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SOX2 Functions to Maintain Neural Progenitor Identity

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Summary

Neural progenitors of the vertebrate CNS are defined by generic cellular characteristics, including their pseudoepithelial morphology and their ability to divide and differentiate. SOXB1 transcription factors, including the three closely related genes *Sox1*, *Sox2*, and *Sox3*, universally mark neural progenitor and stem cells throughout the vertebrate CNS. We show here that constitutive expression of SOX2 inhibits neuronal differentiation and results in the maintenance of progenitor characteristics. Conversely, inhibition of SOX2 signaling results in the delamination of neural progenitor cells from the ventricular zone and exit from cell cycle, which is associated with a loss of progenitor markers and the onset of early neuronal differentiation markers. The phenotype elicited by inhibition of SOX2 signaling can be rescued by coexpression of SOX1, providing evidence for redundant SOXB1 function in CNS progenitors. Taken together, these data indicate that SOXB1 signaling is both necessary and sufficient to maintain panneural properties of neural progenitor cells.

Introduction

Neural progenitor/stem cells throughout the central nervous system (CNS) are defined by common cellular properties: they proliferate, self-renew, and give rise to differentiated progeny. Cells defined by these panneural properties are present during the development of the CNS and persist into adulthood in certain locations. These cellular properties correlate with the expression of general molecular markers supporting the likelihood of common/generic molecular mechanisms shared by neural stem cells throughout their ontogeny (Barres, 1999; Pevny and Rao, 2003). These conserved signaling pathways may serve to maintain generic cellular properties that define the stem cell state, such as the ability to divide and multilineage differentiation. For example, one of the better-characterized molecular pathways shared by neural progenitor cells is the Notch signaling pathway. This pathway appears to play an essential role in the maintenance of a stem/progenitor cell pool. During embryogenesis and in adulthood, expression of Notch1 or its downstream regulators, such as HES-1, inhibits neuronal differentiation and results in the maintenance of a progenitor state. The exact mechanism by which Notch signaling regulates cell fate is not completely determined. Recent studies have suggested that rather than simply inhibiting neuronal differentiation and maintaining a neural progenitor state, Notch may in some contexts promote the acquisition of glial identity (Gaiano and Fishell, 2002).

Superimposed on these generic characteristics is the regional differentiation of neural progenitors in the ventricular zone (VZ). For example, in the spinal cord, neural progenitors take on distinct dorsoventral identities in response to opposing diffusion gradients of Sonic Hedgehog (Shh) and BMPs. These are reflected by region-specific expression of homeodomain transcription factors (Briscoe and Ericson, 2001). This transcriptional regionalization of neural progenitors has been linked with a general program of neurogenesis under the regulation of proneural and neurogenic bHLH transcription factors (Mizuguchi et al., 2001; Novitch et al., 2001). Some bHLH proteins can direct neural progenitors' exit from the cell cycle and promote neuronal differentiation in a coordinately regulated fashion (Guillemot, 1999). In a similar way, the differentiation of glial cells, specifically oligodendrocytes, appears to be dependent on the interaction between homeodomain and bHLH factors (Kessaris et al., 2001).

Thus, although significant progress has been made in the identification of transcription factors that direct neural differentiation, much less is known of the transcriptional mechanisms that function to maintain neural progenitor properties. In the peripheral nervous system (PNS), it has recently been demonstrated that SOX10, a member of the SOX family of transcription factors, plays a role in maintaining multipotency of neural crest stem cells (Kim et al., 2003). In this study we set out to determine if members of the SOXB1 subfamily of transcription factors may play an analogous role in CNS progenitor cells.

Sox1, *Sox2*, and *Sox3* share more than 90% amino acid residue identities in an HMG-DNA binding domain and are classified as subfamily group B1. The *Drosophila*, *Amphioxus*, *Xenopus*, zebrafish, avian, and rodent orthologs of *SoxB1* genes all show broad expression throughout the neural primordium (Bowles et al., 2000; Cremazy et al., 2000; Hardcastle and Papalopulu, 2000; Mizuseki et al., 1998; Nambu and Nambu, 1996; Penzel et al., 1997; Pevny et al., 1998; Rex et al., 1997a; Russell et al., 1996; Streit et al., 1997; Uchikawa et al., 1999, 2003; Uwanogho et al., 1995; Wood and Episkopou, 1999). Functional studies of *SoxB1* genes in murine cells and in *Xenopus* and *Drosophila* embryos suggest that they function to consolidate early neural fate; the misexpression of SOX1 in murine cells results in the activation of early markers of neural progenitors (Pevny et al., 1998). Disruption of SOXB1 signaling in *Xenopus* embryos inhibits CNS formation, primary neurons, and neural crest cells (Kishi et al., 2000), and genetic ablation of both *SoxN* and *Dichaete* in fly embryos results in

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neural hypoplasia (Buescher et al., 2002; Overton et al., 2002). These experiments have established a role for SOXB1 factors in the initial specification and consolidation of neural fate. Moreover, while in each case SOXB1 factors play a general role in neural fate consolidation, they do not appear to be involved in specification of regional identity. Consistent with the possibility of functional redundancy, phenotypic consequences of mutations in *SoxB1* genes in the mouse are only apparent at sites where each gene is uniquely expressed; *Sox2* homozygotes fail to form primitive ectoderm (Avilion et al., 2003), and chimeric mice generated with *Sox3* null ES cells display severe abnormalities during gastrulation and posterior truncations (Parsons, 1997), while *Sox1* homozygotes survive until adulthood without significant defects in CNS development (Malas et al., 2003; Nishiguchi et al., 1998). In each case, the phenotypes displayed by the single mutant mice have precluded the analysis of the role of these factors in neural development. In vertebrates, SOXB1 factors continue to be widely expressed in proliferating neural progenitors, including neural stem cells, throughout development and adulthood (Uchikawa et al., 2003; Zappone et al., 2000; Pevny and Rao, 2003), suggesting a role in the maintenance of neural progenitor fate. However, the role of SOXB1 factors in CNS progenitors and their relationship with the known regionalization and neurogenic transcriptional pathways remains poorly understood.

To examine the role of SOX2 directly in proliferating neural progenitors and to bypass the early phenotypes displayed by *Sox2* mutant mice and *Xenopus* embryos, we have examined the phenotypic and molecular consequences of constitutive maintenance and dominant interference of SOX2 signaling specifically in neural progenitors by in ovo chick electroporation. These data provide evidence that constitutive expression of SOX2 inhibits neuronal differentiation, resulting in the maintenance of progenitor characteristics. Conversely, inhibition of SOX2 signaling results in neural progenitor cells delaminating from the VZ and exiting from cell cycle. This is associated with a general loss of panneural and regional progenitor markers and the onset of expression of early neuronal differentiation markers. The phenotype elicited by this inhibition can be rescued by coexpression of SOX1, providing support for redundant SOXB1 function in neural progenitor cells. Together, these data indicate that SOX2, and more generally SOXB1 factors, are both necessary and sufficient for maintaining panneural properties of neural progenitor cells.

Results

SOX2 Is Expressed in Proliferating CNS Progenitors and Downregulated during Their Final Cell Cycle

To address the role of SOX2 in CNS progenitors, we performed a detailed study of SOX2 protein expression at thoracic levels of the chick neural tube (HH15–27) (Hamburger and Hamilton, 1951). The HH15 neural tube is composed mostly of proliferating progenitors organized into a pseudostratified epithelium in which the processes of the progenitor cells extend from the inner lumen to the outer mantle surface. At this stage, SOX2 expression is detected throughout the cells of the early neural tube (Figure 1A). At later stages, with the exception of the dorsomedial roof plate and ventromedial floor plate, proliferating progenitors are largely restricted to the inner VZ and delaminate toward the mantle zone (MZ) after completing their final mitosis (Altman and Bayer, 1984). Coincident with their exit from cell cycle, neural progenitor cells differentiate into defined classes of neurons at specific dorsoventral (DV) positions in the MZ. SOX2 expression is excluded from these regions of differentiation. SOX2 expression is first excluded from the ventral motor horns (Figure 1B) and later from more dorsal regions (Figure 1C). Eventually, expression is restricted to the thin VZ surrounding the lumen of the embryonic spinal cord (Figure 1D). An exception to this precise ventral to dorsal downregulation is the maintenance of SOX2 in floor plate cells (Figures 1B and 1C). Thus, the expression of SOX2 precisely correlates with *Sox2* mRNA in chick (Kamachi et al., 1999; Rex et al., 1997a; Uchikawa et al., 2003; Uwanogho et al., 1995) and mouse (Collignon et al., 1996; Wood and Episkopou, 1999; Zappone et al., 2000) embryos. The restriction of SOX2 expression is reminiscent of cell proliferation in the developing spinal cord. To provide direct evidence that mitotic cells express SOX2, we exposed HH15–27 embryos to bromodeoxyuridine (BrDU) and compared expression of SOX2 and BrDU. All of the cells that incorporated BrDU lie within the SOX2 expression domain (Figures 1E–1H).

To determine the timing of SOX2 expression downregulation in CNS progenitors, we examined SOX2 expression in relation to molecular markers of proliferating but regionalized progenitors (PAX6 and PAX7) (Bellefroid et al., 1996; Walther and Gruss, 1991), markers transiently expressed in proliferating and postmitotic neurons (Nkx2.2 and MNR2), and markers of terminally differentiated postmitotic neurons (Islet1 and β-tubulin type III) (Briscoe et al., 2000; Ericson et al., 1997; Kawakami et al., 1997; Price et al., 1992; Tanabe et al., 1998). SOX2 (Figures 1I–1L) is expressed in proliferating and regionalized progenitors that express PAX6 (Figure 1I) and PAX7 (Figure 1J) but is mutually exclusive with β-tubulin type III (Figure 1K) and Islet1 (Figure 1L). SOX2 is coexpressed with Nkx2.2-positive cells except those situated in the most lateral aspect of the motor horns (Figures 1M and 1N, arrow). MNR2 expression is initiated during the final cell cycle of motor neuron progenitors and then persists transiently in postmitotic neurons. Only a subset of the MNR2 population located toward the lumen of the neural tube coexpress SOX2 (Figures 1O and 1P, arrow). Thus, within the CNS, SOX2 is expressed by proliferating progenitors up to their final cell cycle and is only downregulated as they exit mitosis and not at the onset of DV regional progenitor differentiation. SOX1 and SOX3 are coexpressed with SOX2 in proliferating progenitors in the embryonic spinal cord (Kamachi et al., 1999; Pevny et al., 1998; Uchikawa et al., 2003; Uwanogho et al., 1995; Wood and Episkopou, 1999).

These data indicate that the downregulation of SOX2 expression is independent of the acquisition of DV regional patterning of neural progenitors but coincides precisely with their exit from cell cycle. One prediction of this hypothesis would be that SOX2 expression is maintained in proliferating progenitors in situations

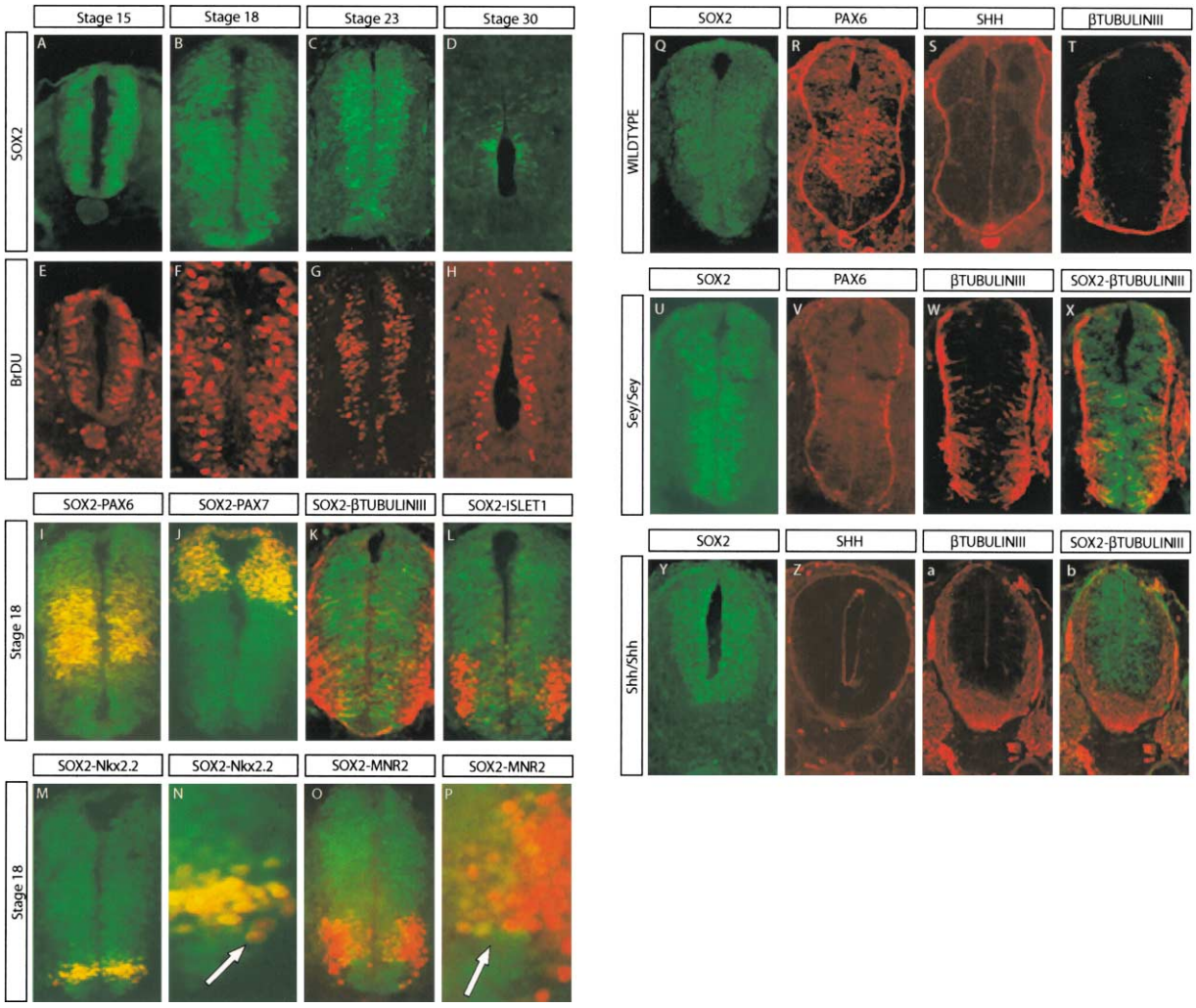


Figure 1. Expression of SOX2 in the Chick Spinal Cord

Localization of SOX2 in transverse thoracic sections through the spinal cord of chick embryos HH15–27 as detected by rSOX2Ab ($n > 3$).
(A) At HH15, SOX2 is expressed throughout the majority of cells in the neural tube.
(B and C) From HH19 to HH23, SOX2 expression is restricted to the medial VZ and excluded from lateral regions of the neural tube.
(D) By HH27, SOX2 expression is restricted to cells around the lumen of the neural tube.
(E–H) BrDU incorporation in proliferating cells within the neural tube in HH15–27 embryos.
(I and J) SOX2 (green) is coexpressed (yellow) with regional markers of proliferating neural progenitors Pax6 (red, I) and Pax7 (red, J).
(K and L) SOX2 (green) expression is mutually exclusive of markers of differentiated neurons marked by β-tubulin type III (red, K) and Islet1 (red, L).
(M and N) SOX2 (green) is coexpressed with medially located Nkx2.2-positive cells (yellow) but excluded from laterally located Nkx2.2-positive cells (red cells, arrow).
(O and P) SOX2 (green) is coexpressed with some medially located MNR2-positive cells (yellow cells, arrow) but is mutually exclusive of laterally located MNR2-positive cells (red cells). Expression of SOX2 in transverse sections of the spinal cord taken at thoracic levels from E10.5 wild-type, $Pax6^{-/-}$, and $Shh^{-/-}$ mouse embryos ($n > 3$).
(Q–T) In E10.5 wild-type control embryos (Q), SOX2 is expressed in proliferating progenitors along entire DV axis.
(R) Pax6 is expressed in medial proliferating progenitors.
(S) Shh is expressed in the floor plate and notocord.
(T) β-tubulin type III is expressed in laterally located postmitotic neurons.
(U–b) E10.5 $Pax6^{-/-}$ mice do not express Pax6 (V), and E10.5 $Shh^{-/-}$ mice do not express Shh (Z). SOX2 expression (green, Q, U, and Y) is mutually exclusive of postmitotic cells expressing β-tubulin type III (red, W, X, a, and b).

where their DV identity is altered. To address this, we examined the expression of SOX2 in the spinal cord of two mutant mouse strains, $Sey^{-/-}$ and $Shh^{-/-}$. In $Sey^{-/-}$ mutant embryos, the elimination of the homeobox transcription factor Pax6 results in a dorsal to ventral transformation in progenitor cell identity (Ericson et al., 1997; Osumi et al., 1997). Conversely, the spinal cord of $Shh^{-/-}$ mutant embryos is dorsalized such that ventral progenitors take on a more dorsal fate (Chiang et al., 1996; Litingtung and Chiang, 2000). In $Sey^{-/-}$ mutant embryos (Figures 1U–1X), as in wild-type embryos (Figures 1Q–1T), SOX2 is expressed in progenitor cells along the

entire DV axis of the developing spinal cord. Moreover, expression of SOX2 is downregulated as $Sey^{-/-}$ mutant progenitors exit the cell cycle, as illustrated by the mutually exclusive expression of SOX2 and β-tubulin type III (Figures 1W and 1X). Similarly, in $Shh^{-/-}$ mutant embryos, SOX2 is expressed in proliferating progenitors but not postmitotic neurons, which express β-tubulin type III (Figures 1Y and 1b). Thus, SOX2 expression is maintained in proliferative progenitors of $Sey^{-/-}$ and $Shh^{-/-}$ mutant spinal cords regardless of their DV identity and is only downregulated as these cells exit mitosis.

SOX2 Is Expressed in the CNS Multipotent Neural Stem Cell

Our in vivo analysis demonstrates that SOX2 is expressed in proliferating progenitor cells, so we next addressed whether it is also expressed in the self-renewing and multipotent (gives rise to neurons, oligodendrocytes, and astrocytes) neural stem cell populations. To date, the most widely accepted method of defining a stem cell is by cloning cells in vitro and showing that a single cell can self-renew and give rise to multiple phenotypes (Gage, 1998). To assay if SOX2 is expressed in the multipotential neural stem cell, we followed a previously described protocol for the isolation of mouse embryonic neuroepithelial (NEP) cells (Mujtaba et al., 1999). The majority of the isolated NEP cells incorporated BrDU and were Nestin immunoreactive, and all of the Nestin immunoreactive cells expressed SOX2, indicating that they were actively dividing precursors (Figures 2A–2E). After withdrawal of mitogens, SOX2+ NEP cells differentiate into β-tubulin type III-positive neurons, GFAP-positive astrocytes, and GALC-positive oligodendrocytes (Figures 2F–2H). Upon differentiation of NEP cells, like in vivo, SOX2 expression became confined to proliferative neural progenitors, whereas downregulation occurred in postmitotic neurons, as illustrated by the mutually exclusive expression of SOX2 and β-tubulin type III (Figures 2I–2K). Sequential clonal analysis of the SOX2+ NEP cells confirmed that individual SOX2+ cells are multipotent. The differentiation of single cells was assayed by double labeling with β-tubulin type III and GFAP (Figures 2L–2N). These in vitro clonal analyses of NEP cells show that within the SOX2+ NEP population there are multipotential neural stem cells, thereby confirming that single SOX2+ cell could self-renew and generate both neuronal and glial cells (Zappone et al., 2000).

SOX2 Inhibits Terminal Neuronal Differentiation of CNS Cells

The restriction of SOX2 expression to proliferating neural progenitors, including neural stem cells, along the entire DV axis of the spinal cord and its precise downregulation during their last cell cycle indicates that SOX2 may play a role in the maintenance of panneural characteristics of CNS progenitors by inhibiting terminal differentiation. To address this question, we asked whether constitutive expression of SOX2 is sufficient to maintain these characteristics in vivo. Specifically, we transfected an expression vector with SOX2 cDNA under the regulation of CMVβactin promoter and linked by an IRES sequence to the Green Fluorescent Protein (GFP) directly in neural progenitors by in ovo chick electroporation. The pCIG-SOX2-IRES-GFP construct was electroporated into the presumptive spinal cord region at HH12–14, and embryos were examined after 12 hr (HH15), 24 hr (HH18–19), or 48 hr (HH22–23). Following electroporation of pCIG-SOX2-IRES-GFP, the right side of the neural tube is GFP positive (Figure 3A, GFP), whereas the left is GFP negative and thereby serves as an internal control. All of the GFP+ cells coexpress the SOX2 protein as assayed by antibody staining (Figures 3B and 3C). At HH15, when the neural tube is composed mostly of proliferating progenitors, SOX2-transfected cells were evenly distributed throughout the neuroepithelium (Figure 3D). By HH18–23, when a differentiating MZ can be clearly distinguished, the majority of SOX2-GFP+ cells were restricted to the VZ; however, small clusters of cells morphologically resembling neural progenitors were located in the MZ (Figures 3H and 3L). The MZ of the spinal cord is composed of postmitotic neurons; by immunostaining for the postmitotic marker neural β-tubulin type III, we examined if the SOX2-GFP-expressing cells had differentiated. By the time the first postmitotic neurons appear in the developing neural tube (HH15), the SOX2-GFP-expressing cells were mutually exclusive of cells expressing β-tubulin type III, and by HH18–23, neuronal differentiation was inhibited in regions of ectopic SOX2 expression (Figures 3E–3O). We also performed marker analysis to determine if differentiation of regionalized neurons along the DV axis of the spinal cord is inhibited by maintenance of SOX2 expression. Staining with cell type-specific antibodies shows that SOX2 expression reduced neuronal differentiation across the entire DV axis, including ventral motor neurons marked by Islet1 (Figures 3P–3S), V1 interneurons marked by En1 (Figures 3T–3W), and interneurons marked by Lim 2 (data not shown). These data indicate that the maintenance of SOX2 expression results in concaminant loss of panneuronal as well as regional markers of neuronal differentiation.

To assess the fate of the SOX2-expressing cells that had migrated to the MZ but were inhibited from terminal neuronal differentiation, we examined whether they retained progenitor characteristics such as proliferative capacity, as assayed by PCNA (proliferative cell nuclear antigen), and progenitor marker expression, as assayed by the general progenitor marker SOX1. To address this question, we focused on the MZ of the ventral spinal cord that at HH23 is mostly composed of differentiated motor neurons. As demonstrated by the mutually exclusive expression of Islet1 and GFP (Figures 4A–4C), constitutive expression of SOX2 inhibits motor neuron differentiation. Misexpression of SOX2 in the MZ was coincident with the ectopic expression of PCNA (Figures 4D–4F) and SOX1 (Figures 4G–4I). Moreover, these ectopic progenitor cells retain the capacity to acquire regional characteristics. For example, in the motor horns a subset of SOX2-transfected cells ectopically expressed two ventral markers, Nkx2.2 and Olig2 (Figures 4J–4O). Intriguingly, at this stage of development both Nkx2.2 and Olig2 mark glial progenitor cells, suggesting that the inhibition of neuronal differentiation by SOX2 is permissive for acquisition of glial cell character (see Discussion).

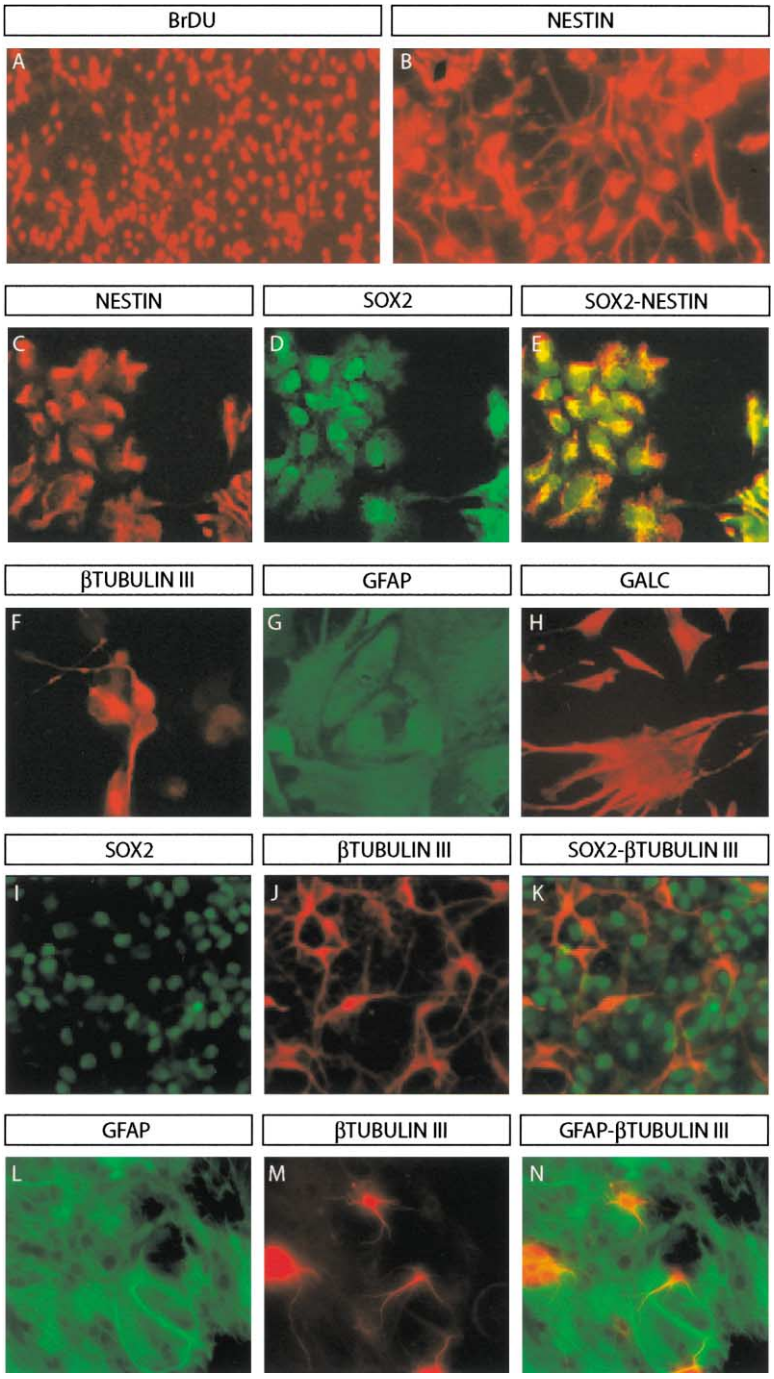


Figure 2. Multipotential CNS Neural Stem Cells Express SOX2

(A–E) E10 mouse neural tubes were dissociated and plated onto fibronectin-coated dishes at low density in NEP basal medium with bFGF alone. Cells were allowed to grow overnight, pulsed with BrDU for 6 hr, and processed for BrDU incorporation (A), Nestin (B), and SOX2 (C–E) expression. Panel (E) shows that these cells coexpress (yellow) Nestin (red) and SOX2 (green).

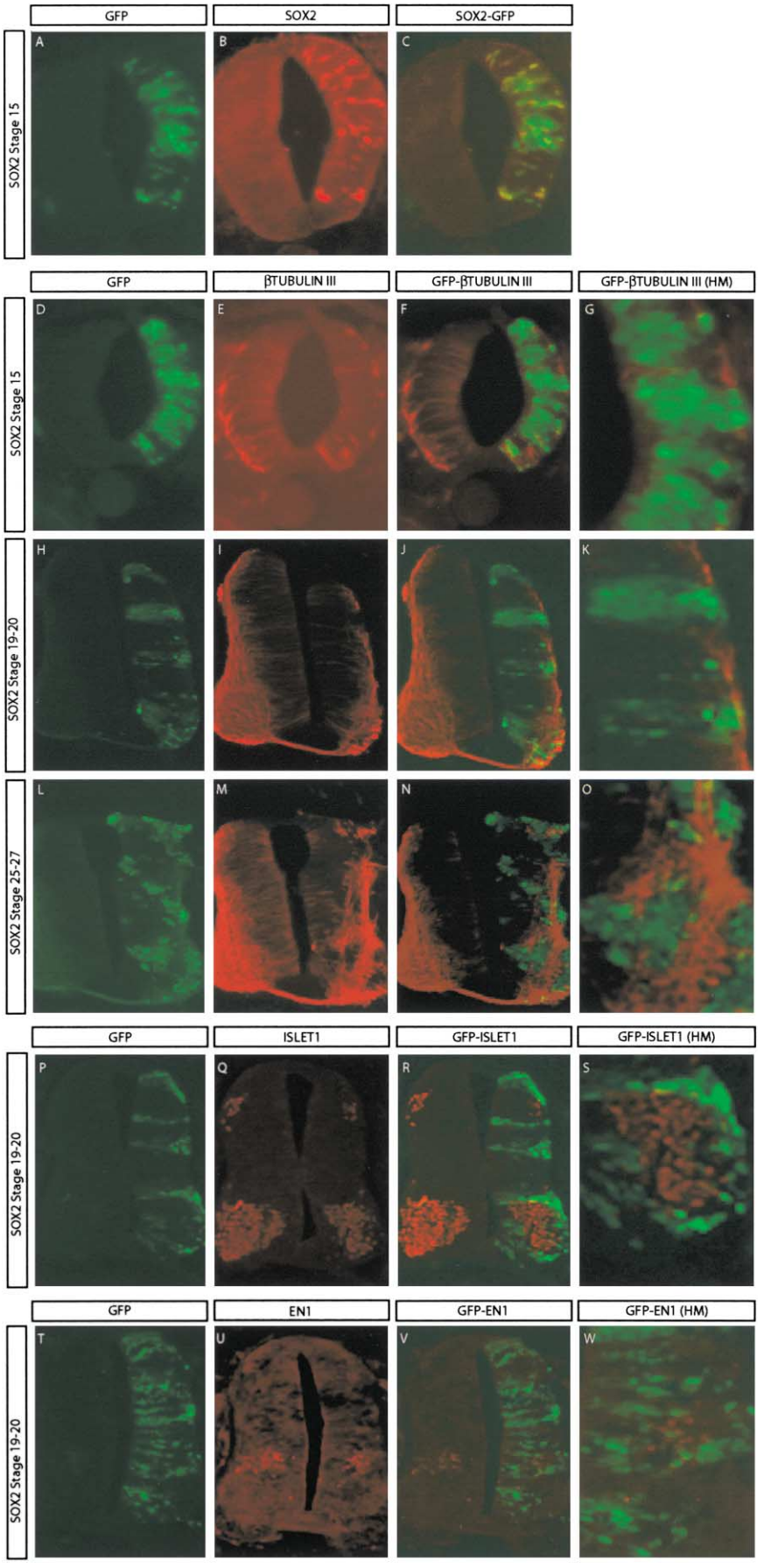
(F–H) SOX2+ mouse NEP cells differentiate into neurons, astrocytes, and oligodendrocytes, β-tubulin type III immunoreactive neurons (F), GFAP-positive astrocytes (G), and Gal-C immunoreactive oligodendrocytes (H).

(I–K) Double labeling with SOX2 and β-tubulin type III shows that these two markers are mutually exclusive.

(L–N) In secondary clonal cultures, SOX2+ cells can give rise to multipotential clones with GFAP-positive glial cells (L and N) and β-tubulin type III-positive neurons (M and N).

Inhibition of SOX2 Activity Results in Lateral Migration and Exit from Cell Cycle of Chick Neural Progenitors

These data indicate that constitutive expression of SOX2 in CNS cells is sufficient to maintain two characteristics that define a neural progenitor cell: proliferative capacity and inhibition of differentiation. To examine the requirement of SOX2 directly in proliferating progenitors, we expressed a dominant-interfering form of SOX2 specifically in neural progenitors by in ovo chick electroporation (Itasaki et al., 1999). We constructed a fusion protein containing the DNA binding domain of SOX2 fused to a myc epitope-tagged Engrailed-Repressor (ER) domain, SOX2ER^{myc}, converting SOX2 from a transcriptional activator to a transcriptional repressor. Past studies have demonstrated that the ER functions as an active repressor. That is, it does not function by occlusion but rather interferes with transcription initiation at any promoter to which it is directed (Han and Manley, 1993; Jaynes and O'Farrell, 1991). This approach has been successfully used to generate dominant-interfering forms of a number of transcription factors including the *Xenopus* ortholog of SOX2 (Conlon et al., 1996; Furukawa et al., 2000; Kishi et al., 2000). The SOX2ER^{myc}


GFP
SOX2
SOX2-GFP
SOX2 Stage 15
A
B
C
GFP
βTUBULIN III
GFP-βTUBULIN III
GFP-βTUBULIN III (HM)
SOX2 Stage 15
D
E
F
G
SOX2 Stage 19-20
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SOX2 Stage 25-27
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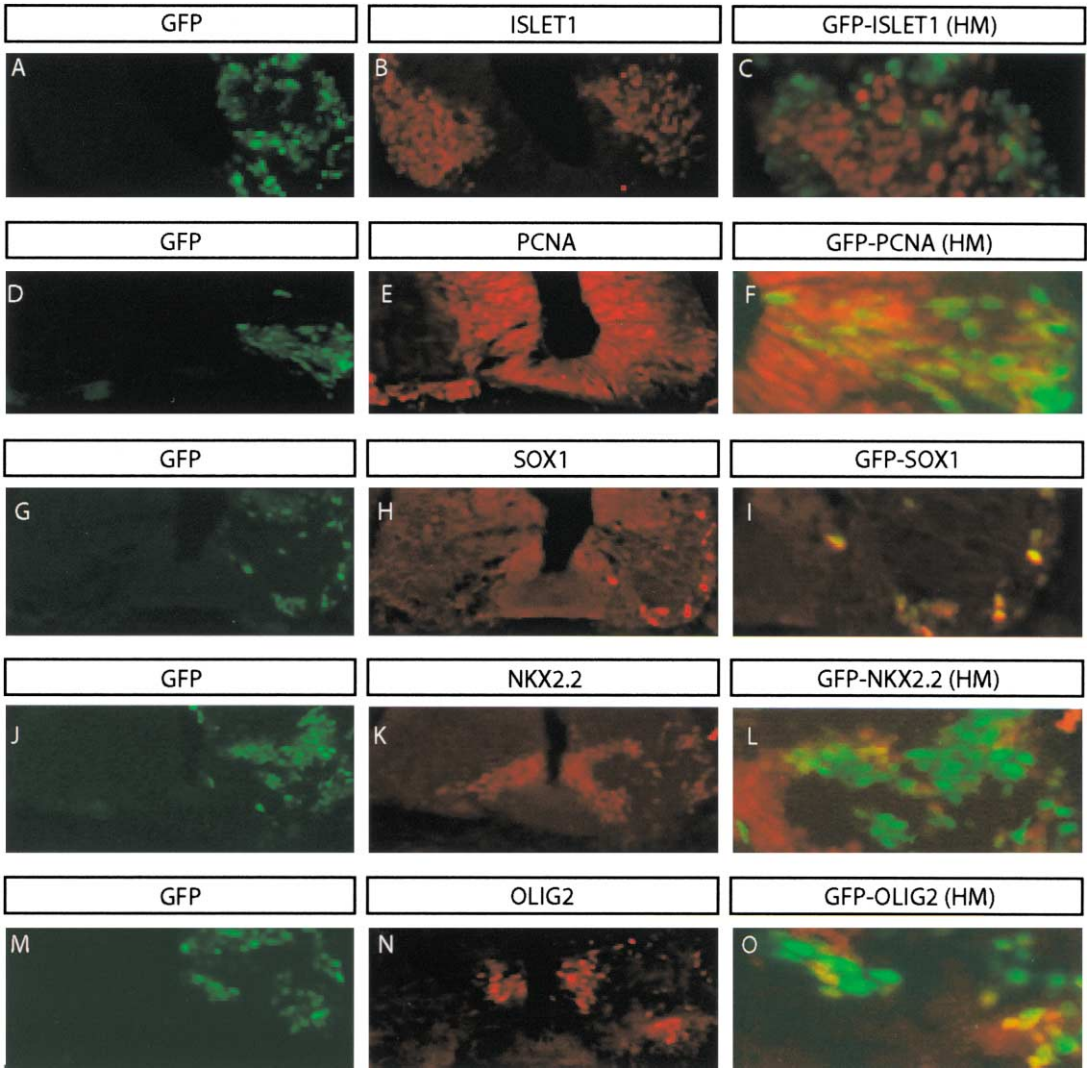

Figure 4. SOX2 Expression Maintains Neural Progenitor Characteristics

(A, D, G, J, and M) Unilateral expression of GFP in HH23 ventral motor horns of chick spinal cord, obtained by electroporation of pCIG-SOX2 at HH12–14.

(B and C) Loss of ventrally located Isl1+ motor neurons after unilateral SOX2 electroporation.

(E–O) Ectopic ventral expression of PCNA (E and F), SOX1 (H and I), Nkx2.2 (K and L), and Olig2 (N and O) in SOX2-positive cells.

cassette was placed under the regulation of the constitutively active CMV promoter and linked by an IRES sequence to the Green Fluorescent Protein (GFP). Following electroporation of SOX2ER^{myc}IRES-GFP, all of the GFP-positive cells coexpress the fusion protein as assayed by myc antibody staining (Figures 5A–5D).

As a first step toward assessing the requirement of SOX2 in proliferating neural progenitors, we electropor-

Figure 3. Forced Expression of SOX2 Inhibits Overt Neuronal Differentiation of CNS Progenitor Cells

Embryos were electroporated with 3 μg/μl of pCIG-SOX2 at HH11–12 and transverse sections through the neural tube analyzed at HH15 ($n > 5$), HH18–19 ($n > 5$), and HH23 ($n = 4$).

(A–C) Visualization of cells expressing SOX2 in HH15 chick neural tube by (A) GFP fluorescence (green), (B) SOX2 antibody staining (red), and (C) colocalization of GFP and SOX2 (yellow).

(D, H, and L) Expression of GFP on the right side of the spinal cord after pCIG-SOX2 electroporation.

(E–O) Loss of β-tubulin type III-positive neurons after pCIG-SOX2 electroporation.

(P) Ectopic expression of GFP after pCIG-SOX2 electroporation.

(Q–S) Loss of Isl1+ motor neurons in SOX2-transfected cells.

(T) Ectopic expression of GFP after pCIG-SOX2 electroporation.

(U–W) Loss of medially located En1+ interneurons in SOX2-transfected cells.

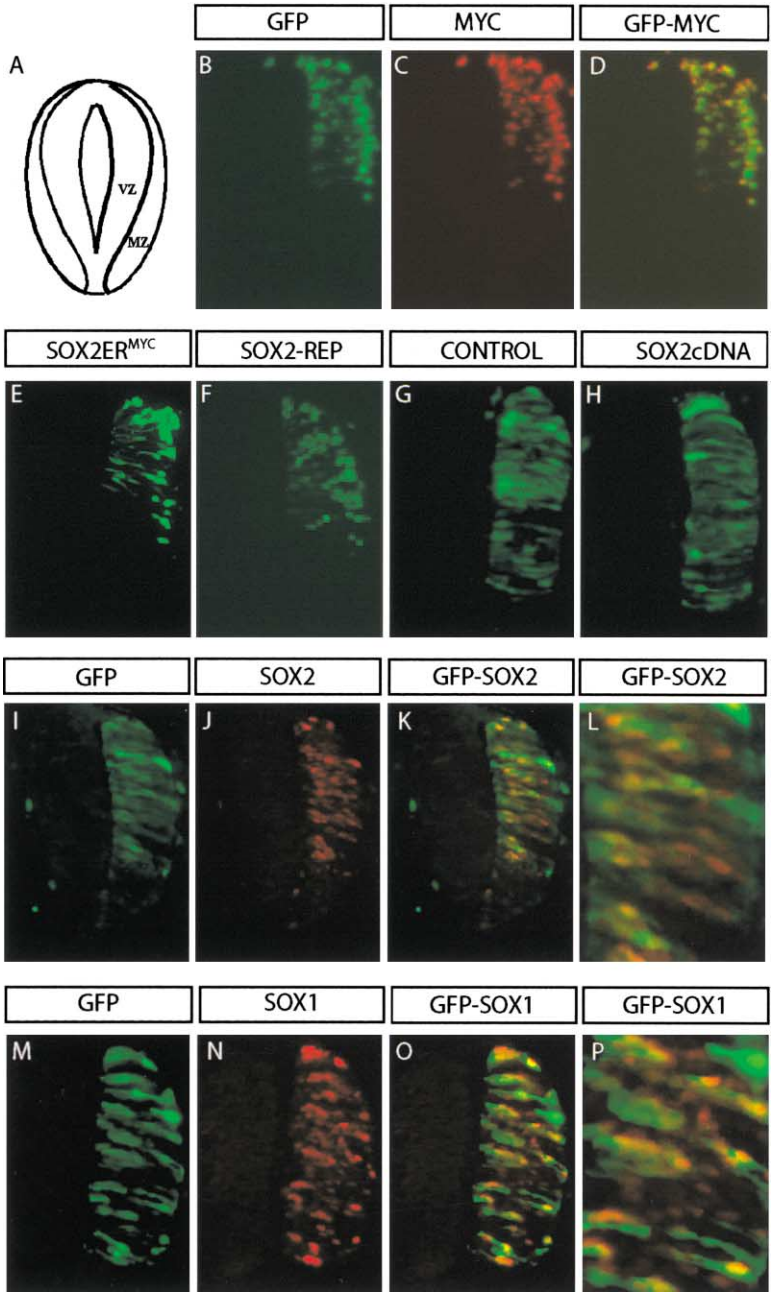

Figure 5. Inhibition of SOX2 Function Results in Lateral Migration of Transfected Cells That Can Be Rescued by Coexpression with SOX1 and SOX2

Embryos were electroporated with 3 μg/μl pSOX2ER^{myc} at HH11–12 and transverse sections through the neural tube analyzed at HH18–19 ($n > 5$).

(A) Schematic representation of the chick neural tube at HH18–19. The VZ is composed of proliferating neural progenitors, whereas postmitotic cells are located in the MZ.

(B–D) Visualization of SOX2ER^{myc}+ cells in HH18–19 chick neural tube by (B) GFP fluorescence (green), (C) Myc antibody staining (red), and (D) colocalization of GFP and Myc (yellow).

(E) Inhibition of SOX2 activity with 3 μg/μl pSOX2ER^{myc} results in lateral migration of GFP-positive cells.

(F) Inhibition of SOX2 activity with 3 μg/μl pSOX2-REP results in lateral migration of GFP-positive cells.

(G and H) In embryos electroporated with 3 μg/μl of pCAGGS GFP control vector (G) or 3 μg/μl p*Sox2* cDNA IRES-GFP (H), GFP-positive cells are distributed throughout the neuroepithelium.

(I–P) Coelectroporation of pSOX2ER^{myc} with pCAGGS *Sox2* cDNA or pCAGGS *Sox1* cDNA at a 3:2 molar ratio ($n > 5$). The SOX2ER^{myc} phenotype is rescued by SOX2 (I–L) or SOX1 expression (M–P). SOX2ER^{myc} expression is visualized by GFP fluorescence (green, I, K–M, O, and P). SOX2 (red, J–L) and SOX1 (red, N–P) expression were detected using polyclonal antibodies to SOX2 and SOX1.

ated embryos with the SOX2ER^{myc} expression vector at the onset of neurogenesis, HH10, and analyzed the embryos at HH18–19 for their location, mitotic activity, and expression of neural markers. We found that by HH18–19, most of the SOX2ER^{myc}+ cells were located lateral to the VZ (Figure 5E). These cells lost their connection with the ventricular surface and delaminated to the MZ. In contrast, the majority of cells transfected with control GFP vector (Figure 5G) or a vector containing the SOX2 cDNA (Figure 5H) were distributed between the VZ and MZ and displayed a typical bipolar morphology of neuroepithelial cells. These data support the idea that SOX2 functions as a transcriptional activator in vivo and show that this activity requires the COO^- transcription activation domain (Kamachi et al., 1999; Kishi et al., 2000). To confirm this further, we constructed an additional fusion protein (SOX2Repressor) in which the DNA binding domain of SOX2 was fused to the repressor domain derived from SOX14. SOX14, along with SOX21, is a member of the SOXB2 subgroup that is very similar to SOXB1 genes in its HMG-DNA binding domain and can bind identified SOX2 target sites. However, the COO^- terminal portions of SOX14 and SOX21 act as transrepression domains (Uchikawa et al., 1999). Expression of the SOX2Repressor resulted in a similar lateral migration to the MZ of SOX2ER^{myc}-transfected cells (Figure 5F). To test the specificity of this SOX2ER^{myc} phenotype, the SOX2ER^{myc}IRES-GFP plasmid was coelectroporated with a plasmid containing the murine *Sox2* cDNA under the regulation of the constitutively

active CMV promoter, and expression of SOX2ER^{myc} was assessed by GFP fluorescence and by SOX2 antibody staining (Figures 5I and 5J). Increasing levels of wild-type *Sox2* cDNA rescued the lateral migration caused by SOX2ER^{myc} in a dose-dependent manner such that doubly transfected cells were evenly distributed along the mediolateral axis of the neural tube (Figures 5I–5L).

SOX2 shares more than 90% amino acid residue identities with SOX1 and SOX3 in its DNA binding domain. This homology appears to be functional, since all three members of the SOXB1 subgroup can bind to the same DNA sequence and switching the C-terminal transcriptional activation domain has no effect on the proteins function in tissue culture assays. Moreover, all three SOXB1 factors are coexpressed in proliferating neural progenitors of the embryonic CNS. Consistent with a possible redundancy in function between the members of the SOXB1 subfamily, cotransfection of SOX2ER^{myc}-IRES-GFP plasmid (Figure 5M) with a plasmid containing the mouse *Sox1* cDNA under the regulation of CMV promoter (Figure 5N) rescued the lateral migration caused by SOX2ER^{myc} (Figures 5O and 5P) in a similar manner to *Sox2* cDNA (Figures 5K and 5L). These results indicate that SOX2ER^{myc} protein may block transcription of all targets the B1 SOX factors in neural progenitor cells and support the idea that group B1 *Sox* genes can act redundantly.

The delamination of progenitor cells from the VZ is coupled with their exit from cell cycle. Thus, the location of SOX2ER^{myc}-expressing cells on the external side of the VZ suggests that these cells are postmitotic. To resolve this issue, we pulsed labeled SOX2ER^{myc}-electroporated embryos with BrDU in order to label S phase nuclei. At HH15, there was already a marked reduction of BrDU incorporating cycling progenitors in the SOX2ER^{myc} transfected side (right) as compared to the control side of the neural tube (left) (Figures 6A–6D) as well as control transfection (Figures 6I–6L). By HH18–19, the cells that incorporated BrDU were mutually exclusive of the cells in the MZ expressing SOX2ER^{myc} (Figures 6E–6H). The majority of SOX2ER^{myc}-transfected cells do not incorporate BrDU, whereas incorporation was observed in neighboring nontransfected cells, showing that the dominant-interfering SOX2 cell autonomously blocks entry into S phase of the cell cycle.

Inhibition of SOX2 Signaling Results in the Loss of Progenitor Marker Expression and Initiation of Neuronal Differentiation

Lateral migration and exit from mitosis by neural progenitor cells is associated with a loss of adhesive connections with the ventricular surface and the onset of differentiation. The above results indicate that SOX2 plays a role in maintaining cells in a proliferative neuroepithelial state, and the inhibition of SOX2 function results in migration and exit from cell cycle. These data suggest that inhibition of SOX2 function alters the progenitor state. To address this, we analyzed the expression of the general neuroepithelial marker N-Cadherin and three regional progenitor markers that define the dorsal, medial, and ventral domains of the developing neural tube (PAX6, PAX7, and Nkx2.2, respectively) in SOX2ER^{myc} electroporated embryos. In chick embryos, N-Cadherin is expressed from the beginning of neural tube formation and remains expressed in the proliferative neuroepithelium (Redies, 2000). In embryos electroporated with SOX2ER^{myc}, we detected a marked decrease in N-Cadherin expression within the tranfected cells (Figures 6M, 6N, 6Q, and 6R). Neuroepithelial cells transfected with the control GFP vector showed wild-type levels of N-Cadherin expression (data not shown).

Consistent with the coexpression of SOX2 with PAX6, PAX7, and Nkx2.2, the inhibition of SOX2 function results in their downregulation. For example, by HH15 in chick embryos electroporated with SOX2ER^{myc}, there is a reduction in the number of PAX6-positive cells on the transfected as compared to the control side (Figures 6O and 6P). By HH18–19, the expression of PAX6 was excluded from the laterally located SOX2ER^{myc} cells (Figures 6S and 6T). The expression of PAX6 was diminished in most of the cells that expressed SOX2ER^{myc} but maintained in neighboring progenitors, arguing for a cell-autonomous action of SOX2. A similar cell-autonomous downregulation of PAX7 expression was detected in SOX2ER^{myc} transfected cells (data not shown).

Within the spinal cord, neural progenitors are initially specified by inductive cues originating from the ventral floor plate and dorsal roof plate. SHH plays a critical role specifying ventral neural cell types by regulating the expression of a group of homeodomain transcription factors such that the dorsoventral patterning of the neural tube relies on the crossrepression of homeodomain proteins. For example, the downregulation of Pax6 in the progenitor domain has been shown to be associated with the expansion of ventral markers (such as Nkx2.2) more dorsally. The expression of Nkx2.2 expands dorsally in the Pax6$^{-/-}$ mutant mouse spinal cord, while overexpression of Pax6 downregulates Nkx2.2 expression. In the chick spinal cord, Pax6 and Nkx2.2 are reported to repress expression of each other (Briscoe et al., 2000). In neural tubes electroporated with SOX2ER^{myc}, Pax6 expression is downregulated (Figures 6O and 6P); however, the expression of Nkx2.2 did not expand dorsally (Figures 6U–6X). Thus, the inhibition of SOX2 signaling caused a general loss of regional progenitor marker expression but did not alter the dorsoventral boundaries of progenitor expression, supporting the conclusion that SOX2 is regulating the differentiation of neural progenitors but not their patterning.

The exit of progenitor cells from mitosis leads to the formation of postmitotic neuroblasts that differentiate into terminal neurons. Our observation that BrDU-negative, SOX2ER^{myc}-expressing cells migrate out of the VZ suggests that they have initiated differentiation. To test this, we assayed SOX2ER^{myc} cells for the expression of neuronal markers. In many regions of the vertebrate CNS, neural progenitors that exit cell cycle early differentiate early and assume early fates. In the development of the spinal cord, the first progenitors to exit the cell cycle are located in the ventral neural tube and differentiate into motor neurons. In chick embryos, MNR2 is a marker of this early specification and differentiation of motor neurons and is switched on by chick motor neuron progenitors just before their final division. Therefore, we tested if SOX2ER^{myc} induced the expression of MNR2. At HH15, we observed a marked increase in MNR2-expressing cells in the SOX2ER^{myc}-transfected cells (Fig-

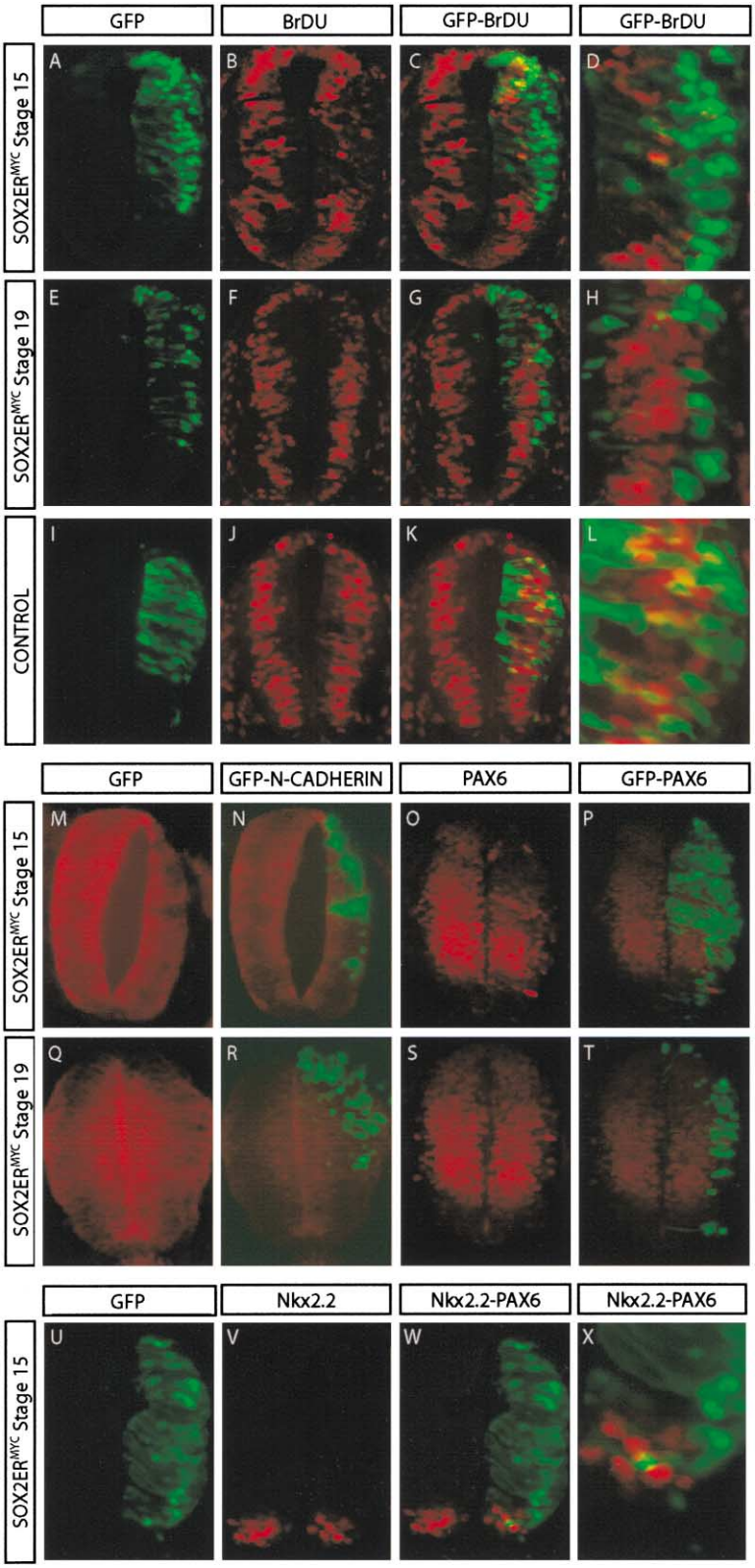

Figure 6. Inhibition of SOX2 Signaling in Neural Progenitor Cells Results in Their Exit from Cell Cycle

(A–L) Embryos were electroporated at HH11–12 with 3 μg/μl pSOX2ER^{myc} (A–H) or 3 μg/μl pCAGGS GFP control vector (I–L). Transverse sections through the spinal cord (between thoracic and lumbar levels) were analyzed at HH15 ($n > 5$) (A–D) or HH18–19 ($n > 10$) (E–L).

(A–D) At HH15, SOX2ER^{myc}+ cells (green, A, C, and D) have begun to exit cell cycle, and BrDU incorporation (red, B–D) is downregulated.

(E–H) In HH18–19 embryos, BrDU incorporation (red, F–H) is mutually exclusive of SOX2ER^{myc}+ cells (green, E, G, and H).

(I–L) In HH18–19 control embryos, BrDU incorporation (red, J–L) is seen in GFP-positive cells (I, K, and L). Inhibition of SOX2 results in downregulation of general and regional markers of proliferating neural progenitors.

(M–T) Transverse sections through the spinal cord (between thoracic and lumbar levels) of embryos electroporated at HH11–12 with 3 μg/μl and analyzed using markers of proliferating progenitors at HH15 ($n > 5$) (M–P) or HH18–19 ($n > 10$) (Q–T).

(M and N) At HH15, SOX2ER^{myc}+ cells (green) have begun to downregulate N-Cadherin (red).

(O and P) At HH 15, SOX2ER^{myc}+ cells (green) have begun to downregulate Pax6 (red).

(Q and R) In HH18–19 embryos, N-Cadherin (red) is mutually exclusive of SOX2ER^{myc}+ cells (green).

(S and T) In HH18–19 embryos, Pax6 staining (red) is mutually exclusive of SOX2ER^{myc}+ cells (green).

(U–X) By HH15, Nkx2.2 expression (red) does not expand dorsally into Pax6-negative domain.

ures 7A–7C), and by HH18–19 the majority of cells transfected with SOX2ER^{myc}-transfected cells ectopically expressed MNR2 (Figures 7D–7F). While MNR2 expression was confined to the ventrally differentiating motor neurons in control transfection and on the nontransfected side, it was detected dorsoventrally throughout the SOX2ER^{myc}-transfected area. The ectopic expression of MNR2 was also associated with a transient coexpres-

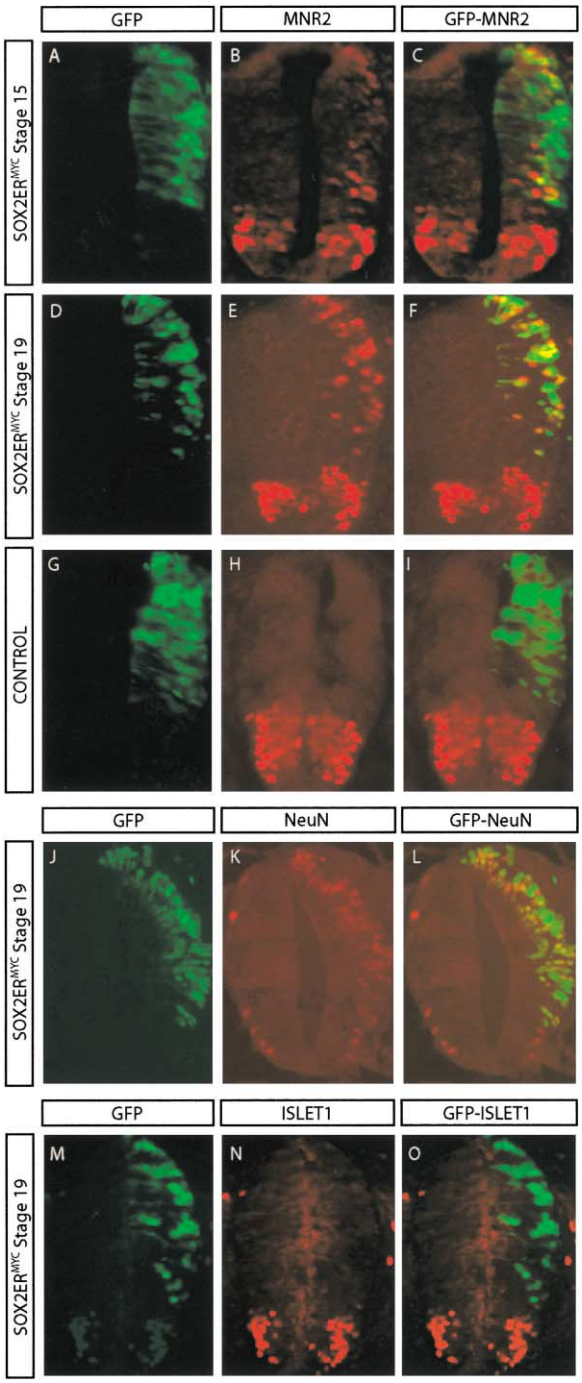

Figure 7. Inhibition of SOX2 Signaling Results in Ectopic MNR2 Expression but Is Insufficient for Terminal Differentiation

(A–C) By HH15, MNR2 (red, B and C) begins to be ectopically expressed in SOX2ER^{myc}+ cells (green, A and C) ($n > 5$).

(D–F) At HH18–19, SOX2ER^{myc}+ cells (green, D and F) ectopically express MNR2 (red, E and F) ($n > 10$).

(G–I) In embryos electroporated with a control GFP construct, MNR2 expression (red, H and I) is not seen in GFP-positive cells (green, G and I) ($n > 5$).

(J–L) At HH18–19, SOX2ER^{myc}+ cells (green, J and L) show ectopic expression of the early neuronal differentiation antigen, NeuN (red).

(M–O) At HH18–19, SOX2ER^{myc}+ cells (green, M and O) show unaltered expression of Islet1 (red, N and O) ($n > 10$).

sion of the neuronal nuclear antigen NeuN (Figures 7J–7L). Cells transfected with control GFP vector never showed ectopic MNR2 expression (Figures 7G–7I) or NeuN expression.

As motor neurons terminally differentiate, MNR2 expression is replaced by the expression of Islet1, a marker of postmitotic motor neurons (Ericson et al., 1992; Tanabe et al., 1998). Moreover, the ectopic expression of MNR2 itself can autonomously specify Islet1 expression and motor neuron differentiation only in the context of a general program of neurogenesis. Therefore, we examined whether SOX2ER^{myc}-transfected cells induced the expression of Islet1 and underwent terminal differentiation. Surprisingly, the SOX2ER^{myc} cells, which ectopically expressed MNR2, were Islet1 negative (Figures 7M–7O). This finding suggested that inhibition of SOX2 signaling in neural progenitor cells results in their exit from mitosis and onset of neuronal differentiation but is insufficient for terminal differentiation. Consistent with this finding, SOXER cells found at the lateral aspects of the MZ at these later stages lost the expression of NeuN and did not express other generic postmitotic markers such as marker β-tubulin type III (data not shown). This apparent lack of terminal differentiation was not stage dependent; SOX2ER^{myc}-transfected cells examined up to 48 hr after electroporation did not express postmitotic neuronal markers (data not shown).

Inhibition of SOX2 Signaling Blocks Delamination of Cells from the Dorsal Neural Tube

SOX2ER^{myc}-transfected neuroepithelial cells that migrate to the mantle zone are frequently more rounded and tend to form aggregates in the dorsal neural tube. These cells show rounded morphology despite the pseudostratified-neuroepithelial organization of their nontransfected neighbors. Unlike control electroporations, where transfected cells often migrate out of the dorsal neural tube and contribute to the neural crest lineage (Figure 8A), SOX2ER^{myc}-transfected cells are rarely found outside the neural tube (Figure 8B). Moreover, the lack of migration of SOX2ER^{myc}-transfected cells out of the neural tube can be rescued by coelectroporation of Sox2cDNA (Figure 8C). We further examined SOX2ER^{myc} electroporated embryos with the marker HNK1. The onset of HNK1 expression corresponds with the exit of neural crest cells from the dorsal neural tube (Tucker and Erickson, 1984). HNK1 was ectopically expressed in dorsal aggregates and scattered ventral cells expressing SOX2ER^{myc} within the neural tube (Figures 8D–8F). Cells transfected with the control GFP vector never showed ectopic HNK1 expression within the neural tube (Figures 8G–8I). These data indicate that inhibition of SOX2 signaling appears to block the delamination of cells from the dorsal neural tube. It has been recently shown that neural crest cells emigrate in the S phase of the cell cycle, and inhibition of G1 to S transition prevents initial delamination of neural crest cells from the neural tube (Burstyn-Cohen and Kalcheim, 2002). Thus, the lack of migration of SOX2ER^{myc}-expressing cells is consistent with their premature exit from cell cycle.

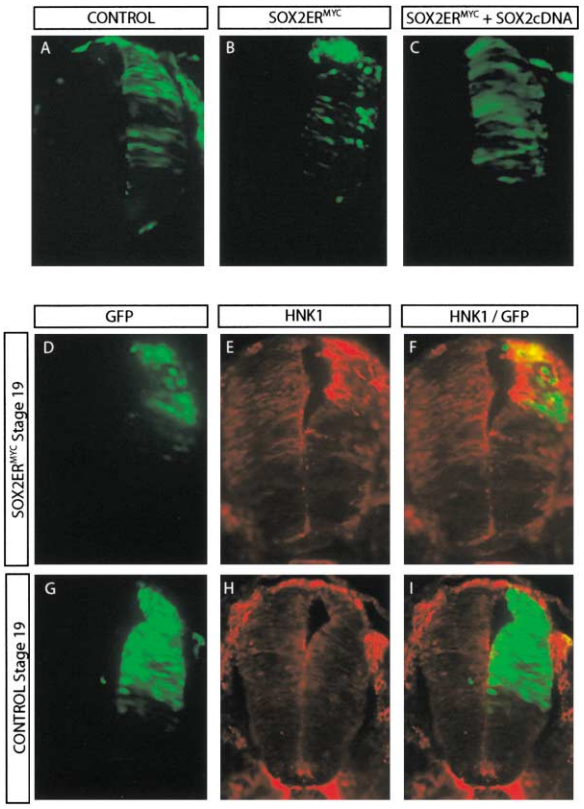

Figure 8. Inhibition of SOX2 Signaling with SOX2ER^{myc} Blocks Delamination of Cells from the Dorsal Neural Tube

Embryos were electroporated at HH11–12, transverse sections through the spinal cord were analyzed at HH18–19 ($n > 10$).

(A) In embryos transfected with 3 μg/μl pCAGGS GFP control vector, transfected cells contribute to neural crest.

(B) In embryos transfected with 3 μg/μl pSOX2ER^{myc}, transfected cells rarely contribute to neural crest.

(C) The SOX2ER^{myc} crest phenotype is rescued by cotransfecting pSOX2ER^{myc} with pCAGGS *Sox2* cDNA at a 3:2 molar ratio.

(D–F) In embryos transfected with 3 μg/μl pSOX2ER^{myc}, HNK1 (red, E and F) is found ectopically within the neural tube in SOX2ER^{myc}+ cells (green, D and F).

(G–I) In embryos transfected with 3 μg/μl pCAGGS GFP control vector, HNK1-positive cells (red, H and I) are not seen in the GFP-positive cells (G and I) within the neural tube.

Discussion

This study provides evidence that SOX2, a SOXB1-HMG protein, is required to maintain two generic characteristics that define a neural progenitor cell: proliferative capacity and inhibition of differentiation. Specifically, forced expression of SOX2 inhibits neuronal differentiation preserving a naive progenitor state. These cells, although ectopically located outside the VZ, retain the capacity to respond appropriately to regional patterning signals. Conversely, neural progenitor cells expressing a dominant-interfering form of SOX2 exit from cell cycle and migrate to the MZ. The phenotype elicited by inhibition of SOX2 signaling can be rescued by coexpression of SOX1, providing evidence for redundant SOXB1 function. We discuss these findings in the context of the cellular and molecular mechanisms that maintain generic characteristics of neural progenitor/stem cells.

A Role for SOX2 in Maintaining Universal Properties of Neural Progenitors

Neural progenitor cells throughout the CNS are defined by common panneural properties: they proliferate, self-renew, and give rise to differentiated progeny. The SOXB1 family of transcription factors represents one group of conserved panneural molecular markers. The expression of SOXB1 factors is suggestive of a similarity among progenitor cells. However, neural progenitor cells are regionally specified and express unique molecular markers. Along the DV axis of the spinal cord, differential expression of Pax and homeobox transcription factors define distinct progenitor domains that subsequently correspond to specific neuronal fates. What remains unclear is whether progenitor cells expressing particular markers are committed in their fate. For example, such cells may remain plastic until they have withdrawn from cell cycle and left the VZ (Anderson, 2001). Moreover, it has recently been demonstrated that embryonic progenitors can maintain expression of markers of regional identity in vitro but can be respecified when grafted to heterologous sites in vivo (Hitoshi et al., 2002). We have shown that SOX2 is coexpressed with regional progenitor markers along the DV axis of the chick spinal cord but is excluded from postmitotic cells. In addition, SOX2 expression is maintained in proliferating progenitors even when regional progenitor identity is altered, such as the mouse mutant for the transcription factor Pax6 or the signaling molecule SHH (Figure 1). Taken together, these data indicate that SOX2 expression marks a common molecular property shared by neural progenitors independent of DV regional patterning and that it is maintained in parallel to regional identity superimposed on progenitors based upon DV position and subsequent specification.

Our data provide evidence that SOX2 functions to maintain panneural properties of CNS progenitor cells. Cells expressing SOX2 retain proliferative capacity and expression of progenitor markers and are inhibited from differentiation (Figure 5). At least in the context of the developing spinal cord, SOX2-expressing cells retain the capacity to respond to dorsal-ventral-inducing signals, as the cells found in the MZ take on molecular markers of corresponding area within the VZ (Figure 4). Consistent with this, inhibition of SOX2 signaling results in neural progenitors exiting cell cycle and the coincident loss of progenitor markers without alteration in regional identity (Figure 6). For example, the loss of Pax6 expression here does not result in the dorsal expansion of ventral markers such as Nkx2.2, and vice versa.

SOX2 Inhibits Neuronal Differentiation of CNS Progenitors

We have shown a temporal and spatial correlation between the differentiation of chick neural progenitor cells and SOX2 expression (Figures 1 and 2). SOX2 is expressed in proliferating neural progenitors, including neural stem cells, and is precisely downregulated as cells exit from their final cell cycle, but not at the onset of regional patterning. Consistent with its restriction to proliferating cells of the CNS, forced expression of SOX2 resulted in the loss of a number of cells expressing panneuronal as well as regional markers and the persis-

tence of proliferative capacity. The precise mechanism by which SOX2 represses neuronal differentiation, however, remains unclear. One possibility is that SOX2 plays a role in maintaining neural progenitors in cell cycle and thus regulates the timing of their exit from cell cycle. The finding that the inhibition of SOX2 function in neural progenitor cells results in their premature exit from mitosis supports this idea. Cells expressing SOX2ER do not incorporate BrDU, do not enter the S phase of the cell cycle, and delaminate to the MZ (Figure 4). Furthermore, SOX2-misexpressing cells maintained proliferative capacity and expressed the proliferative cell antigen PCNA but were not stimulated to overproliferate. A clear example of a cell fate decision in the developing nervous system that is dependent on the timing of the neural progenitors exit from the cell cycle is the programmed sequence of neuron and glial cell differentiation. For example, in the spinal cord, motor neurons and oligodendrocytes are stimulated to differentiate in the ventral region by the signaling molecule sonic hedgehog. Retroviral lineage data point to a common progenitor for these two cell types (Leber et al., 1990). Given that motor neurons are born before oligodendrocytes in vivo, it has been suggested that the progenitor exhibits an order to cell generation (Qian et al., 2000). Recently, an elegant transcriptional mechanism has been proposed for the sequential generation of motor neurons and oligodendrocytes from ventral neural progenitors. At early stages of spinal cord development, the bHLH transcription factor Olig2 cooperates with the neurogenic factor Ngn2 to induce motor neuron differentiation and subsequently with the homeodomain protein Nkx2.2 to induce oligodendrocytes (Rowitch et al., 2002). This mechanism is tightly linked with the regulation of the timing of the neural progenitors exit from cell cycle, as expression of Olig2 and Ngn2 drives cells to exit the cell cycle and relocate to the MZ to take on motor neuron identity. In the ventral spinal cord, the inhibition of motor neuron differentiation as a result of forced SOX2 expression results in the ectopic expression of Olig2 and Nkx2.2 in the ventral MZ (Figure 4). At these later stages of development, Olig2 and Nkx2.2 serve as markers of glial progenitors. Thus, expression of SOX2 in ventral progenitors may play a role in repressing motor neuron differentiation and maintaining proliferative progenitors to later allow the differentiation of progenitor cells into glia. The repression of motor neuron differentiation by SOX2 also provides a possible explanation for the widespread ectopic expression of MNR2, an early motor neuron marker, as a consequence of inhibition of SOX2 signaling (Figure 7).

It remains to be determined if SOXB1 proteins directly interact with cell cycle machinery to regulate timing of cell cycle exit. Previous work in *Xenopus* embryos has shown that misexpression of *Xsox3* with XBF-1 promotes proliferation of neuroectodermal cells, possibly by repressing the expression of cell cycle inhibitor p27XIC1 (Hardcastle and Papalopulu, 2000), establishing a functional correlation between *SoxB1* genes and neural progenitor cell cycle.

Our studies provide evidence that maintenance of SOX2 expression inhibits neuronal differentiation, and thus the downregulation of SOX2 expression appears to be a prerequisite for neuronal differentiation. To date, the molecular mechanisms responsible for the downregulation of SOX2 expression remains unclear. One possibility suggested previously (Uchikawa et al., 1999) is that neural progenitor differentiation relies on a balance of activation and repression of SOXB1 target genes and that this may occur via an auto/crossregulatory SOX loop. Consistent with this regulatory loop for SOXB1 factors, regulatory domains of both *Sox1* and *Sox2* genes contain a number of potential binding sites for SOX transcription factors (Tomioka et al., 2002; Wiebe et al., 2000; our unpublished data). Moreover, expression of SOX2 results in the ectopic activation of SOX1 and vice versa, maintaining neural progenitor fate (Figure 4), whereas the expression of SOX2ER promotes the exit from cell cycle and premature differentiation of neural progenitors. Intriguingly, the SOXB2 subgroup of SOX factors, including *Sox14* and *Sox21,* are very similar to *Sox1*, *Sox2,* and *Sox3* in their HMG-DNA binding domain and can bind identified SOX2 target sites. However, the COO^- terminal portions of SOX14 and SOX21 act as transrepression domains. Moreover, SOX14 and SOX21 are expressed in the developing CNS (Rex et al., 1997b; Rimini et al., 1999; Uchikawa et al., 1999). Support for this hypothesis comes from the observation that ectopic expression of SOX2-REP (a fusion protein containing the SOX2-DNA binding domain and SOX14 COO^- terminus; Figure 5) as well as SOX14 itself (data not shown) in neural progenitors phenocopies the inhibition of SOX2 signaling elicited by $SOX2ER^{myc}$ expression.

Redundant Function *SoxB1* Genes Coexpressed in the CNS

Collectively, several lines of evidence suggest that the members of the SOXB1 subfamily are functionally redundant. First, microinjection of dominant-negative forms of *Sox2* mRNA in *Xenopus* that inhibit neural differentiation of animal caps can be rescued by injection of *Sox3* but not divergent *Sox* genes such as *Sox9* and *SoxD* (Kishi et al., 2000). Second, midline glial defects in *Drosophila Dichaete* mutants can be rescued by directed expression of SOX1 and SOX2 proteins (Sanchez-Soriano and Russell, 1998). Finally, the elimination of both members of the *Drosophila SOXB* subfamily, *SoxNeuro* and *Dichaete*, simultaneously results in much more severe phenotypes in the neuroectoderm than the single mutants (Buescher et al., 2002; Overton et al., 2002). Thus, functional redundancy appears to be confined to SOXB1 subfamily and does not extend to more divergent SOX family members. Our data provides further evidence that the members of the SOXB1 subfamily are functionally redundant: the phenotypic consequences of the inhibition of SOX2 signaling in chick neural progenitors can be rescued by the coexpression of SOXB1 subfamily member SOX1, and the forced expression of SOX1 in CNS cells phenocopies forced expression of SOX2 (data not shown).

Parallel Requirements of SOX Factors in CNS and PNS Neural Stem/Progenitor Cells

Taken together, the data presented here demonstrate that, in addition to their established role in neural cell fate specification, members of the SOXB1 family are both sufficient and essential to maintain characteristics

that define neural progenitor identity, specifically their proliferative capacity and inhibition of neuronal differentiation. Furthermore, SOXB1 factors appear to maintain these characteristics universally in all regionalized progenitors along the DV axis of the spinal cord. Recent studies demonstrate that neural progenitor/stem cells, with the capacity for at least limited self-renewal, are present throughout development of the nervous system. SOX2 continues in proliferating neural progenitor cells throughout vertebrate embryogenesis, and its expression is maintained in proliferating cells in the mouse adult brain, specifically in neurogenic regions such as the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone of the hippocampus (Zappone et al., 2000; P.E. and L.P., unpublished data). It has been hypothesized that adult SVZ cells might be derived from embryonic radial glial cells that retain neuroepithelial stem cell characteristics into adulthood (Alvarez-Buylla et al., 2001). Consistent with this hypothesis, a number of recent data have led to the suggestion that glial cell types (radial glia, astrocytes) may be multipotent progenitors (Gregg et al., 2002). Our data clearly illustrate that SOX2 inhibits neuronal differentiation, but it remains to be determined whether it is permissive for commitment to glial fate. SOX2 is expressed in embryonic radial glial cells and is maintained in a subset of GFAP-positive glia in the adult nervous system, specifically type B astrocytic cells of the SVZ (Pevny and Rao, 2003). Methods, including conditional mutagenesis in the mouse, will resolve whether SOX2 and more generally SOXB1 factors are also essential for the maintenance of progenitor identity in these neurogenic regions of the adult CNS. This direct comparison of SOXB1 function in vivo, in the embryo as well as in the adult, will begin to elucidate similarities and/or differences in the molecular mechanisms during CNS neural progenitor differentiation.

There is now increasing evidence that SOX factors may play a global role in maintaining progenitor/stem cell fates in a variety of tissues including the nervous system. Members of the *SOX* gene family are expressed in a variety of embryonic and adult tissues where their expression, and in some cases function, is associated with the specification and/or maintenance of progenitor identity. For example, SRY is transiently expressed in the progenitor of Sertoli cells of the XY genital ridge and is responsible for triggering development of the male phenotype (Lovell-Badge and Hacker, 1995). SOX9 is expressed in immature chondrocytes and plays a role in their proliferation and differentiation (Akiyama et al., 2002; Morais da Silva et al., 1997; Wehrli et al., 2003; Wright et al., 1993). Intriguingly, a recent report describing the function of SOX10 in the PNS reveals many functional parallels between the role of SOX10 in the PNS stem/progenitor cells and those described here for SOXB1 factors in CNS progenitor cells (Kim et al., 2003). SOX10 is expressed in multipotent neural crest stem cells and is downregulated during their neuronal differentiation. Kim et al. (2003) show that forced expression of SOX10 is able to override both antigliogenic activity of BMP2 and antineurogenic (antiproliferative) activity of TGFβ and thus maintain multipotential differentiation capacity of NCSCs. Furthermore, by directly inhibiting terminal neuronal differentiation, SOX10 appears to provide a permissive environment for glial differentiation. It will be interesting to determine if any of the molecular pathways by which SOX10 maintains neural crest stem cell fate in the PNS are also used in the CNS.

Experimental Procedures

Expression Vectors

The SOX2ER^{myc} expression vector pSOX2ER^{myc} was made by fusing a fragment of mouse *Sox2* cDNA lacking the carboxyl terminus (bases 251–785, Xho1–BspH1) to a myc epitope-tagged fragment of the *Drosophila Engrailed* protein containing the repressor domain (bases 6–894) (Conlon et al., 1996). SOX2ER^{myc} was subsequently inserted into the pIRES2-EGFP vector (Clonetech) between Xho1 and BamH1 sites. The mouse Sox2 cDNA coding region (bases 251–2109, Xho1–EcoRV) was inserted into both pIRES2-EGFP and pCAGGS vectors between Xho1 and Sma1 sites to make the SOX2 expression vectors pCAGGS-*Sox2-cDNA* and *pSox2-cDNA*-IRES-GFP. The mouse *Sox1* cDNA coding region (bases 425–2041, Kpn1–Xho 1) was inserted into pCAGGS between Kpn1 and Xho 1 sites to make the SOX1 expression vector pCAGGS-*Sox1-cDNA*. A control GFP expression vector, pCAGGS-GFP, was made by inserting the GFP coding region into pCAGGS between Xho 1 and EcoR1 sites.

Cloning of SOX14

Overlapping 5′ and 3′ regions of *Sox14* cDNA were obtained by PCR of 11.5 dpc mouse brain cDNA using the Expand Hi-fidelity PCR system (Roche Diagnostics). Reactions were carried out according to manufacturer's instructions. Primers and annealing temperatures used are listed below. Amplification of 5′ *Sox14* fragment: 5′UTR (ggC TCA ggA Cgg ACA gAA Ag) + 3′ INTERNAL (AgT TAC Agg gCA CCA CgT Ag), annealing temp = 57.4°C. Amplification of 3′ *Sox14* fragment: 5′ INTERNAL (CAA gAA ggA CAg gTA TgT CTT CC) + 3′UTR (gTC ggg AgA ggg gAA gAA gA), annealing temp = 58.5°C. MWG Biotech synthesized primers. Fragments amplified using each primer set were cloned using the pGEM-T easy vector system I (Promega) and sequenced in both directions (Big dye sequencing kit, PE Biosystems). 5′ *Sox14* and 3′ *Sox14* fragments without mutations were selected, and each was isolated as an EcoRI/NaeI fragment and coligated back into PGEM-T easy to produce a single cDNA clone spanning the entire ORF and partial UTR regions of *Sox14* (equivalent to positions 289–1349 of published sox14 genomic sequence, accession no. AF193437). The sequence of the joining region at the NaeI site was subsequently verified. Finally, the cDNA fragment was excised as an EcoRI fragment and cloned into the pIRES2-EGFP and pCAGGS vectors. To generate the SOX2-Repressor, a fragment of mouse *Sox2* cDNA lacking the carboxyl terminus (bases 251–785, Xho1–BspH1) was fused in-frame to the COO^- transcriptional repressor domain of mouse *Sox14*.

In Ovo Chicken Electroporation

Hamburger and Hamilton (HH) HH11–12 chick embryos were electroporated in ovo as described previously (Itasaki et al., 1999). Expression vectors or a pCAGGS-GFP control vector (concentrations of 3 μg/ml) were injected into the neural tube lumen with 50 ng/μl fast green and electroporated with 5 pulses of 20 volts for 50 ms using a BTX electroporator (Genetronics). Embryos were left to develop for 8 hr (HH15), 24 hr (HH18–19), or 54 hr (HH23) before analysis. Visualizing GFP expression using a fluorescent dissecting microscope monitored sites of plasmid expression. PCAGGS-*Sox2*-cDNA and pCAGGS-*Sox1*-cDNA expression vectors were coelectroporated with pSOX2ER^{myc} at a molar ratio of 3:2.

Immunohistochemistry

Embryos were fixed at room temperature for 1 hr in MEMFA (Pevny et al., 1998) cryoprotected with 30% sucrose in PBS, and cryosectioned. Frozen sections were incubated overnight at 4°C with primary antibodies. Monoclonal mouse antibodies to Pax6, Pax7, Nkx2.2, MNR2, and Islet1 were used at a concentration of 1:50 (obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD, and maintained by The

University of Iowa, Department of Biological Sciences, Iowa City). Anti-Myc (Invitrogen), anti-β-tubulin type III (TuJ; Covance), and activated Caspase 3 (Cell Signaling Technologies) were used at a concentration of 1:1000. Anti-Olig2 antibody was used at a concentration of 1:8000 (kind gift from Dr. D. Rowitch). Staining solution was made up of PBS, 1% heat-inactivated goat serum (Sigma), and 0.1% Triton (Sigma). For SOX2, double labeling embryos were stained with SOX2 rabbit antisera (rSOX2Ab) at a concentration of 1:1500 overnight at 4°C. rSOX2Ab detects the COO^- terminus of SOX2 3′ to the HMG box (Kamachi et al., 1995). Secondary antibodies anti-mouse Cy3, anti-rabbit Cy3, anti-mouse, and anti-rabbit FITC (Sigma) were used to visualize immunostaining.

Cell Cycle Analysis

For BrdU labeling, 200 μl of 2 mg/ml BrdU (bromodeoxyuridine) was applied on top of the chick embryos in ovo, 45 min prior to dissection. Sectioned embryos were treated with 2 M HCl for 15 min, followed by trypsin for 15 min before being stained with anti-BrDU Ab (Sigma) at a concentration of 1:1000 overnight at 4°C. For BrDU-labeled sections, a polyclonal antibody to GFP (AbCAM) at a concentration of 1:1000 was used to enhance GFP visualization.

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