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The effect of P85 on neuronal proliferation and differentiation during development of mouse cerebral cortex

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ABSTRACT

Proliferation of neural stem cells and differentiation of newly generated cells are crucial steps during the development of mammalian neocortex, which are able to generate suitable number of neurons and glial cells to ensure normal formation of cortex. Any disturbance in these processes leads to structural and functional abnormalities of cerebral cortex, such as epilepsy or intellectual disability. Numerous molecules involved in the development of disorders of the nervous system have been discovered in the recent years. The PI3K/AKT signaling pathway has been shown to be widely involved in the corticogenesis. Recently we could show that overexpression of regulatory subunit P85 of PI3K disrupts neuronal migration. However, it remains unclear whether the regulatory subunit P85 plays a role in the proliferation of neural stem cells and differentiation of newly generated cells during mouse brain development. Here, by using *in utero* electroporation and immunohistochemistry, we show that overexpression of P85 inhibited proliferation of neural progenitor cells and neuronal differentiation. By using 5-bromo-2-deoxyuridine (BrdU) labeling, we reveal that overexpression of P85 extended the cell cycle duration, which may result in developmental retardation during mouse corticogenesis.

1. Introduction

The development of the mammalian neocortex requires an tighly controlled regulation of neuron production, which mainly depends on the balance between proliferation, cell cycle exit and differentiation of neural progenitor cells (NPCs) and neural stem cells (NSCs) (Farkas and Huttner, 2008; Florio and Huttner, 2014). NSCs can produce all cell types of the brain, while NPCs have more restricted potential. In the ventricular zone and subventricular zone (VZ/SVZ), NSCs/NPCs originated from neuroepithelial cells proliferate and generate new neurons continuously to construct the six-layer structure of cerebral cortex (Farkas and Huttner, 2008; McConnell, 1995). NSCs undergo symmetric divisions to expand the precursor pool in the early corticogenesis, and then generate two different types of cells through asymmetric divisions. One of the daughter cells remains a proliferating progenitor, and the other daughter cell starts differentiation after one to three cycles (Homem et al., 2015). Cell cycle parameters influence rates of neuronal generation, which is modulated by many extrinsic and intrinsic factors (Caviness et al., 2003; Hodge et al., 2004). Symmetric divisions generate two progenitors that re-enter the cell-cycle, whereas asymmetric divisions result in at least one daughter cell exiting the cell cycle to undergo differentiation. The balance between NSCs/NPCs proliferation and differentiation ensures an appropriate number of neurons, which is crucial for neocortical neurogenesis (Doe, 2008). Any disturbance in this process gives rise to an aberrant number of neurons, which results in structural abnormalities of the brain, such as microcephaly or macrocephaly, leading to mental retardation in turn (Ohtaka-Maruyama and Okado, 2015). Therefore, the mechanisms of neocortical neurogenesis including proliferation and differentiation of NSCs/NPCs have attracted considerable interest. Several signaling pathways have been proved to be involved in the neurogenesis, such as the Wnt-β-catenin pathway and PI3K/AKT/mTOR pathway (Munji et al., 2011; Zhong, 2016).

It is well known that the PI3K/AKT/mTOR pathway participates in numerous cellular activities, such as nutrient uptake, anabolic reac-

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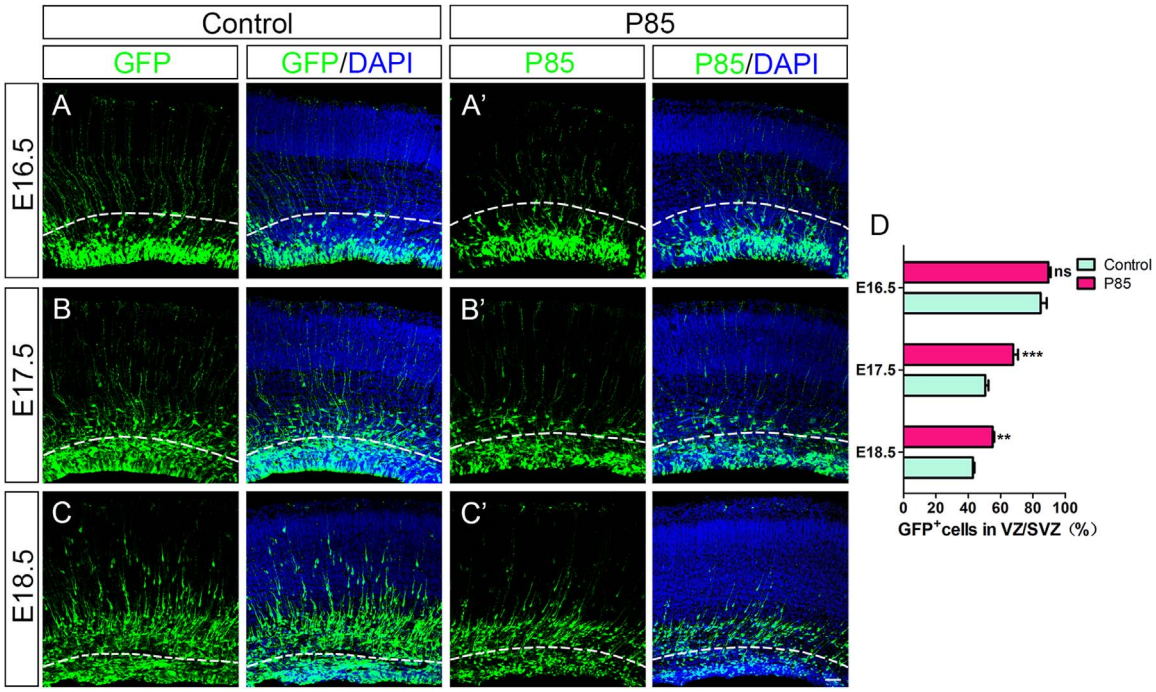

Fig. 1. P85 overexpressed cells were arrested in the ventricular zone and subventricular zone. **A, A'-C, C'** Embryos were electroporated with GFP or P85-GFP expression plasmids at E15.5, and collected at E16.5 (A, A'), E17.5 (B,B'), and E18.5 (C, C'). The brain slices were stained with GFP antibody (*green*) and counterstained with DAPI (*blue*). The regions below dot line indicate the VZ/SVZ. **D:** quantification of of transfected cells in the VZ/SVZ. Two days after IUE, more transfected cells in P85 group stayed in VZ/SVZ than that in control group. Scale bar: 50 μm. Error bars indicate SEM. * *P < 0.01, * **P < 0.001.

tions, cell growth and survival. In recent years, a myriad of studies have revealed that PI3K/AKT is frequently hyperactivated in many cancers (Thorpe et al., 2015). PI3K (phosphoinositide 3 kinase) consists of two subunits, a catalytic subunit (p110α, β, or δ) and a regulatory subunit (P85α, P85β, or p55γ) (Liu et al., 2009). Previous studies indicated that mice with dysfunctional p110α die around E10.5 (embryonic day) (Foukas et al., 2006), and mice lacking p110β die even earlier at E3.5 (Bi et al., 2002). Similarly, lack of the P85 subunit causes perinatal death by increasing insulin sensitivity in mice, which suggested that this signaling pathway is involved in embryonic development (Fruman et al., 2000). Moreover, this signaling cascade is also implicated in neurodegenerative diseases, autism, diabetes and epilepsy (Arachchige Don et al., 2012), indicating that this signaling pathway may be associated with brain development.

mTOR (The mammalian target of rapamycin) is a serine/threonine kinase of PI3K related kinase, which is an important regulator of the balance between self-renewal and differentiation in stem cells. Activation of mTOR promotes NSCs/NPCs generation, followed by neuronal differentiation (Marfia et al., 2011). PI3K initiates the activation of AKT, and activates mTOR as well as downstream targets (Parker et al., 2015). In addition, hyper-activating mutations in the PI3K/AKT/mTOR pathway were found in a large percentage of human patients with cortical malformations, such as focal cortical dysplasia (FCD), megalencephaly (MEG), hemimegalencephaly (HMEG), and tuberous sclerosis complex (TSC) (Crino, 2013; Hevner, 2015; Jansen et al., 2015). These findings suggest that the PI3K/AKT/mTOR signaling pathway plays crucial roles in neocortical neurogenesis. However, the individual effect of PI3K regulatory subunit P85 on neurogenesis is still understated.

In the present study, we demonstrate that overexpression of P85 subunit inhibited NPCs proliferation, but extended the duration of cell-cycle. In addition, overexpression of P85 disturbed the transition from intermediate neuronal progenitors (INPs) to projection neurons and inhibited neuronal differentiation during the development of mouse cerebral cortex.

2. Materials and methods

2.1. Animals and plasmid construction

In the present study, C57BL/6J mice purchased from The Fourth Military Medical University were used and maintained according to the institutional guidelines of Northwest A & F University. The mouse P85 beta gene was cloned by specific primers P85-F (5'-GGAATTCATGGCAGGAGCCGAGGGCTTC-3') and P85-R (5'-CGTCGACGGGCGTGCTGCAGACGGTGGGC-3') from embryonic day 18.5 (E18.5) mouse cerebral cortex using RT-PCR. Inserting the full length P85 into the pCAG-MCS-EGFP frame constructed an expression plasmid encoding mouse P85-GFP fusion protein. The frame of pCAG-MCS-EGFP was used as a control.

2.2. In utero electroporation

In utero electroporation (IUE) was performed as previous (Huang et al., 2017). Briefly, embryos were staged using the virginal plug as E0.5. In this study, pregnant mice at E14.5 or E15.5 with general anesthesia by pentobarbital sodium were used for IUE. Plasmids were purified by Endotoxin-free plasmid maxi kit (Omege) and injected at 2 μg/μl into the ventricle of embryos. This was followed by five 30 V pulses at 950 ms intervals applied by tweezer-style electrodes using a BTX E830. The uterus was replaced into the abdominal cavity, and the peritoneum was sutured to allow normal embryonic development.

2.3. BrdU Labeling

For 5-bromo-2-deoxyuridine (BrdU, Sigma) labeling, electroporated mice were given intraperitoneal injection with 50 mg/kg BrdU. To investigate proliferation of NPCs, BrdU injections were given into pregnant mice 1 h before harvesting. Additionally, to examine cell cycle at E15.5, mice were injected with BrdU 8 h and 14 h prior to sacrifice to label cells in early G1 phase and late G1 phase, respectively. Then, the embryonic brains were harvested after BrdU injection, and fixed with 4% paraformaldehyde (PFA).

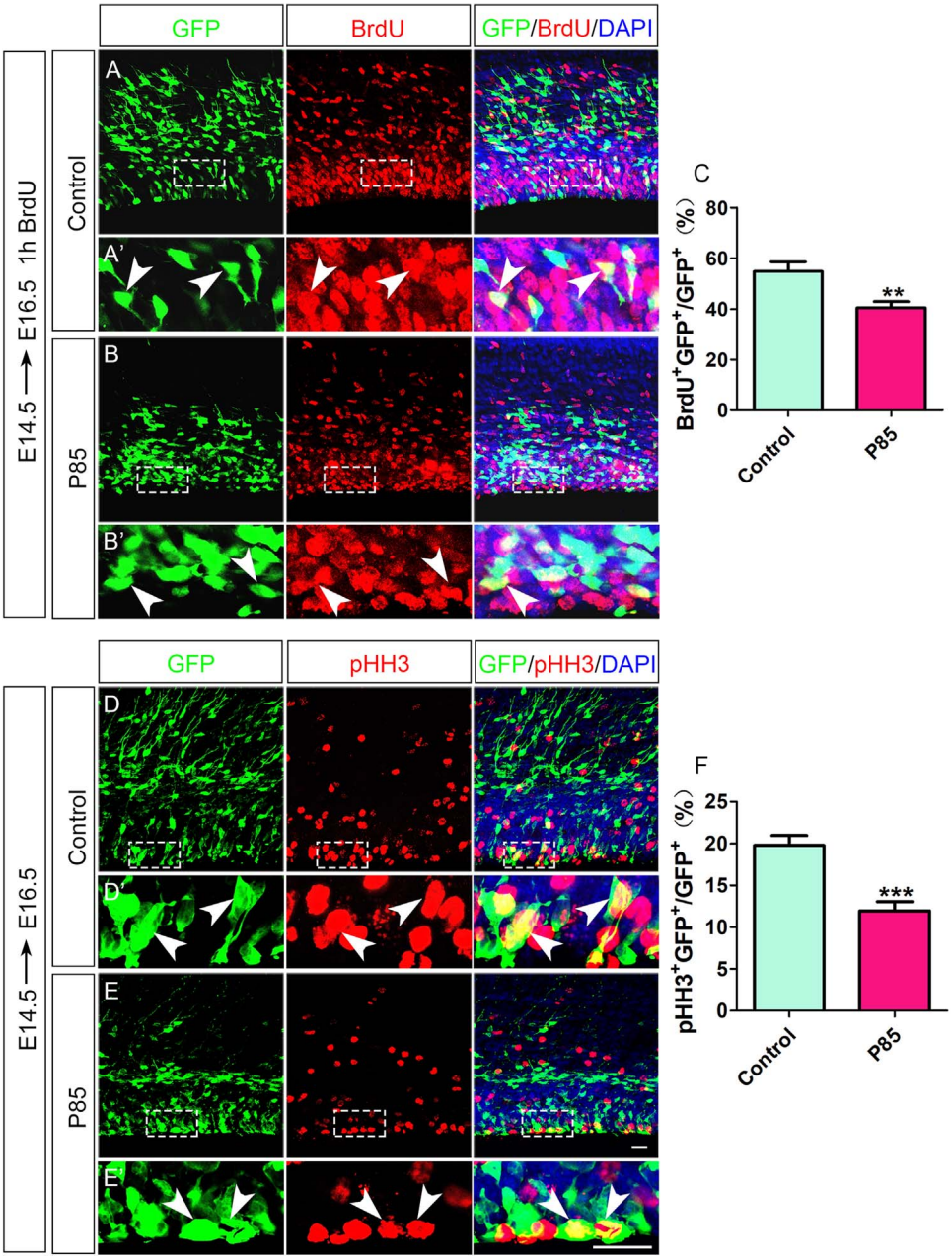

Fig. 2. Overexpression of P85 inhibited proliferation of neural progenitor cells. **A, B:** Embryos were electroporated with GFP or P85-GFP expression plasmids at E14.5 and harvested at E16.5, with BrdU injection one hour before harvesting. **A' B':** Higher magnification images of the boxed area in A and B. Arrow heads pointed out BrdU co-labeling cells in VZ/SVZ (A', B'). **C:** Quantification of BrdU co-labeling cells. The percentage of $BrdU^+GFP^+/GFP^+$ cells in the P85 group decreased, compared with that in control group. **D, E:** Brain slices at E16.5 were stained with pHH3 antibody, showing that less co-labeling cells in the P85 group. **D', E':** Higher magnification images of the boxed area in D and E. Arrow heads indicate pHH3 co-labeling cells in VZ/SVZ. **F:** Quantification of pHH3 co-labeling cells. The percentage of $pHH3^+GFP^+/GFP^+$ cells in the P85 group was reduced. Scale bar: 25 μm. Error bars indicate SEM. **P < 0.01, ***P < 0.001.

2.4. Brain slice preparation and immunofluorescence

Brain sections and immunofluorescence were performed as previous research in our laboratory (Li et al., 2017). After collection, the electroporated brains were fixed with 4% PFA for over 48 h at 4°Cin dark. Before starting to cut the brain into 50 μm slices using a vibratome (VT 1000S, Leica Microsystems), the brains were washed with 0.1 M phosphate buffer (PB) for 30 min at room temperature (RT). In order to perform immunofluorescence, slices were treated with 0.1% Triton X-100 in PB at RT for 1 h, and then blocked in blocking solution with 5% normal goat serum and 1% BSA diluted in 0.1 M PB at RT for 2 h. For BrdU staining, some sections were incubated in 1 N HCl at 37 °C for 30 min and followed with intensive washing in Borate buffer before treated in 0.1% Triton X-100. After blocking solution, slices were incubated with primary antibody at 4 °C overnight. The primary antibodies used in this study were as follows: rabbit anti-GFP, goat anti-GFP (1:1000 dilution, Millipore); Rat anti-BrdU (1:500 dilution, Abcam); rabbit anti- phospho-histone H3 (pHH3), rabbit anti-Ki67, rabbit anti Tbr2, goat anti-Brn2 (1:300 dilution, Santa Cruz Biotechnology). After washing in 0.1 M PB for three times, slices were incubated with secondary antibody at RT for 3 h. The secondary antibody included: Alexa Fluor 488-conjugated donkey anti-rabbit IgG, Alexa Fluor 488-conjugated donkey anti-goat IgG, Alexa Fluor 568-conjugated donkey anti-mouse IgG, Alexa Fluor 555-conjugated donkey anti-rabbit IgG, Cy5-conjugated goat anti-Rat IgG (diluted 1:500; Life Technologies). All slices were counterstained with DAPI (diluted 1:1000, Millipore) or PI (5 mg/ml 1:1000 diluted, Molecular Probes). In the end, slices were washed in 0.1 M

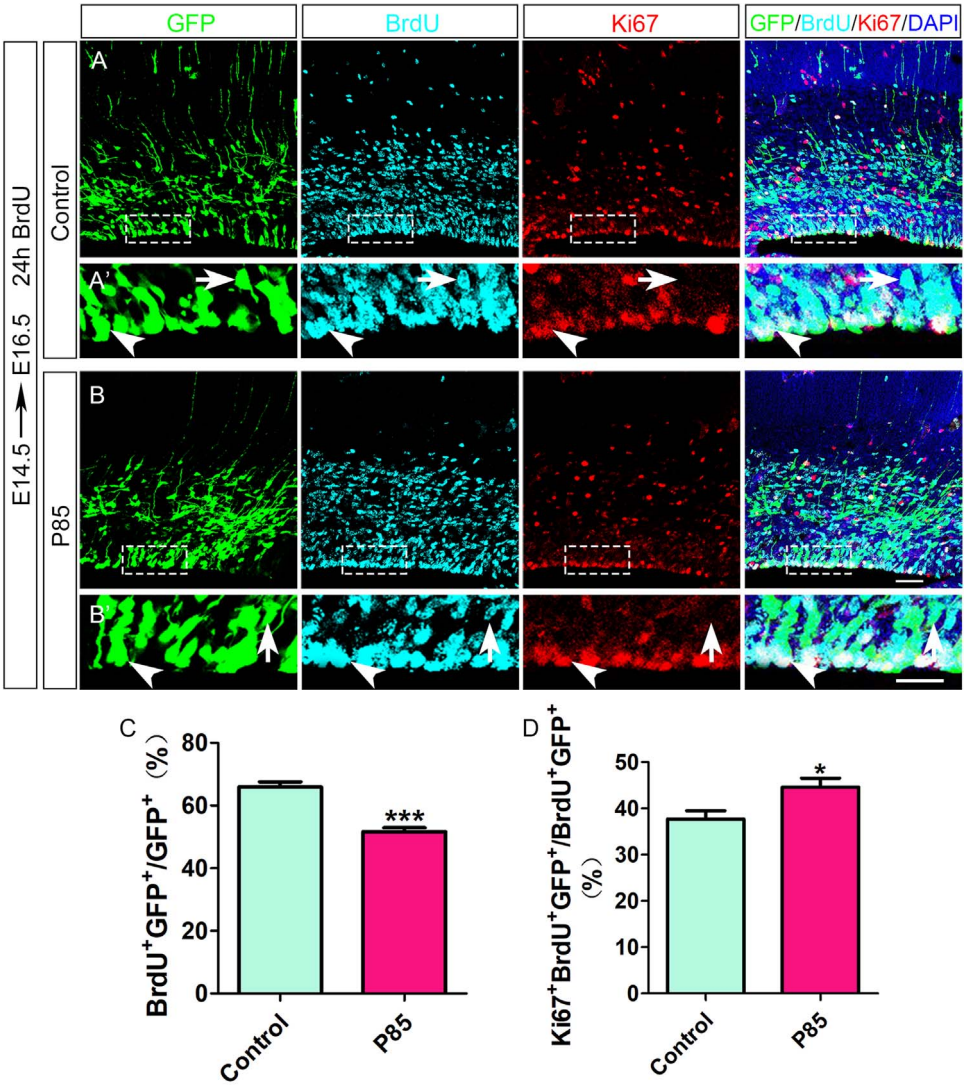

Fig. 3. Overexpression of P85 perturbs the cell cycle. **A, B:** Embryos were electroporated with GFP or P85-GFP expression plasmids at E14.5, and BrdU injection was performed at 24 h after IUE. Brain samples were fixed 24 h after injection, and slices were stained with BrdU antibody (*light blue*) and Ki67 antibody (*red*). **A', B':** Higher magnification images of the boxed area in A and B. Arrow heads indicate triple labeling cells ($GFP^+BrdU^+Ki67^+$),while arrows pointed double positive cells(GFP^+BrdU^+). **C:** The proportion of $BrdU^+GFP^+/GFP^+$ cells in P85 group, was significantly reduced compared to that in control group, consistent with Fig. 2 C. **D:** Quantification of the percentage of $Ki67^+BrdU^+GFP^+/BrdU^+GFP^+$ cells. More cells remained in cell cycle in P85 transfected group than in control group. Scale bar: 50 μm. Error bars indicate SEM. *P < 0.05, * **P < 0.001.

PB and mounted in Fluorescent Mounting Medium (Dako) on glass slides.

2.5. Imaging and statistical analysis

Images were captured with a Leica SP8 laser scanning confocal microscope using LAS AF Lite software. Cell counts were performed on at least 3 embryos per treatment group and a minimum of three cortical sections from each embryo using LAS AF Lite software as well. Statistical significance was calculated using a Student's *t*-test between two values. GraphPad Prism Software was used to produce graphs and statistics. Error bars represent SEM. *p* values were showed as follows: * $p < 0.05$, * * $p < 0.01$, and * ** $p < 0.005$.

3. Results

3.1. Cells overexpressing P85 stagnated in the proliferation zone of the neocortex

Recently we showed that overexpression of P85 inhibited neuronal migration into the cortical plate (CP) during mouse corticogenesis until early postnatal days (Cheng et al., 2017). However, the further function of P85-overexpression in embryonic neurogenesis is still unclear. In the present study, to investigate the role of P85 in neurogenesis, we employed IUE to transfect the P85-GFP plasmid into the NPCs at E15.5. Then the electroporated brains were collected at E16.5, E17.5 and E18.5, respectively. Meanwhile, we used the GFP plasmid as a control. The results showed that at E16.5 the majority of transfected cells remained in VZ/SVZ in both groups: P85 group (89.64 ± 1.312%) and control group (84.37 ± 2.300%) (Fig. 1 A, A'). Nevertheless, the difference emerged in E17.5. About 50% of the transfected cells left VZ/SVZ in control group (50.58 ± 1.983%), whereas over 60% cells still stagnated in VZ/SVZ in P85 group (67.82 ± 3.466%) (Fig. 1 B, B'). Moreover, this difference remained significant at E18.5. In the control group, only less than one half transfected cells were located in VZ/SVZ (42.90 ± 3.633%). On the contrary, more than half of P85-overexpressing cells were stacked in VZ/SVZ (55.15 ± 1.440%) (Fig. 1 C, C'). Since the VZ is the primary germinal zone containing progenitor cells that give rise to projection neurons during neurogenesis, and the SVZ is the secondary germinal zone of the neocortex, these two zones are the proliferation zone of the cerebral cortex (Kwan et al., 2012). This result suggests that overexpression of p85 may impact proliferating cells or their progeny.

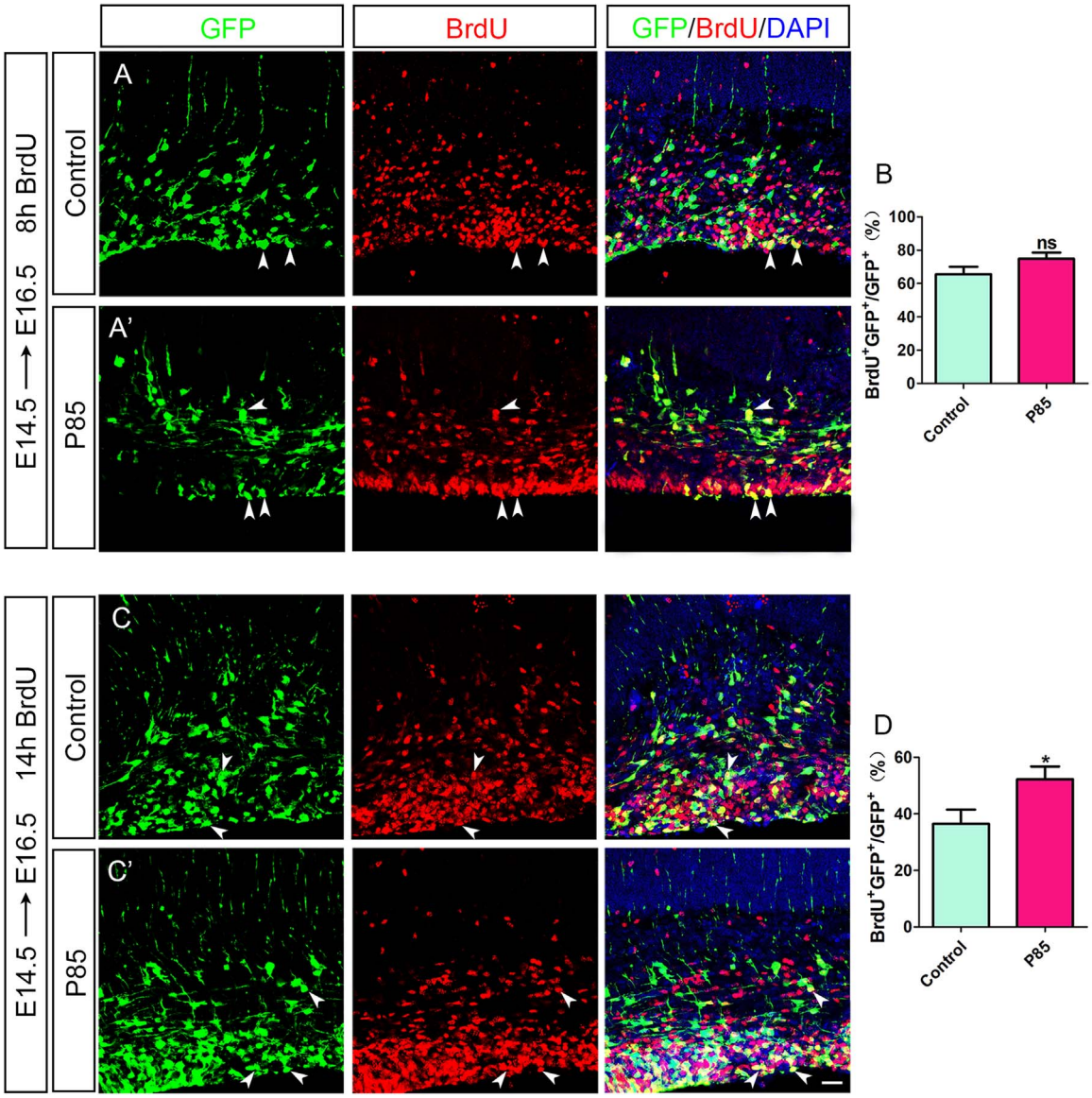

Fig. 4. Overexpression of P85 extended duration of cell cycle. **A, A':** Embryos were electroporated with GFP or P85-GFP expression plasmids at E14.5, and BrdU injection was performed 8 h prior to harvest. $BrdU^+$ cells (*red)* represented those in early G1 phase. **B:** Quantification of $BrdU^+GFP^+/GFP^+$ cells. No significant difference between P85 transfected group and control group was found, although an increasing trend was shown in P85 transfected group. **C, C':** BrdU injection was performed 14 h before harvesting to label cells in late G1 phase. **D:** Quantification results indicate that more cells overexpressing P85 were arrested in G1 phase than that in control group. Arrow heads indicate GFP and BrdU double positive cells. Scale bar: 25 μm. Error bars indicate SEM. *P < 0.05.

3.2. Overexpression of P85 inhibited NPCs proliferation

To examine the possible role of P85 in NPCs proliferation, BrdU labeling combined with IUE was applied. Since proliferating cells can incorporate the thymidine analog BrdU during division, we used BrdU as a marker for cell proliferation and cell cycle analysis. IUE was performed at E14.5. Then BrdU was injected into abdominal cavity of the pregnant mice 48 h after operation at the time when electroporated p85 is still expected in NPCs. One hour after BrdU injection, the embryonic brains were harvested to conduct immunofluorescence staining with BrdU antibody. Both in control group and P85 group, a part of transfected cells were co-labeled with BrdU antibody (Fig. 2 A–B). Quantification of double positive cells showed that 54.82 ± 3.845% GFP-positive cells were also BrdU-positive in control group, while only 40.49 ± 2.421% P85-GFP-positive cells were labeled by BrdU, which suggested that proliferation or cell cycle is altered in P85 group (Fig. 2 C).

To provide additional support for this conclusion, pHH3, a special marker of cells undergoing mitosis (Colman et al., 2006), was used. In, control group, 19.76 ± 1.195% GFP-transfected cells co-expressed pHH3, whereas fewer P85-transfected cells (11.94 ± 1.090%) co-expressed pHH3 (Fig. 2 D-F). These data were consistent with the result of BrdU labeling, which indicates that overexpression of P85 might inhibit NPCs proliferation or affect cell cycle *in vivo*.

3.3. Cells overexpressing P85 did not exit the cell-cycle early

For cortical progenitors, as for other somatic cells, proliferation and growth arrest are regulated by a balance of entering into and exiting from the cell cycle, affected by extrinsic and intrinsic signals (Cunningham and Roussel, 2001; Dehay and Kennedy, 2007). We wondered whether the cells which did not go through S-phase or mitosis in the P85 group did exit from the cell cycle earlier than those in the control group. Therefore, we conducted staining of Ki67, a cell proliferation marker, and BrdU co-labeling of transfected cells. 24 h after transfection, BrdU was injected into pregnant mice, and another 24 h later the fetal mice brains were collected. Calculating the propor-

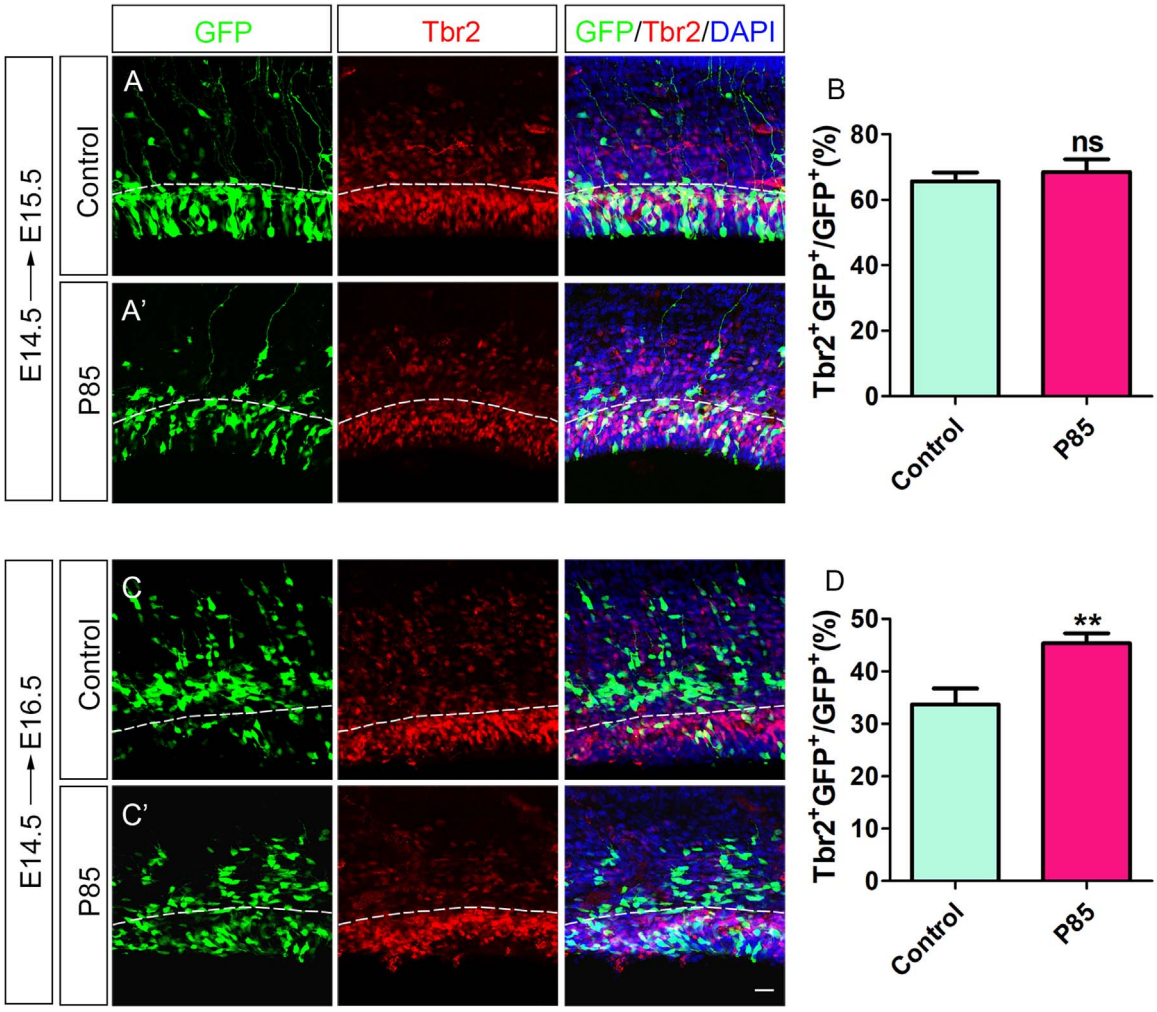

Fig. 5. Overexpression of P85 restrained INPs from transiting to projection neurons. **A, A':** Embryos were electroporated with GFP or P85-GFP expression plasmids at E14.5, and fixed 24 h after IUE. Tbr2 antibody was used to show intermediate neuronal progenitors (INPs, *red*), mainly located in in the SVZ and basal VZ of neocortex, indicated below dot line. **B:** Quantification of $Tbr2^+GFP^+/GFP^+$ cells. No statistically significant difference was found at E15.5. **C, C':** Embryos were fixed 48 h after IUE, and slices were stained with Tbr2. **D:** Quantification of $Tbr2^+GFP^+/GFP^+$ cells at E16.5. Less GFP^+Tbr2^+ double labeled cells were found in control group than that in P85 transfected group. Scale bar: 25 μm. Error bars indicate SEM. * *P < 0.01.

tion of GFP^+ $BrdU^+$ $Ki67^+$cells in $Ki67^-$ cells within the VZ/SVZ revealed how many cells still remained in cell cycle 2 days after transfection (Chenn and Walsh, 2002). The results showed that double labeling (GFP^+ $BrdU^+$ $Ki67^-$) and triple labeling cells (GFP^+ $BrdU^+$ $Ki67^+$) existed in both the control group and the P85 group (Fig. 3 A-B). Also in this experiment, the proportion of $BrdU^+$ cells amongst GFP^+ cells was different: in the P85 overexpression condition only 51.64 ± 1.283% were found, compared to 65.87 ± 1.705% in the control condition (Fig. 3 C). Amongst these $BrdU^+$ GFP^+ cells, a higher proportion expressed Ki67 in the P85 condition (44.62 ± 1.889%) *versus* the control (37.68 ± 1.787%) (Fig. 3 D). The results indicate that inhibition of P85 overexpression on NPCs cell cycle results not from promoting cells to exit the cell cycle, but from keeping cells in the cell cycle, meaning that overexpression of p85 may extend the duration of the cell cycle, since less cells exit.

To study in more detail the cell cycle after P85 overexpression, we performed a series of BrdU injections to label distinct phases of the cell cycle after IUE at E14.5. In the previous studies, the cell cycle has been shown to last 18 h at E15.5 (Takahashi et al., 1995). Thus, to label proliferating cells in the early G1 phase, we conducted a single time injection of BrdU 8 h before harvesting, while labeling 14 h prior to harvest was used to label the late G1 phase (Li et al., 2018). The results showed that in the early G1 phase, no significant difference between control group and P85 group was found (Fig. 4 A–B). However, in the late G1 phase, an increasing percentage of BrdU and GFP double positive cells appeared in the P85-transfected group (52.23 ± 4.531%), significantly higher than that in the control group (36.47 ± 5.074%). This result indicated that overexpression of P85 may extend the cell cycle especially in the late G1 phase (Fig. 4 C–D).

3.4. Overexpression of P85 perturbed transition from INPs to projection neurons

During corticogenesis, neural stem cells develop into neurons through three stages and express distinct transcriptional factors, generating Pax6-positive radial glial cell (RGC) progenitors, Tbr2 (T-box brain protein 2)-positive intermediate neuronal progenitors (INPs), and Tbr1-positive postmitotic projection neurons (Englund et al., 2005). To further dissect whether the tardive cell cycle caused by overexpression of P85 affects the development and differentiation process of neural stem cells to postmitotic neurons, IUE was performed at E14.5 and the electroporated brains were collected at two time points, E15.5 and E16.5. Then, the transfected brain sections were immunostained with an antibody against Tbr2 to investigate the transition of INPs to projection neurons. Twenty-four hours after IUE most of the GFP-positive cells were co-labeled with Tbr2 in both control and P85 groups, indicating that most transfected cells at E15.5 were INPs (Fig. 5 A–B). However, the percentage of INPs decreased

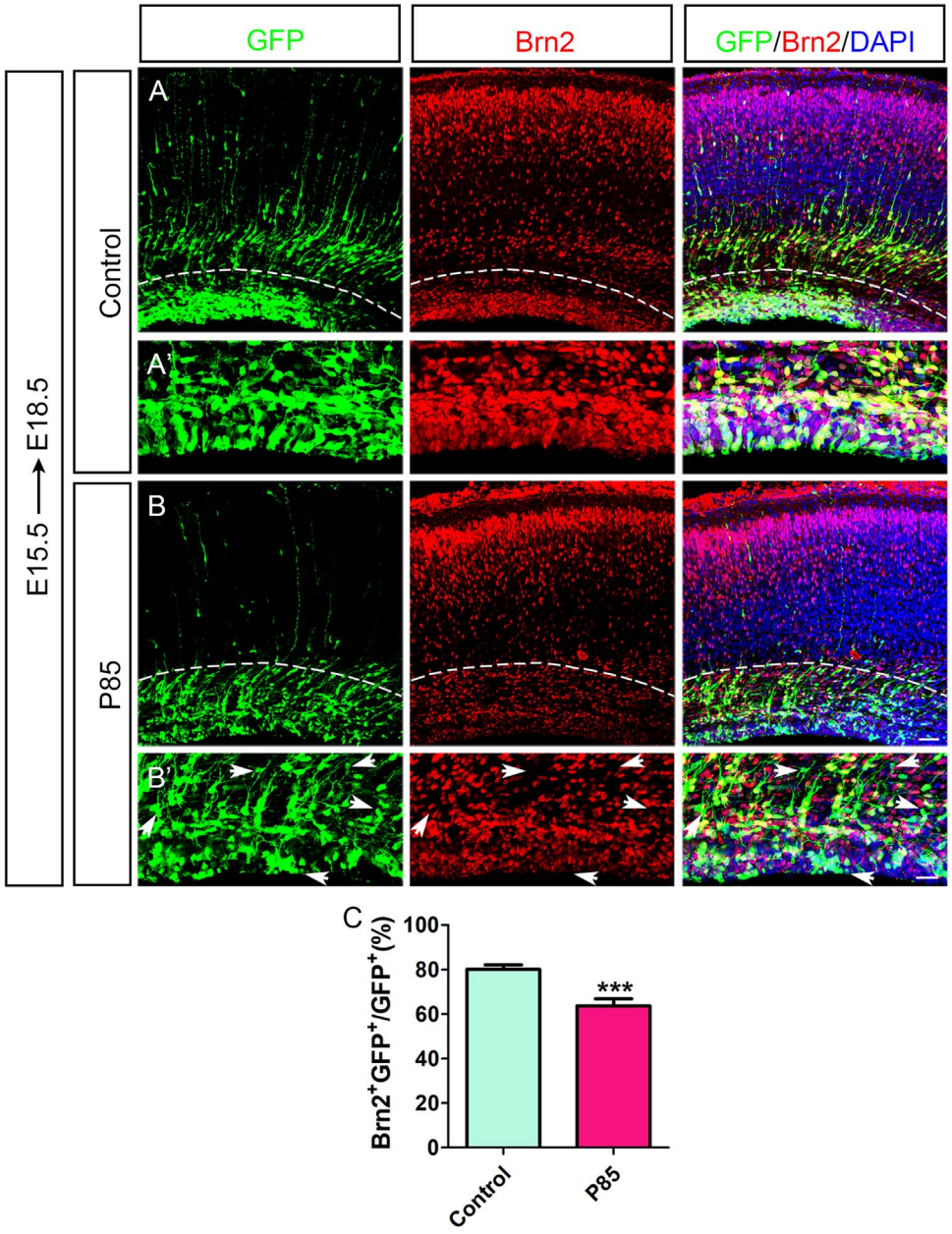

Fig. 6. Overexpression of P85inhibited neuronal differentiation. **A, B:** Embryos were electroporated with GFP or P85-GFP expression plasmids at E15.5, and fixed 3 days after IUE. Brain slices were stained with GFP antibody (*green*), and Brn2 antibody (neurons, *red*) and counterstained with DAPI (*blue*). The regions below dot line indicate the VZ/SVZ. **A', B':** Higher magnification images of VZ/SVZ in A and B. Arrows in B' pointed out cells that were Brn2-negative cells. **C:** Quantification of $Brn2^+GFP^+/GFP^+$ cells. Less transfected cells differentiated into neurons at E18.5 in P85 group than that in control group. Scale bar: 50 μm in A, B and 25 μm in A', B'. Error bars indicate SEM. * **P < 0.001.

rapidly 48 h after IUE in the control group. Only about one third of the transfected-cells expressed Tbr2 and the majority of cells had left the SVZ in the control group at E16.5 (33.66 ± 3.289%). However, half of the transfected cells in the P85 group (45.37 ± 1.840%) remained in the SVZ and expressed Tbr2 (Fig. 5 C-D). These results suggest that fewer INPs had transited into projection neurons in P85 group and overexpression of P85 disturbed the transition of INPs to projection neurons.

3.5. Overexpression of P85 restrained neuronal differentiation in vivo

Neurons are the principal functional cells in the cerebral cortex, and the computations in the cerebral cortex require specific connectivity patterns with precise numbers of diverse types of neurons (Cunningham and Roussel, 2001; Douglas et al., 1995). To further determine whether overexpression of P85 affected neuronal differentiation, we conducted immunofluorescence staining with a marker for newly born neurons, Brn2, and analyzed neurons in VZ/SVZ where Brn2 positive cells are first observed. At E18.5, most of the GFP-positive cells co-expressed Brn2 in the control group (80.07 ± 1.988%), while only about 63% of the P85-transfected cells were Brn2-positive cells (Fig. 6 C). These results suggest that overexpression of P85 inhibited the differentiation of newly generated cells into projection neurons.

4. Discussion

PI3K is categorized as class I, II or III depending on their subunit structure, regulation, and selectivity (Vanhaesebroeck et al., 2001). In the present study, we only considered classI PI3K, which is the unique enzyme able to convert PtdIns (4, 5) P2 to the critical second messenger PtdIns (3, 4, 5) P3. P85β subunit is one of five regulatory

subunits of PI3K and contains all modular domains (Deane and Fruman, 2004). Previously, investigators identified a heterozygous mutation in PIK3R2 (p-Gly373Arg), which encodes the P85β regulatory subunit of class IA PI3K in a patient with MPPH (megalencephaly-polymicrogyria-polydactyly-hydrocephalus syndromes) (Otsu et al., 1991). These results suggested a possible role of P85 in brain development. Our previous study has shown that overexpression of P85 inhibited neuronal migration by promoting the neurite branching during mouse corticogenesis. Therefore, in the present study we investigated the role of P85 on other processes during corticogenesis, including NPCs proliferation and neuronal differentiation. First of all, we found overexpression of P85 impaired proliferation of NPCs. A great deal of previous studies showed that external factor suppress cell proliferation and cell cycle progression by inhibiting PI3K/AKT signal pathway (Wei et al., 2015). Combined with our research, overexpression of P85 may downregulate the activation of PI3K/AKT signal pathway. The fate determination of NPCs between proliferation and differentiation controls the number of neurons generated during brain development and affects brain size in turn (Ohtaka-Maruyama and Okado, 2015). Our results show that overexpression of P85 disturbed the differentiation of INP into neurons. These anomalies may lead to a reduction in the neuronal number in neocortex. However, IUE, the core technology in this study, can only transfect a fraction of cells within neocortex. So oerexpression of P85 using IUE was unable to induce a change in brain size.

PI3K-dependent signaling regulates cell proliferation by promoting G1 to S phase progression during the cell cycle (Dangi et al., 2003). We found that the proportions of BrdU-positive cells and pHH3 positive cells in P85-transfected cells were decreased, indicating that overexpression of P85 inhibited proliferation of neural stem cells. Ki67 labels the cells in the active cell cycle. Expression of Ki67 within cells labeled with BrdU indicates the cells are still remaining in the active cell cycle (Wang et al., 2011). Interestingly, more cells remained in the cell cycle in the P85-transfected group when compared to the control group, suggesting that overexpression of P85 may affect the time duration of cell-cycle. Furthermore, we found that G1 phase was extended after overexpressing P85. PI3K activity has been shown to be required for expressing endogenous cyclin D (1) to promote entry into the S-phase (Gille and Downward, 1999; Stacey and Kazlauskas, 2002). Hence, overexpression of P85 might disturb PI3K activation. However, up to now no study has focused on cortical neurons *in vivo*, to find out if there is a difference between different cell types, or different mechanisms between *in vivo* and *in vitro*. Another possibility is that overexpression of P85 might cause G2 arrest, which may further lead to cell apoptosis. Flow cytometry will be a better way to analyze apoptosis and cell cycle.

Many previous studies have demonstrated that activating the PI3K signaling pathway promotes cell proliferation (Zhang et al., 2017). On the contrary, overexpression of P85 impaired neuronal differentiation in our study, which may be due to feedback inhibition of signal activity. The tumour suppressor PTEN has the same substrate with Class I PI3K, but act as completely opposite role (Maehama and Dixon, 1999). There is an intriguing protein-protein interaction between PTEN and the P85 regulatory subunit of PI3K. P85 interacts with dephosphorylated active form of PTEN in a high molecular weight complex that comprises p110β, which increases the enzymatic activity of PTEN. Therefore, P85 regulates both the increase and the decrease of PIP3 level *via* association with P110 and PTEN, respectively (Chagpar et al., 2010; Rabinovsky et al., 2009). In this study, we speculated that overexpression of P85 in mouse cerebral cortex inhibited PI3K signaling activity.

In summary, our data highlight the role of PI3K in brain development, further supports the involvement of P85, the regulatory subunit of PI3K, in mouse corticogenesis. Here, we found that overexpression of P85 inhibited NPCs proliferation and neuronal differentiation, and also extended time duration of a cell cycle.

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Conflict of interest

The authors declare that they have no conflict of interest.

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