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Inducible Site-Specific Recombination in Neural Stem/Progenitor Cells

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Summary

To establish a genetic tool for manipulating the neural stem/progenitor cell (NSC) lineage in a temporally controlled manner, we generated a transgenic mouse line carrying an NSC-specific nestin promoter/enhancer expressing a fusion protein encoding Cre recombinase coupled to modified estrogen receptor ligand-binding domain (ER^{T2}). In the background of the Cre reporter mouse strain *Rosa26^{lacZ}*, we show that the fusion CreER^{T2} recombinase is normally silent but can be activated by the estrogen analog tamoxifen both in utero, in infancy, and in adulthood. As assayed by β -galactosidase activity in embryonic stages, tamoxifen activates Cre recombinase exclusively in neurogenic cells and their progeny. This property persists in adult mice, but Cre activity can also be detected in granule neurons and Bergmann glia at the anterior of the cerebellum, in piriform cortex, optic nerve, and some peripheral ganglia. No obvious Cre activity was observed outside of the nervous system. Thus, the nestin regulated inducible Cre mouse line provides a powerful tool for studying the physiology and lineage of NSCs.

Keywords

Cre-ER^{T2}; nestin; neural stem cells; tamoxifen; transgenic mouse; recombination

INTRODUCTION

The recognition that the adult brain retains stem cells (NSCs) has fundamentally changed our view of brain plasticity (Lie *et al.*, 2004; Ming and Song, 2005; Zhao *et al.*, 2008). It also raises the hope of cell replacement therapy for neurodegenerative disease (Lie *et al.*, 2004). Adult neurogenesis in the subventricular zone (SVZ) of the lateral ventricles serves to replenish olfactory bulb (OB) interneurons via the rostral migratory stream (RMS). In the dentate gyrus, neurogenesis in the subgranular layer (SGL) generates synaptically active granule neurons and has been implicated in learning, memory and mood disorders in rodents (Li *et al.*, 2008; Ming and Song, 2005; Zhang *et al.*, 2008; Zhao *et al.*, 2008). The development of conditional mutant alleles using the Cre/loxP system has permitted circumvention of early lethality observed when many genes are mutated by traditional knockout, thus offering the opportunity to study gene function with spatial control (Mak, 2007). A further refinement of this technology has been

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Additional Supporting Information may be found in the online version of this article.

the development of inducible Cre transgenes that permit temporal control of gene recombination and inactivation (Feil *et al.*, 1997; Hayashi and McMahon, 2002). Fusion of the Cre recombinase protein with a modified estrogen receptor ligand-binding domain (ER^{T2}) causes sequestering of the fusion protein in the cytoplasm where it cannot mediate loxP recombination. Application of estrogen or estrogen analogs, however, causes translocation of the Cre-ER^{T2} fusion protein to the nucleus where recombination can then be achieved.

To achieve temporal ablation of genes in the neural stem cell lineage, we have constructed a tamoxifen-inducible Cre transgene that is regulated by the neurogenic lineage specific promoter/enhancer of the nestin gene. Nestin is an intermediate filament protein specifically expressed in neural stem/progenitor cells in both developing central nervous system and adult brain. The regulatory element driving neural-specific nestin expression has been mapped to the second intron of the nestin gene (Lendahl *et al.*, 1990; Zimmerman *et al.*, 1994). As detailed in our studies, we show that the transgene is silent in the absence of estrogen analog. Upon activation, the expression is robust and recombination is elicited primarily in the principal neurogenic niches. Additional expression is confined to the cerebellum, certain peripheral nerves, and to the piriform cortex, a potentially novel site of neurogenesis.

RESULTS

Generation of Transgenic Lines

The Cre-ER^{T2} cDNA was placed under the control of a 5.6 kb rat nestin 5' regulatory element and followed by the 668 bp of inversed nestin second intron (Fig. 1a). Six transgenic lines were obtained after pronuclear injection and four underwent germline transmission. To assay Cre recombinase activity after induction, we crossed the *CreER^{T2}* lines with *Rosa26-stop-lacZ* (*Rosa26^{lacZ}*) reporter mice. The *Rosa26^{lacZ}* mice require Cre-mediated recombination for β -galactosidase gene activation due to a stop cassette flanked by loxP sites upstream of the lacZ gene. To assess inducibility of the Cre transgene, sunflower oil vehicle (150 μ l) or the estrogen analog tamoxifen (1 mg) was injected into pregnant mice at embryonic day 12.5 (E12.5) and the embryos were dissected out at E14.5 for whole mount X-gal staining. In a *Rosa26^{lacZ}* reporter background, exposure of the four transgenic lines to tamoxifen revealed that only two of the lines (Line 8 and Line 73) exhibited recombination activity (Fig. 1b and not shown). Moreover, comparison of Cre activity upon induction was similar although Line 8 was leaky, having minor but detectable Cre activity in the absence of tamoxifen. In contrast, Line 73 (*Nes73-CreER^{T2}*) showed no signs of Cre activity in the absence of tamoxifen and the blue X-gal staining was found predominantly in embryonic brain and spinal cord where most nestin-positive neural progenitors are located (Fig. 1b).

Embryonic and Neonatal Stem/Progenitor-Specific Cre Induction

The temporal control of Cre activity allowed us to induce Cre-mediated recombination for the purpose of tracing NSCs and their progeny at various time points. The pattern observed upon embryonic induction closely reflected the course of brain development. Tamoxifen induction at E13.5 labeled almost the entire cortex in the forebrain as well as the entire cerebellum including neurons and glia (Fig. 1d1). This coincides with the initiation of neural progenitor migration that contributes to different cortical layers in embryonic neural development (Sun *et al.*, 2002). Induction at E17.5, when neurogenesis in the forebrain reaches completion, resulted in labeling of only the outer most layers of the cortex (Fig. 1d2), which stands in line with the "inside-out" pattern of cortex layer formation (Sun *et al.*, 2002). Additionally, the thalamus and hindbrain were labeled at this time point. In the neonatal mouse brain, there is persistent mild but widespread lacZ activity, indicative of residual but rare progenitor cells throughout the parenchyma (Fig. 1d3–d4). The most active neurogenic region at this time is the cerebellum (Herrup and Kuemerle, 1997), which showed intense lacZ staining following

induction at E17.5 through P7 (Fig. 1d2–d4). Mouse cerebellum development is considered to be complete by 3 weeks after birth, however our *Nes73-CreER^{T2};Rosa26^{lacZ}* mice showed strong Cre activity in the anterior part of cerebellum when induced 4 and 8 weeks after birth (Fig. 1c, 1d5,d6, and Fig 3a–c; and see below). Nonetheless, in the anterior brain, by 4 weeks of age the SVZ and SGL are the most neurogenic regions as assayed by tamoxifen-induced Cre activity (Fig. 1d5–d6).

Adult Induction and Neurogenesis

Adult NSCs modify their gene expression as they migrate and differentiate. In the SVZ, glial fibrillary acidic protein (GFAP) positive cells are considered to be stem cells (Doetsch *et al.*, 1999). When differentiation starts and neuronal fate of the progenitor cells has been specified, cells begin to express doublecortin (DCX) and migrate into the OB through the RMS to finally become NeuN-positive mature neurons (Doetsch *et al.*, 1999; Ming and Song, 2005). To determine the sites of primary Cre recombinase activity, we examined the SVZ of 4-week-old *Nes73-CreER^{T2};Rosa26^{lacZ}* mice 48 h after a short pulse of tamoxifen, since both GFAP-positive neural stem cells and some transient amplifying progenitor cells express nestin. X-gal staining followed by immunohistochemistry (IHC) with GFAP or DCX antibody revealed that the majority of Cre activity resides in GFAP-positive SVZ cells close to the lateral ventricle, with only rare DCX-positive SVZ or RMS cells showing recombination (Fig. 2a). This was further confirmed using an estrogen receptor antibody to show double labeling of Cre-ER^{T2}-positive cells with the stem cell marker GFAP, and with S100 β , a marker of radial glia-derived ependymal cells (Supp. Info. Fig. 1) (Spassky *et al.*, 2005). These studies indicate that the primary site of tamoxifen-activated Cre recombinase is the GFAP-positive, SVZ stem cell population.

To measure the efficiency of tamoxifen-induced recombination in our *Nes73-CreER^{T2}* mice, we crossed them with the *Rosa26^{YFP}* reporter line to generate *Nes73-CreER^{T2};Rosa26^{YFP}* mice and then induced these mice with tamoxifen at 4 weeks of age. We then harvested brain sections from the induced mice at 6 weeks of age, and performed immunofluorescent double-labeling with GFP and Sox2 antibodies (Supp. Info. Fig. 2). The percentage of GFP/Sox2 double-positive cells divided by the number of Sox2 positive cells in the SVZ was used to determine recombination efficiency. This quantification analysis revealed that $75 \pm 4\%$ of Sox2-positive cells in the SVZ have been targeted 2 weeks after a 5-day tamoxifen induction.

To further study the dynamics of stem/progenitor cell migration and differentiation, *Nes73-CreER^{T2};Rosa26^{lacZ}* mice were induced at 4 weeks of age and examined by X-gal staining 2 or 4 weeks later (Fig. 1d5,d6 and Fig 2b). The dynamics of Cre-active cells in the hippocampus over time was not very dramatic (Fig. 2b,c), however in the SVZ, an increase in the number of Cre active cells in an expanded ventricular area was evident 4 weeks after induction (Fig. 2b). These results suggest a precursor-progeny relationship in which, after 2 weeks of induction, a significant number of new progenitor cells have been generated by stem cells and are beginning to disperse from the SVZ. Similarly, in the OB 2 weeks after induction, the X-gal positive cells were confined to a central cluster, whereas 4 weeks postinduction the cells were dispersed throughout the OB (Fig. 2b). We interpret this result to indicate that at 2 weeks postinduction, cells are just arriving to the OB via the RMS and are confined to this central area, whereas at 4 weeks postinduction, these labeled cells have now dispersed throughout the OB. A similar, although more restricted, migration was also observed in hippocampus, where β -Gal and NeuN double-positive neurons first appear close to the SGL 2 weeks after induction but by 4 weeks postinduction have migrated deeper into the granular layer (Fig. 2c).

To explore the identity of the Cre-active cells, immunofluorescent double labeling was used to characterize *Nes73-CreER^{T2};Rosa26^{lacZ}* mice 4 weeks after induction (Fig. 2d–f). β -Gal immunoreactivity was found in nestin and GFAP-positive neural stem/progenitor cells in the

SVZ and SGL (Fig. 2d). In the anterior part of the SVZ and SGL, DCX-positive neural progenitors also showed Cre activity (Fig. 2e). In addition, a majority of the cells in the RMS express both β -Gal and DCX (Fig. 2e). Furthermore, NeuN-positive mature neurons that also retained β -Gal immunoreactivity could be found in the HP and OB (Fig. 2f). A small number of GFAP-positive astrocytes in the OB and the corpus callosum (CC) also expressed the reporter gene β -Gal (Fig. 2f), indicating the presence of Cre activity in multiple cell types in the NSC lineage. This result is consistent with recent quantitative lineage tracing studies (Lagace *et al.*, 2007).

Additional Tamoxifen-Inducible Cre Activity

The significant amount of Cre activity induced in anterior cerebellum of adult mice was unexpected (Fig. 1c,d). Figure 3a shows a representative eight-week-old brain from a mouse that was induced with tamoxifen at 4 weeks of age. The β -Gal positive cells were mostly NeuN-positive inner granular layer (IGL) granule cells and Bergmann glia that extend long processes to the surface of the cerebellum (Fig. 3a–c). Consistent with previous reports that Bergmann glia express NSC markers such as nestin and Sox2 (Mignone *et al.*, 2004; Sottile *et al.*, 2006), we found that Cre-active Bergmann glia also expressed the NSC marker nestin (Fig. 3b). However, the *Cre-ER^{T2}* fusion transgene was also expressed in some Sox2-negative cells in the IGL (Fig. 3d, middle panel), suggesting potential aberrant expression of the *Nestin-CreER^{T2}* transgene. Mild but reproducible tamoxifen-induced Cre activity was also observed in the piriform cortex (Fig. 3e,f), which has also been reported to be a potential neurogenic region (Pekcec *et al.*, 2006). We next assessed tamoxifen-induced Cre activity in other regions using whole mount X-gal staining, and found that the dorsal root ganglia (DRG) but not the spinal cord showed Cre activity (Fig. 3g). Histologic examination revealed that less than half of the DRG neurons undergo Cre-mediated recombination (Fig. 3h). In addition, Cre activity was detected in the optic nerve and trigeminal ganglia in mice induced at neonatal (Fig. 3i, middle panel) or adult stages (Fig. 3i, right panel). Collectively these data indicate that the nestin promoter/enhancer employed to generate this tamoxifen inducible transgene, exhibits remarkable fidelity to the endogenous neural expression with only a few potential sites of discrepancy.

Detailed analysis of traditional *Nestin-Cre* transgenic lines has revealed Cre activity outside the CNS, for example, in the kidney and in somite-derived tissues (Dubois *et al.*, 2006). To determine whether Cre activity in the *Nes73-CreER^{T2}* mice was restricted to the nervous system, *Nes73-CreER^{T2};Rosa26^{lacZ}* mice were induced for 5 days starting at P0 and analyzed at 8 weeks of age by whole-mount X-gal staining of internal organs including the heart, lung, liver, thymus, spleen, kidney, pancreas and stomach. With the exception of the esophagus, where neonatal but not adult exposure to tamoxifen induced Cre activity (Fig. 4, Supp. Info. Fig. 3) and stomach, where spontaneous lacZ activity is present in controls (Fig. 4, Supp. Info. Fig. 3) (Kwon *et al.*, 2006), we found no evidence of obvious reporter expression in the absence or presence of tamoxifen (see Fig. 4).

DISCUSSION

The rediscovery of neurogenesis in the adult brain has led to reawakened interest in the role of new neurons in the mature brain. The SVZ is a major site of neurogenesis for OB interneurons, although emerging evidence suggests additional roles. In the hippocampus, neurogenesis has been implicated in mood modulation and in learning and memory (Li *et al.*, 2008; Lie *et al.*, 2004; Zhao *et al.*, 2008). On the dark side, stem/progenitor cells in the CNS have been implicated as the source of glioblastoma (Kwon *et al.*, 2008; Sanai *et al.*, 2005; Zhu *et al.*, 2005). Specific ablation or activation of genes implicated in hippocampal function and in glioma can be achieved with our tamoxifen-inducible Cre transgene and we have developed

successful models of both SVZ stem/progenitor cell-dependent induction of glioma and hippocampal stem/progenitor cell-dependent antidepressant insensitive mice using this tamoxifen-inducible Cre mouse line (Li *et al.*, 2008; Llaguno *et al.*, submitted).

Still, there is much to be learned about the precise role of neural stem cells in normal brain function and in associated pathologies. For example, in this report we describe novel sites of nestin-Cre recombinase activity. Whether this activity identifies previously undetected sites of neurogenesis or simply ectopic Cre expression remains to be rigorously determined. Of note, a second, independently derived transgenic line, *Nes8-CreER^{T2}*, shows a similar pattern of inducible expression (data not shown) leading us to favor the conclusion that the expression outside the SVZ and SGZ is not due to position effects at the site of transgene insertion but rather is a reflection of the properties of the transgenic construct. Stem cells have been isolated from neonatal cerebellum and they are reported to be prominin/CD133-positive and Math1-negative (Klein *et al.*, 2005; Lee *et al.*, 2005). We observe Cre activity in the cerebellum from E17.5 through 8 weeks of age. Although diminishing over time, a clear gradient is observed that becomes progressively more anterior. The lacZ positive cells resulting from activation of the *Rosa26* reporter possess the characteristic morphology of granule cells. In adult cerebellum, the Bergmann glia retain a morphology reminiscent of radial glia which can generate neurons and adult NSCs during brain development (Gotz and Barde, 2005; Merkle *et al.*, 2004). In addition, Bergmann glia still express stem cell markers such as Sox2 and nestin (Mignone *et al.*, 2004; Sottile *et al.*, 2006). On the other hand, only rarely have cells with BrdU incorporation been observed in adult cerebellum, even after growth factor infusion (Grimaldi and Rossi, 2006). We also found that a number of cells in the anterior cerebellum targeted 2 days after acute tamoxifen administration were positive for NeuN but not GFAP or nestin (Supp. Info. Fig. 4), suggesting that the cre activity in the IGL was more likely due to promoter leakiness (Supp. Info. Fig. 4). Further study is needed to resolve this issue.

A series of similar inducible *Nestin-Cre* transgenes has recently been reported, although the extent of expression over time and expression outside the nervous system was not described (Supp. Info. Table 1) (Balordi and Fishell, 2007; Burns *et al.*, 2007; Imayoshi *et al.*, 2006; Kuo *et al.*, 2006; Lagace *et al.*, 2007). Eisch and co-workers recently described a tamoxifen-inducible Cre transgenic mouse line with no obvious Cre activity in the cerebellum upon tamoxifen induction (Lagace *et al.*, 2007). The fact that our transgenic construct included only intron 2 of the nestin gene whereas their construct contained nestin exons 1–3 could account for this discrepancy (Zimmerman *et al.*, 1994). It is possible that our more limited nestin construct might lack cerebellar-specific repressor sequences. Another potentially significant variation is the use of a *Rosa26^{lacZ}* reporter line versus the *Rosa26^{YFP}* reporter used by Lagace *et al.* (2007). Both the sensitivity of the reporter and perhaps the recombinogenic efficiency could in principle differ, leading to these discrepancies. We also observe Cre activity in the adult piriform cortex. This is in accordance with previous reports of BrdU incorporation in this region, leading to the suggestion of additional neurogenic niches (Pekcec *et al.*, 2006).

We examined our mice for leakiness as well as for inducible transgene expression in the peripheral nervous system (PNS) and multiple organs. In contrast to many other Nestin reporter transgenic mice (Day *et al.*, 2007; Dubois *et al.*, 2006; Gleiberman *et al.*, 2005; Li *et al.*, 2003; Ueno *et al.*, 2005), we found no evidence of obvious leakiness or of inducible transgene activation outside the CNS except in the PNS, where inducible expression was found both in the DRG and trigeminal ganglion, and in the esophagus. It is possible that our *Nestin-CreER^{T2}* transgene has a more restricted expression pattern or that the tamoxifen induction efficiency is lower in certain tissues. In addition, whole mount X-gal staining of the organs makes it difficult to capture rare Cre-positive cells if they do exist. DRG have been used to culture neurospheres (Li *et al.*, 2007), and it will be of interest to determine whether our transgene is active in these progenitor cells, which would provide supportive evidence for the

existence of additional neural stem/progenitor niches. Subsequent detailed lineage tracing of the Cre expressing cells will more clearly address this issue.

MATERIALS AND METHODS

Transgenic Mice

A 2.0 kb fragment of CreER^{T2} and SV40 polyA sequence of the pCre-ER^{T2} vector (Feil *et al.*, 1997) were amplified using a PCR technique that also generated 5' NotI and 3' SpeI sites. After enzymatic digestion, purified fragment was ligated to an 8.9 kb fragment from pNerv (Panchision *et al.*, 2001; Yu *et al.*, 2005) digested with NotI and XbaI. The resulting pNes-CreER^{T2} construct contains a 5.6 kb rat nestin 5' genetic element from pNerv, a 2.0 kb CreER^{T2} and SV40 polyA sequence from pCre-ER^{T2} and a 668 bp of reversed second intron of rat nestin from pNerv (Fig. 1a). After SalI digestion, an 8.3 kb band was purified and microinjected into the pronuclei of fertilized eggs from B6D2F1 mice. Among 28 pups born after two rounds of transgenic injection, six contained the transgene, and four of them transmitted to germline. *Rosa26^{lacZ}* mice were obtained from Jackson Laboratories (Bar Harbor, ME), *Rosa26^{YFP}* mice were kindly provided by Dr. Jane Johnson. All the mice were maintained in a mixed genetic background of C57BL/6, SV129 and B6/CBA. *Nestin73-CreER^{T2}; Rosa26^{lacZ}* mice were generated by crossing male *Nestin-CreER^{T2}* mice with female *Rosa26^{lacZ}* mice. Genotyping of the mice was performed as described previously (Kwon *et al.*, 2006). All mouse protocols were approved by the Institutional Animal Care and Research Advisory Committee at the University of Texas Southwestern Medical Center.

Tamoxifen Induction

Tamoxifen (Sigma-Aldrich, St. Louis, MO) was dissolved in a sunflower oil (Sigma-Aldrich, St. Louis, MO)/ethanol mixture (9:1) at 6.7 mg/ml. For initial screening of the embryonic induction of the transgenic lines, 150- μ l tamoxifen (1 mg) or vehicle (sunflower oil/ethanol mixture only) was injected intraperitoneally into pregnant mice at embryonic day E12.5 (E12.5 hereafter). Embryos were dissected out 2 days later and subjected to X-gal staining. For in utero induction, 150- μ l tamoxifen (1 mg) or vehicle was injected intraperitoneally into pregnant mothers at E13.5 or E17.5, and pups were analyzed 1 month after birth. For neonatal induction, 12.5- μ l tamoxifen (83.5 mg/kg body weight) or vehicle per gram of mouse body weight was injected into lactating mothers (tamoxifen can be delivered to pups through the mother's milk) at P0 or P7, once a day for 5 days and the pups were analyzed 4 weeks after the first induction. For induction in adult mice, 12.5- μ l tamoxifen (83.5 mg/kg) or vehicle per gram of body weight was injected intraperitoneally into 4- or 8-week-old mice twice a day for five consecutive days and then analyzed 2 or 4 weeks after the first induction.

Histology and X-gal Staining

Mice were dissected and perfused as previously described (Kwon *et al.*, 2006). For whole mount X-gal staining, the embryos or organs were carefully dissected out, washed with phosphate-buffered saline (PBS), and then fixed in 2% (w/v) paraformaldehyde (PFA; in PBS) for 1 h at 4°C. Postnatal brains were postfixed in 2% PFA overnight (O/N) at 4°C, embedded in 2.5% chicken albumin sagittally or coronally, and then cut into 50- μ m thick sections by vibratome (Leica, Nussloch, Germany). Every fifth sagittal section or 12th coronal section was chosen to perform X-gal staining and comparable sections were selected for further immunostaining according to the X-gal staining result. X-gal staining of organs and sections was performed as described (Kwon *et al.*, 2006).

Induction Efficiency Quantification

Four *Nestin73-CreER^{T2};Rosa26^{YFP}* mice were induced at 4 weeks of age as described above and perfused with 2% PFA at 6 weeks of age. The brains were dissected out, postfixed in 4% PFA O/N at 4°C, processed and embedded in paraffin blocks. Five-μm thick sagittal sections were cut until the lateral ventricle was gone. H&E staining was performed on every fifth slide to determine comparable sections. Every 10th of comparable sections was subjected to GFP (Aves Labs, Tigard, OR) and Sox2 (Chemicon, Temecula, CA) immunofluorescence staining, and three random regions of the frontal SVZ of each section were selected for counting. The efficiency was determined by the percentage of GFP (mean 203)/Sox2 (mean 270) double-positive cells out of the total Sox2-positive cells in SVZ.

Immunostaining

Free-floating immunofluorescence staining was performed on 50-μm thick sections. Antibodies used for the staining were against β-galactosidase (ICN, Aurora, OH), GFAP, nestin (BD Biosciences, Bedford, MA), doublecortin (Santa Cruz Biotechnology, Santa Cruz, CA), NeuN (Chemicon, Temecula, CA), Mash1 (BD Biosciences, Bedford, MA), S100β (Sigma-Aldrich, St. Louis, MO). Alexar-488 or Alexar-555 conjugated goat anti-mouse or anti-rabbit (Molecular Probes, Eugene, OR) and Cy2 or Cy3 donkey anti-goat, anti-rabbit antibodies (Jackson ImmunoResearch, West Grove, PA) were used to visualize primary antibody staining. Images were taken on a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany). For ER and Sox2 staining, 5-μm thick paraffin sections were first stained with estrogen receptor antibody (Lab Vision, Fremont, CA) and visualized by DAB substrate with nickel solution (Vector Laboratories, Burlingame, CA). The slides were then washed with PBS three times, stained with Sox2 antibody (Chemicon, Temecula, CA), and visualized by Vector NovaRED (Vector Laboratories, Burlingame, CA). Images were taken with a Nikon 2000 CCD camera (Nikon, Japan). All images were assembled using Adobe Photoshop CS and Illustrator CS (Adobe Systems Incorporated, San Jose, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.

Transgene construct and tamoxifen inducibility. **(a)** Structure of the *Nestin-CreERT²* transgene consisting of the rat nestin promoter/enhancer, cDNA encoding the CreERT² fusion protein and inversely oriented *Nestin* second intron. **(b)** Transgene induction during embryogenesis. Representative whole-mount, X-gal-stained E14.5 embryos (induced at E12.5) show no X-gal signal in vehicle-treated (Veh) *Nes73-CreERT²; Rosa26^{lacZ}* embryos (top panels) or tamoxifen-treated (Tmx) *Rosa26^{lacZ}* embryos (middle panels). Only Tmx-treated *Nes73-CreERT²; Rosa26^{lacZ}* embryos (bottom panels) show blue staining in the developing CNS (black arrowheads). **(c)** Transgene induction in adult. Representative X-gal-stained brain sections from 10-week-old mice show that 5 days of Tmx injection into 8-week-old *Nes73-*

CreER^{T2};Rosa26^{lacZ} mice (top panel) induced recombination as evidenced by X-gal signal in the hippocampus (HP), lateral wall of lateral ventricle (LV), rostral migratory stream (RMS), olfactory bulb (OB), and anterior cerebellum (CB). Vehicle-injected *Nes73-CreER^{T2};Rosa26^{lacZ}* mice (bottom panel) have no X-gal activity. Diffuse, low-level X-gal signal was observed in the thalamus (TH) of both Veh- and Tmx-injected mice (black arrow). **(d)** Tmx induction at various time points during mouse development reflects neurogenesis at different stages. In utero induction of *Nes73-CreER^{T2};Rosa26^{lacZ}* (**d1, d2**) results in Cre activity in the cerebral cortex (CTX) and cerebellum (CB); neonatal induction (**d3, d4**) shows labeling of the whole cerebellum (CB); adult induction (**d5, d6**) results in signal that is restricted to the neural stem cell niches and their migration targets as well as to the anterior part of cerebellum (CB).

FIG. 2.

Cre activity in adult NSC niches and migration targets. (a) Representative X-gal stained brain sections from mice 48 h after two tamoxifen administrations at P28 (12-h interval). X-gal signal was mainly restricted to SVZ (a1), with little or no signal observed in rostral migratory stream (RMS) (a1, a4). Immunohistochemistry following X-gal staining revealed that the majority of X-gal positive cells express glial fibrillary acidic protein (GFAP). A few X-gal positive cells (black arrows) also express doublecortin (DCX) in subventricular zone (SVZ) (a3) and RMS (a4). (b) Representative X-gal-stained brain sections from 6- or 8-week-old *Nes73-Cre^{ERT2}; Rosa26^{lacZ}* mice that were induced at 4 weeks of age show the dynamics of X-gal-positive cells in the hippocampus (HP), SVZ and olfactory bulb (OB). (c) Representative β -

galactosidase (β -Gal) and NeuN staining of hippocampus 2 or 4 weeks after tamoxifen induction at 4 weeks of age. Newly generated β -Gal-positive neurons slowly migrate into the granular cell layer (white arrows). **(d–f)** Representative immunofluorescence staining showing the presence of β -galactosidase (β -Gal)-expressing (hence, Cre active) cells in *Nes73-CreER^{T2};Rosa26^{lacZ}* mice 4 weeks after tamoxifen induction. **(d)** Expression of β -Gal was observed in NSCs that also express nestin and GFAP (white arrows, for example). **(e)** Doublecortin-positive neural progenitor cells also expressed β -Gal in the SVZ, HP, rostral migratory stream (RMS), and OB (white arrows, for example). In the OB, some β -Gal-positive cells were Doublecortin-negative, and thus possibly represent mature neurons (white arrowheads, for example). **(f)** Multiple lineages of differentiated cells with β -Gal expression. Top panels: mature neurons (generated by adult NSCs) that migrated into the hippocampal (HP) granular layers were rare (white arrows, for example), while the majority of β -Gal-positive cells were NeuN-negative (white arrowheads, for example). In the OB, a significant number of mature neurons were contributed by adult neurogenesis as shown by β -Gal and NeuN double labeling. Bottom panels: adult NSCs were also found to differentiate into GFAP-positive astrocytes (white arrows, for example) in the OB and corpus callosum (CC).

FIG. 3.

Novel Cre activity. *Nes73-CreERT²;Rosa26^{lacZ}* mice were treated with tamoxifen at 4 weeks of age and analyzed at 8 weeks (**a–c**, **e–h**, left and right panel of **i**). Abundant β -Gal expression was detected in the anterior part of cerebellum of tamoxifen-treated mice (**a–c**): X-gal staining (**a**) and immunostaining (**b**, **c**) reveal that in cerebellum a majority of β -Gal-expressing cells are nestin-positive Bergmann glia in the molecular layer (ML) (**b**) or NeuN-positive granule neurons in the inner granular layer (IGL) (**c**). (**d**) Representative estrogen receptor (ER) and Sox2 immunohistochemistry staining from 4-week-old *Nes73-CreERT²;Rosa26^{lacZ}* mice. The *Cre-ERT²* fusion transgene was expressed in Sox2-positive cells in SVZ (black arrows in left panel), anterior part of cerebellum (black arrows in folia II in middle panel, for example) and

also some Sox2-negative cells in cerebellum (black arrowheads in middle panel). No Cre-ER^{T2} fusion protein was detected in the IGL of posterior cerebellum in the same animal (folia IX in right panel). (e, f) Weak β -Gal expression (black arrow) was also found in the piriform cortex (e) and co-localized with NeuN-positive neurons (white arrows) (f). Outside of the brain, X-gal signal was detected in the dorsal root ganglia (DRG) (g, h), optic nerve (black arrows) and trigeminal ganglia (black arrowheads) (i). No obvious signal was detected in the spinal cord by whole mount X-gal staining (**g2, g3**). Further sectioning of DRG showed that some small, medium, and large-sized DRG neurons were positive for β -Gal staining (h). Vehicle-treated controls were negative for β -Gal expression (**g1** and i, left panel).

FIG. 4.

Cre activity is not observed in internal organs. *Nes73-CreERT2;Rosa26^{lacZ}* mice were treated with vehicle (Veh) or tamoxifen (Tmx) at P0 for 5 days. Different organs were then dissected out at 8 weeks and subjected to whole mount X-gal staining. Endogenous X-gal signal is present in the stomachs of both treatment groups. Except for the Cre activity shown in the esophagus of the Tmx-treated mice (black arrows), no obvious difference was found elsewhere between genotypes (not shown) or treatments.