Developmental Biology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

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

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ARTICLE INFO

Keywords:
P85
Cerebral cortex
Proliferation
Differentiation
Cell cycle

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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https://doi.org/10.1016/j.ydbio.2018.06.016
Received 22 March 2018; Received in revised form 23 May 2018; Accepted 23 June 2018

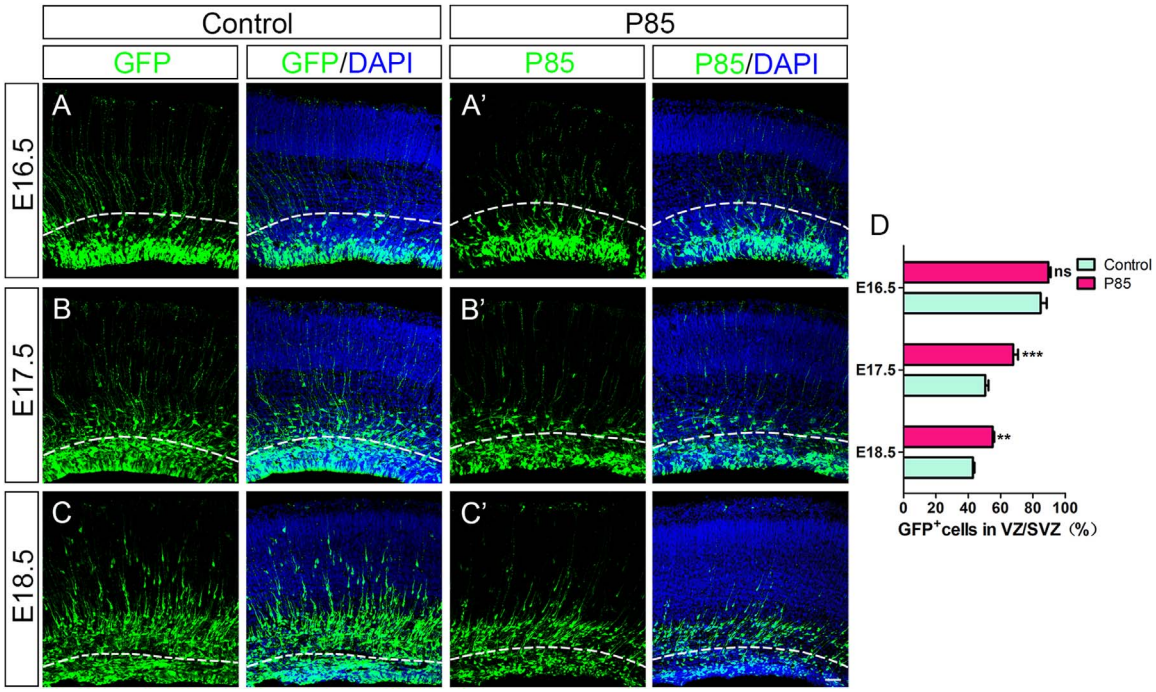

Fig. 1. P85 overexpressed cells were arrested in the ventricular zone and subventricular zone. **A, A'-C, C'** Embryos were electroporated with GFP or P85-GFP expression plasmids at E15.5, and collected at E16.5 (A, A'), E17.5 (B,B'), and E18.5 (C, C'). The brain slices were stained with GFP antibody (*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).