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Sox1 acts through multiple independent pathways to promote neurogenesis

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Abstract

Although Sox1, Sox2, and Sox3 are all part of the Sox-B1 group of transcriptional regulators, only Sox1 appears to play a direct role in neural cell fate determination and differentiation. We find that overexpression of Sox1 but not Sox2 or Sox3 in cultured neural progenitor cells is sufficient to induce neuronal lineage commitment. Sox1 binds directly to the Hes1 promoter and suppresses Hes1 transcription, thus attenuating Notch signaling. Sox1 also binds to β-catenin and suppresses β-catenin-mediated TCF/LEF signaling, thus potentially attenuating the wnt signaling pathway. The C-terminus of Sox1 is required for both of these interactions. Sox1 also promotes exit of cells from cell cycle and up-regulates transcription of the proneural bHLH transcription factor neurogenin1 (ngn1). These observations suggest that Sox1 works through multiple independent pathways to promote neuronal cell fate determination and differentiation.
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Keywords: Sox1; Sox2; Sox3; Hes1; Notch; Neurogenesis; β-catenin; TCF/LEF signaling; Cell cycle; bHLH; Neurogenin1; Retinoic acid (RA)

Introduction

Sox domain proteins are a class of developmentally important transcriptional regulators related to the mammalian testis determining factor SRY (Bowles et al., 2000). The 20 Sox genes that have been identified in the mammalian genome are divided into eight different groups (A through H) according to sequence similarities (Schepers et al., 2002). Group B Sox genes are most closely related to SRY, sharing over 85% sequence identity between their DNA binding domains (Collignon et al., 1996; Harley et al., 1994). The SoxB1 gene subfamily including Sox1, Sox2, and Sox3 has been evolutionarily conserved (Bowles et al., 2000). The *Drosophila* (Buescher et al., 2002; Cremazy et al., 2000; Overton et al., 2002), *Xenopus* (Mizuseki et al., 1998a,b), and avian (Rex et al., 1997; Uwanogho et al., 1995) putative orthologues of Sox1, Sox2, and Sox3 all show expression throughout the neural primordium. Several lines of evidence suggest that Sox-B1 factors play a role in neural cell fate determination and differentiation. Mutation of the *Drosophila* Sox-B1 proteins, SoxNeuro, and Dichaete leads to defects in the specification and differentiation of midline and lateral neural cells, and SoxNeuro or Dichaete double mutants have severe hypoplasia of the entire central nervous system (Buescher et al., 2002; Overton et al., 2002). *Xenopus* Sox2 can synergize with FGF signaling to initiate neural differentiation, and injection of a dominant interfering form of Sox2 mRNA inhibits neural differentiation of animal caps (Mizuseki et al., 1998a). In fact, a Sox2-βGeo insertion construct has been used to select neural precursors from stem cell populations, suggesting that Sox2 is a marker for early neural fate (Li et al., 1998).

In the mouse, the differing expression profiles of Sox1, Sox2, and Sox3 during embryogenesis suggest that these genes may function differently in the control of neural cell fate. Sox2 and Sox3 begin to be expressed at preimplantation and epiblast stages, respectively, and later become restricted to the neuroepithelium (Collignon et al., 1996; Wood and Episkopou, 1999). Targeted deletion of Sox2 leads to death before implantation (Collignon et al., 1996), and chimeric

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mice generated with Sox3 null ES cells display severe abnormalities during gastrulation and posterior truncations (Parsons, 1997). Sox2 and Sox3 expression may help to maintain neural progenitor cell identity by inhibiting neurogenesis (Bylund et al., 2003; Graham et al., 2003). By contrast, Sox1 is expressed later in development coincident with formation of the neural plate (Collignon et al., 1996; Pevny et al., 1998; Wood and Episkopou, 1999). After neural induction, Sox1 expression is confined to neural precursors along the entire anteroposterior axis of the developing embryo and subsequently to adult neural stem cells. Overexpression of Sox1 is sufficient to promote neural differentiation of the P19 cell line (Pevny et al., 1998). Moreover, the brains of Sox1 null mutant mice have a severe loss of neurons in the ventral striatum including loss of the majority of the Gad and pre-proenkephalin-expressing neurons in ventral striatal structures, the olfactory tubercle, and the nucleus accumbens shell (Malas et al., 2003). Further experiments with ectopic expression of Sox1 in mice and the analysis of Sox1-null mice harboring Sox1 expressed only in precursors and not postmitotic neurons indicate that Sox1 is both necessary and sufficient for neuronal differentiation in the ventral telencephalon (Dr. Vasso Episkopou, MRC, London, UK, personal communication).

Sox proteins bind sequence-specifically to DNA by a high-mobility group (HMG) domain that allows them to function as transcription factors (Sinclair et al., 1990 and see Wilson and Koopman, 2002). However, unlike most transcription factors, binding occurs in the minor groove of DNA resulting in the induction of a bend within the DNA helix. The HMG domain also appears to be involved in binding of Sox proteins to other partner proteins including POU proteins (see Wilson and Koopman, 2002). Such protein–protein interactions appear to be critical for defining the specificity of functions of Sox proteins. For example, Sox2 and Pax6 bind to each other and act cooperatively to activate transcription from the DC5 enhancer of the lens-specific δ-crystallin gene (Kamachi et al., 2001). Similarly, Sox2 and Sox3 interact with Oct3/4 to regulate gene expression in the pregastrulation embryo (Nishimoto et al., 1999; Yuan et al., 1995). However, not all protein–protein interactions involving Sox family members involve the HMG domain. For example, the C-terminus mediates interactions of Sox17 with the signaling molecule β-catenin (Zorn et al., 1999). Interaction of Sox17 or Sox7 with β-catenin inhibits TCF-mediated signaling activity thereby interfering with Wnt signaling (Takash et al., 2001; Zorn et al., 1999). Thus, Sox family members regulate developmental events not only by acting as direct transcriptional regulators, but also by forming protein–protein interactions and acting as either coactivators or cosuppressors.

This study examines mechanisms by which Sox1 may regulate neural differentiation and determination. We find that overexpression of Sox1, but not Sox2 or Sox3, in neural progenitor cells is sufficient to induce neuronal lineage commitment. Sox1 binds to the promoter of Hes1 thereby suppressing Notch signaling while it suppresses β-catenin-mediated TCF/LEF signaling by binding to β-catenin itself. The C-terminus of Sox1 is required for both interactions. Overexpression of Sox1 in neural cell lines activates the expression of the proneural gene neurogenin1 and promotes exit from cell cycle and neuronal differentiation. Thus, Sox1 works through multiple independent pathways to promote neuronal cell fate determination and differentiation.

Materials and methods

Plasmids and viruses

A Sox1 full-length expression vector was constructed by inserting a full-length PCR product into a pLenti6/V5 expression vector using a pLenti6/V5 Directional TOPO Cloning Kit (Invitrogen). The primers were as follows: SOX-lenti-5′: CAC CAT GTA CAG CAT GAT GAT GGA GAC; and SOX-lenti-3′: CTA GAT GTG CGT CAG GGG CAC.

An IRES-GFP fragment was then inserted into the Sfu site of this vector, thus creating a Sox1-IRES-GFP expression vector. A control vector containing GFP alone was then created by excising Sox1 and religating the vector. Constructs with deletion of the C-terminus (ΔCSox1), deletion of the N-terminus (ΔNSox1), or the C-terminus alone (C-Sox1) were constructed by replacing the full-length Sox1 coding region with the indicated fragments (*Bam*HI + *Xho*I). The primers were as follows: C-Sox1: *Bam*-SOX1c-term5′: CG GGA TCC AAG ATG GAC AAG TAC TCG CTG G, Sox1ΔN3′: CGG CTC GAG CTA GAT GTG CGT CAG GGG; ΔNSox1: Sox1ΔN5′: GGG GAT CCC CCATGA CCG CCT TCA TGG TG, Sox1ΔN3′: CGG CTC GAG CTA GAT GTG CGT CAG GGG; ΔCSox1: Sox1ΔC5′: GGG GAT CCA TGT ACA GCA TGA TGA TGG AG, Sox1ΔC3′: CGG CTC GAG CTA CGT CTT GGT CTT GCG GCG.

An HMG box (flag-tagged) expression vector was constructed by inserting the HMG box into a pIRES-hrGFP-1α vector (Stratagene, inframe with flag), amplifying the HMG box with flag tag as one fragment and then replacing full-length Sox1 with this fragment (*Bam*HI + *Xho*I).

The primers were as follows: 5′HMG-bm: CG GGA TCC AAC ATG GAT CGG GTC AAG CGG CCC; 3′HMG-e: T CTT GGT CTT GCG GCG CGG CC; and 3′FLAG-x: CCG CTC GAG TTA TTT GTC GTC ATC ATC CTT.

Lentivirus was made for these vectors using a Virapower lentiviral support kit (Invitrogen), followed by titration of viral in 293FT cells.

Hes1 promoter luciferase reporter construct (Hes1-luc) was generously provided by Prof. Alain. ngn1 and the Hes1-truncated promoter were amplified from mouse genomic DNA by PCR and then cloned into a pGL3-Basic vector

(Promega). The Hes1 promoter without the consensus Sox1 binding site was made by deleting the 6 bp consensus sequence from the Hes1-luc construct, using a QuikChange Site-Directed Mutagenesis Kit (Stratagene).

The primers for ngn1 promoter cloning were as follows: ngn1 pro-kpn1 5′: ACG GTA CCG TCA GTG TTC AGT TTG ACG GA, and ngn1 pro-*Bam*HI 3′ ATG GAT CCC CGA GTG TGG CAC ACG AC.

The primers for Sox1 promoter cloning were as follows: Sox1M5′-H: CCC AAG CTT CAT AGC AAG GGA GCA ACG GCG; and Sox1M3-BM′: GCG GAT CCG GGC GGC TAG CGG GTT CAC CG.

The primers for cloning of the truncated Hes1 promoter were as follows: H152: GG GGT ACC AGA TAT ATA TAG AGG CCG CCA; and H131: GA AGA TCT GCT TAC GTC CTT TTA CTT GAC.

The primers for HES1 ΔS (for deletion of one potential Sox1 binding site) were as follows: Ds1: CGT GTC TCT TCC TCC CTG AAA GTT ACT GTG; and Ds2: CAC AGT AAC TTT CAG GGA GGA AGA GAC ACG.

All constructs were confirmed by sequencing and expression vectors were also confirmed by Western blotting using anti-tag antibodies or gene-specific antibodies.

Cell culture

Cell lines: 293FT (Invitrogen), HEK293T, P19 and Neuro-2a (ATCC), and R1 ES cell lines (Nagy et al., 1993) were used in this study. Cells were maintained in recommended medium and propagated according to standard protocols (Graham et al., 1977; Klebe and Ruddle, 1969; McBurney et al., 1982; Nagy et al., 1993).

Neural progenitor cells: Neurosphere cultures were established as described by Tropepe et al. (1999). Briefly, telencephalons of embryos (E17) were dissected and mechanically dissociated in serum-free neurosphere culture medium (Vescovi et al., 1993). After 4 days of culture, primary neurospheres were spun down and dissociated and were further expanded by transfer into fresh neurosphere culture medium for generation of secondary spheres. Secondary spheres were mechanically dissociated and transduced with lentivirus constructs (GFP only and Sox1-IRES-GFP, respectively) for 2 days, then plated into 24-well plates containing cover slips coated with PDL and laminin. The medium was then changed to neurosphere culture medium without EGF, which was changed every third day until 7 days after infection when analyses of cell phenotype were performed. For comparing the effects of different Sox1B family members on neuronal lineage commitment, E14 neurosphere progenitor cells were electroporated with the same amounts (10 μg) of different DNA constructs (Sox1-IRES-GFP, Sox2-IRES-GFP, and Sox3-IRES-GFP), using the Mouse NSE Nucleofector Kit (Amaxa Inc.) according to the manufacturer's instructions. Lineage analyses were performed 7 days later as described above.

Cell cycle and growth curve analysis

HEK293T cells were transduced with 10× multiplicity of infection (MOI) of lentivirus that expressed HMG-IRES-GFP, Sox1-IRES-GFP, or GFP, respectively. One day later, transduced cells were selected with blasticidin (5 μg/ml). The cells were selected for six more days (to kill non-transduced cells) before cell cycle analysis using a Beckman Coulter Epics XL-MCL followed by the standard PI staining protocol (Larsen et al., 1986). Since cells overexpressing Sox1-IRES-GFP cannot survive for prolonged times in culture, the drug-selected cells were immediately subjected to cell cycle and growth curve analysis. For growth curve analysis, the same batch of virally transduced cells were split and 10^5 HEK293T cells overexpressing each of the different constructs were plated into 6-well plates under normal drug selection. Cell numbers were counted daily for 7 days. Mixed clones were used to do these experiments to minimize possible effects caused by the sites of integration of the foreign genes.

Colony formation and rescue assay

In normal liquid medium: Neuro-2A cells were transduced with the indicated expression vectors, and 24 h later they were split to clonal density in new 10-cm plates. After another 24 h, blasticidin (5 μg/ml) was added and the cells were maintained under selection for 7 days with daily changes of medium.

In semisolid medium: Neuro-2A cells were transduced with the indicated expression vectors, and 24 h later the cells were split to clonal density in new 10-cm plates. After another 24 h, the medium was changed to a semisolid one (10 ml 0.8% agarose dissolved in regular medium with blasticidin, 5 ug/ml). Ten milliliters of regular medium with blasticidin (5 μg/ml) was layered on top of this, and the top medium was changed daily for 7 days.

For rescue experiments: Neuro-2A cells were cotransfected with Dsred-tagged Sox1 (red cells) and GFP-tagged ΔN-β-catenin (green cells), and 24 h later the cells were split to clonal density in new 10-cm plates. After 24 h, blasticidin (5 μg/ml) was added and the cells were maintained under selection for 7 days with daily changes of medium.

Luciferase assays

For promoter reporter experiments, HEK293T or P19 cells were cotransfected with 10 ng TK-renilla luciferase and 0.3 μg gene-specific promoter reporter constructs [Hes1-luc (Jarriault et al., 1995) or ngn1-luc] or artificial reporter constructs (TOPFLASH or FOPFLASH) (kind gifts of Prof. Hans Clevers) (Korinek et al., 1997) with or without 0.3 μg indicated expression vectors using FuGENE 6 (Roche). The total amount of DNA was normalized by adding empty vector. Cells were lysed 36 h later, and

Dual-Luciferase activities were measured using Dual-Luciferase reporter 1000 assay system (Promega) according to the guide from the manufacturer. All luciferase assays were performed in duplicate, and normalized relative luciferase activities were expressed as folds of control activity or ratio of two different Luciferase activities: Luc/Ren. All assays were repeated at least three times. Typical results are shown in the figures.

Chromatin immunoprecipitation (ChIP) assay

Co-immunoprecipitation experiments examining protein–DNA interactions were performed using the Chromatin Immunoprecipitation (ChIP) Assay Kit (Upstate Biotechnology). Briefly, P19 cells were split into 10-cm dishes and 5 μg of the indicated DNA was transfected into the cells on the following day. Thirty-six hours later, the cells were cross-linked by adding formaldehyde directly to culture medium to a final concentration of 1% and incubating for 10 min at 37°C (for the ChIP experiment using E10 mouse embryos, the whole embryo was dissected from the uterus and washed in PBS twice before fixing in the same final concentration of formaldehyde). The medium was then removed and the cells were washed twice with ice-cold PBS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, and 1 μg/ml pepstatin A). The cells were scraped into a conical tube, pelleted for 4 min at 2000 rpm at 4°C, resuspended in 200 μl of SDS lysis buffer containing the protease inhibitors, and incubated for 10 min on ice. The lysate was sonicated to shear DNA to lengths between 200 and 1000 bp and then diluted 10-fold in ChIP dilution buffer (with protease inhibitors). Two milliliters of the diluted cell pellet suspension was precleared with 80 μl of Salmon Sperm DNA/Protein A Agarose—50% Slurry for 30 min at 4°C with agitation to reduce nonspecific background. The agarose was pelleted by brief centrifugation and the supernatant fraction was collected. The indicated immunoprecipitating antibody (9E10 antibody for *myc*-tagged RBP/J and *myc*-tagged NICD) was added to 2 ml of the supernatant fraction and was incubated overnight at 4°C with rotation. Agarose was then pelleted by gentle centrifugation (700–1000 rpm at 4°C, approximately 1 min), and the supernatant containing unbound, nonspecific DNA was carefully removed. The protein A agarose/antibody/protein/DNA was washed for 3–5 min on a rotating platform, and the complex was eluted from the agarose by adding 250 μl elution buffer to the pelleted protein A complex, vortexing briefly, and incubating the mixture at room temperature for 15 min with rotation. The agarose was carefully pelleted and the supernatant fraction was carefully transferred to another tube. Ten microliters of 5 M NaCl was added to the combined eluates and the protein–DNA cross-links were reversed by heating at 65°C for 4 h. The eluate was then used as the template for PCR, and the PCR products were detected on a regular agarose gel.

PCR primers: CTC AGG CGC GCG CCA TTG GCC, GCT TAC GTC CTT TTA CTT GAC. In vivo ChIP experiments using E10.5 embryos were done in same way except that the cross-linking was done at 4°C for 4 h.

Co-immunoprecipitations

For co-immunoprecipitation experiments, Sox1 (3 μg) with or without β-catenin (3 μg) (gift of B. Vogelstein) as indicated were cotransfected into HEK293T cells using calcium phosphate. After 36 h, cells were lysed with IP buffer (0.5% Triton, 10 mM Tris pH 7.5, 145 mM NaCl, 5 mM EDTA, 2 mM EGTA, with proteinase inhibitors), and the same amounts of total protein from different extracts were incubated with an affinity-purified rabbit anti-SOX1 antibody (gift of Prof. Kondoh) (Kamachi et al., 1999) directed against the N-terminal peptide MYSMMMETDLHSPGGA or normal rabbit IgG as indicated at 4°C overnight. UltraLink Immobilized protein G beads (40 μl, Pierce) were added for 2 h and then the beads were washed four times with buffer and resuspended in 40 μl Laemmli buffer. Western blotting was performed to probe the blots for specific β-catenin bands (see following for details). In vivo co-immunoprecipitations using E10.5 embryos were done in a similar way with normal rabbit-IgG or no antibody pull-downs acting as negative controls.

Western blot analyses

Western blot analyses were performed according to standard protocol. Briefly, cell lysates or elutes were resolved in 12% PAGE gel. The specific band was detected by indicated first Abs [rabbit anti-sox1 antibody (gift of Prof. Kondoh; Kamachi et al., 1999) 1: 2000; rabbit anti-Hes1 (gift of Prof. Tetsuo Sudo; Hirata et al., 2002) 1:500; rabbit anti-ngn1 (Chemicon) 1:500], followed by desired HRP-conjugated second Abs, chemiluminescence was detected by Western Lighting Chemiluminescence Reagent (PerkinElmer Life Sciences). Mouse β-actin was used as the loading control.

Immunohistochemistry

Hes1 immunocytochemistry and βIII-tubulin/GFP double staining were performed according to standard procedures. Briefly, cells were fixed in 4% formaldehyde for 10 min at room temperature on the cover slips, then washed 3 × 5 min, and blocked by 1:10 diluted normal serum for 30 min. The cells were then incubated with the indicated Abs (1:500 for Hes1 and βIII-tubulin and 1:1000 for chicken anti-GFP; Chemicon) overnight at 4°C, washed 3 × 5 min at room temperature, incubated with the desired second Ab for 2 h at room temperature, and washed again 3 × 5 min. Counter staining for DAPI (1:5000) was performed when

necessary. The cover slips were then mounted on regular glass slides.

Results

Overexpression of Sox1, but not Sox2 or Sox3, in cultured neural progenitor cells promotes neuronal lineage commitment

Overexpression of Sox1 is sufficient to promote neuronal differentiation in the P19 cell line (Pevny et al., 1998). To determine whether Sox1 similarly promotes neuronal lineage commitment by neural stem or progenitor cells, the effects of overexpression of Sox1 were examined in progenitor cells cultured from E17 telencephalon (Fig. 1). Lentiviral vectors were used to transduce Sox1-IRES-GFP or GFP alone into mechanically dissociated E17 neurosphere progenitor cells. Approximately 20% of control (nontransduced) progenitor cells differentiated into βIII-tubulin immunoreactive cells. Similarly, only about 20% of cells transduced with GFP alone differentiated into βIII-tubulin positive cells. By contrast, between 70% and 80% of cells overexpressing Sox1 differentiated into βIII-tubulin$^+$ cells (Fig. 1A) that adopted a neuronlike morphology (Fig. 1B). This suggests that Sox1 alone is sufficient to promote neuronal differentiation of neural stem or progenitor cells. To compare the effects of different Sox1B family members, that is, Sox1, Sox2, and Sox3, on neuronal lineage commitment, we electroporated the same amounts of DNA of Sox1-IRES-GFP, Sox2-IRES-GFP, or Sox3-IRES-GFP constructs into cultured E14 progenitor cells (Fig. 1C). Ap-

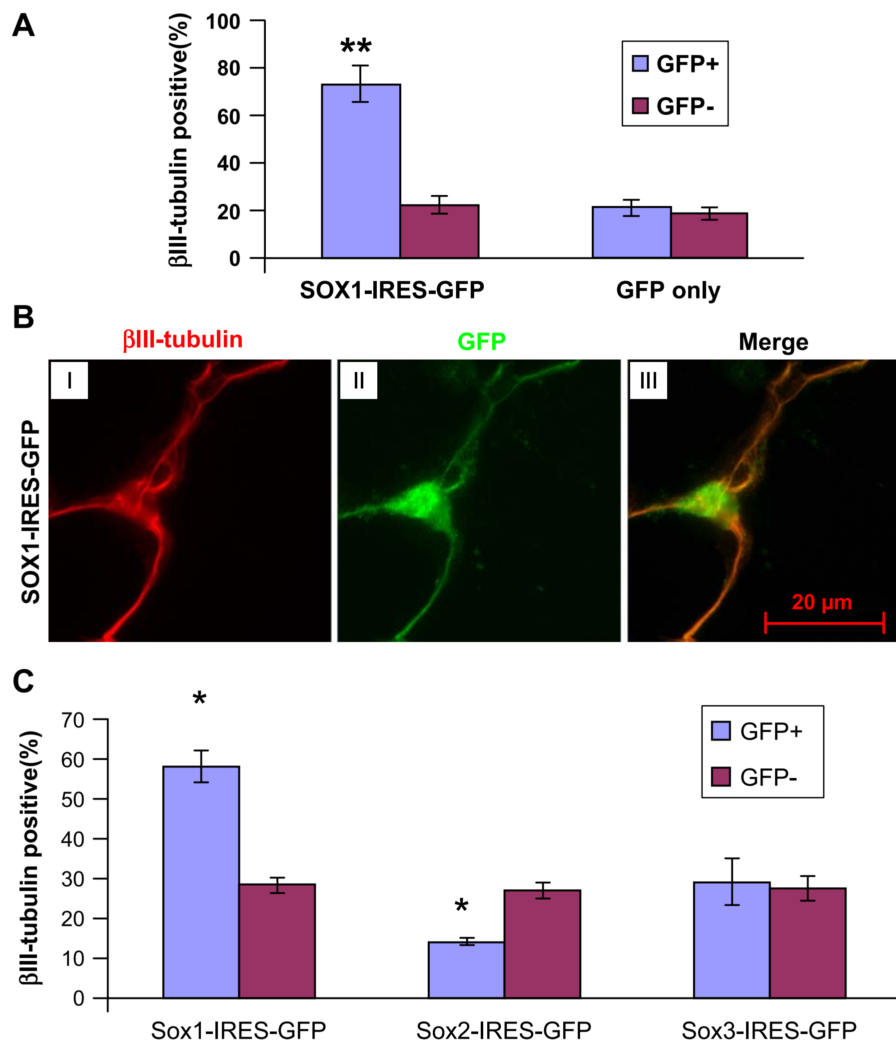


Fig. 1. Overexpression of Sox1, but not Sox2 or Sox3, promotes neuronal lineage commitment by cultured telencephalic progenitor cells. (A) Cultured E17 neural progenitor cells were transduced with lentivirus expressing either Sox1-IRES-GFP or GFP alone and were examined 7 days later for expression of βIII-tubulin. Values represent the means ± SD of the percent of βIII-tubulin$^+$ cells. **Differs from all other groups by ANOVA at $P < 0.01$. (B) Example of a Sox-IRES-GFP-overexpressing cell that is βIII-tubulin$^+$ (I, βIII-tubulin stain; II, GFP stain; and III, merge). Note the typically neuronal morphology. (C) Cultured E14 progenitor cells were electroporated with the same amounts of different DNA constructs including Sox1-IRES-GFP, Sox2-IRES-GFP, Sox3-IRES-GFP, or GFP. Seven days later, the cells were examined for expression of βIII-tubulin. Values represent the means + SD of the percent of βIII-tubulin$^+$ cells. *Differs from control (GFP negative cells) by ANOVA at $P < 0.05$.

proximately 27% of untransduced (GFP negative) progenitor cells differentiated into βIII-tubulin immunoreactive cells in each group. By contrast, a significantly increased number (approximately 60%) of cells overexpressing Sox1 differentiated into βIII-tubulin^{+} cells, whereas a significantly reduced number (approximately 15%) of cells overexpressing Sox2 differentiated into βIII-tubulin^{+} cells. About 28% of cells overexpressing Sox3 differentiated into βIII-tubulin^{+} cells, which did not differ from the GFP negative group. These results indicate that Sox1 promotes neuronal lineage commitment by cultured progenitor cells whereas Sox2 conversely inhibited cells from committing to the neuronal lineage. Sox3 had no significant effect on lineage commitment by neural progenitor cells in this assay.

Sox1 binds directly to the Hes1 promoter in cells and suppresses Hes1 transcription

The mechanisms by which Sox1 promotes neuronal lineage commitment are unknown. Sox family proteins have been shown to function as cosuppressors as well as coactivators depending upon the cellular context (see Wilson and Koopman, 2002). Therefore, one possible mechanism for the proneural effects of Sox1 is suppression of the helix-loop-helix transcription factor Hes1, which is expressed downstream of Notch signaling and which is a potent inhibitor of neurogenesis. To test this hypothesis, we first examined the effects of overexpression of Sox1 on the activities of the Hes1 promoter in P19 and

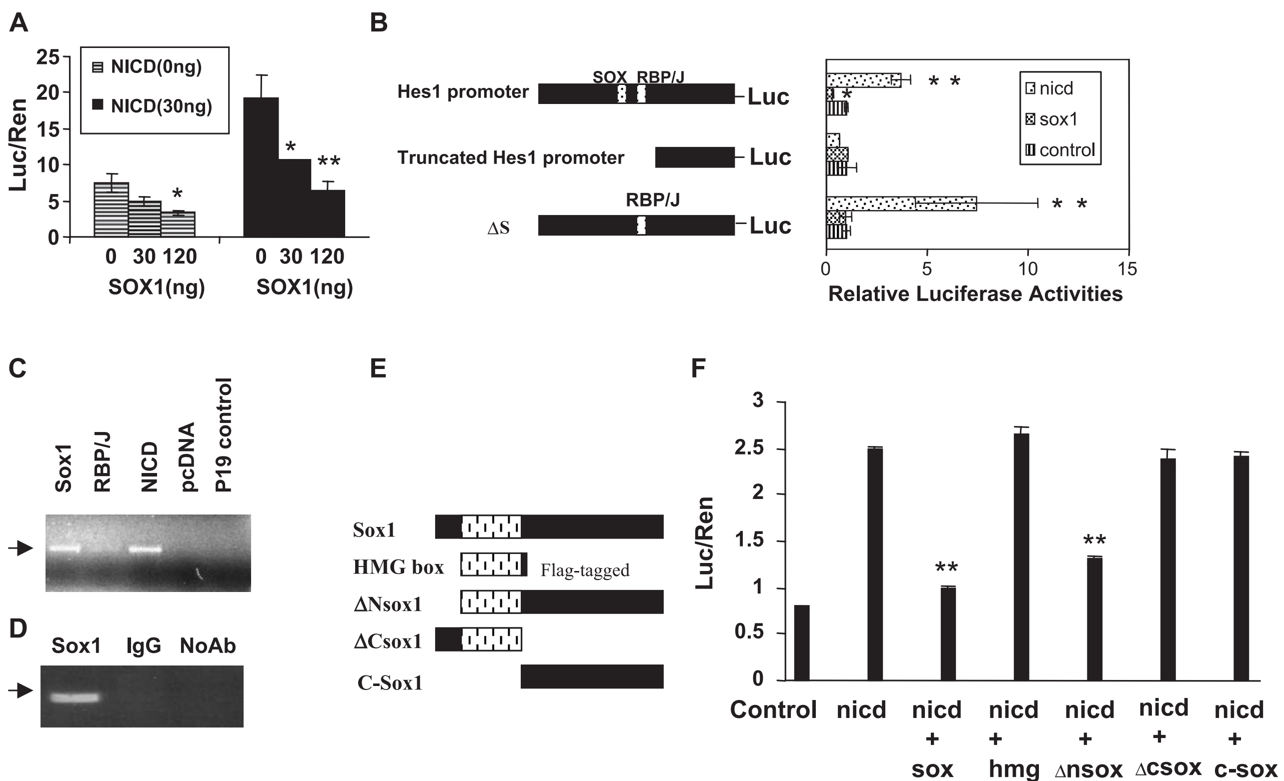


Fig. 2. Sox1 suppresses Hes1 transcription through direct binding to the Hes1 promoter. (A) Luciferase reporter assays using the Hes1 promoter demonstrate that Sox1 suppresses Hes1 promoter activity in a dose-dependent way in HEK293T (shown here) and P19 (not shown) cell lines. Cells were cotransfected with 0, 30, or 120 ng of Sox1 expression vector with or without 30 ng of the NICD expression vector. *Differs from control by ANOVA at $P < 0.05$; **differs from control by ANOVA at $P < 0.01$. (B) Sox1 suppresses Hes1 transcription through the putative Sox binding site. Luciferase assays were performed comparing the wild-type Hes1 promoter with a mutant full-length promoter lacking the potential Sox protein binding site and a truncated form without the RBP/J binding site and upstream sequence. Note that the promoter without the putative Sox binding site does not respond to Sox1 but responds well to NICD, whereas the promoter without both RBP/J and the Sox binding site does not respond to either. *Differs from control by ANOVA at $P < 0.05$; **differs from control by ANOVA at $P < 0.01$. (C) ChIP assay demonstrates that Sox1 binds directly to the Hes1 promoter. P19 cells overexpressing *myc*-tagged NICD and RBP/J were used as positive controls, while cells without any treatment and cells transfected with empty vector served as negative controls. Specific bands were detected only in cells that overexpressed Sox1, NICD, or RBP/J and were not detected in the negative controls. (D) ChIP assays using E10.5 embryos detected a specific band only in the Sox1 antibody lane and not in the control lanes. (E) Truncated and full-length expression vectors of Sox1 proteins. To map which domains play key roles in the suppression of the Hes1 promoter, four truncated SOX1 expression vectors were constructed—HMG box-IRES-GFP only, ΔNSox1-IRES-GFP (Sox1 without the N-terminus), ΔCSox1-IRES-GFP (Sox1 without the C-terminus), andC-Sox1-IRES-GFP (C-terminal portion of Sox1)—by replacing full-length sox1 with indicated PCR fragments in pLenti6/V5 expression vector. Protein expression and stability of the vectors was confirmed by Western blotting (not shown). (F) Intact Sox1 is needed to suppress the Hes1 promoter. P19 cells were cotransfected the indicated constructs with the Hes1 the promoter reporter gene. Note that ΔNSox1-IRES-GFP can suppress Hes1 promoter activity whereas the HMG box-IRES-GFP, ΔCSox1-IRES-GFP, and C-Sox1-IRES-GFP constructs cannot suppress the Hes1 promoter. **Differs from NICD-overexpressing lane by ANOVA at $P < 0.01$. All luciferase assays were done at least in duplicate and were repeated at least three times.

HEK293T cells using Dual Luciferase reporter assays and a well-established Hes1 promoter reporter gene construct, Hes1-luc (Jarriault et al., 1995). We found that overexpression of Sox1 suppresses Hes1 promoter activity in a dose-dependent fashion with as much as a 70% reduction in both cell lines (Fig. 2A). We then transfected the reporter cells with Sox1 alone or along with a Hes activator [the constitutively active Notch intracellular domain, (NICD)] and found that Sox1 suppressed Hes1 promoter activity to a similar extent even in the presence of constitutively active Notch signaling (Fig. 2A). Analysis of the sequence of the mouse Hes1 promoter revealed one ATTGGC sequence, a potential consensus Sox binding sequence (Harley et al., 1994; Wiebe et al., 2000; 2003), located just 8 bp upstream of the RBP/J binding site. To determine whether this is the *cis*-element that actually responds to Sox1, we made a mutant promoter reporter construct that lacks only the putative Sox binding site and a second truncated construct that lacks the RBP/J and Sox binding sites as well as the sequence 5′ to these sites. Luciferase assays in both cell lines indicated that the promoter lacking the putative Sox site failed to respond to Sox1 but did respond to NICD while the truncated promoter construct failed to respond to either Sox1 or NICD (Fig. 2B). These observations suggest that this putative Sox binding site is responsible for the Sox1-mediated suppression of Hes1 expression. To further confirm this finding, we utilized a chromatin immunoprecipitation assay (ChIP assay), which can detect in vivo physical interactions between identified proteins and specific portions of cellular DNA, to examine protein interactions with the Hes1 promoter. P19 cells were transfected with Sox1, *myc*-tagged NICD or RBP/J (positive controls), or empty vector (pcDNA3) (negative control), and untransfected cells served as an additional negative control (Fig. 2C). The proteins were immunoprecipitated and analyzed for co-immunoprecipitation of the Hes1 promoter region. Sox1, NICD and RBP/J each interacted specifically with the Hes1 promoter thus giving a specific band in the ChIP assay whereas no bands were detected in any of the negative controls. Further, a ChIP assay using the same antibodies with tissue from E10.5 mouse embryos also

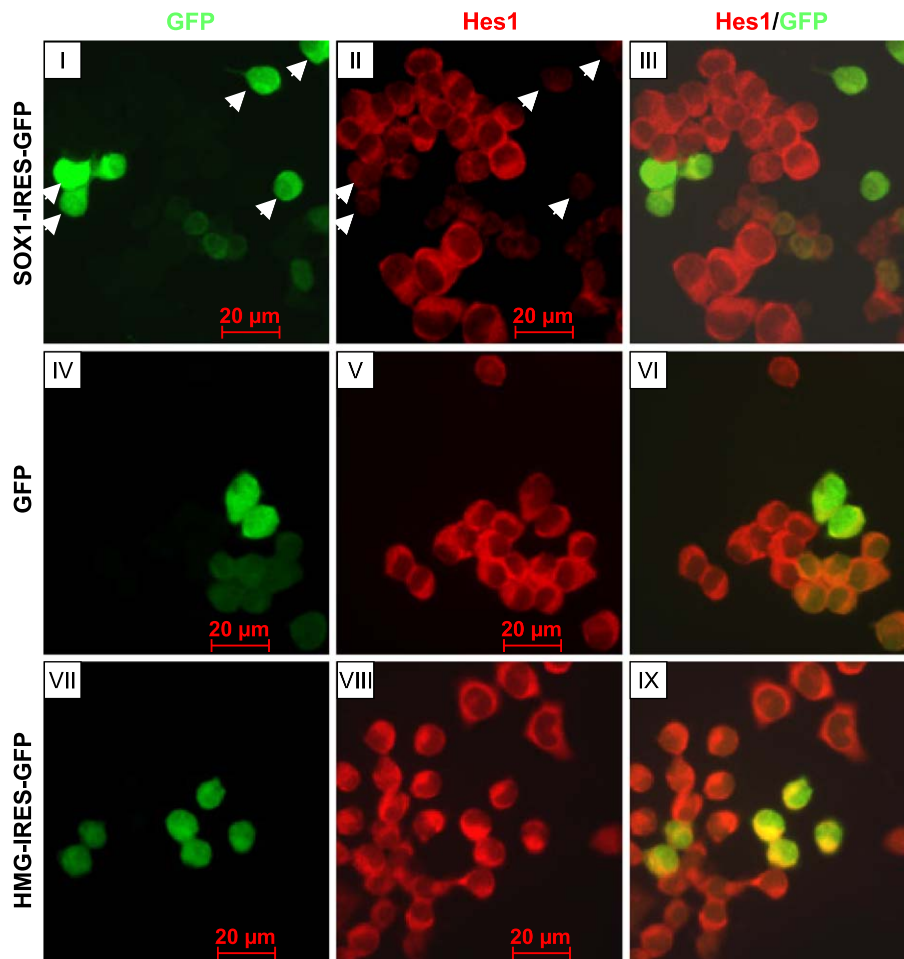


Fig. 3. Sox1 suppresses endogenous expression of Hes1. Neuro-2A cells were transfected with Sox1-IRES-GFP, GFP alone, or the HMG box-IRES-GFP construct and were examined immunohistochemically after 2 days for Hes1 and GFP expression. Hes1 expression was dramatically down-regulated in cells that overexpressed Sox1-IRES-GFP, both in cytoplasm and nucleus (I, II, and III, white arrows indicate Sox1-IRES-GFP-overexpressing cells), whereas Hes1 expression was virtually unchanged in cells that overexpressed GFP only (IV, V, and VI) or HMG-IRES-GFP (VII, VIII, and IX).

detected binding of sox1 to Hes1 promoter region (Fig. 2D). These findings demonstrate that Sox1 interacts directly with the Hes1 promoter in cells.

We next sought to define the domain of Sox1 that is responsible for the suppression of Hes1 expression. To address this issue, we used a luciferase reporter assay to examine the function of four truncated Sox1 expression vectors together with a full-length Sox1 expression vector (Fig. 2E): HMG-IRES-GFP only (flag tagged), ΔNSox1-IRES-GFP (Sox1 without the N-terminus), ΔCSox1-IRES-GFP (Sox1 without the C-terminus), and C-Sox1-IRES-GFP (C-terminal region only). All of the expression vectors expressed specific proteins at similar levels and apparent stability since Western blotting detected similar levels of specific bands after different time points of transient expression (data not shown). Loss of the C-terminus but not loss of the N-terminus significantly impaired the ability of Sox1 to inhibit the Hes1 promoter. However, neither the C-terminus alone nor the HMG box alone was able to suppress the Hes1 promoter. Finally, we examined whether Sox1 suppresses endogenous Hes1 expression in neural lineage cells using Neuro-2A cells, a neural progenitor cell line that expresses moderately high endogenous levels of both Hes1 and ngn1. Cells were transfected with Sox1-IRES-GFP, GFP alone, or the HMG-IRES-GFP and were examined immunocytochemically for expression of Hes1 and of the transgenes using a well-characterized anti-Hes1 antibody (Hirata et al., 2002). This antibody gives a major specific band of about 35KD in Western blotting (Hirata et al., 2002, and data not shown) and specifically immunostains Hes1 in both the cytoplasm and the nucleus (Kabos et al., 2002). Untransfected cells and cells tranfected with either GFP or the HMG-IRES-GFP virtually all expressed Hes1 (Fig. 3). By contrast, more than 80% of cells transfected with Sox1-IRES-GFP were devoid

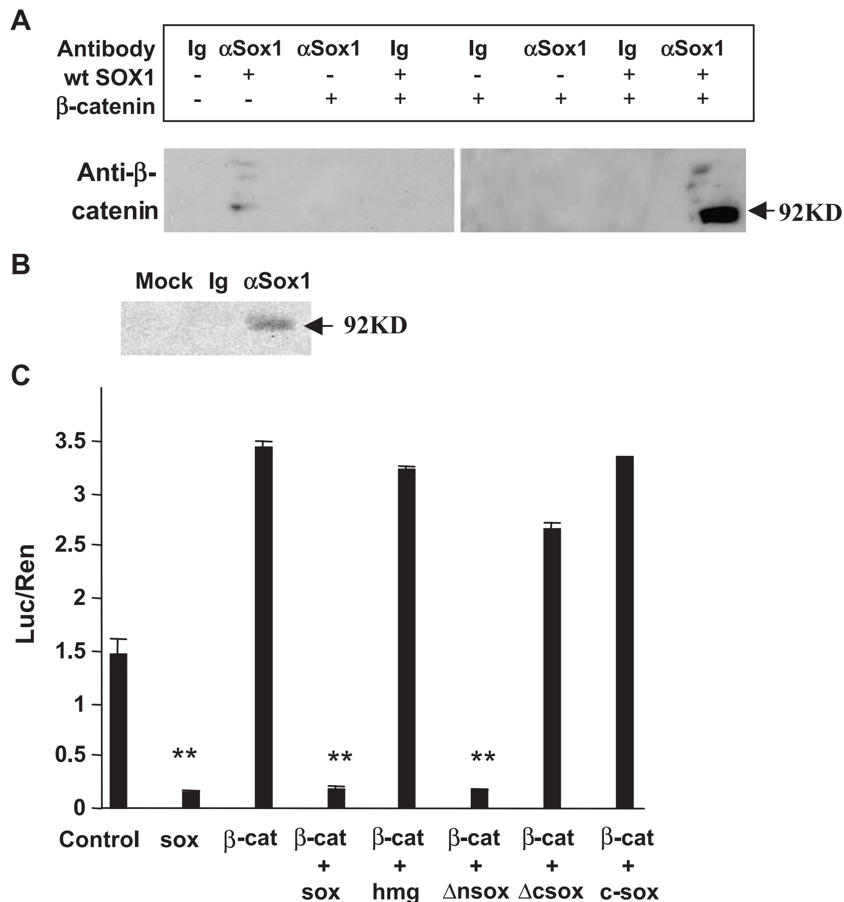


Fig. 4. Sox1 binds to β-catenin and suppresses β-catenin-mediated TCF/LEF signaling. (A) Co-immunoprecipitation demonstrates that Sox1 specifically interacts with β-catenin. HEK293T cells were transfected with empty vector, Sox1, or β-catenin as indicated and were immunoprecipitated with either control immunoglobulin (Ig) or with Sox1 antibody (αSox1). Eluates were resolved on a 12% PAGE gel and were probed with a mouse anti-β-catenin antibody. A specific β-catenin band was detected only in cells that overexpressed Sox1 (with or without β-catenin co-overexpression). (B) Cell lysates from E10.5 embryos were immunoprecipitated with Sox1 antibody, normal rabbit-IgG, or no antibody lanes. Eluates were resolved on a 12% PAGE gel and were probed with a mouse anti-β-catenin antibody. A specific β-catenin band was detected in the Sox1 lane but not in either control lane. (C) TOPFLASH assay indicates that Sox1 suppresses β-catenin-mediated TCF/LEF signaling. HEK293T cells were transfected with truncated or full-length Sox1 with β-catenin, and the TOPFLASH reporter gene and dual-luciferase activities were measured as described above. Note that ΔNSox1-IRES-GFP can suppress β-catenin-mediated TCF/LEF signaling with efficiency comparable to full-length Sox1, whereas HMG-IRES-GFP, ΔCSox1-IRES-GFP, and C-Sox1-IRES-GFP cannot suppress it. **Sox1 lane differs from control at $P < 0.01$ β-cat + Sox1, and β-cat + Δnsox differ from β-cat lanes by ANOVA at $P < 0.01$. Luciferase assays were done at least in duplicate and were repeated three times. Typical results are shown in the figures.

of Hes1 staining in the cytoplasm or the nucleus (white arrows in Figs. 3I and II indicate the typical Sox1-IRES-GFP positive cells) whereas neighboring untransfected cells all displayed Hes1 immunoreactivity. These observations indicate that Sox1 expression suppresses endogenous levels of Hes1 in neuro2A cells. Collectively, these observations indicate that Sox1 binds directly to the Hes1 promoter and down-regulates Hes1 expression, thereby freeing neural progenitor cells from the inhibitory effects of Hes1 on neuronal lineage commitment.

SOX1 binds to β-catenin and suppresses β-catenin-mediated TCF/LEF signaling

The foregoing observations indicated that at least some of the proneural effects of Sox1 may result from suppression of the inhibitory actions of Hes1. We next sought to determine whether Sox1 also regulates neurogenesis through other mechanisms. Two prior studies suggested that other Sox family members may regulate the function of β-catenin (Takash et al., 2001; Zorn et al., 1999), an

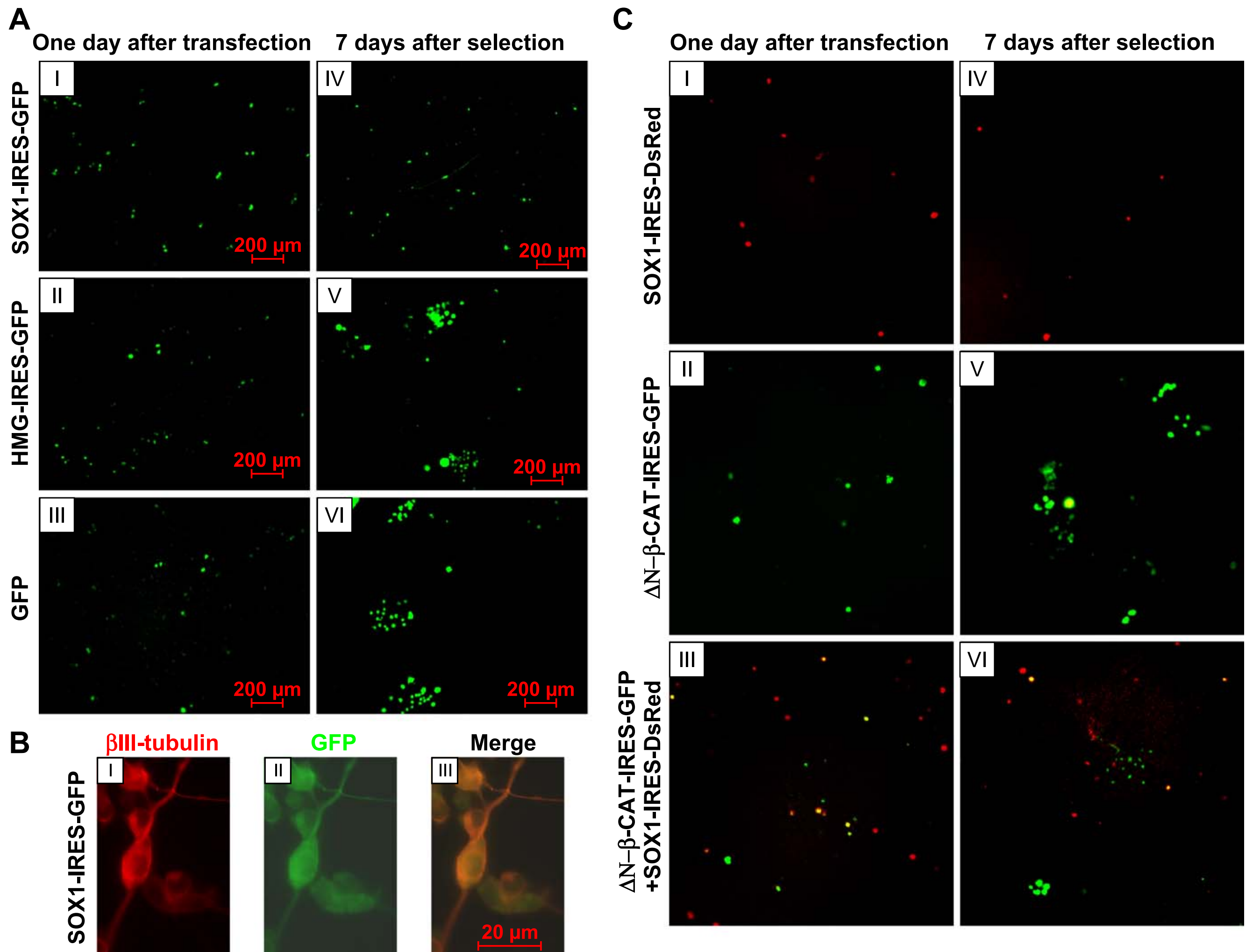


Fig. 5. Neuro-2A cells that overexpress Sox1 do not form colonies and undergo neuronal differentiation instead. (A) Colony formation assay: One day after transfection with the indicated expression vectors, Neuro-2A cells were split to clonal density. The following day, blasticidin (5 ug/ml) was added to the medium and the medium with blasticidin was changed daily. Fluorescent images (low power) were taken 1 day after transfection and 7 days after drug selection. Fluorescent images from 1 day after transfection (I, II, and III) demonstrate that transfection efficiencies were similar for cells overexpressing the different constructs. Fluorescent images taken 7 days after drug selection (IV, V, and VI) demonstrate that cells that overexpressed GFP only (VI) or HMG-IRES-GFP (IV) formed well-defined colonies whereas cells that overexpressed Sox1-IRES-GFP survived but did not form colonies (V). (B) Example of GFP/βIII-tubulin double staining of mature neuron-like cells from a plate of cells overexpressing Sox1-IRES-GFP (I: GFP stain; II: βIII-tubulin; and III: merge). (C) β-catenin signaling could not reverse the effects of Sox1 on cell proliferation. Cells were transfected with Sox1-IRES-Dsred, ΔN-β-CAT-IRES-GFP, or both. Fluorescent images from 1 day after transfection (I, II, and III) demonstrate that transfection efficiencies were similar for cells overexpressing the different constructs. Three types of fluorescent cells were observed in the plates: cells transfected with Dsred-tagged Sox1 (red cells), cells transfected with GFP-tagged ΔN-β-catenin (green cells), and cells transfected with both Dsred-tagged Sox1 and GFP-tagged ΔN-β-catenin (yellow cells). Fluorescent images taken 7 days after drug selection (IV, V, and VI) demonstrate that only green cells (ΔN-β-catenin-GFP positive) formed well-defined colonies whereas red cells (Sox1-IRES-GFP positive) or yellow cells (Sox1-IRES-GFP + ΔN-β-catenin-GFP positive) survived but did not form colonies. See Table 1.

important proneural signaling molecule in the nervous system (see Patapoutian and Richard, 2000). Therefore, to determine whether Sox1 binds to β-catenin expressed in the same cells, lysates of HEK293T cells were immunoprecipitated with an anti-Sox1 antibody and the precipitates were probed by Western analysis with an anti-β-catenin antibody. Since HEK293T cells endogenously express low levels of β-catenin but not Sox1 (data not shown), no band was observed in control cells (Fig. 4). Further, even after transfection of the cells with β-catenin no band was detected in the absence of Sox1. However, after transfection of the cells with Sox1, β-catenin was coprecipitated by the Sox1 antibody (Fig. 4A, lane 2). Moreover, overexpression of β-catenin along with Sox1 greatly enhanced the amount of β-catenin that was co-immunoprecipitated by the Sox1 antibody (Fig. 4A, lane 8), suggesting that Sox1 can bind to β-catenin in vivo. Importantly, using protein lyses from E10.5 mouse embryos, which express both Sox1 and β-catenin, a specific band β-catenin was immunoprecipitated by the Sox1 antibody but not by an irrelevant antibody or in the absence of antibody (Fig. 4B). The effects of this interaction on β-catenin-mediated TCF/LEF signaling were therefore examined using the TOPFLASH reporter assay (Fig. 4C), a well-characterized in vitro system for testing β-catenin-mediated TCF/LEF signaling (Korinek et al., 1997). Expression of β-catenin significantly increased TCF/LEF reporter activity as expected. However, expression of Sox1-IRES-GFP suppressed TCF/LEF reporter activity, and coexpression of Sox1-IRES-GFP and β-catenin not only blocked the stimulatory effects of β-catenin but also significantly reduced activity below control levels, presumably by inhibiting the effects of endogenous β-catenin. Coexpression of the HMG-IRES-GFP along with β-catenin had little effect. However, coexpression of ΔNSox1-IRES-GFP (Sox1 without the N-terminus) along with β-catenin exerted inhibitory effects equivalent to those of full-length Sox1. By contrast, coexpression of ΔCSox1-IRES-GFP (Sox1 without the C-terminus) did not alter the effects of β-catenin, indicating that the C-terminus is necessary for Sox1 to suppress β-catenin-mediated TCF/LEF signaling. Nevertheless coexpression of the C-terminal region alone (C-Sox-IRES-GFP) did not inhibit the effects of β-catenin, indicating that the C-terminus of Sox1 is necessary but not sufficient to suppress β-catenin-mediated TCF/LEF signaling. Coexpression of greatly elevated amounts of β-catenin (4:1 and 8:1) with Sox1 in the TOPFLASH assay partly reversed the Sox1-mediated inhibition of the TOPFLASH reporter (data not shown).

Overexpression of Sox1 inhibits cell proliferation and promotes neuronal differentiation, and the changes are not rescued by β-catenin

β-catenin-mediated TCF/LEF signaling is typically a strong stimulus for cell proliferation. Since Sox1 binds to β-catenin and inhibits TCF/LEF signaling, we sought to determine whether Sox1 expression influences cell cycle. Neuro-2A cells were transfected with Sox1-IRES-GFP, GFP alone, or the HMG-IRES-GFP construct and plated at clonal density, and colony formation assays were performed. At 1 day after transfection, there were no major differences in terms of transfection efficiency or morphology among cells transfected with the different constructs (Figs. 5A, I–III). Note that most GFP-expressing cells were doublets at this time, probably due to one round of division. The cells were then subjected to drug selection so that cells without any constructs would not survive. Daily observations thereafter revealed that cells transfected with Sox1-IRES-GFP ceased proliferating and survived mostly as doublets or singlets, whereas cell transfected with GFP alone or with HMG-IRES-GFP kept proliferating (data not shown). Seven days after selection, the cells transfected with GFP alone or with the HMG-IRES-GFP formed well-defined colonies (or clusters), whereas cells transfected with Sox1 survived as doublets or singlets but did not form colonies (Figs. 5A, IV–VI). More than 90% of the Sox1-IRES-GFP-transfected cells expressed βIII-tubulin, whereas less than 1% of control cells overexpressing GFP alone or HMG-IRES-GFP expressed βIII-tubulin (data not shown). Further, about 10% of the Sox1-IRES-GFP-transfected cells extended long processes and adopted a neuronlike morphology (Figs. 5B, I–III), whereas only rare such cells were detected in the GFP alone or HMG-IRES-GFP groups. The number of GFP^+ cells in the Sox1-IRES-GFP group remained virtually unchanged during the 7 days of selection, whereas GFP^+ cell numbers in control groups increased significantly (data not shown), consistent with the lack of proliferation of Sox1-IRES-GFP-transfected cells demonstrated by the colony forming assay. However, it remained possible that the differences in GFP^+ cell numbers represented differences in cell adhesion among the groups of cells, that is, Sox1-IRES-GFP cells could have proliferated but were detached from the plates for some reason and were washed away during medium changes. To test this possibility, a semisolid medi-

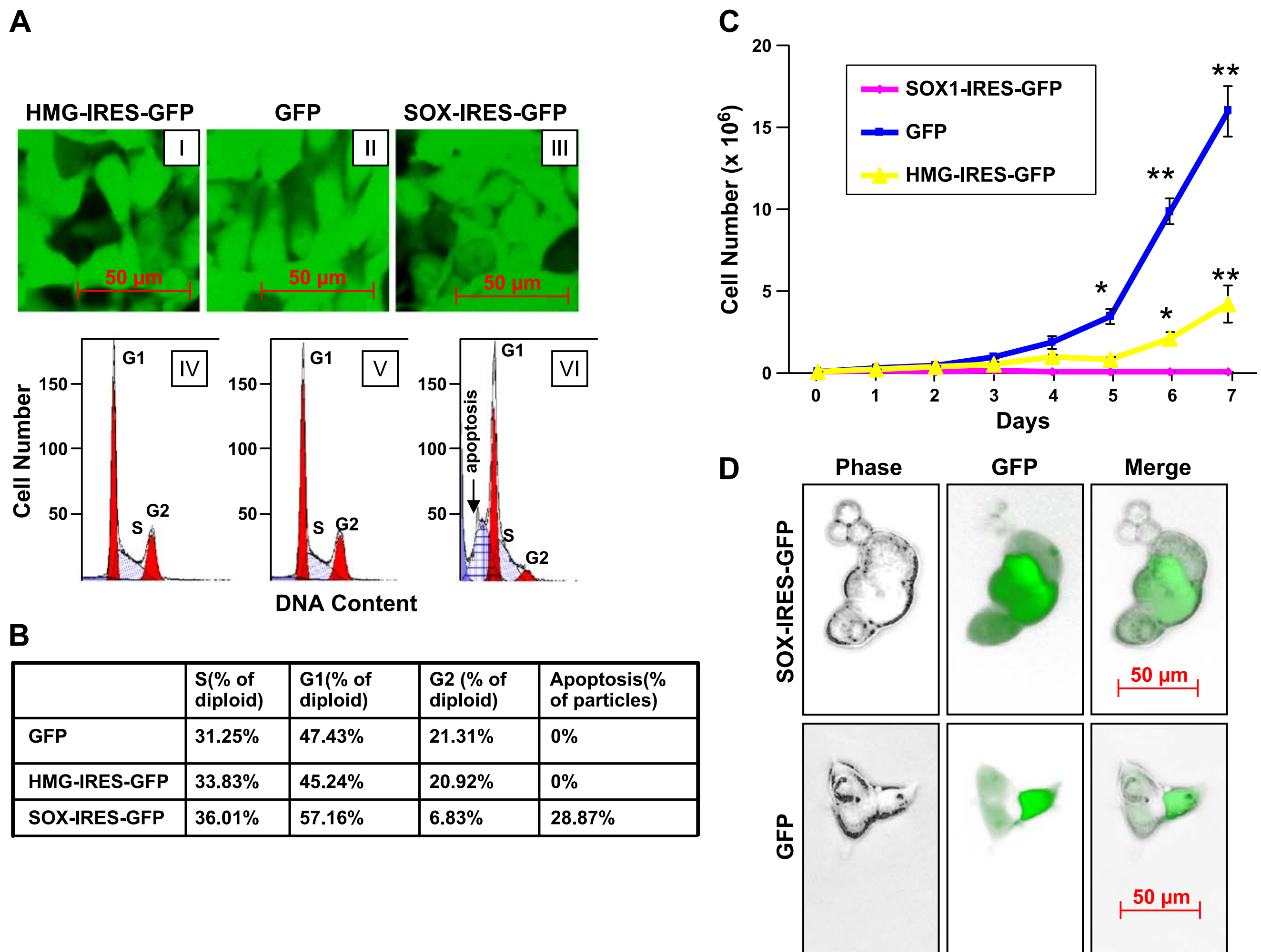
Table 1
Clonogenic activities of different constructs after 7 days of selection (total clones from 20 random low-power fields)

	Sox1-IRESdsRed	β-CAT-IRES-GFP	Sox1-IRES-dsRed + β-CAT-IRES-GFP
Red clones (dsRed)	0	0	0
Yellow clones (dsRed + GFP)	0	0	0
Green clones (GFP)	0	55	47

Quantification of the clonogenic activities of the different constructs showed in Fig. 5C. Clusters of cells containing at least five cells were counted from 20 total random low power (5×) fields. Only cells overexpressing ΔN-β-CAT-IRES-GFP were able to form well-defined clones (green clones). Neither cells overexpressing Sox1-IRES-DsRed (red cells) nor cells overexpressing Sox1-IRES-DsRed + ΔN-β-CAT-IRES-GFP (yellow cells) formed well-defined clones.

um colony formation assay was performed so that the cells were confined to a small area and could not detach and/or be washed away. Similar results were obtained supporting the conclusion that Sox1 promotes exit from cell cycle and cellular differentiation (data not shown). The fact that GFP$^+$ cell numbers in the Sox1-IRES-GFP group remained virtually unchanged and did not decrease significantly during the 7-day selection period argues against the possibility of cell death as the cause of the differences in GFP$^+$ cell numbers among groups.

To determine whether β-catenin signaling could reverse the effects of Sox1 on cell proliferation, Neuro-2A cells were cotransfected with Dsred-tagged Sox1 and GFP-tagged ΔN-β-catenin. One day after transfection, four types of cells were observed in the plates: cells transfected with Dsred-tagged Sox1 (red cells), cells transfected with GFP-tagged ΔN-β-catenin (green cells), cells transfected with both Dsred-tagged Sox1 and GFP-tagged ΔN-β-catenin (yellow cells), and cells without any of these constructs (negative). Daily observations thereafter found that red and yellow cells stopped proliferating while green cells kept proliferating. Seven days later, only the green cells formed well-defined clones (Fig. 5C and Table 1). Thus, enhanced β-catenin signaling was unable to overcome the effects of Sox1 on cell



	S(% of diploid)	G1(% of diploid)	G2 (% of diploid)	Apoptosis(% of particles)
GFP	31.25%	47.43%	21.31%	0%
HMG-IRES-GFP	33.83%	45.24%	20.92%	0%
SOX-IRES-GFP	36.01%	57.16%	6.83%	28.87%

Fig. 6. Constitutive overexpression of Sox1 in HEK293T cells promotes exit from cell cycle. (A) Over 80% of the cells were GFP positive after transduction with HMG-IRES-GFP, GFP only, or Sox1-IRES-GFP for 1 day (I, II, and III). Note that cells overexpressing different constructs have very similar morphologies. Cells overexpressing HMG-IRES-GFP (IV) had a similar cell cycle profile to the cells overexpressing GFP only (V). By contrast, the cells overexpressing Sox1-IRES-GFP (VI) had a marked reduction in the number of cells in G2 and an increase in apparently apoptotic cells. (B) Quantitative analysis of the cell cycle distributions in A. (C) Growth curve analysis supports the hypothesis that constitutive overexpression of Sox1 has an antiproliferative effect. Cells overexpressing GFP (blue line) proliferated normally (cell numbers doubled daily), while cells overexpressing HMG-IRES-GFP (yellow line) also proliferated but at a reduced rate. By contrast, the number of cells overexpressing Sox1-IRES-GFP (red line) remained virtually unchanged over 7 days. *Differs from cells overexpressing Sox1-IRES-GFP by ANOVA at $P < 0.05$; **differs from cells overexpressing Sox1-IRES-GFP by ANOVA at $P < 0.01$. (D) Overexpression of Sox1 for prolonged times leads to morphological changes. HEK293T cells that constitutively overexpress Sox1-IRES-GFP for more than 4 days became larger and rounder, and some cells show signs of apoptosis. Upper panel shows the typical morphology of long-term overexpressing cells (phase, GFP, and merge, respectively), while the lower panel shows the typical morphology of HEK293T cells overexpressing GFP only in the same time point (phase, GFP, and merge, respectively).

proliferation, suggesting that suppression of β-catenin-mediated TCF/LEF signaling may not be the only way that Sox1 regulates cell cycle.

Sox1 promotes exit from cell cycle

To confirm and further define the effects of Sox1 on cell cycle, a different cell line, HEK293T cells, was used to create cell lines overexpressing Sox1-IRES-GFP, GFP alone, or the HMG-IRES-GFP construct. The advantage of using this cell line is that we were able to have cultures with more than 80% positive cells, thus minimizing the effects of nontransduced cells on the cell cycle analysis. Since the Sox1-IRES-GFP-overexpressing cells could not survive in the long term, we transduced the cells and drug selected them for 7 days, and the selected cells were then immediately subjected to cell cycle analysis using an automated flow cytometer (Fig. 6). The cell cycle profiles of cells overexpressing GFP alone or the HMG-IRES-GFP were similar (Fig 6A). By contrast, the profile of cells overexpressing Sox1-IRES-GFP was significantly different with a small increase in the percentage of cells in G1 and a very large decrease in the percentage of cells in G2 (Figs. 6A and B). This indicates that cells overexpressing Sox1-IRES-GFP remained in G1 and were blocked from entering the G2 phase. There was also an increase in apoptosis of cells overexpressing Sox1-IRES-GFP, whereas no apoptotic cells were detected in the GFP-alone or the HMG-IRES-GFP groups (Figs. 6A and B).

Growth curve analyses were then performed with the same cells (Fig. 6C). The GFP-overexpressing cells proliferated normally whereas the Sox1-IRES-GFP-overexpressing cells did not proliferate at all and cell numbers actually decreased, though not significantly, after 4 days in culture, consistent with the previous cell cycle analysis. The cells that overexpressed the HMG-IRES-GFP proliferated but at a slower rate than the control. This may indicate that overexpression of the HMG box alone has some subtle effects, which the cell cycle analysis was not sensitive enough to detect. Morphologic analysis of the cell lines indicated that the Sox1-IRES-GFP-overexpressing cells enlarged in size and became more ovoid with time in culture, but significant morphologic changes were detectable only after 3–4 days in culture, indicating that this may reflect an indirect secondary effect (Fig. 6D). There were also some cells that appeared to

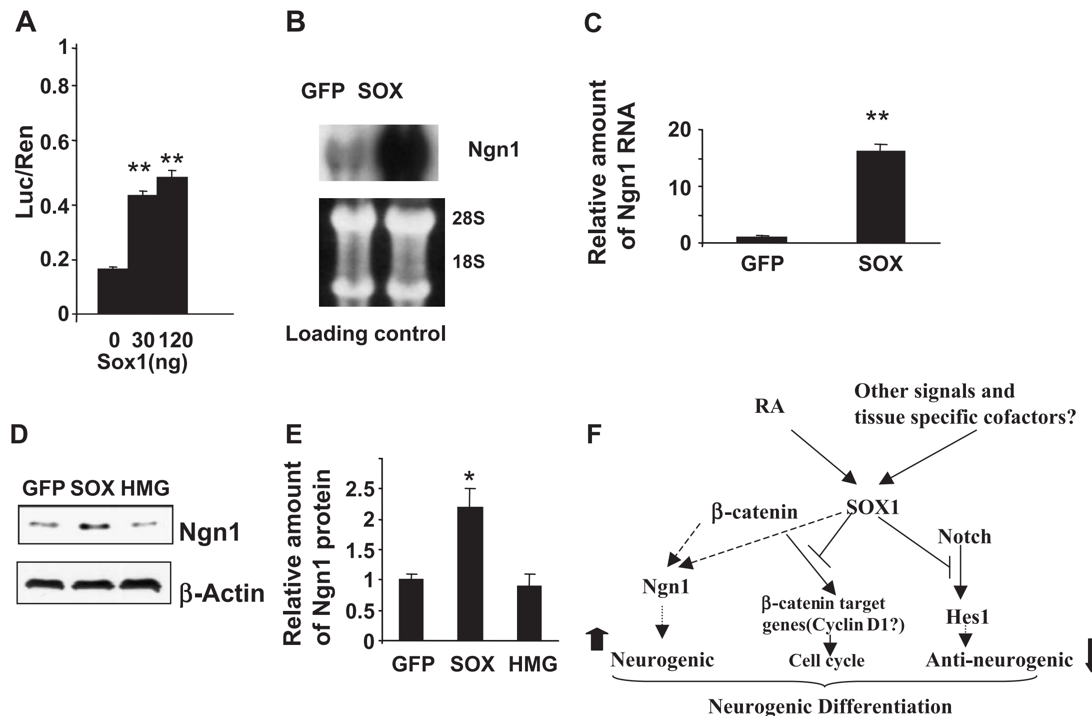


Fig. 7. Sox1 activates expression of neurogenin1. (A) Luciferase reporter assays using the neurogenin1 promoter demonstrated that Sox1 up-regulates ngn1 promoter activity in a dose-dependent way. P19 cells were transfected with different dosages of Sox1 along with the ngn1 promoter reporter, and dual-luciferase activities were measured as described above. **Differs from control by ANOVA at $P < 0.01$. (B) Sox1 up-regulates endogenous ngn1 mRNA expression. Northern blot analysis of total RNA extracted from Neuro-2A cells transiently overexpressing GFP only or Sox1-IRES-GFP indicates that endogenous ngn1 mRNA expression is up-regulated in the Sox1 overexpressing cells. The lower panel is an RNA loading control. (C) Quantitative analysis of the relative amount of ngn1 mRNA. **Differs from control by Student's *t* test at $P < 0.01$. (D) Sox1 up-regulates endogenous ngn1 protein. Western blot analysis of endogenous ngn1 protein expression in Neuro-2A cells transiently overexpressing GFP, Sox1-IRES-GFP, or HMG-IRES-GFP, respectively, demonstrates that Sox1 up-regulates levels of endogenous ngn1 protein. β-actin served as the loading control. (E) Quantitative analysis of the relative amount of ngn1 protein. *Differs from control by ANOVA at $P < 0.05$. (F) Working model of Sox1 functions on neurogenesis. Direct effects are shown as solid lines, unknown, or indirect effects as dashed lines. See text for detailed descriptions.

be undergoing apoptosis, consistent with the cell cycle analysis.

Sox1 activates transcription of the proneural gene neurogenin1

Although Sox1 expression inhibited β-catenin-mediated TCF/LEF signaling and Sox1 and β-catenin also appear to exert opposing effects on cell proliferation, both reportedly exert proneural effects (Israsena et al., in press; Patapoutian and Richard, 2000; Pevny et al., 1998). The ability of Sox1 to drive the promoter of the proneural transcription factor neurogenin1 (ngn1) was therefore examined using a luciferase reporter assay. The ngn1-luc construct used in this experiment contained about 1.6 kb genomic DNA from the predicted mouse ngn1 promoter region (Israsena et al., in press). Expression of Sox1 up-regulated ngn1 promoter activity in a dose-dependent manner (Fig. 7A). To determine whether Sox1 actually promotes expression of endogenous ngn1 in neural-derived cells, Neuro-2a cells were transfected with Sox1-IRES-GFP, GFP, or the HMG-IRES-GFP, and the cells were examined for levels of ngn1 mRNA (Figs. 7B and C) and protein (Figs. 7D and E) 1 day later. Overexpression of Sox1-IRES-GFP significantly increased levels of ngn1 mRNA about 16-fold and more than doubled levels of ngn1 protein, consistent with the proneural effects of Sox1. Thus, the findings with the luciferase reporter that Sox1 activates transcription of ngn1 were substantiated by both Northern (Figs. 7B and C) and Western blotting (Figs. 7D and E).

Discussion

Our current studies provide evidence that Sox1 may regulate neurogenesis through several different mechanisms (Fig. 7F). Once activated, Sox1 (1) binds directly to the Hes1 promoter and suppresses Hes1 transcription, thus attenuating Notch signaling; (2) binds to β-catenin and suppresses β-catenin-mediated TCF/LEF signaling, thus potentially attenuating the wnt signaling pathway; (3) promotes exit of cells from cell cycle; and (4) up-regulates transcription of the proneural bHLH transcription factor neurogenin1. The net effects of Sox1 signaling are neuronal lineage commitment and exit of the cell from cell cycle.

Sox1 is abundantly expressed in vivo by ventricular zone progenitor cells during the period of neurogenesis, and the temporal and spatial patterns of expression overlap with Notch and Wnt (β-catenin) expression. This suggests that Sox1 is normally involved in neurogenesis in vivo. This conclusion is supported by the observation that Sox1 null mutant mice exhibit a severe loss of neurons in ventral striatal structures, the olfactory tubercle and the nucleus accumbens shell, and that adult mice suffer from spontaneous seizures. (Malas et al., 2003). Further experiments with ectopic expression of Sox1 in mice and the analysis of Sox1-null mice harboring Sox1 expressed only in precursors and not postmitotic neurons indicate that Sox1 is both necessary and sufficient for neuronal differentiation in the ventral telencephalon (Dr. Vasso Episkopou, personal communication). Our results are also consistent with observations that overexpression of Sox1 is sufficient to promote neural differentiation of the P19 cell line (Pevny et al., 1998). However, we found that other SoxB1 family members (Sox2 and Sox3) that are expressed in the developing nervous system do not promote neurogenesis and that Sox2 actually inhibited neuronal lineage commitment. These findings are consistent with recent reports indicating that Sox2 and Sox3 expression may help to maintain neural progenitor cell identity by inhibiting neurogenesis (Bylund et al., 2003; Graham et al., 2003). We do not agree, however, with the extension to Sox1 in one of these reports (Bylund et al., 2003) of their experimental findings with Sox2 and Sox3. The apparent inconsistencies between Bylund et al. (2003) and our findings could be explained in several ways. First, Bylund et al. (2003) never actually presented the data of Sox1 functional analysis, and the extension of their findings with Sox2 and Sox3 to Sox1 may be unwarranted. Our observations that these factors may have divergent effects on neuronal lineage commitment (Fig. 1) suggest that each member of the SoxB1 family may have specific nonredundant functions in the developing CNS. This conclusion is consistent with the different but overlapping temporal and spatial patterns of expression of the different Sox1B family members and it is also consistent with the different phenotypes of null mutant mice (Avilion et al., 2003; Collignon et al., 1996; Nishiguchi et al., 1998; Parsons, 1997). Sox2 and Sox3 begin to be expressed at preimplantation and epiblast stages, respectively, and later become restricted to the neuroepithelium (Collignon et al., 1996; Wood and Episkopou, 1999). Targeted deletion of Sox2 leads to death before implantation (Collignon et al., 1996), and chimeric mice generated with Sox3 null ES cells display severe abnormalities during gastrulation and posterior truncations (Parsons, 1997). By contrast, Sox1 is expressed later in development coincident with formation of the neural plate (Collignon et al., 1996; Pevny et al., 1998; Wood and Episkopou, 1999). After neural induction, Sox1 expression is confined to neural precursors along the entire anteroposterior axis of the developing embryo and subsequently to adult neural stem cells.

Sox family proteins have been shown to function as cosuppressors as well as coactivators depending upon the cellular context (see Wilson and Koopman, 2002). Thus, the effects of Sox1 may be context dependent, and the discrepancies between our findings and those of Bylund et al. (2003) could also reflect differences in the ways that Sox function was examined. However, the observation in Sox1 mutant mice that Sox1 is both necessary and sufficient for neuron differentiation in the ventral telencephalon suggests that our observations reflect the function normally served by Sox1 in vivo.

The suppressive effects of Sox1 on Hes1 expression differ from the findings of Buescher et al. (2002) for the putative SoxB1 homologues in *Drosophila*, Dichaete, and SoxNeuro, which reportedly do not antagonize Notch signaling. This suggests either that Dichaete and SoxNeuro are not precise homologues of the SoxB1 family or that the murine proteins act through different mechanisms. Mutations of *Drosophila* SoxNeuro lead to defects in the specification and differentiation of midline and lateral neural cells, and SoxNeuro or Dichaete double mutants have severe hypoplasia of the entire central nervous system (Buescher et al., 2002; Overton et al., 2002). Regardless of the reasons for the species differences, the presence of the consensus Sox binding site on the mouse Hes1 promoter, the direct demonstration that Sox1 binds to the Hes1 promoter, and the inhibition of Notch signaling in cells that overexpress Sox1 strongly support the biologic importance of this regulatory mechanism.

Our findings regarding interactions of Sox1 with β-catenin are consistent with prior observations that Sox17 physically interacts with β-catenin (Zorn et al., 1999) and that Sox7 as well as Sox17 inhibits wnt signaling (Takash et al., 2001). The interaction of Sox1 with β-catenin depends upon the C-terminus rather than the HMG box, indicating that different domains of the Sox1 molecule may mediate different regulatory functions during mouse development. β-catenin is a potent signal for maintaining neural progenitor cells in a proliferative state (Chen and Walsh, 2002), and interactions between the Sox proteins and β-catenin may be important for maintaining a balance between proliferation and differentiation of neural progenitor cells. Our conclusions regarding the effects of Sox1 on cell proliferation differ somewhat from the conclusions of Pevny et al. (1998). We both find that Sox1 is a potent neurogenic factor that is expressed by dividing progenitor cells and that Sox1 expression is down-regulated concurrent to exit from cell cycle and commitment to the neuronal lineage. However, our findings suggest that Sox1 expression may be part of an in vivo feedback mechanism for the control of cell number in the CNS, that is, that Sox1 up-regulation is the result of proliferation of neural progenitors rather than the cause. Our finding that Sox1-transfected cells appear to undergo one additional round of division before exiting cell cycle and committing to the neuronal phenotype would be consistent with such a role. Moreover, although Sox1 is down-regulated in most postmitotic neurons, it is still found in ventral striatal structures, the olfactory tubercle, and the nucleus accumbens shell (Malas et al., 2003). Sox1 is also expressed in scattered neurons in other adult brain regions (unpublished observations).

In addition to the effects of Sox1 in blocking the inhibitory effects of Notch signaling, Sox1 promotes expression of the proneural gene neurogenin1. Although the neurogenin1 promoter has a Sox consensus binding site, it is not clear that there is a direct effect of Sox1 on the neurogenin gene. Chromatin precipitation assays failed to identify a direct interaction between Sox1 and the ngn1 promoter, and gel shift assays using the potential Sox1 binding site in the promoter region of ngn1 also failed to demonstrate an interaction (data not shown). This suggests that Sox1 could regulate ngn1 promoter activity through indirect mechanisms. Regardless of whether the effects on neurogenin1 are direct or indirect, it is apparent that Sox1 expression may promote neuronal lineage commitment through multiple pathways.

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