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Role of Sox2 in the development of the mouse neocortex

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Abstract

The mammalian neocortex is established from neural stem and progenitor cells that utilize specific transcriptional and environmental factors to create functional neurons and astrocytes. Here, we examined the mechanism of Sox2 action during neocortical neurogenesis and gliogenesis. We established a robust Sox2 expression in neural stem and progenitor cells within the ventricular zone, which persisted until the cells exited the cell cycle. Overexpression of constitutively active Sox2 in neural progenitors resulted in upregulation of Notch1, recombination signal-sequence binding protein-J (RBP-J) and hairy enhancer of split 5 (Hes5) transcripts and the Sox2 high mobility group (HMG) domain seemed sufficient to confer these effects. While Sox2 overexpression permitted the differentiation of progenitors into astroglia, it inhibited neurogenesis, unless the Notch pathway was blocked. Moreover, neuronal precursors engaged a serine protease(s) to eliminate the overexpressed Sox2 protein and relieve the repression of neurogenesis. Glial precursors and differentiated astrocytes, on the other hand, maintained Sox2 expression until they reached a quiescent state. Sox2 expression was re-activated by signals that triggered astrocytic proliferation (i.e., injury, mitogenic and gliogenic factors). Taken together, Sox2 appears to act upstream of the Notch signaling pathway to maintain the cell proliferative potential and to ensure the generation of sufficient cell numbers and phenotypes in the developing neocortex.

Keywords: Astrocytes; Gliogenesis; Neural stem and progenitor cells; Neurogenesis; Precursors; Notch1; Proliferation; Sox2 degradation

Introduction

The mammalian central nervous system (CNS) arises from progenitor cells that undergo a well-orchestrated program of cell division, fate determination and differentiation (Jacobson, 1991; Qian et al., 1998, 2000; Rubenstein et al., 1998; Bertrand et al., 2002). One of the earliest transcription factors expressed in the developing CNS is Sox2 (Sex determining region of Y chromosome (*S*ry)-related high mobility group b*ox2*), a member of the extended Sox family (Gubbay et al., 1990; Laudet et al., 1993; Koopman, 1999; Sasai, 2001). Sox proteins contain a characteristic HMG domain that facilitates their interaction with the minor groove of the DNA helix, causing DNA bending and a transcriptionally permissive change in chromatin structure (Scaffidi and Bianchi, 2001; Weiss, 2001). Although they share similar DNA-binding properties, the individual Sox proteins use specific partners to regulate different sets of target genes (Kamachi et al., 2000; Tanaka et al., 2004).

There is accumulating evidence that Sox2 controls the expression of several developmentally important genes (Sox2, Oct4, Nanog, nestin, δ-crystalline, fibroblast growth factor 4, undifferentiated embryonic cell transcription factor 1 and F-box-containing protein 15), hence, it plays a crucial role in embryonic development (Pevny and Lovell-Badge, 1997; Wegner, 1999; Kamachi et al., 2000; Avilion et al., 2003; Uchikawa et al., 2003, 2004; Catena et al., 2004; Miyagi et al., 2004; Wegner and Stolt, 2005). In particular, studies on the chick spinal cord show that the inhibition of Sox2 activity in neural progenitors leads to their exit from the cell cycle followed by early neuronal differentiation (Bylund et al., 2003; Graham et al., 2003; Tanaka et al., 2004). While several reports suggest a role for Sox2 in the development of the CNS (Uwanogho et al., 1995; Zappone et al.,

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2000; Ferri et al., 2004), it remains to be determined, which of the fundamental biological processes (i.e., proliferation, fate determination or differentiation) depend on the Sox2 function. Specifically, it is not known whether its role in neurogenesis differs from that in gliogenesis or how the gene responds to the factors that control the generation of neurons and glia. Furthermore, although Sox2 inhibits neurogenesis (Bylund et al., 2003; Graham et al., 2003; Tanaka et al., 2004), the molecular mechanisms involved in this process are not known.

To address some of these questions, we examined the role of Sox2 in the developing mouse neocortex. We established that Sox2 expression was restricted to the proliferating cell populations including neural stem and progenitor cells, glial precursors and proliferating astrocytes. Neurons not only turned the gene off, but also utilized a proteolytic serine-specific pathway to eliminate the protein prior to terminal differentiation. In contrast, terminally differentiated astrocytes, which maintain a proliferative competence, were capable of switching the gene on in order to transit from quiescence to proliferation. Constitutive overexpression of Sox2 in neural progenitors effectively inhibited neurogenesis and caused upregulation of the molecular components of the Notch signaling pathway, known for its role in the development of the CNS.

Materials and methods

Tissue isolation

Timed pregnant CD1 mice (Charles River, St. Constant, QC) were sacrificed by CO_2 inhalation at gestational (embryonic) days 9.5–18 (E9.5–E18), in accordance with a protocol approved by the Canadian Council on Animal Care. The uteruses were aseptically removed and transferred sequentially to two Petri dishes containing calcium- and magnesium-free Hank's balanced salt solution (HBSS, Invitrogen Corporation, Burlington, ON) to rinse away blood. Embryos were dissected out of the amniotic sacs and examined for morphological hallmarks to ensure the accuracy of the gestational timing. The heads and the dorsal telencephalons were sequentially isolated under a dissection microscope (Wild Heerbrugg, Germany) and transferred into new plates containing HBSS. The dorsal telencephalons were freed of meninges and dissected further to isolate the ventricular zone (VZ) or the neocortex, depending on the experiment (i.e., cell culture, single cell clonal assay or biochemical, histochemical and molecular analyses). Similar procedures were followed for the preparation of adult and postnatal day 0–3 (PN0–PN3) neocortices.

Cell cultures

Tissues were mechanically dissociated in Dulbecco's Modified Eagle Medium, high glucose, L-glutamine (DMEM; Invitrogen) and filtered through a 40-μm nylon cell strainer (Falcon, VWR, Mississauga, ON). The dissociated cells were quickly assessed for viability by the trypan blue exclusion assay and used as described below.

Neural stem cells and neurosphere assay

Neural stem cells were derived from the E13.5 cortical VZ and examined for the self-renewal and multipotential properties (modified from Tropepe et al., 1999; also see Seaberg and van der Kooy, 2003). In brief, cells were deposited into the uncoated 96-well plates (Nunc) in DMEM (Invitrogen) + N2 supplement (Invitrogen) + fibroblast growth factor 2 (FGF2, 20 ng/ml, Invitrogen) at a density of 1 cell/well (plating efficiency: ~40%). Single cells were repeatedly monitored under a light microscope for the neurosphere formation, using the same culture condition. After 7 days in vitro (DIV), a total of 70 ± 9.2 floating primary neurospheres were generated from 10,000 cells (three independent experiments). The primary neurospheres were divided into three groups: (a) 20 neurospheres were used for immunocytochemical analysis of Sox2 and neuronal and glial phenotypes; (b) another 20 neurospheres were transferred onto the PLL-coated coverslips (Invitrogen) in DMEM + 5% fetal bovine serum (FBS; Hyclone, Logan, UT) + N2 supplement and examined 1–10 days later for the expression of Sox2 and neuronal and glial markers; (c) 25 of the remaining primary neurospheres were dissociated separately into single cells to generate the secondary neurospheres in DMEM + N2 supplement + FGF2 (i.e., one primary neurosphere generated 32 ± 8.7 secondary neurospheres after 7 DIV).

Similar to the primary neurospheres, secondary neurospheres were divided into three groups (25 each) and examined for: (a) Sox2, MAP2 and GFAP expression; (b) multipotential properties by differentiation into neurons and astrocytes; and (c) self-renewal capacity (i.e., one secondary neurosphere generated 18 ± 5.2 tertiary neurospheres).

Neural progenitor, neuronal and astroglial cultures

Neural progenitors were obtained from the E13.5 VZ, plated onto PLL-coated coverslips (9×10^5 living cells/ml) in DMEM + 5% FBS + N2 supplement and examined 4–8 h after plating. Neocortical neurons were generated from neural progenitors ($3–9 \times 10^5$ living cell/ml) by reducing the serum concentration (i.e., 0.5% FBS) and adding 1 μM cytosine arabinoside (Sigma-Aldrich Ltd., Oakville, ON) to the cultures between day 3 and 7 to limit the generation of glial cells. Medium was replenished every 48 h during the course of the experiment, and cells were examined at 3–7 DIV.

Astroglial cultures were generated from neural progenitors of the E13.5 VZ ($0.5–5 \times 10^5$ living cells/ml) in DMEM + 10% FBS (replenished every 48 h) and were analyzed after 1–16 weeks. In some experiments, the 16-week-old astrocytes were mechanically injured with a sterile p200 pipet tip and cultured further for up to 7 days in the presence or absence of 40 ng/ml of FGF2, 100 ng/ml of ciliary neurotrophic factor (CNTF; Sigma), 15 ng/ml of leukemia inhibitory factor (LIF; Invitrogen) or 1 mM dibutyryl cAMP (dbcAMP; Sigma) in DMEM + 5% FBS (replenished every 48 h). The cultures were analyzed for Sox2 expression and BrdU immunoreactivity, as described below.

Isolation of neuronal and glial precursors by immunopanning

Neuronal and glial precursors were isolated, as previously described (Mayer-Proschel et al., 1997; Rao and Mayer-Proschel, 1997). In brief, separate 100-mm tissue culture plates were coated with 1 ml of either 5 μg/ml sterile A2B5 monoclonal antibody (Chemicon International, Inc., Temecula, CA) or 10 μg/ml sterile E-NCAM monoclonal antibody (5B8 clone, Developmental studies Hybridoma Bank, University of Iowa, IA) in PBS for 2–4 h. The plates were washed three times with PBS just prior to the addition of cells. To isolate neuronal precursors, the dissociated E13.5 dorsal telencephalic cells were added to the A2B5-coated plate for 30 min (37°C, 5% CO_2) and non-attached cells (i.e., remaining in suspension) were, subsequently, transferred into the E-NCAM-coated plate. After 45 min (37°C, 5% CO_2), the cells attached to the E-NCAM-coated plate (i.e., A2B5-negative NCAM-positive) were scraped off, transferred onto PLL-coated coverslips, placed in DMEM (Invitrogen) + 0.5% FBS (Hyclone) + N2 supplement and were used for further analysis of the neuronal cell lineage. To isolate glial precursors, the cells attached to the A2B5-coated plate were scraped off and placed in the E-NCAM-coated plate for 45 min (37°C, 5% CO_2). The non-attached (i.e., A2B5-positive NCAM-negative) cells were collected, plated onto PLL-coated coverslips in DMEM + 10% FBS and were used for analysis of the glial cell lineage. The cultures were examined for the expression of A2B5, E-NCAM, Sox2, βIII tubulin, MAP2 and GFAP to determine the efficiency of immunopanning and the fate of immunopanned cells.

Mouse embryonic stem cells

ES-D3 cells (ATCC, Manassas, VA) were seeded at a density of 5×10^5 living cells/ml in a gelatin-coated T-25 flask in DMEM + 10% FBS (Gemini BioProducts, Woodland, CA) + 1% non-essential amino acids (NEAA, Invitrogen) + 2000 U/ml leukemia inhibitory factor (LIF, 10^5 Units/μg, Invitrogen) + 0.001% β-mercaptoethanol (Sigma). To induce differentiation, cells were removed from the substrate with trypsin–EDTA, gently dissociated into a mixture of single cells and small clumps and transferred into four 100-mm bacterial plates, each containing 10 ml of DMEM + 10% FBS + 1% NEAA. Cell aggregates were grown for 4 days, during which the medium was refreshed once (after 2 days). Following this period, cell aggregates were treated with DMEM + 10% FBS + 1% NEAA + 1 μM RA for 4 days, during which the

medium was refreshed once. After the 8-day induction period, aggregates were gently dissociated with trypsin–EDTA and plated into the laminin-coated tissue culture dishes (that is, approximately 100 aggregates were plated into each 35-mm plate, containing a coverslip). Cells were treated with DMEM + 10% FBS + 1% NEAA and examined for neuronal and astroglial phenotypes at day 14, counting the first day of aggregation as day 1.

N2a neuroblasts

N2a neuroblastoma cells (ATCC) were plated at the density of 5×10^5 living cells/ml in DMEM + 10% FBS and a day after plating were used for transient transfections or retroviral infections, as described in Supplementary materials.

Human embryonic kidney (HEK) 293GPG cells

See Supplementary materials.

Inhibition of proteolysis

N2a cells were transfected with Sox2-EGFP or Sox2-cMyc constructs as described in Supplementary materials. Twenty four hours after transfection, cells were treated with individual protease inhibitors [i.e., 1 mM AEBSF (Calbiochem, EMD Biosciences Inc., San Diego, CA), 10 μM E-64 (Sigma) or 25 μM MG132 (Calbiochem)] for up to 30 h. The cell lysates were collected and the proteins were resolved by 10% SDS-PAGE for Western blotting.

Inhibition of the Notch pathway

The retrovirally infected (Sox2-EGFP or EGFP alone) E13.5 VZ neural progenitors (see Supplementary materials) were incubated in the absence or presence of γ-secretase inhibitors X (final concentration: 10 μM; Calbiochem) or II (final concentration: 150 μM; Calbiochem), starting 6 h after infection (Li et al., 2000; Schroeter et al., 2003). Fresh inhibitor was added to the culture every 24 h for 5 days in DMEM + 0.5% FBS + N2 supplement. Inhibitors were solubilized in Dimethylsulfoxide (DMSO, Sigma) and diluted in medium. An equal concentration of DMSO was also used in control cultures.

Tissue injury

In some experiments, CD1 mice (1 month old) were placed in a stereotaxic frame and the skull was opened. The prefrontal association cortex was injured using a Pasteur pipet connected to a vacuum pump. Animals were sacrificed by CO_2 inhalation at 0 and 5 days post-lesion and perfused with 4% paraformaldehyde for 10 min. Brains were removed, fixed overnight, processed, paraffin-embedded, sectioned and immunostained with Sox2 and GFAP antibodies as described in Supplementary materials.

BrdU incorporation

Pregnant CD1 mice (13.5 day gestation) were injected intraperitoneally with 5-bromo-2-deoxyuridine (BrdU, Sigma), using 50 μg BrdU/g body weight. After 2 h, animals were sacrificed and embryos were prepared for immunohistochemical analysis, as described above. Following fixation, sections were denatured in 4 N HCl (15 min), neutralized in 100 mM sodium tetraborate pH 9.0 (15 min) and stained with monoclonal BrdU antibody.

Similarly, cells were incubated with 10 μM BrdU (final concentration) at 37°C, 5% CO_2 (8–12 h), fixed in 4% paraformaldehyde (20 min), denatured, neutralized and stained, as described above. The BrdU-immunoreactive cells were visually scored against the Hoechst and Sox2-positive nuclei. Confluent monolayers of quiescent astrocytes were scratched and incubated with 10 μM BrdU (final concentration) and the factors (see Cell cultures) for 8–12 h (37°C, 5% CO_2). Cultures were fixed at 15 min, 3 days and 7 days post-lesion, and their Sox2 and BrdU immunoreactivity was determined, as described above.

Generation of SOX2 antibody

The antibody was raised against a full-length human SOX2, cloned into the pBAD/His A vector (Invitrogen) and expressed as a $(His)_6$-tagged SOX2 fusion protein in *E. coli* strain LMG 194. The recombinant SOX2 was purified on a His-Trap nickel column (Amersham Health, Oakville, ON). New Zealand rabbits were injected with 500 μg of recombinant SOX2 and boosted three times (250 μg each) within 4 weeks. The antibody titer was tested 10–14 days after each boost and collected 14 days after the third injection. The specificity of SOX2 antibody was validated in several tissues and cell lines.

Other antibodies

See Supplementary materials.

Statistical analysis

Data were derived from a total of three to five independent experiments (each in triplicate). Cells were quantified in a uniform random fashion by scoring the number of cells per field of view. Final values were presented as means ± SEM, and their significance was determined by χ^2 test, one-way ANOVA and post hoc Dunnett's test, using GraphPad Instat (GraphPad Software Inc., San Diego, CA).

Other methods

Gene delivery (including the detailed description of vectors), RT-PCR, immunocytochemical, immunohistochemical and Western blotting methods have been described in detail in the Supplementary materials.

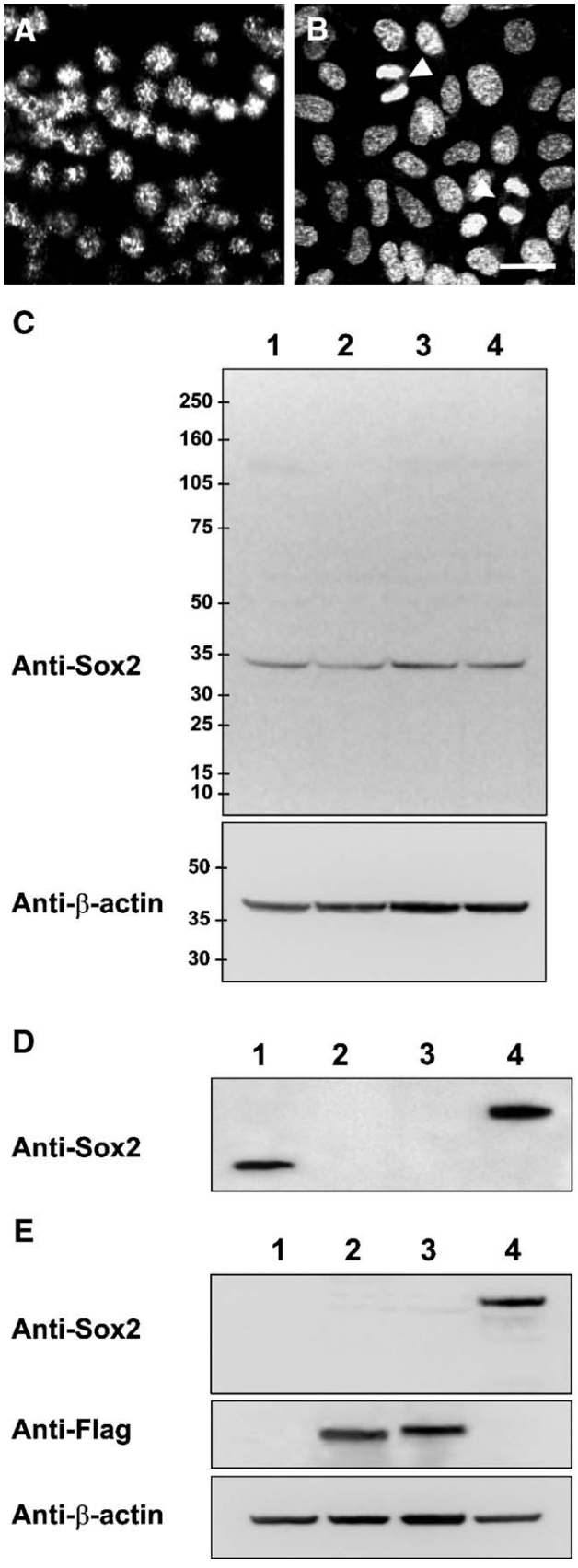
Results

Characterization of Sox2 antibody

Analysis of Sox2 expression in the developing neocortex requires a well-characterized antibody suitable for immunochemistry and Western blotting. Therefore, in order to proceed with this study, we generated a rabbit polyclonal antibody raised against the full-length Sox2 protein and tested its immunoreactivity in tissues in which Sox2 is known to be present (i.e., the blastocyst and the brain, Wegner, 1999). Our antibody showed high specificity for Sox2 in immunocytochemical analysis, revealing punctuate nuclear localization of Sox2 and no cross-reactivity with the cytoplasmic components (Figs. 1A and B). On Western blots, the antibody recognized the endogenous Sox2 (34 kDa) as well as the overexpressed Sox2-EGFP fusion protein (56 kDa), both as single protein bands (Figs. 1C–E). Furthermore, the antibody did not cross react with other SoxB1 subfamily members, i.e., Sox1 and Sox3 (Fig. 1E). The immunodetection capability of this antibody allowed us to carefully examine Sox2 expression in the subsequent sets of experiments.

Sox2 is spatially and temporally expressed in the neocortex

Western blot analysis showed abundant Sox2 expression in the embryonic neocortex (Fig. 2A) during a time window in which the pool of neural progenitors expands extensively to satisfy the need for the generation of tens of thousands of cortical neurons and astrocytes. These results were further supported by immunohistochemical staining that showed Sox2 expression in neural stem and progenitor cells residing in the ventricular zone (Figs. 2B and D). Sox2 expression was dramatically downregulated after birth to a negligible level in the adult mouse neocortex (Figs. 2A, F). The low level of Sox2

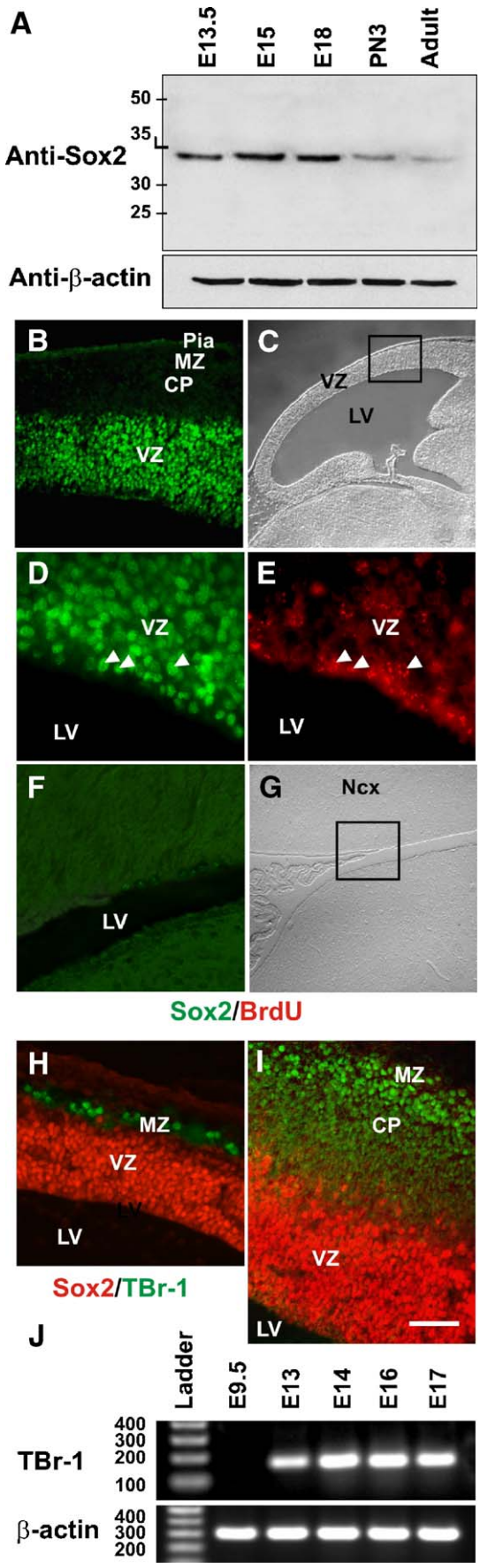


in the adult neocortex (Fig. 2A) likely reflected its expression by the SVZ neural stem cells, ependymal cells and a limited number of dividing astrocytes (Ellis et al., 2004). Using a 2 h BrdU exposure of the E13.5 neocortex, we established that the vast majority (i.e., 92 ± 6%, one-way ANOVA, $P < 0.001$) of the BrdU-positive cells also showed Sox2 immunoreactivity (Figs. 2D and E). However, cells leaving the ventricular zone and acquiring neuronal identity did not stain with the Sox2 antibody (Figs. 2H and I). Instead, they were labeled with an antibody specific for the transcription factor TBr-1, a marker of newborn neurons (Fig. 2H). Neocortical neurons expressed TBr-1 immediately after they exited the cell cycle and migrated out of the ventricular zone at E11 (Fig. 2H). Later, at E13.5, TBr-1 staining displayed a gradient across the developing neocortex, labeling the marginal zone and the cortical plate, but not the ventricular zone (Fig. 2I). Furthermore, RT-PCR analysis confirmed that TBr-1 was expressed from the onset of neocortical neurogenesis at E11 until its completion at E17 (Fig. 2J). Thus, neocortical cells switched from Sox2-positive to Sox2-negative TBr-1-positive status as they acquired neuronal identity.

Sox2 is downregulated as neocortical cells acquire neuronal fate

To better understand the role of Sox2 in neural stem cells and neuronal precursors, we examined Sox2 expression in these subpopulations in the brain (Fig. 3A) and throughout their differentiation in culture (Figs. 3B–M). The E13.5 ventricular zone was dissociated into single cells (Figs. 3B and C) and evaluated for self-renewal capability by neurosphere assays (Figs. 3D and E). Sox2 was prominently expressed in both single neural stem cells (Fig. 3B) and neurospheres (Fig. 3D). The primary neurosphere cells maintained Sox2 expression after dissociation and formation of the secondary neurospheres, i.e., throughout the entire self-renewal process (data not shown). In parallel experiments, we examined the generation of neurons and astrocytes in more than 120 neurospheres and established that neural stem cells turn off Sox2 as they differentiate into neurons (Fig. 3F) but maintain its expression during gliogenesis (Fig. 3H). The number of Sox2-negative MAP2-positive neurons and Sox2-positive GFAP-positive astrocytes varied depending on the size of neurospheres and the time at which

Fig. 1. The in-house generated rabbit polyclonal antibody specifically detects Sox2 protein. (A and B) Immunocytochemical examination of mouse embryonic (A) and neural (B) stem cells reveals nuclear localization of Sox2. Arrowheads: Sox2 in daughter cells at telophase. Scale bar: 10 μm. (C–E) Western blot analyses with Sox2 antibody show a single 34-kDa band of endogenous Sox2 in adult mouse brain (C, lane 1, whole brain; lane 2, cerebellum; lane 3, hippocampus; lane 4, olfactory bulbs), mouse embryonic stem cells (D, lane 1) and a single 56 kDa band of Sox2-EGFP fusion protein in transfected 293 cells (D, lane 4; E, lane 4). Untransfected 293 cells (D, lane 2; E, lane 1) and EGFP-transfected 293 cells (D, lane 3) were used as negative controls. The expression level of β-actin (42 kDa) validates an equal protein loading. (E) Sox2 antibody does not cross react with either Sox1 (E, lane 2) or Sox3 (E, lane 3), as evidenced by the lack of immunoreactivity in samples expressing mouse recombinant Sox1 (Flag)-hrGFP (42 kDa) and Sox3(Flag)-hrGFP (43 kDa) proteins. Anti-Flag (E) and anti-β-actin (E) blotting was used to verify transfection and equal protein loading, respectively.



they were immunostained. These results were further supported by experiments on embryonic stem cells, in which the same Sox2 behavior was observed during the generation of neurons and astrocytes (Supplementary Fig. 1).

We applied a well-established immunopanning technique with E-NCAM (PSA-NCAM) and A2B5 antibodies to isolate the NCAM-positive A2B5-negative neuronal precursors (Mayer-Proschel et al., 1997; Rao et al., 1998). Since NCAM is a cell surface glycoprotein involved in the adhesion and migration of neuronal precursors, it is routinely used to sort and isolate these cells (Raff et al., 1983; Noll and Miller, 1993; Mayer-Proschel et al., 1997; Rao and Mayer-Proschel, 1997; Rao et al., 1998; Dietrich et al., 2002). These cells displayed several important features. First, they were detected in a transitory location, adjacent to the ventricular zone, where both Sox2 and NCAM were present in the same cells (Fig. 3A). Secondly, the level of Sox2 protein/cell was significantly lower in neuronal precursors (44 ± 0.12%) than that of the cells located in the VZ. Thirdly, the isolated NCAM-positive A2B5-negative cells contained a small number of BrdU-positive (Fig. 3J) and Sox2-positive βIII tubulin-negative (13 ± 5%) cells after 24 h in culture. However, nearly all cells were Sox2-negative after 3 DIV. Double immunostaining confirmed that over 90% of cells were Sox2-negative βIII tubulin-positive after 3 DIV (Fig. 3L) and Sox2-negative MAP2-positive after 7 DIV (Fig. 3M). Together, these results suggested that Sox2 downregulation may be an essential step for neocortical cells to complete neurogenesis. To validate this concept and better understand the mechanism, we examined the fate of neural progenitors following constitutive Sox2 overexpression.

Sox2 overexpression inhibits neurogenesis via the Notch signaling pathway

Neural progenitors were efficiently (>90%) infected with the retroviral particles carrying EGFP or Sox2-EGFP and carefully

Fig. 2. Sox2 displays a spatiotemporal expression pattern in the developing neocortex. (A) Western blot shows that Sox2 is highly expressed in the embryonic neocortex, but it is significantly downregulated after birth. Expression of β-actin (42 kDa) shows equal protein loading. (B) The immunohistochemical analysis of E13.5 neocortex clearly demarcates Sox2 expression (green) in the ventricular zone (VZ), where neural stem cells and progenitors are residing. (C) Hoffmann modulation contrast image of E13.5 neocortex. Inset: the region examined in panel B. (D–E) Sox2-positive neural progenitors (D, green) reside in the proliferative E13.5 VZ, as demonstrated by BrdU staining (E, red). Arrowheads: proliferating cells. (F) Most regions of the adult neocortex do not express Sox2, with the exception of few cells in the ependymal layer. (G) Hoffmann modulation contrast image of the adult neocortex. Inset: the region examined in panel F. (H–I) Cells downregulate Sox2 (red), as they leave VZ and adopt a TBr-1-positive neuronal identity (TBr-1, green). Note that the TBr-1-positive neurons are Sox2-negative. This transition from Sox2-positive to TBr-1-positive status coincides with an increase in neocortical neurogenesis from E11 (H) to E13.5 (I). (J) RT-PCR analysis confirms the absence of TBr-1 transcript (175 bp fragment) at E9.5 and its subsequent presence during neurogenesis (E13 to E17). The β-actin transcript (297 bp fragment) was used as a control for RNA input. E9.5–18: embryonic days 9.5–18; PN3: postnatal day 3; CP: cortical plate; LV: lateral ventricle; MZ: marginal zone; Ncx: neocortex; VZ: ventricular zone. Scale bar: B and F 400 μm; C and G, 100 μm; D and E, 50 μm; H and I, 75 μm. All sections are sagittal.

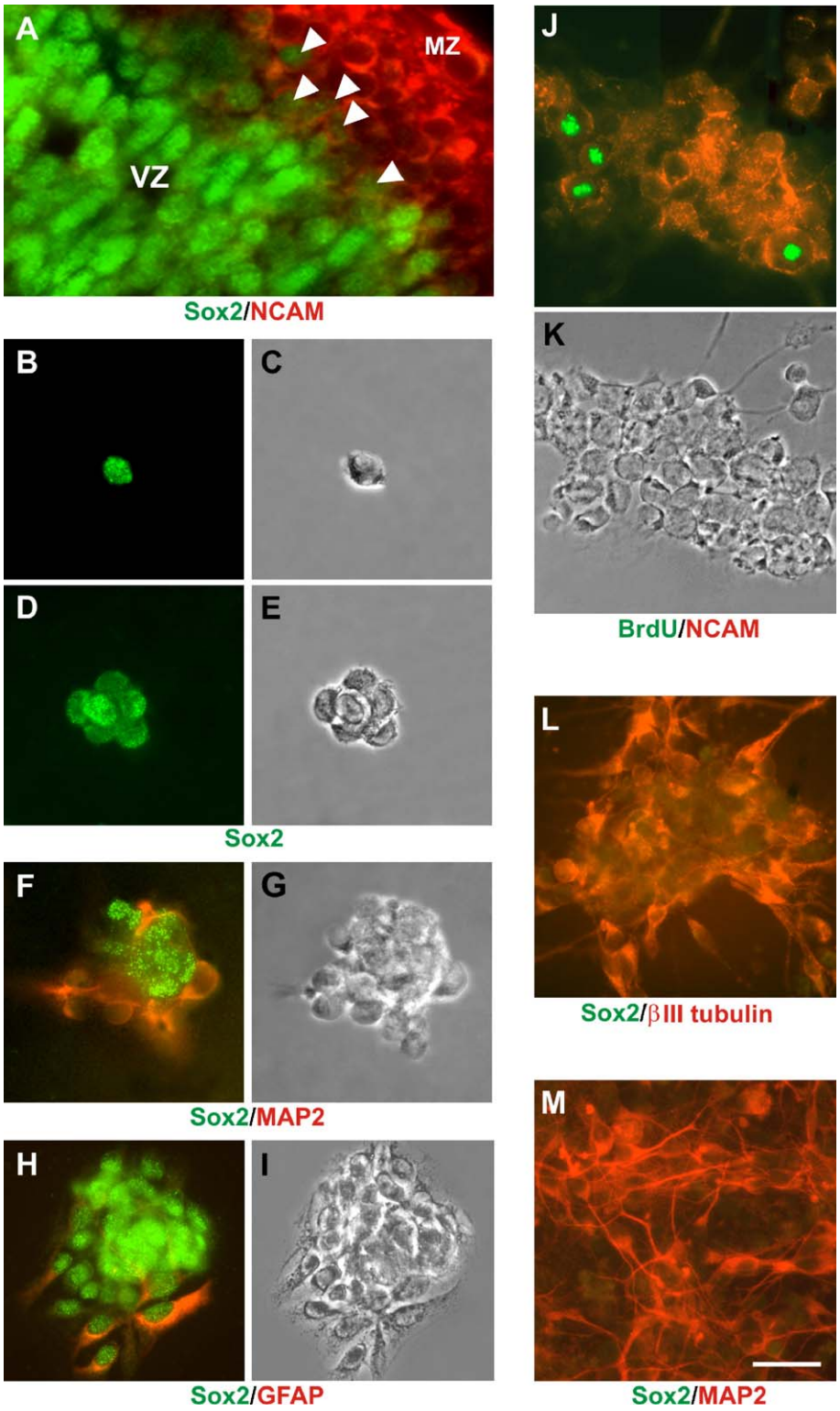
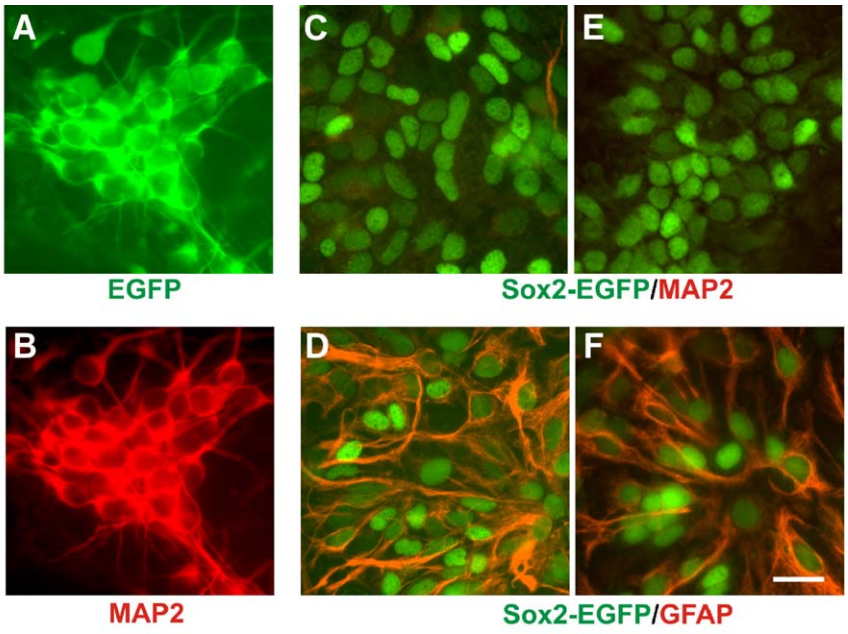


Fig. 3. Neuronal precursors downregulate Sox2 as they exit the cell cycle. (A) The immunostaining of E13.5 neocortex (sagittal) demonstrates the presence of a Sox2-positive NCAM-positive subpopulation (marked with arrowheads) at the outer boundary of the VZ (Sox2—green, NCAM—red). (B–I) A single Sox2-positive neural stem cell (B) from VZ forms a Sox2-positive neurosphere (D), which, in turn, gives rise to neurons (F, MAP2—red, Sox2—green) and astrocytes (H, GFAP—red, Sox2—green). While both cell types are derived from the same neural stem cell, only astrocytes maintain Sox2 expression (H). (C, E, G and I) Corresponding Hoffmann modulation contrast images of panels B, D, F and H, respectively. (J) The NCAM-positive neuronal precursors (red) include a cell subpopulation that even 24 h after sorting still incorporates BrdU (green). (K) The phase contrast image of panel J. (L) The isolated NCAM-positive cells completely lose the Sox2 signal within 3 days of sorting, as shown by double staining with Sox2 and neuronal βIII tubulin antibodies (Sox2—green, βIII tubulin—red). (M) Sox2 continues to be absent in the MAP2-positive neurons at day 7 (Sox2—green, MAP2—red). MZ: marginal zone; VZ: ventricular zone. Scale bar: A, 20 μm; B–I, 10 μm; J–K, 12 μm; L–M, 18 μm.

examined throughout their differentiation in vitro (Fig. 4). The control EGFP-tagged cells readily differentiated into both neurons (Figs. 4A, B and G, 5 days after infection) and astrocytes (Fig. 4G, 8 days after infection). By contrast, neurogenesis (Figs. 4C and G), but not gliogenesis (Figs. 4D and G), was severely inhibited in cultures expressing Sox2(FL)-EGFP. The examination of Sox2-EGFP-positive cells after two additional weeks in culture confirmed this outcome and ruled out the possibility of delayed neurogenesis (data not shown). We also used several truncated Sox2 constructs that carried the HMG domain but lacked serine-rich region (i.e., amino acids 203–261), carboxyl tail (i.e., amino acids 262–317) or both (i.e., amino acids 203–317). The results showed that the N-terminal fragment containing the HMG domain [Sox2(1–202)-EGFP] was sufficient to confer the Sox2 inhibitory effect on neurogenesis (Figs. 4E and G, 5 DIV) without a significant effect on gliogenesis (Figs. 4F, G, 8 DIV). The percentages of MAP2-positive neurons and GFAP-positive astrocytes within the Sox2-overexpressing populations (EGFP-positive cells) are presented in Fig. 4G. The majority of the remaining cells (>70%, 5 DIV) in these cultures were nestin-positive (data not shown).

Using Sox2 overexpression, we also identified downstream molecules that might underlie the molecular mechanism of Sox2 function. Among the pathways involved in neocortical development, Notch1 signaling plays a key role in inhibiting neurogenesis (Beatus and Lendahl, 1998; Hitoshi et al., 2002). Using the same retroviral system, we established that the levels of Notch1, RBP-J and Hes5 transcripts were significantly upregulated in response to Sox2 overexpression (Fig. 5A, representative results from one of three experiments). Again, the N-terminal fragment carrying the Sox2 HMG domain [Sox2(1–202)] was sufficient for these effects.

In order to confirm that the upregulation of Notch pathway by Sox2 is involved in the inhibition of neurogenesis, we both repressed (Fig. 5) and activated (Supplementary Fig. 2) the Notch signaling. Notch is activated through the binding of Delta/Serrate/Jagged ligands, which triggers proteolytic events requiring γ-secretase activity (Beatus and Lendahl, 1998; Kimberly and Wolfe, 2003). The action of γ-secretase generates a free Notch intracellular domain (Notch ICD) that translocates into the nucleus and activates the transcription of target genes. As shown in Figs. 5B–D, neurogenesis was partially restored in



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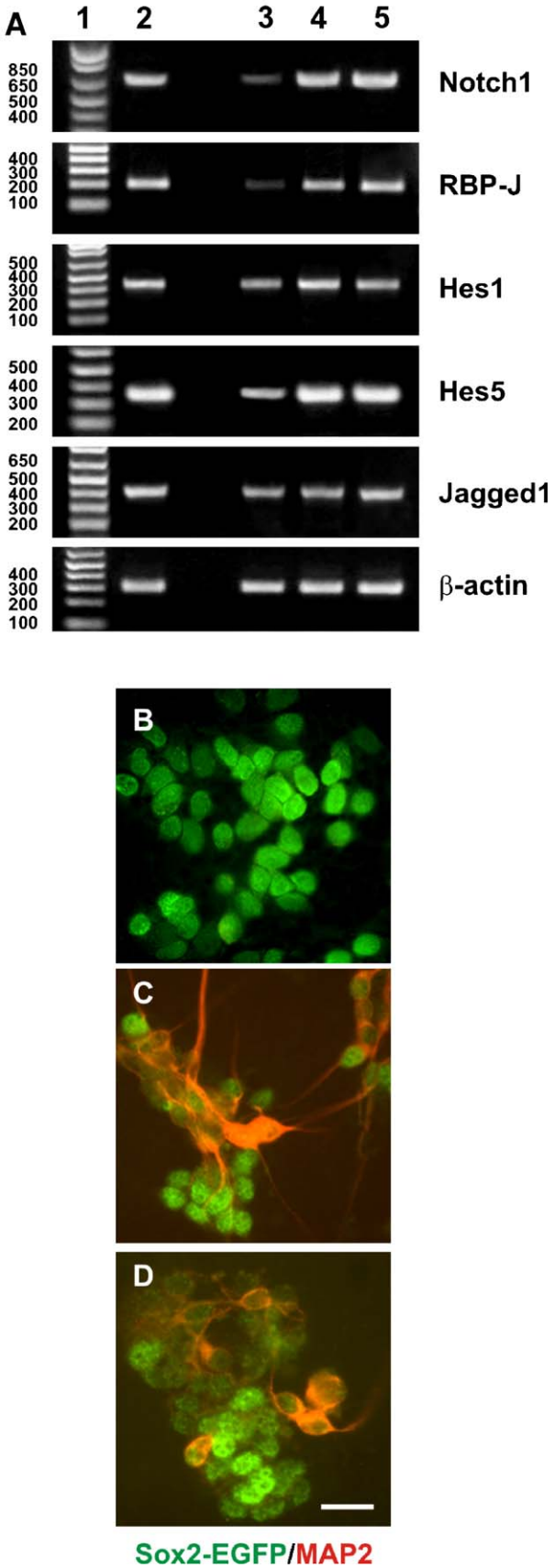
Cell types	Vector used		
	EGFP	Sox2(FL)-EGFP	Sox2(1-202)-EGFP
Neurons (MAP2)	45.2 ± 3.2	0.3 ± 0.1	0.9 ± 0.3
Astrocytes (GFAP)	58.8 ± 3.3	52.6 ± 3.6	54.9 ± 2.2

Fig. 4. Sox2 overexpression inhibits neurogenesis, but not gliogenesis. (A–B) The EGFP-tagged neural progenitors (A, EGFP—green) readily differentiate into mature neurons (B, MAP2—red). (C–D) Cultures infected with Sox2(FL)-EGFP do not form MAP2-positive neurons (C, Sox2—green, MAP2—red), while they readily give rise to the GFAP-positive astrocytes (D, Sox2—green, GFAP—red). (E–F) Similarly, cultures infected with N-terminal fragment of Sox2(1–202)-EGFP do not differentiate into neurons (E, MAP2—red), but form astrocytes (F, GFAP—red). Scale bar: 10 μm. (G) Statistical analysis of infected cultures (one-way ANOVA, $P < 0.001$) verifies the inhibition of neurogenesis by Sox2 and confirms that the N-terminal fragment of Sox2 protein (amino acids 1–202) is sufficient to confer this inhibition. Percentages of cell types within the infected populations are presented as mean ± SEM.



the E13.5 cell cultures transduced with either Sox2(FL)-EGFP (Fig. 5C) or N-terminal Sox2(1–202) fragment (Fig. 5D) upon treatment with the cell permeable γ-secretase inhibitor X. Statistical analyses further confirmed that 19.7 ± 1.7% of the Sox2(FL)-EGFP-positive cells were also positive for MAP2 in the presence of γ-secretase inhibitor X, compared with 0.4 ± 0.15% in the control cultures (i.e., infected with Sox2(FL)-EGFP and incubated in the absence of the inhibitor) at 5 DIV (one-way ANOVA, $P < 0.001$). Similarly, 16.8 ± 1.5% of the cells were Sox2(1–202)-EGFP-positive MAP2-positive in the presence of γ-secretase inhibitor X at 5 DIV (one-way ANOVA, $P < 0.001$). Noticeably, the intensity of nuclear Sox2 appeared to be lower in the MAP2-positive cells in both cases. In addition, the overexpression of Notch1-ICD or HES5 in neocortical progenitors also prevented their differentiation into neurons (Supplementary Fig. 2), similar to that of Sox2 (Fig. 5). Taken together, these results confirmed the involvement of Sox2-activated Notch pathway in the inhibition of neurogenesis.

Neuronal precursors and neuroblasts utilize serine protease(s) to degrade the exogenous Sox2

Thus far, we showed that neural progenitors downregulated Sox2 as they acquired neuronal fate, but the question still remained how cells deal with constitutively active Sox2, which clearly interfered with the terminal differentiation of neurons. To address this issue, we extended the overexpression studies to the NCAM-positive A2B5-negative neuronal precursors and N2a neuroblasts, using similar transduction conditions. Western blot analyses revealed that, within 48 h of infection/transfection, both neuronal precursors and N2a neuroblasts effectively degraded the exogenous Sox2 (Fig. 6A). This phenomenon was unique to neuronal precursors (Fig. 6A, lane 3) and neuroblasts (Fig. 6A, lanes 5 and 7), as it was not observed in the infected neural progenitors (Fig. 6A, lane 2). Parallel experiments showed that other transcriptional factors/transducers (i.e., MASH1 and Smad5) were not degraded and further confirmed that the proteolytic process was specific to Sox2 (Fig. 6B). Using specific groups of protease inhibitors, we were able to establish that neuroblasts engage serine protease activities to eliminate Sox2 protein (Fig. 6C). In contrast, neither cysteine protease nor proteasome inhibitors appeared to stop Sox2 degradation in neuroblasts.

Fig. 5. Sox2 utilizes the Notch signaling pathway to inhibit neurogenesis. (A) Specific components of Notch signaling pathway (i.e., Notch1, RBP-J and Hes5) are upregulated in the cells transduced with Sox2 after 7 days in culture as shown by RT-PCR analysis. Lane 1—molecular size markers (1 kb plus DNA ladder), lane 2—E13.5 telencephalon (positive control; Hitoshi et al., 2002), lane 3—cells infected with EGFP (negative control), lane 4—cells infected with Sox2(FL)-EGFP and lane 5—cells infected with the N-terminal Sox2(1–202)-EGFP. (B) In the absence of γ-secretase inhibitors, the neural progenitors transduced with Sox2(FL)-EGFP do not form MAP2-positive neurons (Sox2—green, MAP2—red). (C–D) Inactivation of the Notch pathway by γ-secretase inhibitor X restores neurogenesis in the cells overexpressing either Sox2(FL)-EGFP (C, Sox2—green, MAP2—red) or Sox2(1–202)-EGFP (D, Sox2—green, MAP2—red). Scale bar: 10 μm.

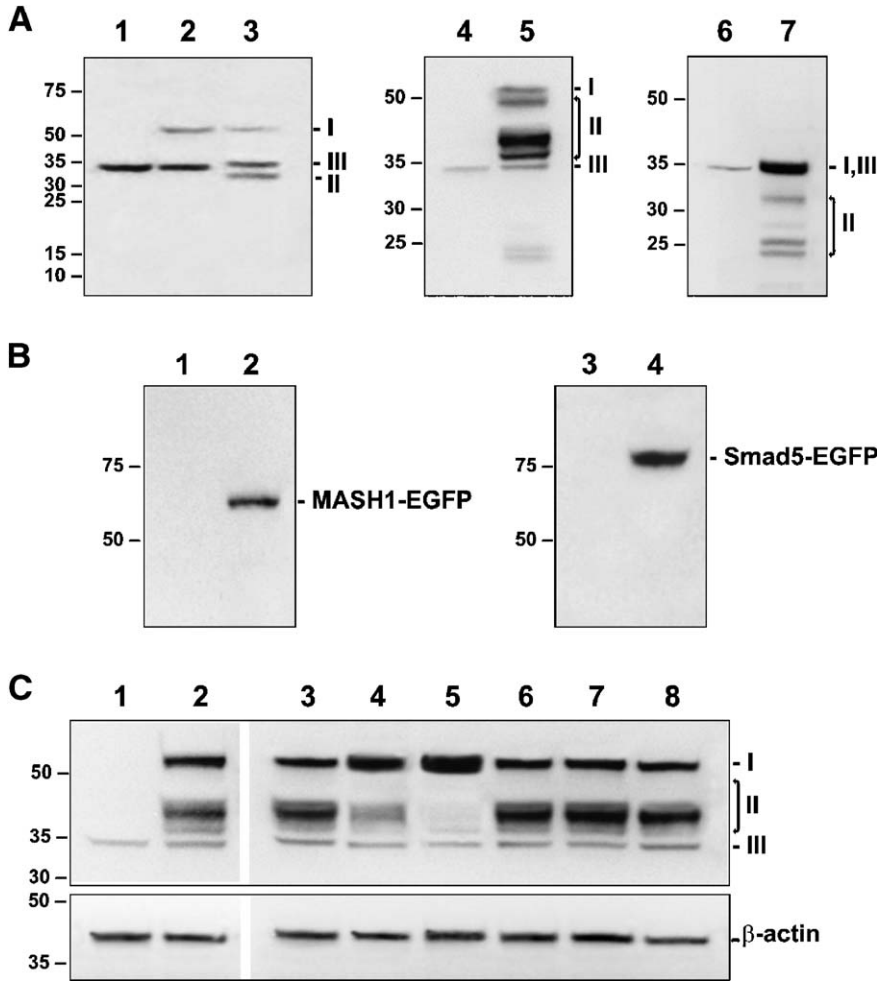


Fig. 6. Neuronal precursors and neuroblasts utilize the serine-specific protease activity to degrade the exogenous Sox2. (A) Western blot analyses show that both endogenous 34 kDa Sox2 (III) and exogenous 56 kDa Sox2-EGFP fusion protein (I) are intact in neural progenitors (lanes 1 and 2). However, the overexpressed Sox2-EGFP protein undergoes extensive degradation in the NCAM-positive A2B5-negative neuronal precursors (lane 3, II). Similarly, N2a neuroblasts selectively degrade overexpressed Sox2 (lanes 4 and 6—untransfected, lane 5—transfected with Sox2-EGFP, lane 7—transfected with Sox2-cMyc; I, II and III indicate overexpressed, degraded and endogenous Sox2, respectively). (B) In contrast to Sox2, N2a neuroblasts do not degrade other CNS transcription factors and transducers such as MASH1 (lane 1—untransfected, lane 2—transfected with MASH1-EGFP) or Smad5 (lane 3—untransfected, lane 4—transfected with Smad5-EGFP). (C) Sox2 degradation is inhibited by a selective inhibitor of serine protease in N2a cells (lane 1—untransfected; lanes 2, 3 and 8—transfected with Sox2-EGFP, no inhibitors; lanes 4 and 5—transfected with Sox2-EGFP and treated with 1 mM AEBSF for 2 or 4 h, respectively), but not cysteine protease (lane 6—treated with 10 μM E-64 for 4 h) or proteasome (lane 7—treated with 25 μM MG132 for 4 h) inhibitors. I, II and III indicate overexpressed, degraded and endogenous Sox2, respectively. β-actin (42 kDa) validates an equal protein loading.

Sox2 expression is maintained during gliogenesis but is turned off in quiescent astrocytes

The A2B5 antigen is a commonly used cell surface marker that identifies glial precursors (Mayer-Proschel et al., 1997; Rao, 2004; Rao and Mayer-Proschel, 1997; Rao et al., 1998). Using this characteristic, we showed that neural progenitors maintained Sox2 throughout gliogenesis (Fig. 7). The mitotically active NCAM-negative A2B5-positive glial precursors (Figs. 7A–D) readily differentiated into Sox2-positive GFAP-positive astrocytes (Fig. 7E) of flat (Fig. 7F) or stellate (Fig. 7G) shapes. However, as the long-term cultures of astrocytes (i.e., 12–16 weeks in vitro) stopped proliferating, they also turned off Sox2 expression (Fig. 7H). The accompanying Western blot analyses confirmed that, unlike neurons, astrocytes maintained Sox2 expression after they acquired glial fate, until they became quiescent (Fig. 7I). Additional studies on cell cultures at other developmental time points (i.e., E11 for neurons, and E17 or PN0 for astrocytes) consistently demonstrated the absence of Sox2 in post-mitotic neurons and quiescent astrocytes (data not shown), thus establishing a link between astroglial Sox2 expression and cell cycle. This link was further confirmed in the quiescent astrocytic cultures that resumed proliferation after injury (Fig. 8). Within 3 days post-lesion, Sox2-positive astrocytes appeared in the proximity of the damaged area and increased in number after 7 days (Fig. 8D). The significance of injury-induced Sox2 reactivation was more obvious with the application of CNTF (Fig. 8E), FGF2 (Fig. 8F) or a combination of CNTF + FGF2 + dbcAMP (Fig. 8G). Furthermore, these treatments clearly triggered cell proliferation and resulted in the formation of stellate astrocytes, which filled and repaired the damaged area (Figs. 8E–J). Therefore, Sox2-positive cells clearly re-entered the cell cycle.

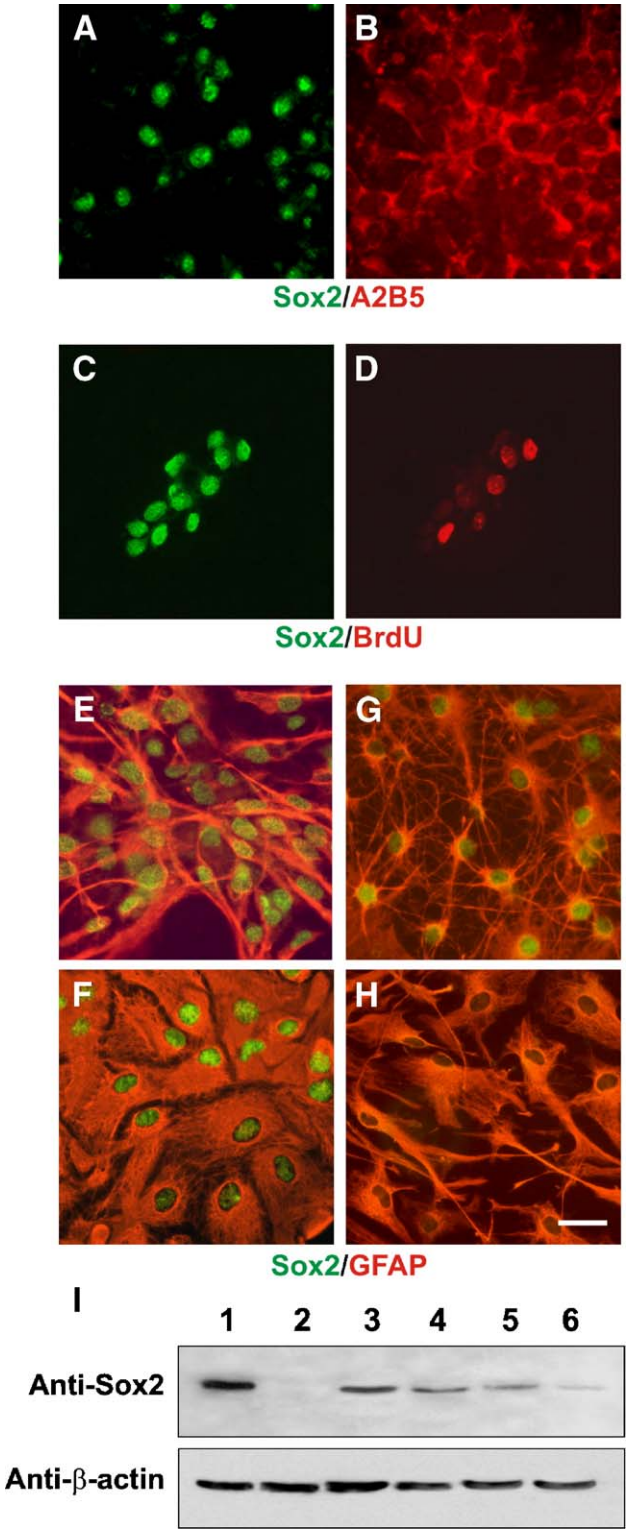


Fig. 7. Glial precursors maintain Sox2 expression as they differentiate into astrocytes. (A–D) The immunopanned glial precursors express Sox2 (A and C, green) and A2B5 (B, red) as they proliferate (D, BrdU—red). (E–G) Sox2 continues to be expressed in the GFAP-positive astrocytes (E, Sox2—green, GFAP—red), regardless of their flat (F) or stellate (G) morphology. (H) In contrast, the quiescent GFAP-positive astrocytes (red) do not express Sox2. Scale bar: 10 μm. (I) Western blot analysis further confirms that neural progenitors (lane 1) and astroglia (lanes 3–6), but not neurons (lane 2), express Sox2 and that its expression level decreases with the age of astrocytes (compare lane 3 and 6). Lanes 3–6 represent astrocytes after 1, 2, 4 or 8 weeks in culture, respectively.

Sox2 expression is regulated by mitogenic and gliogenic factors

The re-expression of Sox2 was also observed in vivo upon a mechanical injury of mouse brain tissue (Figs. 8K–N). Within 5 days of the injury, a significant number of Sox2-positive cells were observed around the site of damage (Fig. 8M). The majority of these cells were also GFAP-positive, which further strengthens the link between Sox2 expression and gliogenesis. These results also indicated that the expression of Sox2 gene may be modulated by the brain microenvironment (i.e., the mitogenic and gliogenic factors involved in the development of the CNS). Among the trophic factors present in the brain, FGF2 exerts mitogenic effects on neural progenitors to increase the cell number and expand the ventricular zone (Ford-Perriss et al., 2001), whereas factors such as CNTF and LIF directly contribute to the generation of astroglia by instructing a glial fate (Panchision and McKay, 2002). We have examined the effects of these factors on the neocortical cultures at the peak of neurogenesis (i.e., E13.5) and the onset of gliogenesis (i.e., E18). A robust increase in the total number of Sox2-positive cells was observed in the presence of FGF2 (Fig. 9A), consistent with its mitogenic effects on neural progenitors. Meanwhile, the number of Sox2-positive GFAP-positive astrocytes remained nearly unchanged (Fig. 9A). Neither CNTF nor LIF significantly increased the total number of Sox2-positive cells at either time point (Fig. 9). Instead, these factors significantly enhanced the number of Sox2-positive GFAP-positive cells (i.e., the Sox2-positive astrocytic subpopulation). The CNTF- and LIF-induced Sox2 expression in E18 cultures (Fig. 9B) was coincident with the onset of gliogenesis in the neocortex. Finally, the number of total Sox2-positive cells as well as Sox2-positive GFAP-positive cells was synergistically increased in the presence of both FGF2 and CNTF.

Discussion

Our study showed that Sox2 was expressed mainly in the expanding cell population of the ventricular zone, in which the appropriate pool of neural progenitors is generated to subsequently establish a functional neocortex (Jacobson, 1991; Qian et al., 1998, 2000). The number of Sox2-positive cells diminished postnatally as only ependymal cells and the SVZ neural stem cells maintained Sox2 expression. A similar reduction in the Sox2 level has also been documented during the development of chicken spinal cord (Uwanogho et al., 1995; Pevny and Rao, 2003). Using TBr-1 as a marker of early-born cortical neurons (Bulfone et al., 1995; Donoghue and Rakic, 1999; Hevner et al., 2001, 2003; Chenn and Walsh, 2002), we have shown a clear switch from a Sox2-positive to a TBr-1-positive state during neocortical development. TBr-1 was first detected at E11, when neurogenesis commences with the generation of a single layer of neurons in the dorsal telencephalon. TBr-1 expression was maintained until E17, when the majority of the neocortical neurons have been formed. Both this study and others (Bulfone et al., 1995; Donoghue and Rakic, 1999; Chenn and Walsh, 2002) describe a TBr-1 gradient

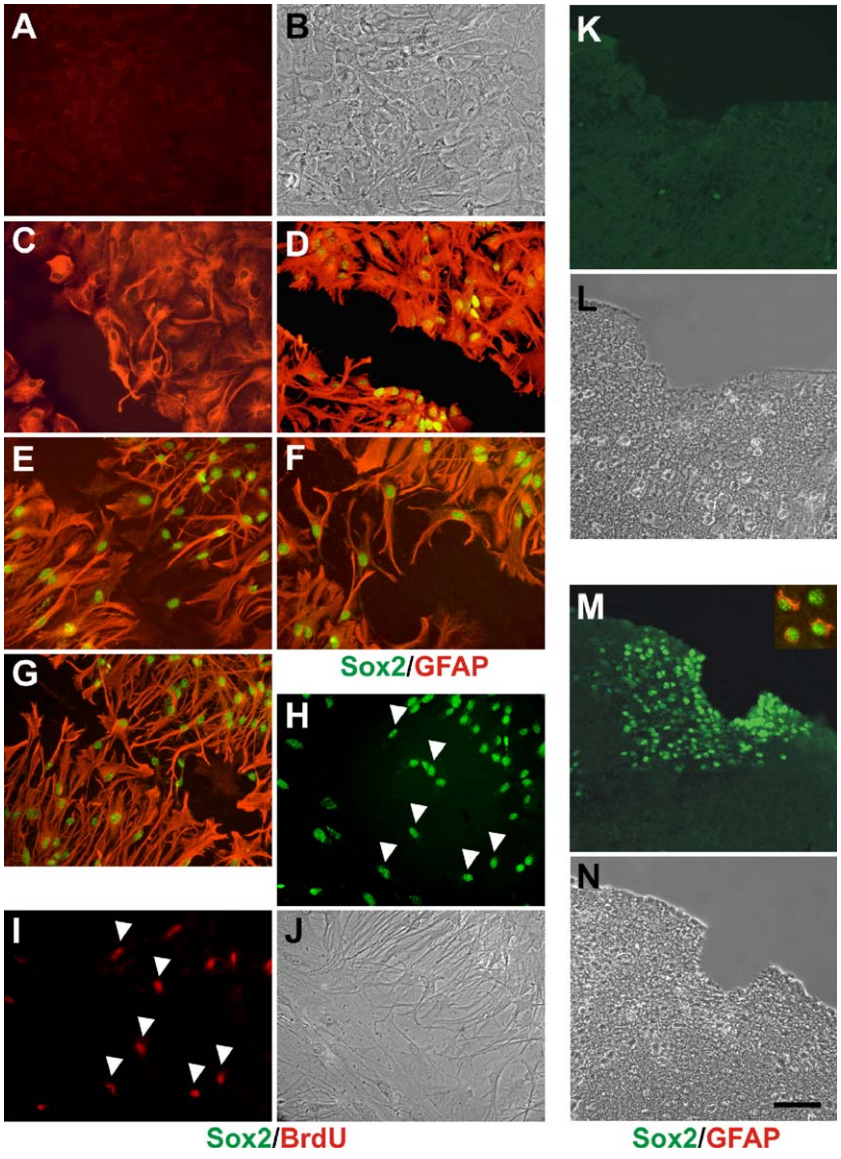


Fig. 8. Quiescent astrocytes re-express Sox2 upon re-entry into the cell cycle. Neither BrdU (A) nor Sox2 (C) staining is present in the 16-week-old quiescent GFAP-positive (red) astrocytes (C, 15 min post-lesion). (B) Phase contrast image of panel A. (D–G) Sox2 immunoreactivity is restored in the injured astrocytic cultures (D, 7 days post-lesion), especially upon treatments with CNTF (E, 3 days post-injury), FGF2 (F, 3 days post-injury) or the combination of CNTF, FGF2 and dbcAMP (G, 3 days post-injury). (H–I) The re-expression of Sox2 (H, 7 days post-injury + FGF2, Sox2—green) is accompanied by the cell cycle re-entry, as evidenced by BrdU staining (I, red). (J) Phase contrast image of panels G, H and I. (K–N) The immunohistochemical analysis shows an abundant Sox2 re-expression (M, green) near the injury site 5 days after the neocortex was lesioned. A similar section shows the level of Sox2 15 min after the injury (K). Panels L and N represent the Hoffmann modulation contrast images of panels K and M, respectively (all sections are sagittal). Scale bar: A–J 10 μm; K–N 60 μm; inset 20 μm.

across the neocortex. In this study, we also showed that Sox2 was absent in the TBr-1-positive cells populating the newly formed E11 marginal zone. The non-overlapping Sox2 and TBr-1 expression patterns indicate that cells must turn off Sox2 before they adopt the TBr-1-positive identity in the neocortex. Indeed, Sox2 was irreversibly turned off in terminally differentiated post-mitotic neurons.

The upregulation of Notch pathway by Sox2 inhibits neurogenesis

Several lines of evidence indicate that Sox2 is important for the maintenance of neural potential in embryonic and neural stem cells (Zappone et al., 2000; Graham et al., 2003; Ellis et al., 2004; Zhao et al., 2004; Pevny and Placzek, 2005). Here, we have demonstrated, for the first time, that Sox2 acts upstream of the Notch signaling pathway. Indeed, Sox2 overexpression caused the upregulation of Notch1, RBP-J and Hes5 genes and blocked neurogenesis. Furthermore, γ-secretase inhibitors (Kimberly and Wolfe, 2003) nullified the effects of Sox2 and restored neurogenesis, whereas the overexpression of either Notch1-ICD or HES5 mimicked the inhibitory effect of Sox2 overexpression.

Notch1 has been shown to preserve the pool of progenitors and repress neurogenesis (but not gliogenesis) in the developing CNS (Ishibashi et al., 1994; Nye et al., 1994; Kageyama and

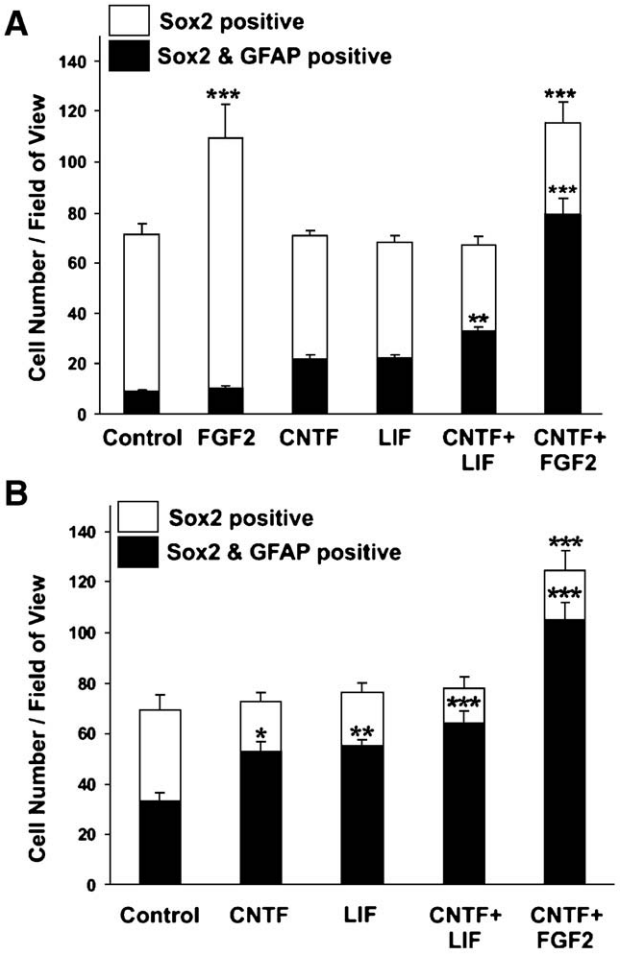


Fig. 9. Sox2 expression is regulated by environmental factors. (A–B) The size of Sox2-positive cell populations is regulated by environmental factors. FGF2 (a mitogenic factor) significantly increases the total number of Sox2-positive cells in the E13.5 (A) and E18 (B) neocortical cultures within a week. In contrast, the CNTF and/or LIF gliogenic factors specifically increase the number of Sox2-positive GFAP-positive astrocytes. The significance of data (mean ± SEM) was determined by one-way ANOVA (*$P < 0.05$; **$P < 0.05$; ***$P < 0.001$).

Nakanishi, 1997; Ohtsuka et al., 1999; Ge et al., 2002; Hitoshi et al., 2002; Grandbarbe et al., 2003; Tokunaga et al., 2004). The activation of Notch signaling occurs in response to ligand binding (i.e., a member of Delta/Serrate/Jagged family), and the proteolytic activity of γ-secretase is required to generate the free Notch1 ICD, which in turn, translocates into the nucleus and activates the transcription of target genes such as cyclin D1 and cyclin-dependent kinases (Castella et al., 2000; Ronchini and Capobianco, 2001; Hansson et al., 2004). Notch1 has also been shown to upregulate Hes genes via interaction with RBP-J (Artavanis-Tsakonas et al., 1995; Beatus and Lendahl, 1998; Beatus et al., 2001).

The Sox2 HMG domain, a signature motif for DNA binding and potential interactions with other partners (Yuan et al., 1995; Johnson et al., 1998; Nowling et al., 2000), was sufficient for the upregulation of Notch pathway and inhibition of neurogenesis. Based on the current knowledge, there is no obvious explanation for the effects of the truncated Sox2. However, it is possible that, in addition to the *cell type- and partner-specific* functional features of the C-terminus (Kamachi et al., 1999, 2000), the truncated protein contains yet unidentified domain(s) that are clearly sufficient to confer its functionality.

Thus far, very little is known about Sox2 involvement in transcriptional regulation of the Notch pathway. However, the fact that Sox1 (i.e., a member of SoxB1 subfamily) regulates the Hes1 promoter in P19 cells (Kan et al., 2004) raises the possibility that Sox2 may regulate the Hes5 promoter through a similar mechanism. Furthermore, recently published genome scale (i.e., chromatin immunoprecipitation coupled with microarray) analysis of OCT4, NANOG and SOX2 target genes in human stem cells has identified NOTCH1 as a direct target of SOX2 and revealed that these transcription factors frequently co-occupy target gene promoters to form a unique transcriptional hierarchy that is essential in early development (Boyer et al., 2005).

The number of identified Sox2 target genes is still relatively small; hence, Sox2-binding element(s) are not well characterized. However, two recent studies report that a short DNA sequence (CTTTGTT in the *Fgf4* enhancer and TATTGTT in the c-*myc* scaffold or matrix attachment region, S/MAR) makes physical contact with Sox2 protein (Remenyi et al., 2004; Lei et al., 2005). Significantly, the binding of SOX2 to the latter sequence correlates with the expression of c-*myc* transcripts in proliferating human NT2 neuronal precursors, consistent with SOX2 being required for the correct level of c-*myc* transcription in cycling cells. We applied the SMARTest module of the Genomatix Suite integrated bioinformatics software package (Genomatix Software GmbH, München, Germany; Liebich et al., 2002; Frisch et al., 2002) to examine the genomic sequences from 10 kbp upstream to 10 kbp downstream of Notch1 acc. # NM_008714), Hes5 (acc. # NM_010419) and RBP-J (acc. # NM_009035) genes for the presence of S/MARs. Using the sequences from UCSC mouse genome version mm6 via the UCSC Genome Browser (Kent et al., 2002), we showed that the mouse RBP-J gene contained seven predicted S/MAR sites. Five of the seven putative RBP-J S/MARs sites contained Sox2-like binding regions, with one (located between nucleotides 60,686 and 61,015) enclosing a putative base unpairing region (BUR) core motif. These features of RBP-J gene are very similar to those of c-*myc*, suggesting a mechanism by which Sox2 may enhance the expression of RBP-J by binding to S/MAR and possibly within a BUR.

Inactivation of Sox2 by proteolysis

We have also examined the turnover of Sox2 protein during terminal differentiation of neuronal (NCAM-positive) and glial (A2B5-positive) precursors. Both populations initially contained Sox2-positive cells. However, the level of Sox2 protein in the cells that followed the neuronal lineage was significantly lower than that of the cells residing in the VZ or the A2B5-positive glial precursors. Furthermore, overexpression of Sox2 in NCAM-positive neuronal precursors resulted in its degradation. Thus, the cells already committed to the neuronal lineage have in place a degradation mechanism to eliminate Sox2. This mechanism appeared to be specific to Sox2 protein as

other transcription factors/transducers (i.e., MASH1 and Smad5) were not affected by proteolysis. These observations revealed, for the first time, the existence of a post-translational mechanism controlling the precise level of Sox2 during neurogenesis. Indeed, a serine-specific proteolytic pathway, sensitive to an irreversible inhibitor (i.e., AEBSF), appeared to be responsible for Sox2 degradation. This activity was different from that affecting another Sox family member, Sox9, which is inactivated by the ubiquitin–proteasome system during chondrogenesis (Akiyama et al., 2005). Thus, it is possible that cells engage cell-type-specific pathways to control the level of Sox proteins according to their functional and phenotypic requirements.

Sox2 in gliogenesis

Sox2 did not interfere with gliogenesis as glial precursors maintained Sox2 expression throughout differentiation and as long as they continued to divide. Astrocytes turned off the gene as they became quiescent. However, they re-expressed Sox2 and resumed proliferation upon stimulation. Thus, Sox2 expression was mainly restricted to mitotically active neocortical cell populations, including neural progenitors and GFAP-positive astrocytes.

Numerous studies (see Silver and Miller, 2004 for a review) show that terminally differentiated astrocytes can resume proliferation upon activation (i.e., after brain injury). Here, we showed that injured astrocytes re-expressed Sox2 both in vitro and in vivo as they re-entered the cell cycle. Furthermore, the exposure of injured astrocytes to either FGF2 or CNTF, whose elevated expression has been demonstrated following injury (Banner et al., 1997; Silver and Miller, 2004), significantly increased the number of Sox2-positive proliferating astrocytes. This phenomenon suggested that both FGF2- and CNTF-mediated pathways (Lillien and Raff, 1990; Bonni et al., 1997; Cavanagh et al., 1997; Vaccarino et al., 1999; Ford-Perriss et al., 2001; Monville et al., 2001; Dallner et al., 2002) may be involved in the regulation of Sox2 expression. However, further studies are required to establish which of the regulatory elements in Sox2 enhancers (Zappone et al., 2000; Uchikawa et al., 2003, 2004; Catena et al., 2004; Miyagi et al., 2004; Rodda et al., 2005) are influenced by these pathways.

In summary, the present study established that Sox2 functions as a key regulator of proliferative potential of neocortical cells (i.e., neural stem and progenitor cells, glial precursors and astrocytes). Its function interfered with neurogenesis, but not gliogenesis. Committed neuronal precursors utilized a serine protease activity to eliminate Sox2 protein. Sox2 acted upstream of the Notch signaling pathway, and the Sox2 HMG domain was sufficient to upregulate Notch and inhibit neurogenesis. Thus, neural progenitors could differentiate into neurons when Sox2 was turned off and/or degraded and the Notch1 signaling was repressed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.03.007.

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