Nestin is essential for mitogen-stimulated proliferation of neural progenitor cells

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Abstract

The intermediate filament (IF) protein nestin is a widely accepted molecular marker for neural progenitor cells (NPCs), but its function during neurogenesis remains largely unknown. We found that in embryonic cortical NPCs down-regulation of the expression of nestin, but not its co-polymer IF protein vimentin, resulted in a G1 cell-cycle arrest and a severe reduction in the generation of neurons. Furthermore, down-regulating nestin expression in cultured cortical NPCs markedly suppressed their colony-formation ability and blocked the elevation of the cyclin D1/E protein level in response to the treatment with bFGF. Interestingly, nestin down-regulation caused a marked suppression in the activation of the phosphoinositide 3-kinase (PI3K) pathway but not the mitogen-activated protein kinase (MAPK) pathway in these NPCs. Moreover, effects in the proliferation of cortical NPCs caused by nestin down-regulation could be prevented by up-regulating PI3K activity. Thus, nestin is essential for the proliferation of NPCs by promoting the activation of PI3K in response to mitogenic growth factors.

Introduction

The progenitors for neurons and glia that build up the central nervous system (CNS) are located at two major germinal zones in the developing brain—the ventricular zone (VZ) lining the cerebral ventricles and the adjacent sub-ventricular zone (SVZ) (McKay, 1997). In the early expansion stage, NPCs enlarge their population primarily by proliferative division. As development proceeds, they undergo more frequent differentiative division to produce cells that progressively initiate the expression of pro-neural genes, and eventually differentiate into post-mitotic neurons or glial cells (Gotz and Huttner, 2005). Due to its specific expression in NPCs, nestin is widely used as a molecular marker for neural progenitor cells. In radial glial cells, a major type of NPCs in developing cortex (Rakic and Lombroso, 1998), nestin expression is tightly controlled during the cell cycle, and has been proposed to contribute to the maintenance of the elongated radial morphology of these cortical progenitors (Sunabori et al., 2008). Recent study in Zebrafish showed that loss of nestin led to apoptosis of NPCs (Chen et al., 2010). However, the exact function of nestin inside mammalian NPCs during brain morphogenesis remains a mystery.

As a member of type IV intermediate filament (IF) proteins, nestin is specifically expressed in NPCs but not in post-mitotic neurons and glia (Lendahl et al., 1990). Unlike most other IF proteins, nestin cannot form stable filaments by itself, but can form co-polymer IFs both in vitro and in vivo by association with type III IF proteins such as vimentin (Marvin et al., 1998; Steinert et al., 1999). In the developing CNS, nestin is expressed concurrently with vimentin in NPCs from neural tube closure till early gliogenesis (Dahlstrand et al., 1995). During the terminal differentiation of NPCs, the IF composition in the cell rapidly switches from nestin/vimentin to α-internexin/neurofilament in neurons (Liem, 1993) and GFAP/vimentin in astrocytes (Zerlin et al., 1995). In adult brain of mammals, nestin is expressed in NPCs at the sub-ventricular zone and the sub-granular layer of the hippocampal dentate gyrus, two regions of active neurogenesis in adulthood (Lagace et al., 2007). Under pathological condition, nestin expression can be re-induced in adult tissues, such as brain tumor and CNS injury (Clarke et al., 1994; Sugawara et al., 2002).

The proliferation of stem cells or progenitor cells is precisely controlled by extrinsic factors, including EGF, FGF, Wnt and BMP (Adachi et al., 2007; Ille et al., 2007; Lie et al., 2005; Ostenfeld and Svendsen, 2004), as well as intrinsic signals like MAPK and PI3K pathways (Peltier et al., 2007; Samuels et al., 2008; Sumi et al., 2008; Torroglosa et al., 2007). In the present study, we found that nestin was required for the activation of PI3K by bFGF. Down-regulation of nestin expression suppressed the proliferation of cortical NPCs both in vivo and in vitro, and this effect could be compensated by up-regulating PI3K activity. Thus, nestin is essential for the proliferation of NPCs in response to the stimulation of growth factors.

Results

Down-regulation of nestin impaired the cortical neurogenesis

To explore the potential function of nestin in brain development, we introduced plasmids coding for small interference RNAs (siRNAs or RNAis) against nestin into progenitors of the embryonic cerebral cortex.
of rat by using the in utero electroporation (Chen et al., 2008; Saito and Nakatsuji, 2001). The efficiency of these siRNAs was verified by examining their effectiveness on down-regulating the nestin protein level in both cell line and primary culture of cortical NPCs (Supplementary Fig. S1), with the RNAi vector (pSuper) or a scramble RNA sequence (sc-RNAi) as the control. These RNAi constructs, together with plasmids encoding EYFP, were electroporated into cortical NPCs in the VZ of rat cortex at embryonic day 16 (E16), the peak stage for the generation of neurons destined for the outmost layers of CP (Jacobson, 1970). If cortical NPCs were transfected with pSuper or sc-RNAi, newborn neurons derived from these progenitors started to enter CP at the first postnatal day (P0) and most of them arrived at the upper layers of CP at around P3 (Fig. 1A and D, Supplementary Fig. S2), consistent with the previous observation (Chen et al., 2008). However, when cortical NPCs were transfected with two effective RNAis against nestin, very few transfected neurons were observed in CP at either P0 or P3 (Fig. 1B and D). The total cell number also markedly reduced in nes-RNAi1-transfected slices comparing with the control (Fig. 1E). This phenomenon was not due to the non-specific effect of RNAis, since no obvious defect was observed after transfection with a mutant form of nes-RNAi1 with three mutated nucleotides (nes-RNAiM), and over-expression of nestin together with nes-RNAi1 prevented the reduction of neurons in CP (Fig. 1C and D) (We use nes-RNAi to represent nes-RNAi1 hereafter). After immunostaining with the anti-cleaved caspase-3 antibody, we calculated the ratio of the number of cleaved caspase-3-positive (caspase-3+) cells in the transfected side over the untransfected side of the same cortical section and found that caspase-3+ signal was not elevated after transfection with nes-RNAi (Fig. 1F). Western blotting analysis of transfected progenitors in vitro also showed that nes-RNAi did not improve the protein level of cleaved caspase-3 (Supplementary Fig. S3). Thus apoptosis of transfected cells does not seem to be a major reason for the neuronal reduction after down-regulation of nestin.

To clarify the reason for the reduction of newborn neurons in CP after down-regulation of nestin, we next examined the cell fate of those cells remained in the VZ/SVZ at P3 cortex by immunostaining. Since cells are highly compacted in VZ/SVZ region, to avoid potential false positive/negative signal during stereological cell counting, we dissociated cells in the VZ/SVZ at P3 cortex, fixed cells, and plated them on laminin-treated cover glass before immunostaining, a method

**Fig. 1.** Down-regulation of nestin impaired the cortical neurogenesis. Progenitors located in the VZ of E16 rat cortex were transfected with pSuper (A), nes-RNAi1 (B), and nes-RNAi1 plus plasmids coding for nestin (C). Coronal sections of P3 cortex at similar anterior-posterior level are shown. EYFP+ cells are shown in green and DAPI staining in blue. Scale bar, 500 μm. The average percentages of newborn neurons in CP under various treatments are shown in (D). The average number of cells derived from NPCs transfected with pSuper or nes-RNAi is shown in (E). The thickness of each slice is 30 μm. Numbers in brackets indicate the amount of pups (one slice per rat) examined in each experiment. Slices were stained for cleaved caspase-3 at E18 and P0. A ratio of the number of caspase-3+ cells in VZ/SVZ of transfected (T.H) over untransfected hemisphere (UT.H) of the same slice was calculated (T.H/ UT.H) (F). Cells remained in VZ/SVZ of P3 cortex were dissociated and stained for different markers with specific antibodies. Percentages of each group of cells are shown in (G). Each group includes more than 200 cells and all experiments have been repeated for four times. Data represent mean ± S.E.M. Student t-test. *P<0.05; **P<0.01; ***P<0.001. VZ: ventricular zone; SVZ: sub-ventricular zone; WM: white matter; CP: cortical plate.
that has been used previously (Sanada and Tsai, 2005) (Supplementary Fig. S4). Cells were stained with different markers including Ki-67 for cells within the cell cycle (from G1 to M phase, except G0), Tuj1 for neurons, S100β for glial lineage cells, GFAP for astrocytes, and vimentin for progenitors. Although the immunostaining for vimentin showed no significant difference between pSuper- and nes-RNAi-transfected cells, there were much less Ki-67+ cells in nes-RNAi-transfected cells comparing with those transfected with pSuper (Fig. 1G), suggesting that these cells were not able to proliferate actively. In addition, the percentage of positive cells for each of the three markers of differentiated cells, Tuj1, GFAP, and S100β, decreased when nestin was down-regulated (Fig. 1G), indicating that the observed reduction in cell proliferation is not caused by a premature differentiation of neural progenitors. Taken together, these results suggest that down-regulation of nestin possibly suppresses the cortical neurogenesis by blocking the active proliferation of NPCs.

**Down-regulation of nestin in NPCs induced a cell-cycle arrest**

Next, we analyzed the short-term effect of the transfection of nes-RNAi into NPCs. We used antibodies against 5-bromodeoxyuridine (BrdU) and cell cycle markers including Ki-67 and phospho-Histon3 (p-H3) to label transfected cells in the VZ/SVZ of cortex 48 hr after electroporation, with injection of BrdU pulses at 24 hr or 4 hr before tissue fixation (Supplementary Fig. S5). First we found that cortical progenitors transfected with nes-RNAi showed a significant reduction in the proportion of 4-hr BrdU-incorporation rate comparing with control cells (Fig. 2A and D). Then cortical sections were double stained for BrdU, to label cells that were undergoing DNA synthesis (S phase) at the time of BrdU injection, and Ki-67, to label cells that remained in the cell cycle at the time of fixation (24 hr after BrdU injection). Cells that had exited the cell cycle within 24 hr after BrdU labeling were identified as BrdU+, EYFP+, but Ki-67−, and progenitors remained in the cell cycle as BrdU+, EYFP+, and Ki-67+ (Chenn and Walsh, 2002; Sanada and Tsai, 2005). An index of cell cycle persistence was defined as the percentage of BrdU+ / EYFP+ / Ki-67+ cells among all BrdU+ / EYFP+ cells. We found that the transfection with nes-RNAi significantly increased the index of cell cycle persistence, indicating higher percentage of cells that remained in the cell cycle (Fig. 2B and E). This suggests that the observed decrease in the percentage of BrdU+ cells after transfection with nes-RNAi is likely a result of cell-cycle arrest instead of the premature differentiation of progenitors. Next, the mitotic rate of progenitor cells was assessed by double staining for BrdU and p-H3, to mark cells in the M phase at the time of fixation (4 hr after BrdU injection). Cells that had processed from S phase to M phase within the 4 hr of BrdU incorporation were identified as BrdU+, EYFP+, and p-H3+. The proportion of triple-positive cells among all BrdU+/EYFP+ cells was defined as the mitotic rate (Sanada and Tsai, 2005). Interestingly, we found that down-regulating nestin did not affect the mitotic rate for those cells having entered the S phase (Fig. 2C and F), indicating that the cell-cycle arrest may happen during the G1-S transition (see Discussion). Taken together, these data suggest that down-regulation of nestin has caused a cell-cycle arrest and thus suppressed the proliferation of cortical NPCs.

**Fig. 2.** Down-regulation of nestin in progenitors caused a cell-cycle arrest. E18 cortical slices were stained for BrdU (A), BrdU and Ki-67 (B), or BrdU and p-H3 (C). Scale bar, 50 μm. The projections of xz and yz axis are shown. The BrdU-incorporation index, cell-cycle persistence index and mitotic rate index are shown in (D), (E), and (F). Numbers in brackets indicate the amount of pups (one slice per rat) used in each experiment. Data represent mean± S.E.M. Student t-test. *P<0.05; **P<0.01; ***P<0.001.
Down-regulation of nestin did not affect the dividing patterns of NPCs

The VZ/SVZ contains progenitors that are heterogeneous in their proliferative features. Based on the cell fate of two daughter cells, three major types of progenitor division have been observed during early development including proliferative division (P-P, one progenitor produces two progenitors), asymmetric differentiative division (P-N, one progenitor produces a progenitor and a neuron), and symmetric differentiative division (N-N, one progenitor produces two neurons) (Shen et al., 2002). We next explored which type of progenitor division was impaired by monitoring the fate of a pair of daughter cells derived from a single NPC in culture (Sanada and Tsai, 2005). Cortical NPCs were transfected by *in utero* electroporation at E16 and dissociated at E18 and put to culture. Images of transfected cells were captured at 4 hr and 24 hr after plating to ensure that each pair of sister cells was indeed generated from the division of a single NPC. At the end of the 24-hr culture, the fate of the two sister cells was determined by immunostaining with Ki-67 antibody to discriminate progenitors from differentiated cells (Fig. 3A). Interestingly, although the percentage of NPCs that have divided during 24 hr was significantly reduced after down-regulation of nestin (Fig. 3B), the percentage of each type of division of NPCs was not changed (Fig. 3C). Thus, nestin is more likely to be generally required for all types of division of NPCs, but not specifically for one certain type of division.

**Nestin-vimentin co-filament was not required for the neurogenesis**

Nestin and vimentin form co-filament in NPCs. Thus we studied whether down-regulation of vimentin had a similar effect as *nes*-RNAi transfection. In cultured cortical NPCs, nestin co-localized with vimentin in a filamentous pattern (Fig. 4A and A'), suggesting that nestin protein has been incorporated into the vimentin IF network. When nestin was knocked down, there was no apparent change in the structure of vimentin (Fig. 4B and B'), while when vimentin was down-regulated, nestin lost the filamentous distribution at the cell body (Fig. 4C and C'), similar to what was observed in cell lines in previous studies (Marvin et al., 1998). However, in contrast to the significant loss of neurons after

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**Fig. 3.** Down-regulation of nestin did not affect the division patterns of cortical NPCs. Cortical NPCs were dissociated 24 hr after *in utero* electroporation and cultured for 24 hr in vitro. Pictures taken at 4 hr and 24 hr after plating were compared to figure out divided cells. Transfected cells were shown in green. Cells were stained for Ki-67 to identify the three types of division of NPCs (A). The percentage of divided cells among all transfected cells was decreased when nestin was down-regulated (Student t-test) (B). The percentage of each type of cell division in pair-cell analysis was not affected (chi-square Test) (C). Scale bar, 10 μm. Experiments have been repeated for three times and each time more than 40 pairs were analyzed for each group. Data represent mean ± S.E.M. *P<0.05; **P<0.01; ***P<0.001. P-P: progenitor-progenitor; P-N: progenitor-neuron; N-N: neuron - neuron.
down-regulation of nestin, we observed no defects in neurogenesis after down-regulation of vimentin (Fig. 4D and E). Therefore, nestin may exert a different function from vimentin on neurogenesis in cortex.

Nestin was required for proliferation of cultured NPCs

We further studied the proliferative capacity of NPCs transfected with nes-RNAi in vitro. Cortical NPCs transfected by in utero electroporation at E16 were dissociated 24 hr after electroporation and cultured for 48 hr in the presence of BrdU (10 μg/ml). Knockdown of nestin resulted in a significant decrease in the percentage of BrdU-incorporated cells comparing with control cells (Fig. 5A). In order to study the function of nestin in NPCs from earlier developmental stages, cortical NPCs were transfected by exo utero electroporation at E13 (Saito and Nakatsuji, 2001), followed by dissociation, and were plated at a low density (5 × 10^5 cells per 35 mm dish, ~1% transfected cells). Some of these isolated NPCs in culture could divide for several times and form colonies of different sizes (Supplementary Fig. S6). We fixed the cultures at different time points and measured the percentages of colonies of different sizes which were presumably generated from single NPCs transfected with control plasmid or nes-RNAi. As shown in Fig. 5B and C, in the presence of the mitogen bFGF, nes-RNAi-transfected NPCs generated only 24.3 ± 1.3% clones that consisted of 2–3 cells and 7.6 ± 1.5% clones of 4–8 cells, comparing with 34.4 ± 5.6% and 18.9 ± 2.9% in control groups at 48 hr after plating. After 72 hr, there were 18.6 ± 3.8% clones that consisted of more than 8 cells in control group; however, no such big clones were ever detected when nestin was down-regulated. Further experiments were carried out by flow cytometric analysis of the cell-cycle profile in cultured NPCs after

Fig. 4. Nestin-vimentin co-filaments were not required for neurogenesis. Nestin and vimentin co-localized and formed filaments in cultured NPCs (A). Cells were transfected with nes-RNAi (B) or vim-RNAi (C) Scale bar, 10 μm. The selected region is magnified and shown in (A’), (B’) and (C’). Unlike nes-RNAi, transfection of progenitors with vim-RNAi did not cause obvious defect in neurogenesis in cortex (D and E). EYFP+ cells are shown in green and DAPI staining in blue. Scale bar, 500 μm. Numbers in brackets indicate the amount of pups (one slice per rat) used in each experiment. Data represent mean ± S.E.M. Student t-test. ***P<0.001.
transfection with control plasmid or nes-RNAi. As shown in Supplementary Fig. S7, down-regulation of nestin significantly elevated the percentage of cells in G1 stage, supporting a cell-cycle arrest in G1-S transition.

The active proliferation of NPCs depends on the presence of mitogenic growth factors including EGF and bFGF (Erickson et al., 2008). We observed that withdrawal of the growth factor bFGF in NPC culture suppressed the colony formation, as shown by much lower percentage of clones that consisted of 4–8 cells at 48 hr (Fig. 5C). Since there was no significant difference in the percentage of clones of different sizes between nes-RNAi-treated and control cultures in the absence of bFGF (Fig. 5C), nestin may not be required for the basic cell mitosis machinery, but be essential for the mitogen-stimulated proliferation of NPCs. Consistent with this notion, we found that nestin expression in cultured cortical NPCs was markedly increased in response to the stimulation of mitogenic factors, including LIF, EGF and bFGF, but not to the non-mitogenic neurotrophin BDNF (Fig. 5D and E). Thus mitogenic growth factors may promote the proliferation of NPCs, at least in part, through stimulating the expression of nestin.

Nestin is required for activation of PI3K signaling pathway in NPCs

Next, we tried to clarify how nestin may affect the proliferation of NPCs. First we found that in the primary culture of E13 cortical NPCs treated with bFGF, the elevation of the protein level of cyclin D1 and cyclin E, two cyclins essential for the G1-S transition during cell-cycle
Fig. 6. Nestin is essential for activation of PI3K signaling pathway in response to bFGF in NPCs. Transfection with nes-RNAi blocked the bFGF-induced elevation of cyclin D1/E (A). bFGF (20 ng/ml) was added 24 hr after transfection and presented for 24 hr. The down-regulation of nestin specifically impaired the Akt/PKB pathway but not the MAPK pathway of the bFGF signaling (B). The time course of bFGF-induced elevation of p-Akt is shown in (C). All Western blotting results have been repeated for at least three times with similar results and analyzed by Student t-test. Co-transfection with pten-RNAi and nes-RNAi restored the colony formation ability of cultured NPCs (chi-square Test). Scale bar, 50 μm. Numbers in brackets indicate repeated times (>200 clones calculated per experiment). Data represent mean± S.E.M. *P<0.05; **P<0.01; ***P<0.001.
progression, was blocked by the down-regulation of nestin (Fig. 6A), supporting the notion that nestin may mediate the pro-proliferative signal of bFGF. Mitogenic growth factors are known to promote cell-cycle progression by elevating the intracellular level of G1-cyclins through the PI3K and MAPK signaling pathways (Blagosklonny and Pardee, 2002). Consistently, in cultured cortical NPCs, we observed the activation of these two signaling pathways in response to bFGF (20 ng/ml), as indicated by Western blotting using antibodies against the active form of their downstream targets Akt and ERK1/2, respectively (Fig. 6B). After down-regulation of nestin, activation of Akt but not ERK1/2 in response to bFGF was significantly reduced (Fig. 6B). Time-course analysis further showed that Akt activation in response to bFGF was markedly attenuated after down-regulation of nestin (Fig. 6C). Thus, nestin is essential for the activation of the PI3K signaling pathway, rather than the MAPK signaling pathway in NPCs.

The lipid and protein phosphatase PTEN represents a major negative regulator of PI3K and Akt/PKB signaling pathway (Leslie et al., 2008; Manning and Cantley, 2007). Although up-regulating PI3K pathway by pten-RNAi did not significantly affect the proliferation of NPCs, co-transfection with pten-RNAi and nes-RNAi restored the colony formation ability of cultured NPCs (Fig. 6D). Taken together, these data indicate that nestin supports NPC proliferation by promoting the PI3K activity in response to mitogenic growth factors.

Discussion

Proliferation and differentiation of stem cells and/or progenitor cells are tightly regulated processes during development. By balancing these processes, proper size of various organs (Chenn and Walsh, 2002) and composition of cell types can be achieved (Molyneaux et al., 2007). As a widely used marker for NPCs, nestin is also highly expressed in cells that undergo active proliferation, including somatic stem cells and tumor cells from various tissues (Dahlstrand et al., 1992; Kachinsky et al., 1995; Lardon et al., 2002; Li et al., 2003; Sejersen and Lendahl, 1993). Although nestin expression has been long considered to correlate with active cell proliferation, whether it is directly involved in regulating cell proliferation has been unknown. In the present study, we provided evidence that nestin indeed directly participates in the control of NPC proliferation by its regulation of PI3K, a major regulator of cell proliferation.

As a component of IF in progenitors, nestin may contribute to the mechanical support and cytoskeletal dynamics of the cell. However, down-regulation of vimetin, the essential co-ploymer protein for nestin filament, had no effect on the production of neurons, consistent with what was observed in vimetin-null mice (Colucci-Guyon et al., 1994). Interestingly, recent studies have shown that nestin actively participates in diverse cellular functions. For example, nestin was found to bind to the Cdk5/p35 complex and regulate its subcellular localization, and thereby works as an anti-apoptosis factor when cells are under oxidative stress (Sahlgren et al., 2006). Knockdown of nestin by siRNA was also shown to reduce the growth and proliferation of the C6 glioma cell line (Wei et al., 2008). It has been shown that down-regulation of transtin, an avian homologue of nestin, resulted in a decrease in the BrdU-incorporation in avian neuroepithelial cells (Wakamatsu et al., 2007). The authors showed that transtin can bind with an important cell fate-determinant molecule numb and thus regulation of numb distribution in mammalian NPCs, the existence of other nestin downstream effectors for the regulation of NPC proliferation can not be ruled out. Upon NPC differentiation, nestin becomes down-regulated and is replaced by tissue-specific IF proteins. However, nestin expression is re-induced in the adult during pathological situations, such as in reactive astrocytes after CNS injury and in certain glia-derived tumor cells (Dahlstrand et al., 1992; Douen et al., 2004). The discovery that nestin promotes NPC proliferation helps to explain its specific expression in fast-growing cells and sheds light on the tumorigenesis and potential therapy of cancers related to the dysfunction of growth factor signaling.

Experimental methods

Animals

All timely pregnant Sprague–Dawley (SD) rats used in the present study were provided by SLAC Laboratory Animal Co. Ltd. All experimental procedures involving rats were carried out under the guideline and permission of the Bioethics Committee of the Institute of Neuroscience at the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (NA-100410-1).
Cell culture

Cultured NPCs were derived from E13 rat cortex. The NPCs were cultured in DMEM supplemented with 2% B27, 1% N2, and 10 ng/ml bFGF. In some experiments, transfection was done by Amaza Nucleofector following the protocol provided by the manufacturer (Li et al., 2005) and 20 ng/ml bFGF were added 24 hr after transfection.

Plasmid construction

The siRNA sequences were designed using an online design tool (http://www.dharmacon.com) and cloned into a pSuper vector under the control of H1 promoter. The siRNA sequences are given as following: 5′-GAAGATGACTGATCAAATA-3′ for nes-RNAi2; 5′-GCTGGAAGCCAACTTCCTT-3′ for nes-RNAi3; 5′-AAAGACACCTGTTAA-3′ for vim-RNAi; 5′-GGATGGAT TCAGCTTAGC-3′ for PTEN RNAi; and 5′-GAGTGGGATTCGGCTTGTCAGTG-3′ for sc-RNAi. Nestin cDNA was cloned into the MCS of pCAG-IRES-EGFP vector. The sizes of plasmids used in the current studies are shown as follows: CAG-EYFP 4.7 kb, plasmids for siRNA 4.9 kb, pCAG-nestin-IRES-EGFP 10.3 kb.

In utero electroporation

The in utero electroporation was carried out as previously described (Chen et al., 2008, Zhao et al., 2009). In brief, SD rats at E16 were anesthetized with 10% chloral hydrate (3.5 ml/kg body weight, intraperitoneally). For different purposes, a mixture of EYFP, siRNA and/or overexpression constructs was prepared at a final concentration of 5 pM for each plasmid. After exposing uteruses, 1–2 µl plasmids mixed with Fast Green (2 mg/ml; Sigma) were injected trans-uterusly into the lateral brain ventricle of embryos with a sharp glass pipette by air pressure. Successful injection was verified by visualizing a crescent staining of the ventricle by Fast Green. Next, electric pulses were generated by an ElectroSquareporator T830 (BTX) and applied to the cerebral wall trans-uterusly at five repeats of 60 V for 50 ms, with an interval of 100 ms. In this way, cortical NPCs lining the ventricle wall of the hemisphere close to the anode of the electric field could be transfected. After electroporation, uterus was placed back into the abdomen of the dam. Warm PBS was supplemented into the abdomen of the dam during the surgery to compensate the loss of body fluid. Antibiotics was included in PBS to suppress potential inflammation. Post-surgery animals were maintained in a warm animal room (25 °C) with plenty of water supply and food. In some experiments, BrdU (Sigma) was injected at 100 mg/kg intraperitoneally into the dam twice with 30 min interval 24 hr or 44 hr after in utero electroporation. For exo utero electroporation, brains of embryos were taken out and electroporated as described above before dissociation.

Pair-cell analysis

The pair-cell analysis was performed basically as previously reported (Sanada and Tsai, 2005). Progenitor cells in the V2 of E16 rat cortex were transfected by in utero electroporation. The dorsolateral cortices derived from the transfected telencephalons were dissected at E18. Transfected tissues at V2/SVZ/I2 were collected under fluorescent stereo microscope (Olympus sxz12). Cells were dissociated and cultured at a density of 5 × 10^5 cells per 35 mm dish. Divided cells among all GFP+ cells were identified through comparing the images taken at 4 hr and 24 hr after plating. Cells were fixed and stained for Ki-67 (polyclonal, Novocastra, 1:500) or TuJ1 (polyclonal, Sigma, 1:2000).

Colonial formation assay and flow cytometric analysis

Brains of E13 rat embryos were taken out of the uterus. Plasmids were injected into the ventricle and electroporated with pulses of 60 V for 50 ms, with an interval of 100 ms. Cortices were dissected immediately after the electroporation and dissociated cells were cultured at a density of 5 × 10^3 cells per 35 mm dish. The percentage of transfected cells under this culture condition was around 1% (5 × 10^3 cells per dish). Cells were fixed at different times after plating. Clusters of transfected cells with EFYP fluorescence signal were identified as colonies derived from single progenitors. For flow cytometric analysis, cultured cells were digested with 0.1% trypsin-EDTA after cultured for 60 hr and fixed with 1% PFA at 4 °C overnight. All samples were treated with 0.2% Triton X-100 for 10 min and stained with DAPI for 30 min at 37 °C. The flow cytometric analysis was done by MoFlo XDP (Beckman Coulter) and the data were analyzed by Summit 5.2.

Immunohistochemistry

Brains were fixed with 4% paraformaldehyde at appropriate ages and cryopreserved in O.C.T. (Sakura). For cell-cycle analysis, fetal brains were cut into coronal sections of 15 µm. Slices were treated with 2 N HCl for 45 min followed by treatment with 0.1 M Na2Ba4O7 (pH 8.5) for 45 min at room temperature. After washing with PBS, slices were blocked with 10% BSA plus 0.3% Triton X-100. Primary antibodies used were anti-BrdU (monoclonal, Sigma, 1:100), anti-Ki-67 (polyclonal, Novocastra, 1:100) and anti-p-H3 (polyclonal, Upstate, 1:100). For cell fate identification, the cortices from electroporated animals were dissected at P3. Cells remained in the V2/SVZ were collected under fluorescent stereo microscope and dissociated in PBS. After being fixed with 4% paraformaldehyde, cells were plated and attached to the cover glass (Fisher). The primary antibodies used are anti-Tuj1 (polyclonal, Sigma, 1:2000), anti-GFAP (polyclonal, DAKO, 1:500), anti-Ki-67 (polyclonal, Novocastra, 1:1000), S100β (monoclonal, Sigma, 1:500), anti-vimentin (monoclonal, Sigma, 1:100), anti-Cux1/CDP (polyclonal, Santa Curz, 1:50). Fluorescently conjugated monoclonal or polyclonal IgG Alexa 488, or Alexa 633 (Molecular Probes, 1:1000) were used as secondary antibodies. Other primary antibodies used are anti-nestin (monoclonal, Chemicon, 1:500), anti-nestin (polyclonal, a gift from Dr N-H Jing), anti-cleaved caspase-3 (polyclonal, Cell Signaling Technology, 1:100) and anti-GFP (polyclonal, Molecular Probes, 1:1000). Sections were also stained for 4′-6-diamidino-2-phenylindole (DAPI) to visualize nuclei. Images were acquired on an Olympus F1000 confocal system or a NIKON E600FN microscope, and processed using Adobe Photoshop CS 8.0.

Immunoblotting

Cells were lysed in RIPA buffer (20 mM HEPES, pH 7.4, 140 mM NaCl, 10 mM pyrophosphate, 5 mM EDTA, 0.4% NP-40, 100 mM PMSF, and protease inhibitor cocktail) for 30 min on ice and centrifuged at 14,000 rpm for 15 min. Nestin was separated with 8% SDS-polyacrylamide gels (SDS-PAGE) and other proteins were on 10% SDS-PAGE. The primary antibodies used are anti-nestin (monoclonal, Chemicon, 1:1000), anti-cyclin D1 ( polyclonal, Lab Vision, 1:100), anti-cyclin E ( polyclonal, Santa Cruz, 1:100), anti-Cdk2 ( polyclonal, Santa Cruz, 1:100), anti-vimentin (monoclonal, Sigma, 1:500), anti-cleaved Caspase-3 ( polyclonal, Cell Signaling Technology, 1:100), anti-phospho-Akt ( polyclonal, Cell Signaling Technology, 1:400), anti-Akt ( polyclonal, Cell Signaling Technology, 1:400), anti-ERK ( polyclonal, Cell Signaling Technology, 1:100), anti-α-tubulin (monoclonal, Sigma, 1:3000) and anti-β-actin (monoclonal, Chemicon, 1:3000). Secondary antibodies are peroxidase-conjugated goat anti-mouse, and goat anti-rabbit IgG (Bio-Rad, 1:10,000). Chemiluminescent detection was performed with the ECL kit (Pierce).

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Statistics and data presentation

All slices were stained with DAPI to distinguish the structure of VZ/SVZ, WM and CP. For cleaved caspase-3 staining, z-stack images of optic sections (15 μm in thickness) from the somatosensory cortex of each brain were collected. A ratio of the number of caspase-3+ cells in VZ/SVZ of transfected over untransfected hemisphere of the same slice was calculated. Average ratios were calculated based on data from five independent experiments. For cell-fate detection using immunostaining, 3–5 random images (20× objective) were acquired from each cover glass to calculate the percentage of positive cells among all transfected cells, and four independent experiments were carried out to obtain the average results. For pair-cell analysis, each time about 40 pairs were randomly chosen to calculate the percentage of each division type, and three independent experiments were carried out. For colony formation assay, 36 random images (10× objective) were captured from each cover glass to calculate the percentages of colonies of different sizes, and the experiments were repeated for 3 to 8 times to obtain the average percentages. Western blotting experiments have been repeated for at least three times. Densitometry quantification was done by using the software Image-Pro Plus. The band signal was normalized by the actin level of the same sample. The statistical test for pair-cell analysis and colony formation assay is the chi-square Test. All other data were analyzed by the two-tail Student t-test and shown as mean ± SEM.

Acknowledgments

We thank Drs. R. Goldman and N.-H. Jing for providing the nestin cDNA, Q. Hu for technical support in confocal microscopy, Y. Wang and A.-q. Geng for plasmid construction. This work was supported by 973 projects (2006CB806800, 2006CB949303), the National Natural Science Foundation of China (30625023, 30721004), and the Chinese Academy of Sciences (KSCX2-YW-R-103).

Appendix A. Supplementary data

Supplementary data related to this article can be found in the online version, at doi:10.1016/j.mcn.2010.05.006.

References


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