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Visualization of embryonic neural stem cells using Hes promoters in transgenic mice

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In the central nervous system, neural stem cells proliferate in the ventricular zone (VZ) and sequentially give rise to both neurons and glial cells in a temporally and spatially regulated manner, suggesting that stem cells may differ from one another in different brain regions and at different developmental stages. For the purpose of marking and purifying neural stem cells to ascertain whether such differences exist, we generated transgenic mice using promoters from *Hes* genes (pHes1 or pHes5) to drive expression of destabilized enhanced green fluorescent protein. In the developing brains of these transgenic mice, GFP expression was restricted to undifferentiated cells in the VZ, which could asymmetrically produce a Numb-positive neuronal daughter and a GFP-positive progenitor cell in clonal culture, indicating that they retain the capacity to self-renew. Our results suggest that pHes-EGFP transgenic mice can be used to explore similarities and differences among neural stem cells during development.

Introduction

During the development of central nervous system (CNS), multipotent neural progenitor cells in the ventricular zone (VZ) initially expand their own population by symmetric cell divisions, in which both progeny re-enter the cell cycle. By mid-gestation, the cells initiate neurogenesis by adopting a mode of asymmetric cell division, in which one daughter differentiates into a neuron while the other continues to cycle (Takahashi et al., 1994; Chenn and McConnell, 1995). Many of the progenitor cells in the VZ exhibit characteristics of neural stem cells, which include the capacity to generate many cell types, including neurons, astrocytes, and oligodendrocytes (multipotentiality), and the ability to divide symmetrically to duplicate their own population or divide asymmetrically to continually replenish the progenitor pool (self-renewal) (Weiss et al., 1996a,b; Weissman et al., 2001).

The nature and identity of neural stem cells in embryonic development remain poorly understood. It has been difficult to draw a clear distinction between neural stem cells and progenitor cells with relatively restricted developmental potentials, and it can be challenging to define and identify neural stem cells in vitro because neural stem cells can change their properties during a prolonged time in culture (Seaberg and van der Kooy, 2003). Investigations of the properties and types of neural stem cells would be significantly enhanced by the development of methodologies by which to identify, isolate, and purify these cells. For example, stem cell purification methods would enable the identification of molecules that are expressed differentially by distinct subsets of neural stem cells. In addition, methods for the isolation and expansion of neural stem cells would be extremely valuable for the progress of cell replacement and regeneration therapies in the treatment of neurodegenerative diseases and CNS injury. Despite the potential importance to basic and clinical research, such purification methods remain elusive due to the lack of suitable markers by which one can identify neural stem cells in culture or distinguish stem cells from other progenitor cells that may commingle within the VZ.

In an effort to visualize neural stem cells within the developing CNS, we used the promoters from two *Hes* genes. *Hes1* and *Hes5* are expressed within the VZ throughout the developing CNS, where they function downstream of Notch signaling as negative regulators of neuronal differentiation (Ishibashi et al., 1994; Tomita et al., 1996; Ohtsuka et al., 1999; Kageyama and Ohtsuka, 1999; Nakamura et al., 2000) and promotes a neural stem cell identity (Ohtsuka et al., 2001). Thus, we hypothesized that *Hes1* and *Hes5* are expressed preferentially or exclusively by neural stem cells and that the identification of *Hes*-expressing cells might enable the isolation of neural stem cells. We therefore established transgenic mouse lines in which *Hes* promoters directed the expression of destabilized enhanced green fluorescent protein (d2EGFP) to undifferentiated VZ cells. Because asymmetric divisions were

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visualized in clonal density culture, in which GFP expression was retained exclusively in undifferentiated daughter cells, and because the GFP-labeled cells exhibited the capacity for self-renewal, we propose that pHes-d2EGFP expression can be used to mark embryonic neural stem cells.

Results

The Hes1 and Hes5 promoters are active in VZ cells

To examine the activities of the *Hes1* and *Hes5* promoters in the developing brain, we constructed expression vectors that utilize promoter regions from the *Hes1* or *Hes5* gene to direct the expression of GFP (pHes1-EGFP, pHes5-EGFP) and introduced these constructs into the telencephalon of E14.5 mouse embryos using in utero electroporation. After 12–72 h, the fates of the GFP-positive transfected cells were determined. Following expression of a control vector, in which GFP expression was directed by CMV promoter, the majority of GFP-positive cells migrated out of the VZ and into the cortical plate after 24–72 h (Supplementary Figs. 1A–C). In contrast, when pHes1-/pHes5-EGFP was misexpressed, most GFP-expressing cells were observed in the VZ 48 h later (Supplementary Figs. 1E, H). However, after 72 h, GFP labeling was observed in cells that had migrated away from the VZ (Supplementary Figs. 1F, I). Previous in situ hybridization analyses of *Hes1* and *Hes5* mRNAs revealed that their expression is confined to VZ cells, suggesting that promoter activity is low or absent in migrating neurons. We speculated that GFP expression was observed after the *Hes1/Hes5* promoters were downregulated because of the stability of EGFP protein.

We therefore constructed vectors in which the expression of destabilized EGFP (d2EGFP), which has a half-life of ~2 h, is driven by the *Hes1* or *Hes5* promoter and assessed these vectors by in utero electroporation. In this case, GFP expression at 24 h was confined to VZ cells, as was seen with the pHes1- and pHes5-EGFP constructs (Supplementary Figs. 1J, M). In contrast to these latter vectors, however, expression of d2EGFP at 48–72 h after electroporation remained confined to the VZ and was never detected in migrating cells (Supplementary Figs. 1K, L, N, O), suggesting that the shorter half-life of d2EGFP enabled a more accurate visualization of *Hes1* and *Hes5* promoter activity.

pHes1 and pHes5 confer complementary expression patterns in the CNS of transgenic mice

Based on the results of these pilot experiments, we generated transgenic mice carrying the pHes1-d2EGFP or pHes5-d2EGFP transgenes (Figs. 1A, B). Five or more transgenic founder mice were obtained for each vector, and all successfully transmitted the transgene to their offspring to establish breeding lines. Mice from both lines developed normally into adulthood and were fertile, indicating that transgene expression had no deleterious effects. Lines generated from the same promoter construct showed nearly

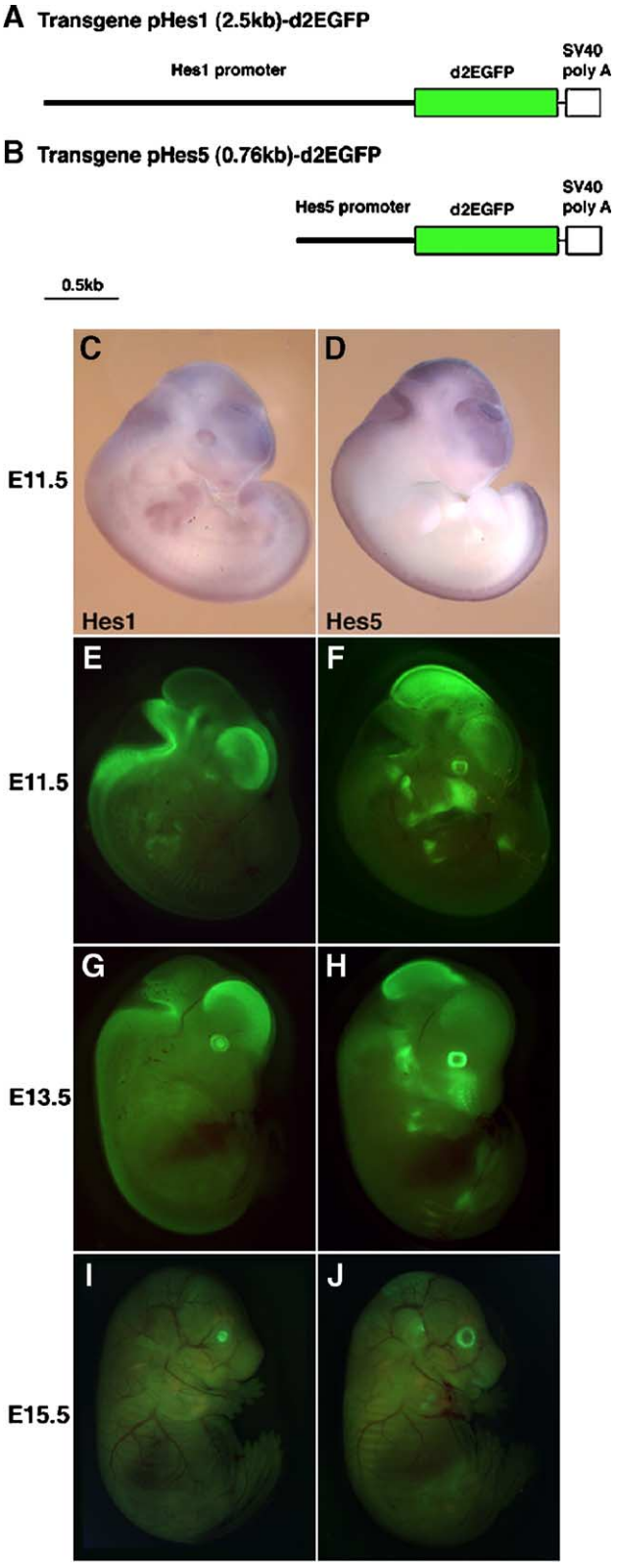

Fig. 1. Generation of transgenic mice. (A, B) Structure of the pHes1-d2EGFP (A) and pHes5-d2EGFP (B) transgenes. The 2.5-kb *Hes1* promoter or the 0.76-kb *Hes5* promoter was inserted into the upstream of sequences encoding destabilized EGFP (d2EGFP) and a polyadenylation signal. (C, D) Whole mount in situ hybridization of wild-type mouse embryos at E11.5. The expression of *Hes1* and *Hes5* mRNAs was detected throughout the CNS. (E–J) GFP expression (green) in transgenic mice at E11.5 (E, F), E13.5 (G, H), and E15.5 (I, J). GFP was highly expressed in telencephalon and hindbrain of pHes1 Tg embryos (E, G, I) and in the midbrain of pHes5 Tg mice (F, H, J). GFP was also expressed in the retina and limbs in both transgenic lines, in the lens of pHes1 Tg, and in the branchial arches and otic placode of pHes5 Tg animals.

identical patterns of GFP expression in all tissues examined, with the exception of one line of pHes5-d2EGFP mice.

Whole mount in situ hybridization in wild-type mice at E11.5 demonstrated that *Hes1* mRNA was expressed throughout the CNS (including the retina) and in the limbs (Fig. 1C). *Hes5* mRNA showed higher levels of expression within the CNS and was confined to neural tissues (Fig. 1D). Complementary expression patterns were also seen in transgenic mice. In pHes1-d2EGFP mice (pHes1 Tg), GFP was expressed more strongly in the telencephalon and hindbrain than in the midbrain (Fig. 1E). In contrast, GFP expression was prominent in the midbrain of pHes5-d2EGFP mice (pHes5 Tg) and weak in the telencephalon (Fig. 1F). These expression patterns were maintained at E13.5 and E15.5, when GFP expression in telencephalon of pHes1 Tg and midbrain of pHes5 Tg mice was particularly striking (Figs. 1G–J).

We noted several instances in which the patterns of pHes-driven GFP expression did not mimic the endogenous pattern of *Hes* mRNA expression. For example, in pHes1 Tg mice, GFP was expressed in lens, where endogenous *Hes1* expression is not detected (Figs. 1E, G, I). Conversely, although *Hes1* mRNA is expressed in the limb buds, GFP was not present in pHes1 transgenics. In pHes5 Tg mice, GFP was expressed in the branchial arches, otic placode, and limbs (Figs. 1F, H, J), all regions that lack detectable *Hes5* mRNA expression by in situ hybridization. We do not know why there was an imperfect match between the patterns of mRNA expression and GFP in these lines, although it seems likely that the promoter fragments used to drive d2EGFP expression lack certain elements required for the precise regional control of *Hes* gene expression. Nevertheless, as described below, the promoters can confer the temporal–spatial patterns of expression that are likely to distinguish neural stem cells from other progenitors and differentiating cells within the VZ.

GFP expression in pHes1 and pHes5 transgenic mice mimics the pattern of endogenous Hes1 and Hes5 mRNA expression in the developing CNS

The expression of GFP in the pHes1 and pHes5 Tg mice was analyzed using anti-GFP antibodies on sectioned tissue from both lines (Figs. 2A–F) and compared to the patterns of endogenous *Hes* gene expression (Figs. 2G–L). Most or all VZ cells in the telencephalon of pHes1 Tg mice expressed GFP (Fig. 2A), a pattern seen after in situ hybridization to reveal endogenous *Hes1* (Fig. 2G). In contrast, GFP labeling in pHes5 Tg animals generated a speckled pattern within the telencephalic VZ (Fig. 2B), while the endogenous *Hes5* was expressed more uniformly in the VZ (Fig. 2H). In the midbrain at E13.5, pHes1-GFP was expressed strongly in the ventral VZ and within the roof plate, with weaker expression

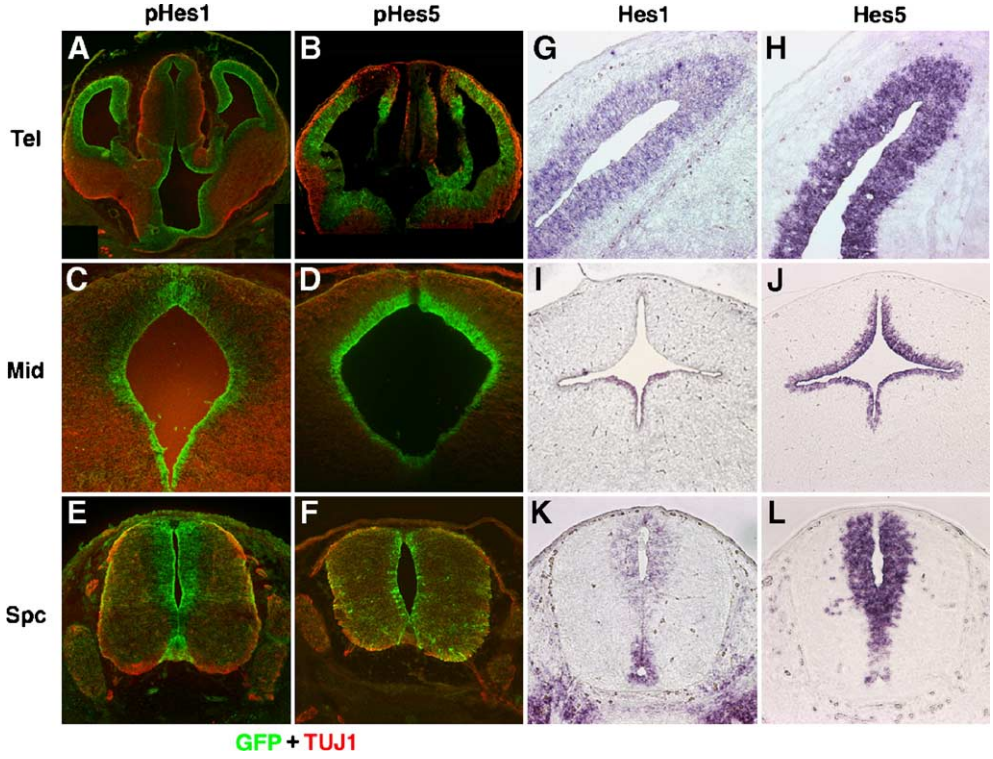

Fig. 2. Comparison of GFP expression in the CNS of pHes1 and pHes5 Tg mice. (A–F) GFP (green) was expressed in the ventricular zone (VZ) throughout the CNS. TUJ1 staining is shown in red. (A, B) Coronal sections through the forebrain at E11.5. GFP was expressed homogeneously in the telencephalic VZ of pHes1 Tg mice and in a speckled pattern in pHes5 Tg mice. (C, D) Coronal sections through the midbrain at E13.5. In pHes1 Tg mice (C), GFP was expressed more strongly in the ventral VZ than in the dorsal half, although expression was also strong near the roof plate. Conversely, GFP was highly expressed in the dorsal VZ of the pHes5 Tg midbrain (D). (E, F) Axial sections through the spinal cord at E13.5. GFP was expressed in the spinal cord VZ in both lines, but the expression appeared speckled in pHes5 Tg mice. Only pHes1 Tg mice showed GFP expression in the floor plate. (G–L) In situ hybridization of wild-type mouse embryos at E13.5. *Hes1* and *Hes5* mRNA expression was detected in the VZ throughout the CNS. (G, H) *Hes* genes are expressed throughout the telencephalic VZ. (I, J) *Hes1* expression is detectable in the ventral midbrain and roof plate, whereas *Hes5* is expressed more strongly in dorsal VZ and is absent from the roof plate. (K, L) *Hes1* expression in the spinal cord is weak dorsally but strong in the ventral VZ, including the floor plate. In contrast, *Hes5* is highly expressed in the dorsal VZ but is absent from the roof plate or floor plate.

in the dorsal VZ (Fig. 2C). In situ hybridization for *Hes1* mRNA showed a similar pattern (Fig. 2I). Within the midbrain of pHes5 Tg mice, both GFP and *Hes5* mRNA were expressed most strongly in the dorsal VZ with the exception of the roof plate, which lacked labeling (Figs. 2D, J). Finally, in the E13.5 spinal cord, GFP expression in pHes1 Tg animals mimicked that of *Hes1* mRNA (Figs. 2E, K), with strong labeling in the floor plate and dorsal VZ cells. GFP expression in the spinal cord of pHes5 Tg mice showed a speckled pattern within the VZ (Fig. 2F), which contrasted with the strong expression of *Hes5* in this area (Fig. 2L). However, both GFP and *Hes5* mRNA expression were absent from both the roof plate and floor plate. Thus, the patterns of GFP expression in early pHes1 and pHes5 Tg embryos largely mimicked the patterns of endogenous *Hes* expression, particularly in being confined to VZ cells in all regions of the CNS analyzed.

GFP was expressed in a variety of tissues other than the CNS in both pHes1 and pHes5 Tg mice, including the olfactory epithelia, support cells in the inner ear, tooth primordia, salivary glands, glomeruli in the kidney, the enteric nervous system, myoblasts, and somites, among other cell types (see Supplementary Fig. 2 for a more complete description). Although the expression patterns were interesting and suggestive in terms of monitoring Notch signaling in vivo, some of the patterns did not mimic those of the endogenous *Hes* genes, particularly in the pHes5 Tg line.

GFP is expressed in mitotically active VZ cells, including radial glial cells

To ascertain the identities of GFP-expressing cells in transgenic animals, neurons were marked with TUJ1 antibodies, actively cycling cells were identified by expression of Ki67, and radial glia were identified with RC2 antibodies. We focused on two regions in which GFP was strongly expressed: the telencephalon of pHes1 Tg mice and midbrain of pHes5 Tg mice. In the telencephalon of pHes1 Tg animals, most VZ cells appeared to express GFP, and many extended radial fibers toward the pial surface (Figs. 3A, D), suggesting that these cells are radial glia. In support of this hypothesis, the expression of GFP and TUJ1 was complementary rather than overlapping, clearly demarcating the VZ and cortical plate (Figs. 3A–C). Immunostaining with RC2 confirmed that many GFP-positive cells were radial glial cells (Figs. 3D–F), many of which were also positive for Ki67, indicating that they were proliferating (Figs. 3G–I).

Similar findings were obtained for GFP-labeled cells in the midbrain of pHes5 Tg mice: many or most of these cells had radial processes (Figs. 3J, M, P) and were not labeled with TUJ1 antibodies (Figs. 3J–L) but did co-express the RC2 (Figs. 3M–O) and Ki67 (Figs. 3P–R). Thus, it appears that both the pHes1 and pHes5 transgenes mark mitotically active radial glial cells during early embryonic development.

A subpopulation of OPCs expresses GFP at late embryonic stages in pHes5 Tg mice

In the telencephalon of E11.5 pHes5 Tg mice, many VZ cells expressed GFP; however, numerous GFP-negative or weakly expressing cells were also present in a speckled pattern (Fig. 2B). At E13.5, expression of GFP in the telencephalic VZ was further diminished, apart from the cortical hem, which continued to show relatively high expression (Fig. 4A). After E15.5, few GFP-positive cells were visible in the VZ, although some migrating cells were found in the intermediate zone and cortical plate (Figs. 4B, C). These cells showed elongated shapes reminiscent of those of tangentially migrating neurons (Jimenez et al., 2002) or oligodendrocyte precursor cells (OPCs) (Miller and Ono, 1998).

To identify these cells, we performed immunohistochemistry using antibodies against the OPC markers NG2 and PDGFR-α (Levine et al., 1993; Hall et al., 1996). These experiments revealed that the GFP-positive cells are indeed OPCs (Figs. 4D–L), which are presumably migrating from the ventral telencephalon (Noll and Miller, 1993; Pringle and Richardson, 1993). However, not all NG2- and PDGFR-α-positive OPCs were GFP-positive, indicating that a subpopulation of OPCs was marked. The fractions of PDGFR-α-positive cells that expressed GFP were 79.9 ± 2.82% (E17.5) and 44.6 ± 4.21% (P0), and, conversely, almost 100% (99.2 ± 0.14% at E17.5 and 98.7 ± 1.19% at P0) of GFP-positive cells in the dorsolateral telencephalon expressed PDGFR-α. In other regions of the CNS, including the diencephalon, midbrain, hindbrain, and spinal cord, NG2- and PDGFR-α-positive OPCs were present at even earlier stages. Our results support a previous report that *Hes5* is expressed by OPCs and is downregulated as cells differentiate into mature oligodendrocytes (Kondo and Raff, 2000). Interestingly, Belachew et al. (2003) reported that postnatal NG2-expressing progenitors are intrinsically multipotent and able to generate functional neurons, suggesting that the VZ cells and OPCs labeled by GFP in pHes5 Tg mice may represent distinct types of multipotent progenitors or stem cells.

GFP expression in adult brains

Both lines of transgenic mice continued to express GFP in specific subsets of cells into adulthood. During the late embryonic and early postnatal development of pHes1 Tg mice, GFP continued to be expressed by VZ cells (Supplementary Figs. 3A–H). At early postnatal ages, GFP expression gradually diminished within the thinning VZ (Supplementary Figs. 3I–K, M–O), and, at these ages, GFP-positive cells also appeared outside the VZ in regions including the cerebellum and spinal cord, as well as other tissues in the CNS and the body.

The expression of pHes1-d2EGFP was particularly striking in two regions of the mature brain that are associated with ongoing neurogenesis in adulthood (Supplementary Figs. 3L, P). In the mature hippocampus, dentate granule neurons are continually generated from neural stem cells, which are located in subgranular layer (SGL) (Kuhn et al., 1996; Palmer et al., 1997), extend processes that contact the ventricular surface (Alvarez-Buylla et al., 2002), and lie close to blood vessels (Capela and Temple, 2002). Consistent with this notion, many GFP-labeled cells were observed in the dentate gyrus of adult pHes1 Tg mice (Figs. 5A–G). Seaberg and van der Kooy (2002) reported that neural stem cells exist in the ventricular subependymal tissue surrounding the hippocampus and contribute to the replenishment of progenitor cells in both the CA1 region and the dentate gyrus, as seen after experimental brain ischemia (Nakatomi et al., 2002); in agreement with this previous work, many GFP-positive cells were present in the hippocampal arch (Fig. 5A). None of the GFP-labeled cells were NeuN-positive (Figs. 5E–G), and the majority (91.2%) was immunoreactive for the astrocyte marker GFAP (Figs. 5A–D), which is expressed by hippocampal stem cells as well as by astrocytes (Seri et al., 2001). Adult neurogenesis also occurs in the subventricular zone (SVZ), a neurogenic region that seeds the rostral migratory stream (RMS) and produces new inhibitory interneurons in the adult olfactory

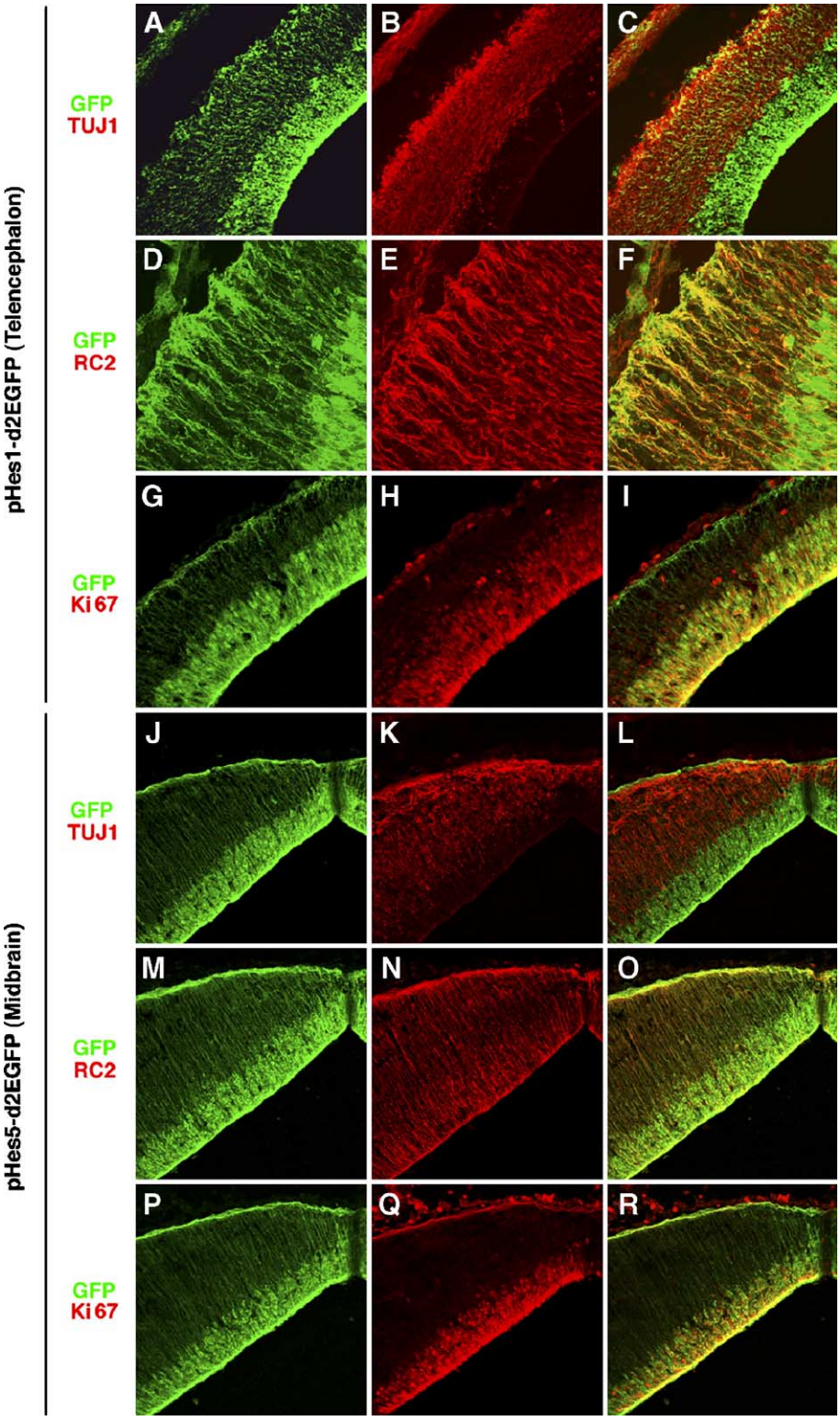

Fig. 3. GFP is expressed by mitotically active VZ cells, including radial glia. Coronal sections of the telencephalon of the pHes1 Tg mice (A–I) and midbrain of the pHes5 Tg mice (J–R) at E13.5. (A–C, J–L) GFP expression (green) in the VZ was segregated from TUJ1 staining (red), which marks postmitotic neurons in the cortical plate. (D–F, M–O) Radial glia were labeled with RC2 antibodies (red), revealing that most or all radial glial fibers co-expressed GFP and RC2. (G–I, P–R) When cycling cells were labeled with Ki67 antibodies (red), GFP expression overlapped extensively with Ki67 staining, suggesting that most GFP-labeled cells are mitotically active.

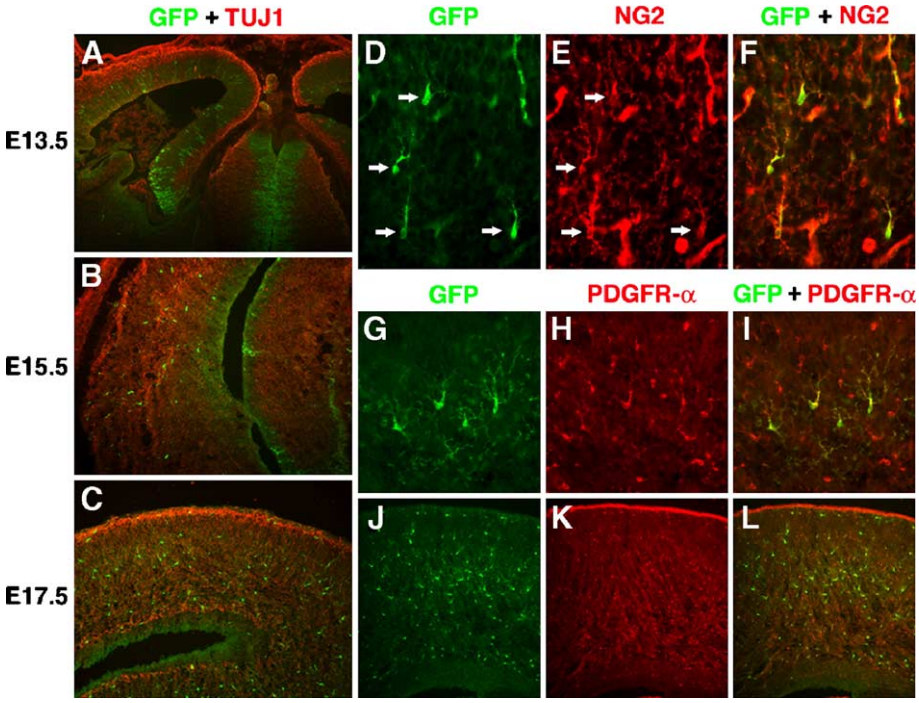

Fig. 4. GFP expression in oligodendrocyte precursor cells in the telencephalon of pHes5 Tg mice. Coronal sections of the telencephalon of pHes5 Tg mice at E13.5 (A), E15.5 (B), E17.5 (C), and P0 (D–L). (A–C) GFP expression in the telencephalic VZ diminished gradually over time, whereas the number of GFP-positive cells (green) migrating outside the VZ increased. Postmitotic neurons were labeled with TUJ1 antibodies (red). Within the P0 neocortex, essentially all GFP-positive cells were oligodendrocyte precursor cells (OPCs), as marked by the expression of NG2 (red, D–F) and PDGFR-α (red, G–L). However, not all OPCs expressed GFP.

bulb (Corotto et al., 1993). GFP expression was also apparent in the SVZ (Figs. 5H–P) and RMS (Figs. 5Q–S), extending into the olfactory bulb. Several lines of evidence suggest that SVZ astrocytes (which express glial markers but are functionally distinct from terminally differentiated astrocytes) show neural stem cell activity (Doetsch et al., 1999). Many of the GFP-positive cells (56.1% in the SVZ, 57.4% in the RMS) were also GFAP-positive (Figs. 5I–M, Q, R) but did not express NeuN (Figs. 5N–P, S) or markers for OPCs or oligodendrocytes, suggesting that most of these cells are undifferentiated neural progenitors.

In adult pHes5 Tg brains, many GFP-positive cells were present in the midbrain and diencephalon. There appeared to be two distinct populations: cells scattered in the ventral midbrain and diencephalon showed a morphology resembling that of OPCs, and these cells were NG2- and PDGFR-α-positive. In contrast, cells located in dorsal regions displayed multipolar shapes with many fine processes, and they were negative for NG2 or PDGFR-α. Some of latter cells could be immunostained with antibodies against GST-π and APC, both of which are expressed by oligodendrocytes. None of the dorsal GFP-labeled cells was positive for TUJ1, NeuN, Nestin, or GFAP (data not shown). These results suggest that a subpopulation of OPCs and some differentiated oligodendrocytes were labeled with GFP even in the mature brains of pHes5 Tg animals.

GFP expression is gradually confined to Müller glial cells in pHes1 Tg mice and to the ciliary marginal zone in pHes5 Tg mice

GFP was expressed in neuroepithelial cells of the embryonic retina in both pHes1 and pHes5 Tg mice. However, the expression patterns were not identical and displayed an interesting divergence postnatally. In E13.5 pHes1 Tg animals, GFP was expressed in the central retina but was absent from peripheral regions (Fig. 6A). In contrast, GFP was expressed more strongly in the peripheral retina in pHes5 Tg mice (Fig. 6G). Between E15.5 and P0, GFP expression expanded peripherally in pHes1 Tg mice (Figs. 6B, C), and the peripheral expression in the pHes5 Tg retina was gradually confined to the most peripheral region, including the ciliary body (Figs. 6H, I). GFP was also expressed in the lens in pHes1 Tg mice.

In postnatal retinas of pHes1 Tg animals, GFP labeled a subset of cells located in the inner nuclear layer (Figs. 6D–F). Further analysis using antibodies against Glutamine Synthetase (GS), a marker for Müller glial cells, demonstrated that GFP is expressed in almost all Müller glia (Figs. 6M–O). In the adult retina, it appeared that fewer Müller glial cells express GFP (Figs. 6P–R). In postnatal and adult pHes5 Tg mice, GFP expression in the retina was confined to ciliary marginal zone (CMZ), the ciliary body, and a small fraction of Müller glia located in the peripheral retina (Figs. 6J–L). It has been reported that retinal stem cells reside in the CMZ (Ahmad et al., 2000; Tropepe et al., 2000; Fischer and Reh, 2000) and that Müller glia have a potential to proliferate and generate neurons (Dyer and Cepko, 2000; Fischer and Reh, 2001, 2002, 2003); thus it could be hypothesized that GFP expression in pHes1 and pHes5 Tg mice marks different populations of retinal stem/progenitor cells (Müller glia and CMZ cells, respectively).

GFP-positive cells generate neurospheres in vitro

The expression patterns described above suggest the hypothesis that pHes-d2EGFP transgenes may specifically mark neural stem cells, an idea that can be tested by isolating these cells and

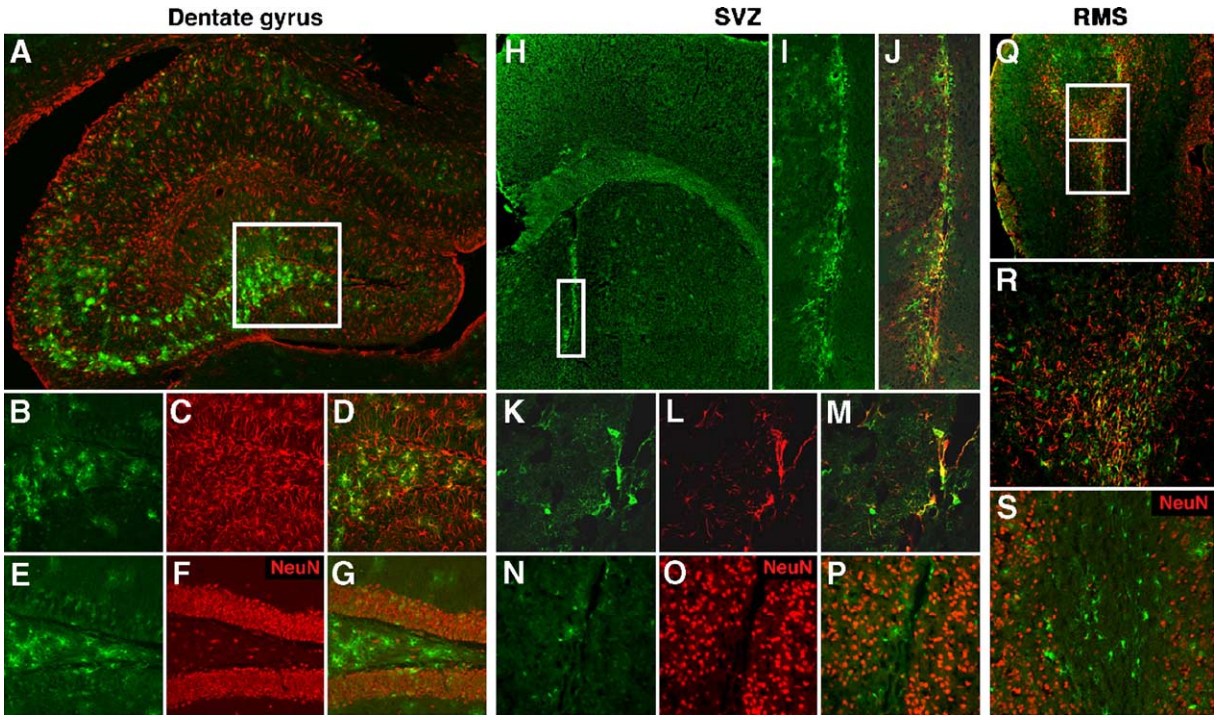

Fig. 5. GFP expression in the brains of adult pHes1 Tg mice. GFP-positive cells were present throughout the CNS, including cerebellum and spinal cord, and were found in many other tissues. In pHes1 Tg mice, GFP-positive cells were visible in neurogenic sites of the adult brain. (A) Within the hippocampus, GFP-positive cells were scattered in the CA3 region and the dentate gyrus. (A–G) The majority (91.2%) of GFP-labeled cells (green) in the dentate gyrus expressed the astrocyte marker GFAP (red). (H–P) GFP-positive cells were found in SVZ, a neurogenic region that contributes to the rostral migratory stream (RMS). Panels I and J show a higher magnification view of the region indicated by the box in panel H. (I–M) Many GFP-labeled cells in the SVZ (56.1%) were GFAP-positive (red). (Q–S) A number of GFP-positive cells were present in the RMS, and many (57.4%) were GFAP-positive (red, Q, R). Panels R and S show a higher magnification view of the region indicated by the box in panel Q (R: upper region, S: lower region). (E–G, N–P, S) None of the GFP-positive cells in the adult brain expressed the neuronal marker NeuN (green: GFP, red: NeuN).

assessing their multipotentiality and self-renewal properties. To ascertain whether GFP-positive cells exhibit the characteristics of neural stem cells in vitro, cells from the telencephalon of pHes1 Tg and midbrain of pHes5 Tg embryos at E11.5 were dissociated and plated onto poly-D-lysine-coated Terasaki wells at clonal density. After 2 h in culture, the percentages of GFP-positive cells derived from the dorsal telencephalon of pHes1 Tg and dorsal midbrain of pHes5 Tg mice were 20.5% and 41.2%, respectively. After 18–24 h in culture, only a small fraction of cells retained GFP expression (pHes1: 2.8%, pHes5: 4.6%); however, essentially all GFP-positive cells were Nestin-positive (pHes1: 96.7%, pHes5: 97.1%) (Figs. 7A–D, Supplementary Fig. 4) and TUJ1-negative. In many cases, GFP-positive cells were found in close association with one or more TUJ1-positive cells (Figs. 7E, F), which may represent the progeny of a GFP-labeled cell (see below).

To ascertain whether GFP-positive cells in the brains of pHes transgenic mice are neural stem cells, we dissociated cells from the E11.5 dorsolateral telencephalon of pHes1 Tg animals, sorted GFP-positive and -negative cells by FACS (Supplementary Fig. 5), and compared the ability of labeled and unlabeled cells to form neurospheres with that of unfractionated cells that passed through the FACS but were not gated. The cells were sorted, plated on ultra-low attachment dishes, and cultured in suspension for 7 days as primary neurospheres. Cells obtained from the GFP^+ fraction efficiently generated neurospheres (Fig. 7G) that contained many GFP-positive cells intermingled with GFP-negative cells (not shown). The average number of primary spheres >50 μm in diameter was 20.43 ± 1.86 (frequency 2.04%, n = 7, representing >3 independent experiments). In contrast, cells from the GFP-negative fraction produced very few neurospheres: only 1.5 ± 0.38 neurospheres were found (frequency 0.15%, n = 8, >3 independent experiments). These spheres also contained scattered GFP-positive cells (Supplementary Fig. 6), suggesting that a small number of cells in the GFP-negative population are capable of reexpressing GFP and pHes1. The number of primary spheres formed from unfractionated cells was 4.75 ± 0.82 (frequency 0.48%, n = 8, >3 independent experiments). Collectively, these results indicate that neural stem cells could be substantially enriched by sorting GFP-positive cells from pHes transgenic mice.

To assess secondary neurosphere formation, GFP-positive and -negative cells were sorted from the E11.5 dorsal midbrain of pHes5 Tg mice, plated on ultra-low attachment dishes, and cultured in suspension for 7 days as primary neurospheres. The primary spheres were then dissociated, and 10,000 cells were plated in each well to generate secondary neurospheres. In accordance with previous reports (Weiss et al., 1996a,b; Kawaguchi et al., 2001), we counted the numbers of secondary neurospheres present after 7 days of culture to assess the self-renewal capacity of the GFP-positive cells. Cells obtained from the GFP^+ fraction efficiently generated secondary neurospheres (Fig. 7H). The average number of secondary spheres >50 μm in diameter was 34.3 ± 2.81 (n = 6, representing >3 independent experiments). The multipotentiality of sphere-forming cells was verified by a differentiation assay, which demonstrated that all three lineages (neurons, astrocytes, and oligodendrocytes) were generated from each neurosphere (data not

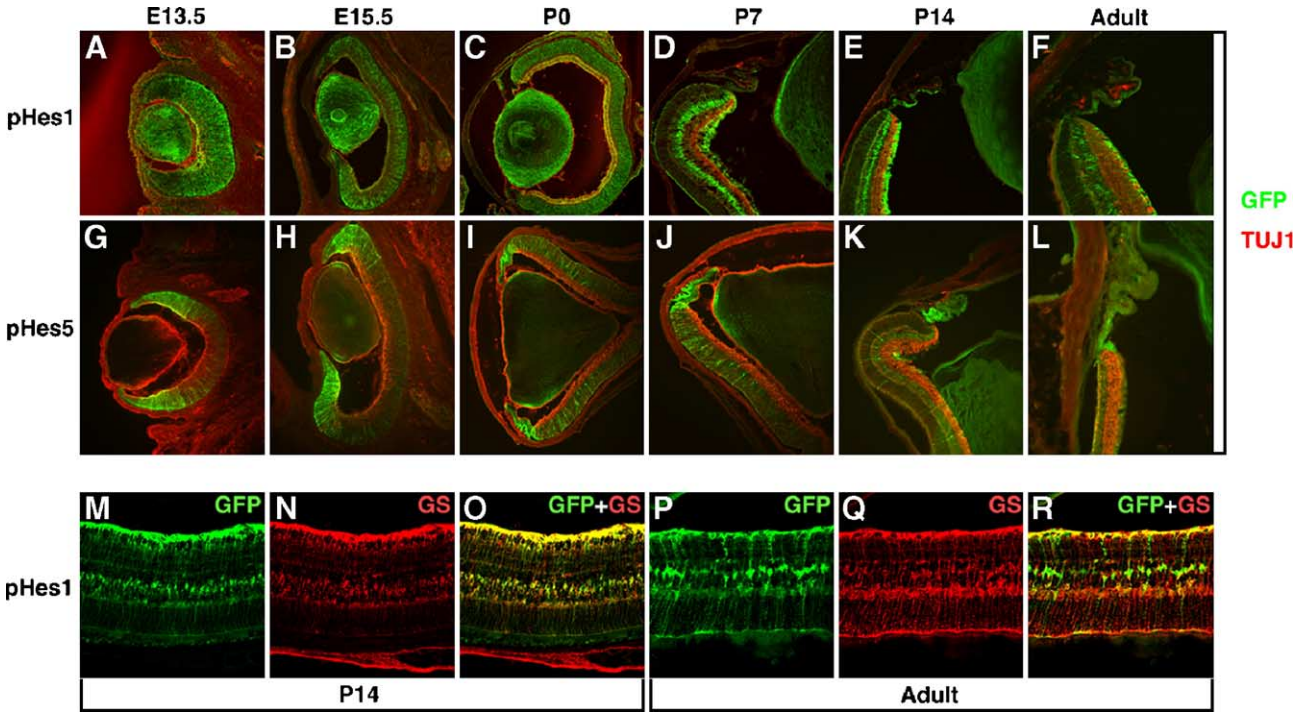

Fig. 6. GFP expression in the retinas of pHes1 and pHes5 Tg mice. In both pHes1 and pHes5 Tg mice, GFP (green) was expressed in the neuroepithelial layer of embryonic retina. (A, B) At E13.5 and E15.5, GFP expression was apparent in the central retina of pHes1 Tg animals but was less strong in the periphery, whereas GFP expression was higher in the peripheral retina in pHes5 Tg mice (G, H). As GFP expression expanded peripherally in pHes1 Tg retinas (C), the peripheral expression in pHes5 Tg was gradually confined to the extreme periphery, including the ciliary body (I). (J–L) In postnatal and adult retinas from pHes5 Tg mice, GFP expression was retained within ciliary marginal zone, ciliary body and in a small fraction of Müller glial cells in the peripheral retina. (D–F, M–O) In the postnatal retina of pHes1 Tg mice, GFP was expressed by almost all Müller glial cells, which were labeled with antibodies to Glutamine Synthetase (GS; red in panels N, O). (P–R) In the adult pHes1 Tg retina, GS-positive Müller glial cells (red) that expressed GFP were fewer in number.

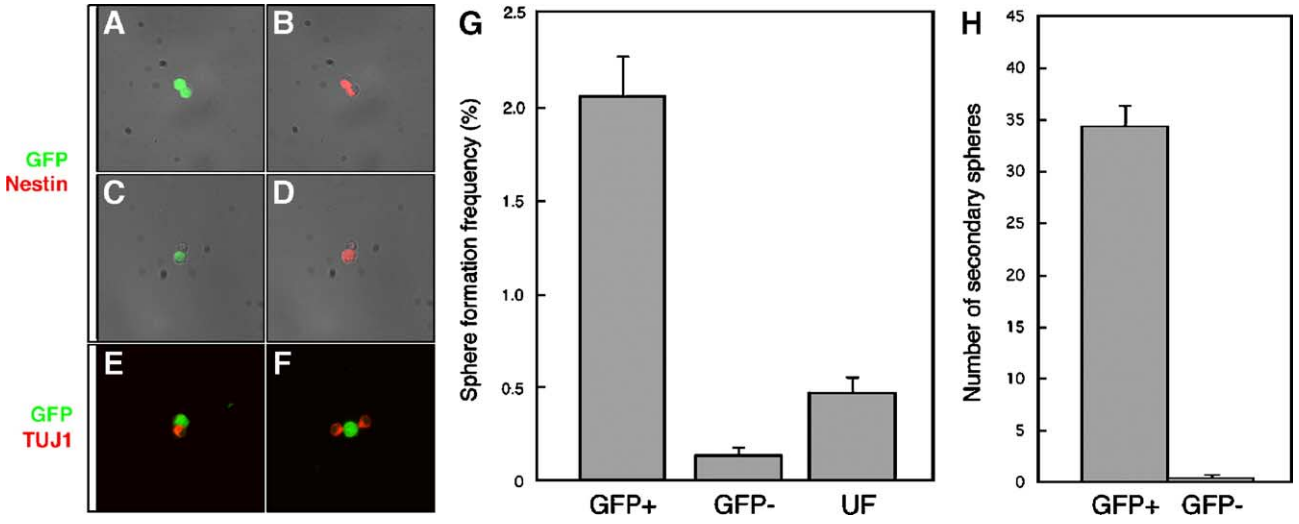

Fig. 7. GFP expression in dissociated cells and neurosphere-forming activity. Neural precursor cells were dissociated from the midbrain of pHes5 Tg mice and cultured at clonal density. (A, B) After 18–24 h in vitro, a pair of daughter cells both co-expressed GFP (A, green) and Nestin (B, red), suggesting that the parental cell had undergone a symmetric division. (C, D) In this case, only one of the pair co-expressed GFP (C, green) and Nestin (D, red), whereas the other daughter failed to express either marker, suggesting an asymmetric division. (E) An asymmetric division in which one daughter expressed GFP (green) and the other expressed the neuronal marker TUJ1 (red). (F) A single GFP-positive cell (green) was sometimes found in close association with one or more TUJ1-positive cells (red), but co-expression of these two markers was never observed. (G) Dissociated cells from the dorsolateral telencephalons of pHes1 Tg mice at E11.5 were sorted into GFP-positive and -negative fractions by FACS, and their ability to form primary neurospheres was compared to that of unfractionated cells (UF) that passed through the FACS but were not gated. Cells obtained from the GFP^+ fraction showed higher sphere formation frequency. The average numbers of primary spheres (larger than 50 μm in diameter) of >3 independent experiments are shown (bars represent SE). (H) Dissociated cells from the dorsal midbrains of pHes5 Tg mice at E11.5 were sorted by FACS, and their ability to form secondary neurospheres was assessed. Cells obtained from the GFP^+ fraction efficiently generated neurospheres. The average numbers of secondary spheres (larger than 50 μm in diameter) of >3 independent experiments are shown (bars represent SE).

shown). In contrast, cells from the GFP-negative fraction essentially failed to produce neurospheres: only 0.3 ± 0.19 neurospheres were found (n = 11, >3 independent experiments), indicating that the GFP-negative fraction contained few stem cells capable of maintaining proliferation in neurospheres. However, these results were not unexpected because many or most cells from the GFP-negative fraction are young neurons, as indicated by TUJ1 staining in Figs. 3C, L.

GFP-positive VZ cells undergo asymmetric cell divisions and self-renewal in vitro

Our initial culture studies showed that GFP-labeled cells in vitro expressed Nestin and were often closely associated with TUJ1-positive neurons, suggesting that GFP-labeled cells can undergo asymmetric divisions to generate neuronal progeny and undergo self-renewal. To test this hypothesis directly, we observed individual GFP-labeled cells in culture with time-lapse video-microscopy and subsequently immunostained cells using TUJ1 antibodies to reveal neurons. We were able to observe single GFP-positive cells that divided during the imaging period in an asymmetric mode. In these divisions, one daughter cell maintained GFP expression, while the other daughter differentiated into TUJ1-positive neuron (Figs. 8A–D). In these cases, the two daughter cells remained closely associated with one another, and some TUJ1-positive cells extended neurites to contact the GFP-positive sister (Fig. 8D). Alternatively, some single GFP-positive cells divided symmetrically: both daughter cells lost GFP expression, and each differentiated into a TUJ1-positive neuron (Figs. 8E–H). In these cases, the two daughters showed a tendency to separate from one another after division. Long periods of observation revealed that GFP-positive cells have a potential to self-renew. Some GFP-positive cells divided repeatedly for 2–3 successive divisions in which GFP-positive cells gave rise to GFP-positive daughter cells in the next generation (data not shown).

Previous studies showed that Numb is asymmetrically segregated into the neuronal daughter cell when neural stem cells undergo asymmetric division (Rhyu et al., 1994). Numb is thought to suppress the Notch signaling pathway (Shen et al., 2002) through the recruitment of α-adaptin, which promotes the endocytosis of Notch and thereby attenuates signaling (Santolini et al., 2000; Berdnik et al., 2002), or by promoting the ubiquitination of membrane-tethered Notch and the degradation of the Notch intracellular domain (McGill and McGlade, 2003). Thus, it is hypothesized that daughter cells inheriting more Numb protein are biased toward neuronal differentiation, but cells that fail to inherit Numb remain as proliferative stem cells due to higher levels of Notch signaling. Consistent with the idea, double labeling with Numb antibodies revealed that the levels of Numb and GFP expression showed opposing patterns in sister cells. When an asymmetric division occurred during time-lapse observation, the GFP-positive daughter was Numb-negative (arrow in Figs. 8I, J); conversely, the Numb-positive daughter was negative for GFP (or expressed GFP only weakly) (arrow in Figs. 8K, L). Shen et al. (2002) previously demonstrated that, when Numb is asymmetrically inherited by one daughter after division, the Numb-positive cell differentiates into a neuron, consistent with the absence of GFP in the present study.

Collectively, these results are consistent with the view that a daughter cell that inherits Numb downregulates Notch signaling, which regulates the Hes promoter that controls GFP expression. In cells that inherit little or no Numb, Notch–Hes signaling is maintained, and the cell continues to proliferate as a multipotent progenitor that retains the capacity to self-renew. These data suggest that pHes-d2EGFP expression can be used to identify cells that display the hallmark characteristics of neural stem cells.

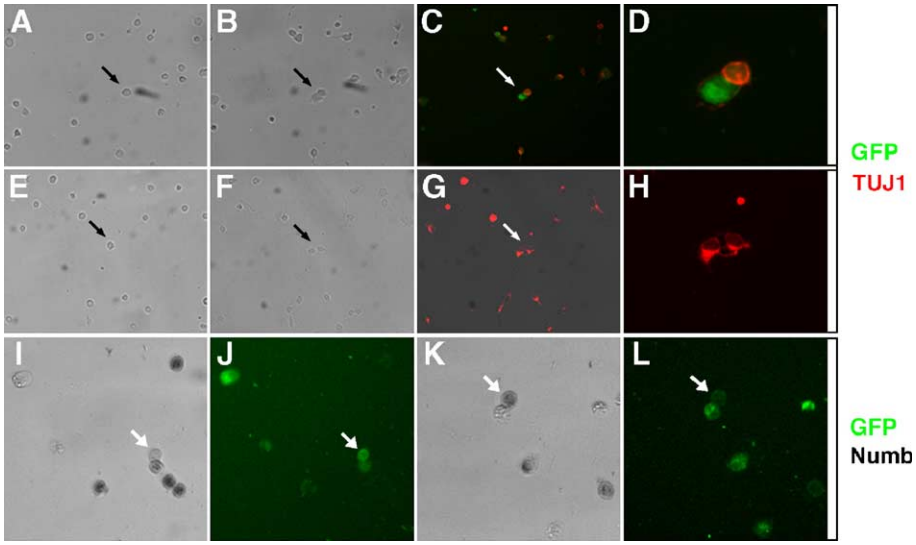

Fig. 8. GFP expression is retained by one daughter cell following asymmetric cell divisions. Time-lapse observation of asymmetric (A–D) or symmetric (E–H) cell divisions in vitro. (A, B) In these phase-contrast images, a single GFP-positive cell (arrow in panel A) divided into two daughter cells (arrow in panel B). (C) Only one of the pair retained GFP expression (green), whereas the other daughter cell was positive for TUJ1 (red). (D) A higher magnification of the cells shown in panel C. (E, F) Phase-contrast view of a single GFP-positive cell (arrow in panel E) that produced two daughter cells (arrow in F). (G) In this symmetric division, both daughters expressed TUJ1 (red) and differentiated into neurons, losing GFP expression during the process. (H) A higher magnification of the cells in panel G. (I, J) An asymmetric division that generated one GFP-positive cell (arrow; green in panel J), whereas the GFP-negative daughter cell was Numb-positive (dark staining in panel I). (K, L) In asymmetric divisions, the Numb-positive daughter cell (dark staining marked by the arrow in panel K) was GFP-negative (green in panel L).

Discussion

During development and in adulthood, neural stem cells retain the potential to generate both neurons and glia. We have used promoters derived from *Hes* genes to drive expression of destabilized EGFP and mark VZ cells of the developing mouse brain. Our studies of pHes transgenic mice revealed that GFP expression was restricted to undifferentiated VZ cells, including radial glia, and that these GFP-positive cells efficiently generated neurospheres. Clonal analysis revealed that cells marked by pHes-GFP expression can undergo asymmetric cell divisions that produce a Numb-positive neuronal daughter and a GFP- and Nestin-positive progenitor cell. Because self-renewal is a key attribute of stem cells, our data collectively suggest that pHes-GFP expression identifies cells that function as neural stem cells during development. pHes1-GFP expression in the telencephalic VZ persisted until early postnatal life, and some GFP-positive cells were still present in the SVZ and the dentate gyrus in the adult brain. Our results suggest that cells that express pHes-EGFP show enriched neural stem cell activity.

Hes gene function in CNS development

Ventricular zone cells in the developing CNS express the *Hairy/Enhancer of Split* homologs *Hes1* and *Hes5*, members of the basic helix–loop–helix (bHLH) family of genes, which regulate cell fate decisions and the differentiation of a variety of tissues including the CNS. *Hes1* and *Hes5* function downstream of Notch signaling as negative regulators of neuronal differentiation and maintain cells as progenitors (Ohtsuka et al., 1999). Transient misexpression of *Hes1* or *Hes5* in the developing telencephalon prevents the differentiation of VZ cells and preserves cells as radial glia, which exhibit the properties of neural stem cells; when *Hes* expression is downregulated, the cells produce both neurons and astrocytes, suggesting that *Hes1* and *Hes5* may promote a neural stem cell identity (Ohtsuka et al., 2001). In further support of this view, it has been reported that Notch signaling is essential for the maintenance of neural stem cells (Hitoshi et al., 2002a) and promotes radial glial identity (Gaiano and Fishell, 2002; Gaiano et al., 2000). During CNS development, radial glial cells normally generate both neurons and glia (Miyata et al., 2001; Noctor et al., 2001, 2002), can give rise to neurospheres (Hartfuss et al., 2001), a commonly used barometer of stem cell identity, and persist in the postnatal VZ with a capacity to generate neurons (Tramontin et al., 2003). It is unclear, however, whether all neural stem cells at early stages have a radial glial morphology (Hartfuss et al., 2001, Anthony et al., 2004) or whether radial glia represent only one of several stem cell types in the developing brain.

Endogenous *Hes1* and *Hes5* are expressed in distinct patterns in some different brain regions, and this complementary expression pattern seemed to be enhanced in pHes-d2EGFP transgenic mice. During early gestation in pHes1 Tg mice, GFP was strongly expressed in the VZ of the telencephalon, ventral midbrain, and hindbrain; in contrast, pHes5 Tg animals showed strong expression in the dorsal midbrain. The pattern of GFP expression in pHes1 Tg mice closely mimicked that of endogenous *Hes1*. GFP expression in pHes5 Tg mice, however, differed from that of endogenous *Hes5* in some parts of the body, probably due to the shorter length of *Hes5* promoter sequences used (760 bp) compared to the *Hes1* promoter (2.5 kb). Nevertheless, as expected, GFP was expressed in undifferentiated VZ cells, including radial glia, during mid- to late stages of gestation in pHes1 Tg mice and until mid-gestation in pHes5 Tg mice. In the telencephalon of pHes1 Tg and the midbrain of pHes5 Tg mice, GFP was expressed strongly and homogeneously in almost all VZ cells, and expression persisted in telencephalic VZ of pHes1 Tg animals until early postnatal life.

GFP expression marks neural stem cells during asymmetric cell divisions

In both pHes1 and pHes5 Tg embryos, GFP expression appears to mark mitotically active cells including radial glia, raising the possibility that the transgenes might be expressed specifically in neural stem cells within the developing CNS. To test this hypothesis, we performed two assays that are thought to assess stem cell activity: the neurosphere assay and a time-lapse analysis to ascertain whether GFP-labeled cells undergo asymmetric cell divisions in vitro. Indeed, we found that GFP-positive cells from pHes Tg brains showed high levels of sphere-forming activity (14–100 times greater than that observed for GFP-negative cells), and differentiation of the spheres resulted in the production of all three lineages (neurons, astrocytes, and oligodendrocytes). Observation of individual GFP-labeled cells in culture using time-lapse videomicroscopy revealed single GFP-positive cells that divided asymmetrically during the imaging period, yielding one daughter that maintained GFP expression and another that differentiated into a TUJ1-positive neuron. Longer observations visualized GFP-positive cells that divided repeatedly for 2–3 successive divisions, showing that these cells are capable of self-renewal. Collectively, these observations suggest that pHes transgenic mice enable the visualization and enrichment of cells that can display the characteristics of neural stem cells in vitro.

After dissociation into single cells and plating onto dishes, many cells extinguished GFP expression and differentiated into neurons. Presumably, this was due to a loss of cell–cell contacts and consequent loss of stimulation by Notch ligands (such as Delta) that are expressed on neighboring neurons or precursors, thus diminishing signaling through the Notch–Hes pathway. In contrast, GFP-positive cells in aggregates composed of >2 cells retained GFP expression more efficiently, presumably because of the continued influence of cell–cell interactions on *Hes* gene expression.

Our observations of asymmetric cell divisions in vitro revealed that daughter cells that retain GFP expression remain as undifferentiated proliferative cells, whereas daughters that extinguish GFP express Numb at high levels. Using similar clonal density cultures, Shen et al. (2002) previously demonstrated that cells that inherit Numb following asymmetric divisions differentiate into neurons. Recent studies have demonstrated that Numb downregulates Notch signaling through endocytosis by recruiting α-adaptin (Santolini et al., 2000; Berdnik et al., 2002) or degradation following ubiquitination (McGill and McGlade, 2003). Our results reinforce the theory that, when asymmetrically distributed into one daughter cell, Numb represses Notch signaling upstream of the *Hes* promoter and thereby promotes neuronal differentiation, whereas the other daughter, which expresses little or no Numb, maintains Notch–Hes signaling (as indicated by retention of GFP expression) and remains as a neural stem cell. Taken together, our data suggest that GFP expression can be used as an indicator of cells that remain as neural stem cells following asymmetric cell division.

pHes-GFP expression also marks latent progenitors in the postnatal retina

pHes-GFP transgenic mice may provide an excellent opportunity to enhance our general comprehension of stem cell identity and activities in various tissues. Even in older animals, GFP expression was found in defined populations of cells, some of which may show stem cell activity. For example, it has been reported that stem cells reside in the ciliary marginal zone (CMZ) of the retina (Ahmad et al., 2000; Tropepe et al., 2000; Fischer and Reh, 2000) and that Müller glia have the potential to proliferate and generate neurons in damaged retinas (Dyer and Cepko, 2000; Fischer and Reh, 2001, 2002, 2003). Indeed, both we and others have reported that expression of *Hes1* and *Hes5* promotes Müller glial fates in the retina (Hojo et al., 2000; Furukawa et al., 2000). It is intriguing to speculate that GFP expression in CMZ cells and Müller glia may mark multipotent stem cells within this tissue, a hypothesis that remains to be tested directly.

GFP expression as a marker of embryonic neural stem cells

Neural stem cells display distinct capacities for self-renewal and the generation of multiple cell types, with differences apparent among cells from different developmental stages and spatial locations within the CNS (Temple, 2001). For example, stem cells derived from different anterior–posterior levels of the neural axis produce progeny that express markers characteristic of their region of origin (Hitoshi et al., 2002b; Ostenfeld et al., 2002; Jain et al., 2003; Horiguchi et al., 2004). To analyze such differences or alterations, it is necessary to mark and isolate the neural stem cells as purely as possible. However, the lack of suitable markers makes the identification of neural stem cells difficult, although some specific markers have been reported to distinguish them. For example, Capela and Temple (2002) reported that LeX marks SVZ stem cells, Uchida et al. (2000) sorted human neural stem cells using cell surface markers, Rietze et al. (2001) purified pluripotent neural stem cells from adult mouse brains, and two groups used the nestin promoter to mark stem cells in embryonic brains (Kawaguchi et al., 2001; Mignone et al., 2004). However, there are currently no available markers by which one can distinguish neural stem cells from other progenitor cells that may commingle within the VZ or to prospectively identify stem cells in culture.

The generation of pHes-d2EGFP transgenic mice has provided a positive step in this direction. While our results do not enable us to conclude that every GFP-positive cell in the embryonic neural tube has the characteristic features of a stem cell, the data do demonstrate that these cells can self-renew in culture and thus exhibit one feature that is key to a stem cell identity. Self-renewal is not, of course, sufficient to define a stem cell; the second aspect is the demonstration that the cell can generate a variety of neural phenotypes. With current technologies, it is difficult to ascertain clearly whether every GFP-positive cell is multipotential. However, we adopted the neurosphere assay and the pair assay (clonal analysis) as the best combination of functional assays to assess the developmental capacity of GFP-positive cells. These experiments revealed that GFP-positive cells can form neurospheres efficiently and generate a variety of cell types, suggesting that many of these cells have stem cell activity.

It is difficult to draw a clear distinction between neural stem cells and more restricted progenitors. However, our results strongly suggest that the GFP-positive population includes multipotential stem cells including radial glial cells and show that GFP expression is lost rapidly when the progenitor cells start to express TUJ1, an early marker of postmitotic neurons. Previous studies suggest that multipotential stem cells divide asymmetrically to produce one stem cell daughter and one neuronal daughter, the latter of which inherits Numb and differentiates into a TUJ1-positive neuron (Shen et al., 2002). Thus, the retention of GFP expression by the undifferentiated cell with self-renewal capacity is consistent with the suggestion that this daughter functions as a stem cell.

Thus, we propose that pHes-d2EGFP expression can be used to mark, isolate, and enrich embryonic neural stem cells from the embryonic CNS and that these cells will be useful in identifying molecules expressed differentially by distinct subsets of neural stem cells and to define the properties and types of neural stem cells.

Experimental methods

Transgene construction and generation of transgenic mice

The plasmid pd2EGFP-1 containing the destabilized, red-shifted variant of wild-type GFP (d2EGFP) and SV40 early mRNA polyadenylation signal (SV40 polyA) was purchased from the Clontech Laboratories. pHes1-d2EGFP and pHes5-d2EGFP transgenes were generated by subcloning a 2.5-kb blunt-ended *Hin*dIII–*Hin*dIII fragment containing the *Hes1* promoter from the pHes1-luc vector or a 0.76-kb blunt-ended *Nhe*I–*Nhe*I fragment containing the *Hes5* promoter from the pHes5-luc vector, into the *Sma*I site of pd2EGFP-1 by blunt-end ligation. For microinjection, the 3.6-kb *Hes1* promoter-d2EGFP-SV40 polyA fragment (Fig. 1A) or 1.9-kb *Hes5* promoter-d2EGFP-SV40 polyA fragment (Fig. 1B) was excised using *Kpn*I–*Afl*II, separated by agarose gel electrophoresis, purified using a Qiagen QIAEXII Gel Extraction Kit and eluted with MiTE (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.4).

Transgenic mice were generated by pronuclear microinjection using fertilized eggs from F1 mice (pHes1) or FVB/N mice (pHes5). Founders were identified by Southern blot analysis of tail DNA using probes complementary to *Hes1* or *Hes5* promoter sequences. For genotyping of pHes1-d2EGFP transgenic mice, the 0.79-kb *Bam*HI–*Xho*I fragment was used to detect a 5.9-kb wild-type band and 2.7-kb transgenic band following digestion of genomic DNA with *Bam*HI–*Hin*dIII. For pHes5-d2EGFP mice, the 0.76-kb *Nhe*I–*Sac*I fragment was used as a probe to detect a 1.4-kb wild-type band and 0.76-kb transgenic band following digestion of genomic DNA with *Apa*I–*Sac*I.

In utero electroporation

In utero electroporation was performed using methods described previously (Ohtsuka et al., 2001). Embryos were harvested 24–72 h after electroporation. Brains were excised, fixed in 4% paraformaldehyde, cryoprotected, embedded in OCT, and sectioned on a cryostat at 16 μm.

In situ hybridization

In situ hybridization was performed as previously described (Sakamoto et al., 2003). Digoxigenin-labeled antisense RNA probes corresponding to the full-length mouse *Hes1* and *Hes5*

cDNAs were synthesized in vitro and hybridized to whole-mount embryos or cryosections. Labeled preparations were imaged using a Zeiss Axiophot microscope equipped with an AxioCam color CCD camera.

Immunohistochemistry

Fixed cryosections were washed with PBS, preincubated in PBS containing 5% normal goat serum and 0.1% Triton X-100, then incubated in 1% normal goat serum and 0.1% Triton X-100 with the following primary antibodies: mouse and rabbit anti-GFP (1:500; Molecular Probes), mouse anti-TUJ1 (1:1000; Covance), mouse anti-RC2 (IgM; 1:5; Developmental Studies Hybridoma Bank, Iowa City, IA), mouse anti-Ki67 (1:100; BD Pharmingen), rabbit anti-NG2 (1:200; Chemicon), rat anti-PDGFR-α (1:400; BD Pharmingen), mouse anti-GFAP (1:400; Sigma), mouse anti-NeuN (1:500; Chemicon), mouse anti-Glutamine Synthetase (1:400; Chemicon), mouse anti-Nestin (1:500; BD Pharmingen), and rabbit anti-Numb antibodies (1:200; kindly provided by Dr. Weimin Zhong). Primary antibodies were detected with Cy2-conjugated goat anti-rabbit IgG (1:200; Jackson), Cy2-conjugated goat anti-mouse IgG (1:200; Jackson), Cy3-conjugated goat anti-mouse IgG (1:1000; Jackson), Texas red-conjugated goat anti-mouse IgM (1:200; Jackson), biotinylated goat anti-mouse, rabbit or rat IgG (1:200; Vector), Texas red avidin D (1:2000; Vector), and ABC-VIP kit (Vector). Fluorescent sections were imaged using a Zeiss LSM510 confocal microscope.

Cell sorting

Brain tissues were excised from transgenic embryos, digested with 0.25% trypsin–EDTA and 50 μg/ml DNaseI and dissociated completely by pipetting. 0.25% trypsin inhibitor and DMEM were added, and the cells were spun down, re-suspended in serum-free culture medium [DMEM/F-12 (1:1) supplemented with B-27, N-2, 20 ng/ml EGF, and 20 ng/ml bFGF], and filtered with a cell strainer.

FACS sorting was performed using a FACS Vantage flow cytometer/cell sorter or a FACSAria cell sorter (BD Biosciences). Dead cells were excluded by gating on forward and side scatter and by elimination of cells stained with propidium iodide. Cells in GFP^+ fraction and GFP^- fraction were sorted and collected separately into culture medium. A fluorescence intensity of $>2 \times 10^1$ was used to define cells as GFP^+ and $<5 \times 10^0$ as GFP^- (Supplementary Fig. 5).

Neurosphere assay

Neurosphere formation assays were performed essentially as described previously (Reynolds and Weiss, 1996; Weiss et al., 1996a,b; Nakamura et al., 2000; Ohtsuka et al., 2001). The neuroepithelium of the dorsal telencephalon or midbrain of E11.5 transgenic embryos was digested with trypsin–EDTA and DNaseI as described above. Following FACS separation, cells were collected into neurosphere culture medium [DMEM/F-12 supplemented with 100 μg/ml transferrin, 25 μg/ml insulin, 20 nM progesterone, 30 nM sodium selenite, 60 μM putrescine, 20 ng/ml EGF, and 20 ng/ml bFGF] or NeuroCult™ NSC basal medium supplemented with NeuroCult™ NSC proliferation supplements (StemCell Technologies) and 20 ng/ml EGF. For primary sphere formation assay, 100 μl of cell suspension (1×10^4 cells/ml) containing ~1000 cells was plated into each well of a 96-well ultra-low attachment plate (Corning). The numbers of primary spheres larger than 50 μm in diameter were counted at day 7. For secondary sphere formation assay, primary spheres derived from 50 μl of cell suspension (1×10^6 cells/ml) were collected at day 7, digested with trypsin–EDTA and DNaseI, and dissociated completely by pipetting. Fifty microliters of cell suspension (2×10^5 cells/ml) containing ~10,000 cells was plated into each well of a 96-well plate and cultured for 7 days. The numbers of secondary spheres larger than 50 μm in diameter were then counted at day 7.

Time-lapse observation of cells in clonal density cultures

For clonal density cultures, the neuroepithelium of the dorsal telencephalon or midbrain of E11.5 embryos was digested with trypsin–EDTA, and dissociated cells were plated at clonal density onto poly-D-lysine-coated Terasaki wells or glass base dishes (IWAKI) in serum-free culture medium [DMEM/F-12 supplemented with B-27, N-2, 20 ng/ml EGF, and 20 ng/ml bFGF]. Cells that divided during the first 18–24 h in culture were observed by time-lapse microscopy or were automatically monitored under an inverted microscope equipped with a CO_2 incubator and Photometrics CCD camera for up to 48 h. Cultures were then fixed in 4% paraformaldehyde and examined immunocytochemically. Time-lapse images were analyzed and processed with a MetaMorph Version 4.6.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mcn.2005.09.006.

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