Developmental Biology 352 (2011) 40–47

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

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

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ARTICLE INFO

Article history:
Received for publication 5 November 2010
Revised 10 January 2011
Accepted 11 January 2011
Available online 21 January 2011

Keywords:
Neural stem cell
Neurosphere
Radial glia
Intermediate progenitor
Enhanced green fluorescent protein

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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doi:10.1016/j.ydbio.2011.01.015

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