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Supplemental Data

In Vivo Fate Analysis Reveals the Multipotent and Self-Renewal Capacities of *Sox2*$^+$ Neural Stem Cells in the Adult Hippocampus

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Supplemental Results

Establishment of a lentivirus-meditated fate mapping system *in vivo*

To directly test the differentiation potential of *Sox2*$^+$ cells in the adult hippocampus, fate mapping via lentivirus was designed. A *Sox2* promoter containing 2.7 kb of proximal promoter and 0.4 kb enhancer that confers *Sox2* expression in CNS (Ferri et al., 2004; Uchikawa et al., 2003; Zappone et al., 2000) was cloned to drive GFP-fused CRE recombinase in *Sox2*$^+$ cells (Figure 3S-A). The fidelity of the promoter and CRE recombinase was thoroughly tested.

Lentivirus successfully transduced GFP expression in the mouse NSCs but not fibroblasts (FB), consistent with a specific expression of *Sox2* mRNA in the NSCs (Figure 3S-B). FACS analysis further showed GFP transduction in the NSCs, although a weak GFP expression was detected in a minor population (2%) of infected fibroblasts (Figure 3S-C). CMV promoter-driven lentivirus was used to demonstrate that both NSCs and fibroblasts were subject to lentiviral transduction (Figure 3S-C).

Lentivirus was also injected into the dentate gyrus of ROSA26 reporter mice to examine the specific viral transduction in *Sox2*$^+$ cells and the functionality of CRE

recombinase. CRE recombinase recognizes two loxP sites and excises intervening “stop” sequences, which results in activation of a β–gal reporter gene. Since CRE-mediated recombination occurs at the genomic level, targeted cells as well as their progeny can be permanently labeled and traced. GFP^+ cells were restricted to the SGZ, recapitulating endogenous *Sox2*-expression in the SGZ, whereas $\beta\text{–gal}^+$ progeny expanded throughout the granular layer when animals were examined 3 months after virus injection (Figure 3S-D). This finding is consistent with our expectation of the continuous generation of new neurons from the targeted $Sox2^+$ cells and migration of neurons to the granular layer.

Finally, IHC with SOX2 antibody was used to confirm specific transduction of lentivirus in $Sox2^+$ cells. When this lentivirus was transduced in C57BL/6 mice analyzed 7 days later, 70% of the GFP^+ cells (indicative of targeted cells) co-localized with SOX2, confirming targeting in $Sox2^+$ cells (Figure 3S-E). However, this system showed some off-targets only in differentiated neurons ($NeuN^+$, 26±3%, n=800), presumably due to the integration sites of virus to a host genome. We did not observe any GFP expression in other cells that have the potential to proliferate, including DCX^+ or PSA-$nCAM^+$ cells in C57BL/6 mice. Since our lentivirus had unspecific expression in the differentiated neurons, we included the BrdU paradigm to trace the fate of $Sox2^+$ cells that only underwent cell division and to rule out following non-dividing, unspecifically targeted cells (see main text).

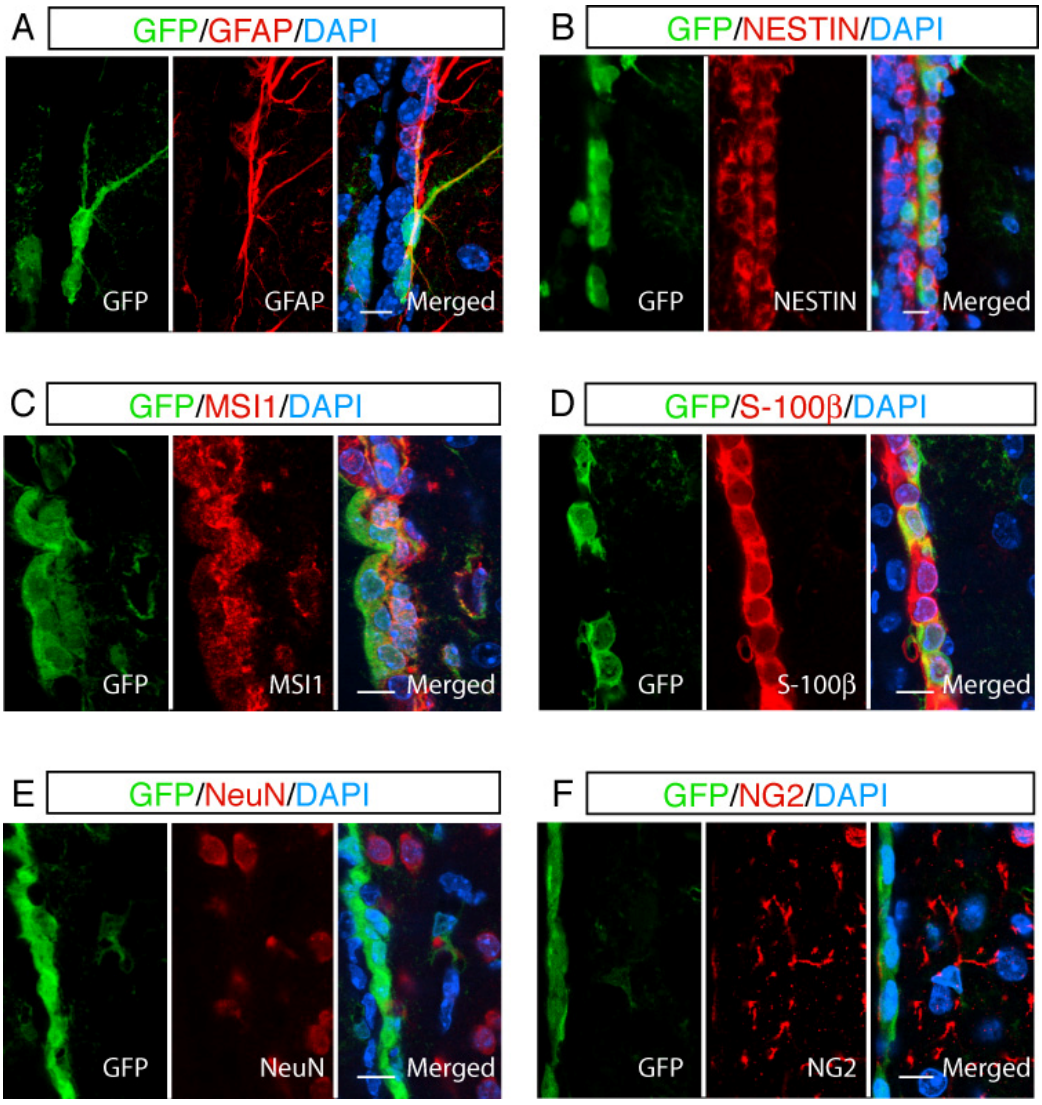

Figure S1. *Sox2*-GFP cells in the SVZ

Double IHC with cell-type specific markers showed that *Sox2*-GFP cells co-localized with undifferentiated cell markers such as GFAP (A), NESTIN (B) and MUSASHI-1 (C) in the SVZ. *Sox2*-GFP cells express S-100β, a marker for ependymal cells, which have also been suggested to be NSCs in the SVZ (D) (Johansson et al., 1999). However, *Sox2*-GFP cells did not co-localize with differentiated markers, including NEUN (E) and NG2 (F).

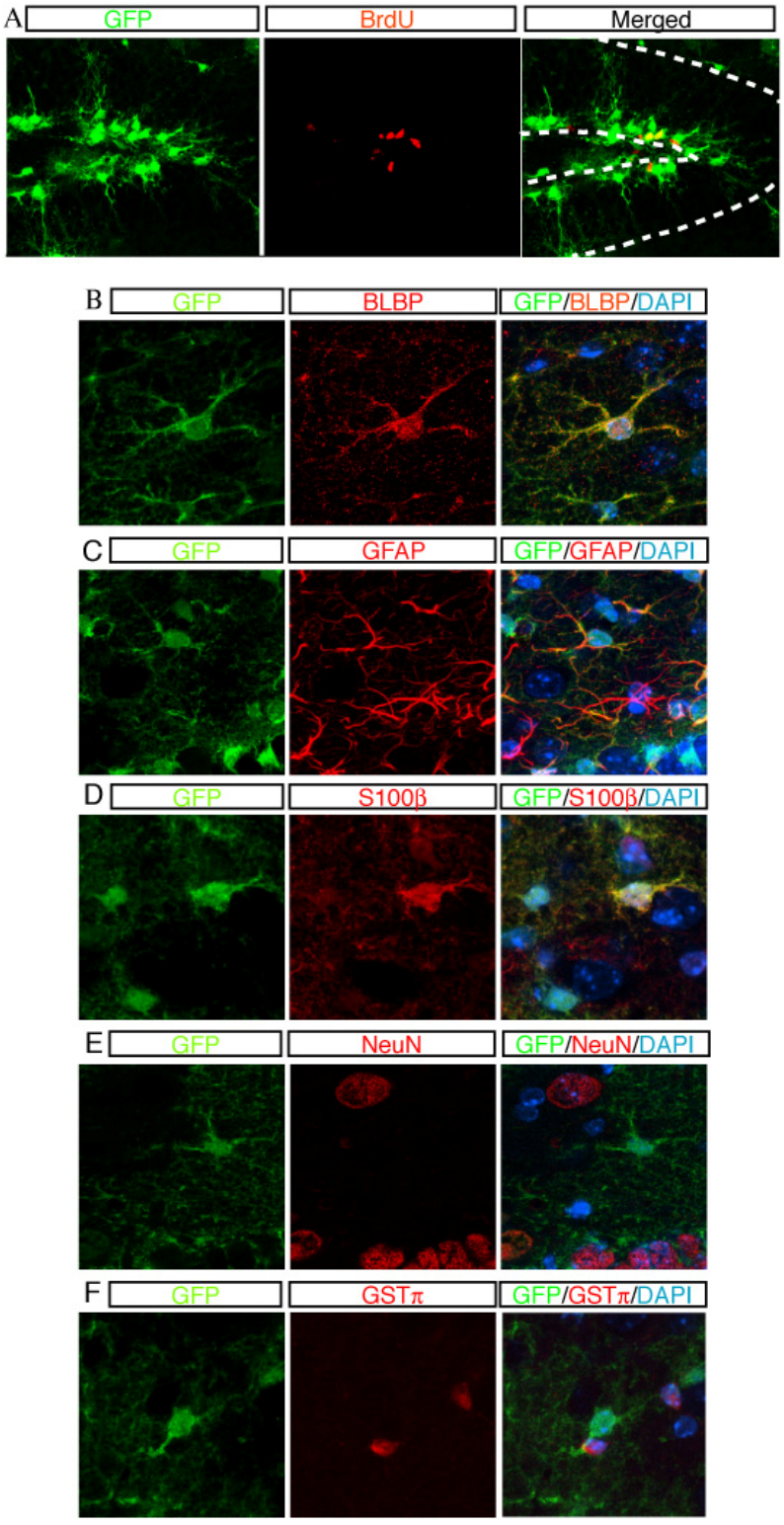

Figure S2. Dividing *Sox2*-GFP cells and *Sox2*-GFP cells in the hilus

Among two morphologically distinct *Sox2*-GFP cells, only non-radial *Sox2*-GFP cells are positive for BrdU (A). *Sox2*-GFP cells in the hilus showed the expressions of BLBP (B), GFAP (C), and S-100β (D). However, *Sox2*-GFP cells did not co-localize with NEUN (E) and GST-π (F), indicating that they are not differentiated neurons or oligodendrocytes, respectively. These results suggested that *Sox2*-GFP cells in the hilus appeared to show characteristics of astrocytes based upon marker expression and morphology.

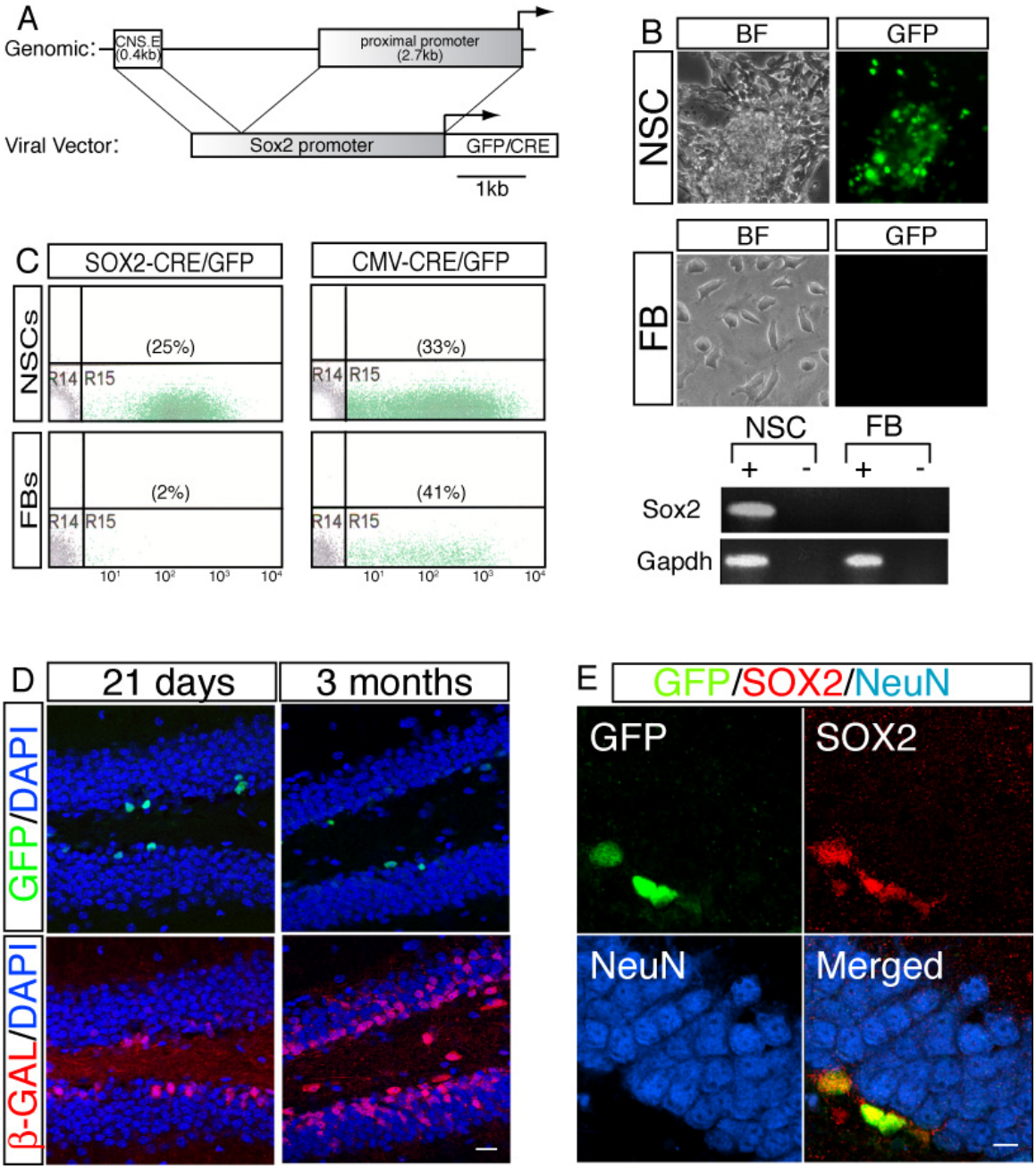

Figure S3. Generation of lentiviral-vector for fate mapping of *Sox2*$^{+}$ cells in the SGZ

A murine *Sox2* promoter containing 2.7 kb proximal promoter and 0.4 kb CNS enhancer (CNS.E) was cloned to express GFP and CRE fusion protein (A). Consistent with endogenous *Sox2* mRNA in NSCs, GFP was transduced only in NSCs but not in fibroblasts (FBs) (B). FACS confirmed specific transduction of GFP in the NSCs (C).

Note that the GFP expression with lower intensity was found only in 2% of FB. CMV promoter was used to show that both NSCs and fibroblasts (FBs) were subject to lentiviral transduction (C). Lentivirus carrying *Sox2*-GFP/CRE was injected into the dentate gyrus of ROSA26R mice. GFP^+ cells were mainly restricted in the SGZ, but their progeny labeled with β-gal reporter expanded into the granular layer when examined 3 months after transduction (D). Specificity of lentivirus was confirmed by examining GFP expression in SOX2-positive cells (E). Scale bars: 20 μm (D) or 10 μm (E).

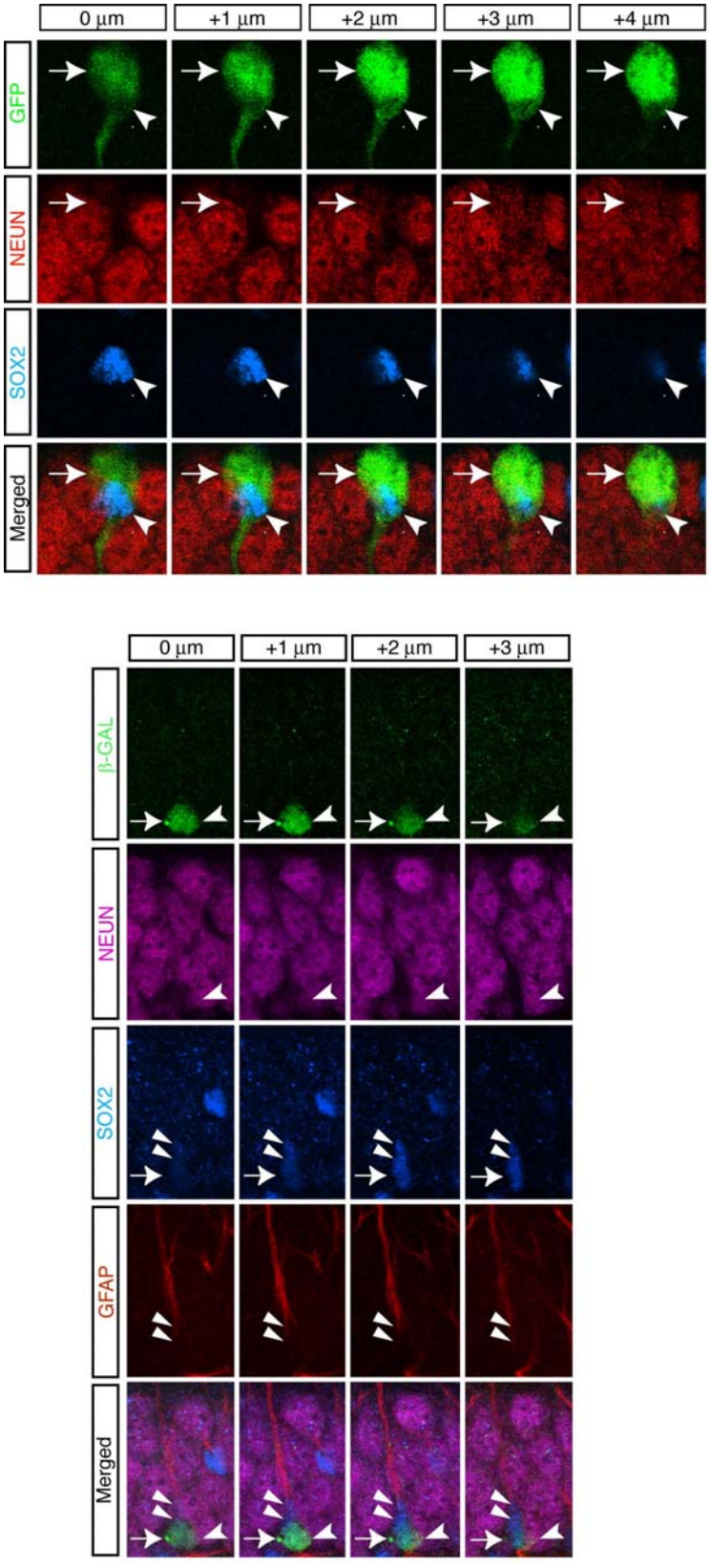

Figure S4. Sequential images of multi-cell clones

Sequential z-series images of the clone containing a *Sox2*$^{+}$cell and a differentiated neuron (NeuN^{+}) demonstrate the multipotency of *Sox2*$^{+}$ NSC in the SGZ. Arrows and arrowheads are pointing to a neuron and a *Sox2*$^{+}$ cell, respectively (A). Sequential images of z-series showed that a targeted *Sox2*$^{+}$ NSC gave rise to a differentiated neuron (NeuN^{+}, right arrowhead) as well as one *Sox2*$^{+}$cell (SOX2^{+}, arrow) that is also positive for GFAP (GFAP^{+}, 2 arrowheads) in the radial process (B).

Supplemental Experimental Procedures

Immunohistochemistry

Immunohistochemistry (IHC) was performed with floating sections as previously described (Lie et al., 2002). Brain sections containing the hippocampus were prepared from 6- to 9-week-old transgenic mice. Brains were isolated after trans-cardiac perfusion with 4% paraformaldehyde (PFA, Sigma) in phosphate buffered saline (PBS) and further post-fixed in 4% PFA for 6 hours to overnight at 4°C. 40-μm coronal sections were prepared using a sliding microtome after cryo-protection in 30% sucrose.

Primary antibodies (Lie et al., 2002; Steiner et al., 2006) are listed as follows: NESTIN (mouse, 1/200, Pharmingen), DOUBLECORTIN (DCX, goat, 1/500; Santa Cruz Biotechnologies), NEUN (mouse, 1/100, kindly provided by Dr. R. Mullen, University of Utah, Salt Lake City, UT), TuJ1 (rabbit, 1/200, Covance), MAP2ab (mouse, 1/200, Sigma), GFAP (rabbit,1/2000, Dako; guinea pig, 1/1000, Advanced Immuno), S-100β (rabbit, 1/500, Swant), SOX2 (rabbit, 1/200, Chemicon; goat, 1/200, Santa Cruz), PROX1(rabbit, 1/500, Chemicon), NG2 (rabbit, 1/500, Chemicon), GST-π (mouse, 1/200, BD sciences) (Hsieh et al., 2004), BrdU (rat, 1/200, Accurate Chemicals), Ki67 (rabbit, 1/500, Novocastra), β-galactosidase (rabbit, 1/500, 5 Prime-3 Prime; goat, 1/500, Biogenesis), GFP (rabbit, 1/1000, Molecular Probes; chick, 1/500, Aves Labs), BLBP (rabbit, 1/2000, a kind gift from Dr. Nathaniel Heintz, Rockefeller University, New York, NY) (Anthony et al., 2004), and MUSASHI-1 (MSI-1, rat, 1/1000, a kind gift from Dr. Okano Hideyuki, Keio University, Japan) (Kaneko et al., 2000; Sakakibara and Okano, 1997). FITC-, Cy3- or Cy5-conjugate secondary antibodies were used (Jackson ImmnoResearch) followed by counterstaining with DAPI (10 μg/ml, Sigma).

The sequential IHC was performed for double or triple staining with BrdU antibody. After the first IHC (with antibodies except BrdU), tissues were fixed in 4% PFA for 15 minutes to stabilize antigen-antibody complex. To retrieve BrdU antigen, sections were treated with 2 N HCl for 30 minutes in a 37°C water bath followed by neutralization with 0.1 M boric acid for 10 minutes. IHC with BrdU was carried out subsequently.

All images from the stained brain sections were captured using a confocal microscope (Bio-rad) and processed for stacking of z-series images and 3D representation by using Metamorph (Molecular Devices) and Photoshop (Adobe). Images from cultured cells were taken by SPOT-CCD camera system (Diagnostic Instrument Inc.).

In situ hybridization

In situ hybridization to detect mRNA of *Sox2* was carried out following the standard method, and the detailed procedures are described elsewhere (Suh et al., 2002). Briefly, an 800 base-pair (bp) *Sox2* probe spanning the open reading frame and 3' untranslated region (UTR) was cloned into pBluescript vector (Stratagene). A Digoxigenin (Roche Applied Science)-labeled antisense probe was generated by *in vitro* transcription (Promega). Adult mouse brain was frozen in 2-methylbutane at –30°C and then 12-μm sections were cut by cryostat. The hybridization was performed at 60°C overnight.

Preparation, culture and differentiation of *Sox2*-GFP NSCs

Mouse NSCs from the adult hippocampus were prepared as described with a minor modification (Ray and Gage, 2006). Briefly, 4 pairs of hippocampi from transgenic mice were isolated and digested in the enzyme mixture containing papain (2.5 U/ml, Worthington), pronage (1U/ml, Roche) and DNase (250 U/ml, Worthington). Cells were plated in the presence of FGF2 (Fibroblast Growth Factor, 20 ng/ml; Peprotech), EGF (Epidermal Growth Factor, 20 ng/ml; Peprotech) and heparin (5 μg/ml; Sigma) in DMEM/F12 (Omega Scientific Inc) basal media supplemented with N2 (Life Technologies) (hereafter referred to as the growth medium) after cell debris and myelin were removed by filtration and sucrose-gradient centrifugation. One half of the growth medium was replaced with fresh growth medium every 3 days, maintaining the final concentration of growth factors and heparin until colonies of NSCs formed as a monolayer. For the differentiation of NSCs to neural lineages, 10^5 cells/cm^2 were plated in the laminin-coated glass chamber slides (Nalge Nunc International) and cultured for 24 hours in growth medium. Then the growth medium was replaced with the differentiation medium consisting of DMEM/F12, N2 supplement, and 5 μM forskolin (Sigma) and cultured for 7 more days. To differentiate NSCs to oligodendrocytes, *Sox2*-expressing NSCs were labeled with lentivirus-expressing β-gal reporter gene under the control of the constitutive promoter, CMV (a kind gift from Dr. Verma, Salk Institute), and co-cultured with the primary neurons as described previously (Song et al., 2002).

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