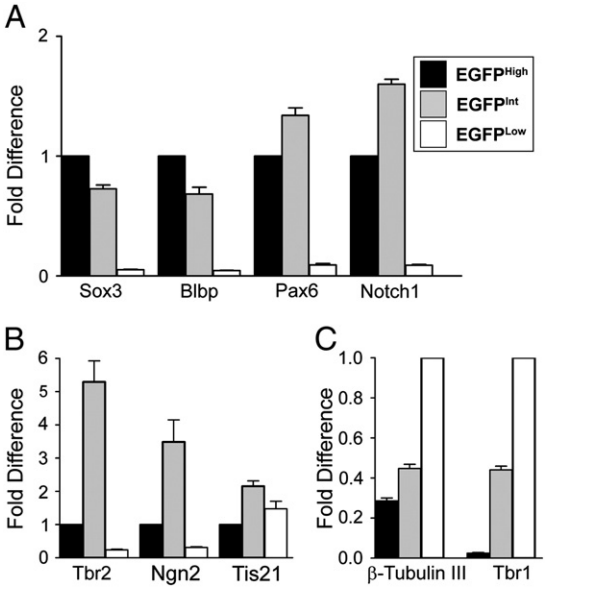

Fig. 5. Quantitative RT-PCR analyses of EGFP^{High}, EGFP^{Int}, and EGFP^{Low} cells isolated from E16.5 SOX2$^{EGFP/+}$ dorsal telencephalon (N = 3 separate sorting experiments). (A) mRNA transcript levels for neural progenitor markers *Sox3* and *Notch1* and radial glial markers *Blbp* and *Pax6*. (B) mRNA transcript levels for intermediate progenitor cell markers *Tbr2* and *Ngn2* and the marker of neuronal-fated cells, *Tis21*. (C) mRNA transcript levels for neuronal markers *β-Tubulin III* and *Tbr1*. Abbreviations: EGFP, enhanced green fluorescent protein; BLBP, Brain Lipid Binding Protein and NGN2, Neurogenin 2.

may also be generating immature neurons (see also Fig. 1H). 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 tissues (Corti et al., 2007; Lee et al., 2005). The cell-surface marker CD15 (SSEA-1, LeX) has also been used to isolate neural progenitor cells from 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 (Fox et al., 1981; Gomperts et al., 1994; Jiang et al., 2002; Mizrak et al., 2008; Solter and Knowles, 1978). 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 viable 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 CNS, as well as other non-neural populations such as the stomach, lung, hair follicle and tongue (Driskell et al., 2009; Ishii et al., 1998; Okubo et al., 2006; Pevny and Placzek, 2005; 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 (2003) enriched for neural progenitor cells by selectively isolating SOX2–EGFP-positive cells from the telencephalon using FACS and also found that SOX2 levels varied dependent upon EGFP intensity. However, the authors categorized all SOX2–EGFP-positive cells from the telencephalon as a single cohort of "neural stem cells" rather than a collection of cellular and molecularly distinct populations of neural progenitor cells and therefore they did not address whether these distinct populations differentially express SOX2. It is also important to note that the particular mouse SOX2–EGFP mouse line used by D'Amour and Gage (2003) does not completely recapitulate endogenous SOX2 expression as EGFP is not expressed in all neural progenitor cells due to the exclusion of select Sox2 regulatory domains in the randomly integrated transgenic construct (D'Amour and Gage, 2003; Zappone et al., 2000). 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 (Ellis et al., 2004). We find that all neurosphere generating cells from the developing dTel express SOX2 and moreover, we demonstrate the power of this genetic 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 RGCs in the dTel 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 is also highly expressed in radial glial cell bodies in the VZ and was largely coexpressed with PAX6 in these cells. In addition to prospectively isolating radial glial populations, we are able to utilize SOX2 expression levels to distinguish between IPCs 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 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.

The observation that many of the $EGFP^{Int}$ and $EGFP^{Low}$ neurospheres were multipotent was surprising, given the enrichment of IPC and neuronal markers in these populations; however the results were not altogether unexpected. By sorting cells based solely upon a continuum of SOX2–EGFP expression, overlap between distinct cell populations was unavoidable. However, these results are more likely the result of inherent limitations of the culture system as the *in vitro* neurosphere culture system does not completely recapitulate the *in vivo* neural progenitor niche of the animal (reviewed in (Jensen and Parmar, 2006)). Previous studies have demonstrated that the regional identity of cells can change under neurosphere culture conditions with progenitor cells from the dorsal telencephalon acquiring a ventral progenitor identity after culturing as neurospheres (Abematsu et al., 2006; Hack et al., 2004). Moreover, the differentiation potential of neural progenitor cells has been shown to change when cultured *in vitro*. For example, the presence of bFGF, a necessary component of the neurosphere culture medium, is able to convert bipotent spinal cord neural progenitor cells to a tripotent state *in vitro* (Gabay et al., 2003). The presence of multipotential neurospheres, especially in the $EGFP^{Int}$ population, may suggest that while *in vivo* these cells are neuronally-specified, *in vitro* they are capable of generating both neurons and glia. Moreover we hypothesize that progenitor cells that are too far along the neuronal differentiation "process" are unable to (re)acquire their multipotential state, instead forming the small, neuronal unipotent neurospheres enriched in our $EGFP^{Int}$ and $EGFP^{Low}$ cultures.

In these studies, we utilized the $SOX2^{EGFP/+}$ mouse line in which endogenous SOX2 expression levels are approximately 50% of wildtype controls (Taranova et al., 2006). Although we cannot exclude the possibility that our functional and molecular analyses are influenced by the lower levels of initial SOX2 expression, our lab and others have generated multiple SOX2 heterozygous mouse lines which genetically demonstrate that heterozygous levels of SOX2 are sufficient for proper nervous system development (Avilion et al., 2003; Ellis et al., 2004; Taranova et al., 2006) and defects in neural progenitor cells are only observed when SOX2 levels drop to 20–30% of wildtype levels (Cavallaro et al., 2008; Ferri et al., 2004; Taranova et al., 2006). However, it remains unclear if SOX2 levels directly influence the normal RPC–IPC–Neuron transition. Mice harboring hypomorphic mutations in SOX2 have shown decreases in cell-proliferation and neuronal differentiation, in addition to defects in neuronal maturation, in the cortex (Cavallaro et al., 2008; Favaro et al., 2009; Ferri et al., 2004; Miyagi et al., 2008; Taranova et al., 2006). Moreover, overexpression of SOX2 in neurosphere cultures inhibits neurogenesis while permitting gliogenesis (Bani-Yaghoub et al., 2006; Bylund et al., 2003; Graham et al., 2003). These data suggest that the precise regulation of SOX2 levels plays an important role in neural progenitor fate specification. If SOX2 expression is too high, neurogenesis is inhibited; however, if neural progenitors fail to reach a certain threshold of SOX2 expression (i.e. SOX2 hypomorphic mice), neurons are unable to properly differentiate (Avilion et al., 2003; Bani-Yaghoub et al., 2006; Bylund et al., 2003; Cavallaro et al., 2008; Graham et al., 2003). Our data support a hypothesis in which a precisely regulated decrease in SOX2 expression therefore is important for the proper RG–IPC–Neuron transition in the dorsal telencephalon.

Conclusion

The identification and characterization of neural progenitor populations *in vivo* have 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.

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