CORRECTION

Correction: Prdm16 is crucial for progression of the multipolar phase during neural differentiation of the developing neocortex

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There was an error published in Development 144, 385-399.

In the data availability section, the accession information for the microarray data was incomplete. The full information is as follows: Microarray data have been deposited at ArrayExpress under accession numbers E-MTAB-5438 and E-MTAB-5646.

The authors apologise to readers for this mistake.
Prdm16 is crucial for progression of the multipolar phase during neural differentiation of the developing neocortex

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ABSTRACT

The precise control of neuronal migration and morphological changes during differentiation is essential for neocortical development. We hypothesized that the transition of progenitors through progressive stages of differentiation involves dynamic changes in levels of mitochondrial reactive oxygen species (mtROS), depending on cell requirements. We found that progenitors had higher levels of mtROS, but that these levels were significantly decreased with differentiation. The Prdm16 gene was identified as a candidate modulator of mtROS using microarray analysis, and was specifically expressed by progenitors in the ventricular zone. However, Prdm16 expression declined during the transition into NeuroD1-positive multipolar cells. Subsequently, repression of Prdm16 expression by NeuroD1 on the periphery of ventricular zone was crucial for appropriate progression of the multipolar phase and was required for normal cellular development. Furthermore, time-lapse imaging experiments revealed abnormal migration and morphological changes in Prdm16-overexpressing and -knockdown cells. Reporter assays and mtROS determinations demonstrated that PGC1α is a major downstream effector of Prdm16 and NeuroD1, and is required for regulation of the multipolar phase and characteristic modes of migration. Taken together, these data suggest that Prdm16 plays an important role in dynamic cellular redox changes in developing neocortex during neural differentiation.

KEY WORDS: Neocortical development, Neural differentiation, Neural stem cells, Multipolar phase, Prdm16, NeuroD1, Mouse

INTRODUCTION

During neocortical development, newborn neurons undergo multiphasic radial migration processes to reach their final position within the cortical plate (CP). During this process, postmitotic immature neurons originate from radial glial progenitors and remain in the ventricular zone (VZ) for more than 10 h (Tabata and Nakajima, 2003; Noctor et al., 2004; Tabata et al., 2009, 2012, 2013) prior to transforming into multipolar cells that move out of VZ towards the intermediate zone (IZ). These multipolar cells remain just above VZ, in the multipolar cell accumulation zone (MAZ), for ~24 h, where they acquire a multipolar morphology and actively extend and retract dynamic processes (Tabata and Nakajima, 2003; Noctor et al., 2004; Tabata et al., 2009, 2012, 2013) in the multipolar phase (Fig. S1A). Subsequently, they transform from a multipolar to a bipolar morphology that is suitable for locomotion along the radial glial fibers before entering the subplate (SP) and CP (Rakic, 1972; Nadarajah et al., 2001). Recently, the importance of the multipolar migratory phase involved in mature cortical network assembly has received substantial attention (LoTurco and Bai, 2006; Torii et al., 2009; Costa and Hedin-Pereira, 2010; Yamagishi et al., 2011; Ohtaka-Maruyama et al., 2013; Inoue et al., 2014). The early postmitotic neuronal marker NeuroD1 is highly localized to MAZ (Tabata et al., 2009, 2012, 2013), and downregulation of NeuroD1 in the early multipolar phase enables cells to initiate Unc5D (a marker for late multipolar cells) expression, which facilitates their transition from the early to the late multipolar phase (Fig. S1A) and is thus crucial for their migration through the IZ (Yamagishi et al., 2011; Miyoshi and Fishell, 2012; Inoue et al., 2014). This dynamic expression change is required for the cells to transition out of the multipolar state and into the CP, although the precise genetic controls at this stage are not well understood.

Controlled delivery and use of oxygen are essential for maintaining the balance between energy generation and avoiding unwarranted oxidation. During oxidative phosphorylation, mitochondria use oxygen to generate ATP from organic fuel molecules, but in the process they also produce intracellular reactive oxygen species (ROS) (Shadel and Horvath, 2015). Although ROS have long been known for their damage-promoting detrimental effects, their roles as signaling molecules are now becoming better understood (Shadel and Horvath, 2015). In particular, mitochondrial ROS (mtROS) signaling has also been implicated in homeostatic processes, including cellular differentiation (Hamanaka and Chandel, 2010; Tornos et al., 2011; Hamanaka et al., 2013). A recent study demonstrated that multipotent neural progenitors maintain a high level of ROS (Le Belle et al., 2011). Furthermore, a gene expression profile from embryonic stem cell-derived neosphere (NS) culture revealed that neuronal differentiation is accompanied by upregulation of pathways related to mitochondrial DNA (mtDNA) contents, mtROS generation and hypoxia, thereby underlying the importance of oxygen sensing in the cellular processes related to neural differentiation (Moliner et al., 2008).

We hypothesized that the transition of progenitors through progressive multipolar migratory phases, which requires drastic morphological changes and characteristic migration modes, probably involves dynamic changes in mtROS, depending on the needs of the cell. In particular, we aimed to elucidate the role of...
RESULTS

Cellular mtROS levels change with differentiation

To determine endogenous mtROS levels in neocortical cells, we performed fluorescence-activated cell sorting (FACS) using the mtROS-sensitive chemical probe MitoSox (Robinson et al., 2008). Specifically, we dissociated embryonic day 14.5 (E14.5) neocortical cells and performed FACS analyses using an anti-CF133 antibody (neural progenitor marker) conjugated to a fluorescent dye (Fig. 1A; Mizutani et al., 2007).

Neural progenitor cells maintained a high level of ROS, which is consistent with a previous study (Le Belle et al., 2011), and cellular MitoSox levels (mtROS levels) were positively correlated with CD133 expression. Therefore, we quantified changes in mtROS levels of neocortical cells at E10.5, E14.5 and E17.5 (Fig. 1A,B). Neocortical cells were clearly separated into cell populations with high (mtROS\textsuperscript{high}) and low levels of mtROS (mtROS\textsuperscript{low}). In addition, relative proportions of mtROS\textsuperscript{high} and mtROS\textsuperscript{low} cells changed during neocortical development (Fig. 1B), and the mtROS\textsuperscript{high} Neuro2a cell populations decreased significantly after treatment with retinoic acid (RA; Fig. 1C). Conversely, other ROS species-sensitive dyes, such as DCFDA for total ROS (Fig. 1D), DAF for peroxides (Fig. 1E) and HPF for hydroxyl radical (data not shown), did not show marked changes during neural differentiation.

To examine changes in mtDNA contents (Puente et al., 2014) during neural differentiation (Fig. 1F), we collected GFP\textsuperscript{+} cells using FACS at 24, 36 and 48 h after in utero electroporation (IUE) (Mizutani et al., 2005, 2007; Inoue et al., 2014, 2015; Yamanishi et al., 2015) of CAG-EGFP plasmid at E14.5. Subsequent comparisons with mtDNA mRNA levels using quantitative polymerase chain reaction (qPCR) (Fig. 1F), showed that mtDNA copy numbers decrease linearly during differentiation. These results suggest that neural progenitor cells have higher mtROS levels, which decrease significantly with differentiation.

Identification of a candidate modulator of mtROS

In further transient transfection experiments in neocortical primary cultures, we used a plasmid containing natural basic helix-loop-helix Hes1, neurogenin 2 (Ngn2) and NeuroD1 promoter regions fused to the luciferase reporter gene. Following culture in the presence of mitoROS inhibitor of lipoic acid (LA), which is an essential co-enzyme involved in mitochondrial multienzyme complexes, Hes1 promoter activity was significantly suppressed, whereas both Ngn2 and NeuroD1 promoter activities were significantly increased (Fig. 2A). Moreover, expression profiling in qPCR, gain-of-function analyses of mt-target catalase (mt-Cat; Schriner et al., 2005) in primary cultures showed that overexpression of mt-Cat increased the expression of pro-neural genes such as Ngn2 and NeuroD1, and decreased Hes1 expression levels (Fig. 2B). Therefore, we hypothesized that neural differentiation (Fig. S1A) occurs in response to dramatic decreases in cellular mtROS levels. To quantify changes in cellular mtROS levels during differentiation, we used FACS to separate E14.5 neocortical cells into mtROS\textsuperscript{high} and mtROS\textsuperscript{low} cell populations (Fig. S1B) and compared expression profiles using qPCR (Fig. 2C). In these experiments, mtROS\textsuperscript{high} cells expressed significantly higher levels of the stem/progenitor marker Pax6, whereas mtROS\textsuperscript{low} cells expressed significantly higher levels of the early multipolar cell marker NeuroD1 (See et al., 2007; Tabata et al., 2009, 2012, 2013; Miyoshi and Fishell, 2012; Inoue et al., 2014) and the late multipolar cell marker Unc5D (Miyoshi and Fishell, 2012; Inoue et al., 2014). In addition, we compared expression profiles after separating three gates (Fig. S1C) and found that gated populations were distinguished by differing markers as follows: Pax6 (gate 1), NeuroD1 (gate 2) and Unc5D (gate 3). Hence, mtROS levels are markedly altered during the transition of progenitor cells to postmitotic multipolar cells.

During the transition of progenitors to postmitotic cells, remarkable changes in mtROS levels between mtROS\textsuperscript{high} and mtROS\textsuperscript{low} cells may reflect regulation of genes that are associated with antioxidative and oxidative activities, respectively. Accordingly, stem cells have unique mechanisms for coping with accumulated ROS, involving increased antioxidant defenses and unique redox-dependent effects on growth and differentiation (Csete et al., 2001; Saretzki et al., 2004; Piccoli et al., 2005). Thus, in further studies, we used FACS to separate E14.5 neocortical cells into mtROS\textsuperscript{high} and mtROS\textsuperscript{low} cell populations, and compared their expression profiles. However, comparisons of mtROS\textsuperscript{high} and mtROS\textsuperscript{low} cells using DNA microarrays revealed no changes in the expression of genes that are known to regulate redox homeostasis, including superoxide dismutase genes (Sod1, Sod2), Cat, FoxO genes, APE-Ref-1 (Apx1), Nrf2 (Gabpa), Atm, Ahsa1 and Trps3 (Table 1), but showed a twofold increase in the expression of PR (PRD1-BF-1-RIZ1 homologous) domain-containing protein 16 (Pdmd16).

Pdmd16 is a transcription factor that regulates energy balance and contributes to brown fat development (Seale et al., 2008; Kajimura et al., 2010; Trajkovski et al., 2012). A recent study showed that Pdmd16 is preferentially expressed by adult neural stem cells and is required for their maintenance, partly by modulating oxidative stress (Chuikov et al., 2010). Therefore, we transfected Neuro2a cells with psh-Pdmd16 plasmids and determined mtROS levels after 24, 36 and 48 h of culture. Subsequently, mtROS levels were significantly decreased in Pdmd16 loss-of-function cells (Fig. 2D). In addition, Pdmd16 loss-of-function vector significantly decreased mtDNA contents (Fig. 2E).

Taken together, these results suggest that the dynamic changes in mtROS levels during differentiation are closely correlated with stepwise regulation of the cell state, and that these levels change markedly when progenitor cells transition into postmitotic multipolar cells. In addition, Pdmd16 was identified as a possible candidate for mtROS modulation in the developing neocortex.
compared with GFP expression (Fig. 3F). Prdm16 expression gradually decreased with the transition of progenitors through the progressive stages of differentiation from the VZ to the IZ. In addition, the developing cortex from the ventricle face to the lower region of the IZ was divided into four bins (Fig. 3B‴), and numbers of cells that were strongly (++) or weakly (+) positive for Prdm16 or
NeuroD1 were quantified and compared (Fig. 3G). In these analyses, cells in the VZ expressed only Prdm16, whereas those in the MAZ expressed both NeuroD1 and Prdm16; those in the lower part of the IZ expressed only NeuroD1, indicating that neural progenitor cells specifically express Prdm16. However, Prdm16 expression declined with the transition into NeuroD1-positive multipolar cells from the MAZ to the lower region of the IZ.

Prdm16 regulates the development of postmitotic multipolar cells

In accordance with in vitro gain- and loss-of-function analyses of Prdm16, NS frequency and EdU labeling assays (Fig. 4A,B) showed decreased percentages of proliferating cells, suggesting that precise expression of Prdm16 is crucial for the transition of progenitors into postmitotic cells.

Subsequently, we performed Prdm16 gain- and loss-of-function experiments to test in vivo function within the developing neocortex. Three days after IUE at E12.5 (Fig. 4C and Fig. S3B), most Prdm16 gain-of-function cells possessed multipolar morphologies within the VZ and the IZ. By contrast, electroporated control vector cells were broadly distributed within the VZ, the IZ and the CP, and many differentiated neurons were observed. Statistical analyses revealed the significant effects of Prdm16 gain of function in the lower IZ (Bin 2; \(P<0.001\)), and the CP (Bin 4 and 5; \(P<0.01\)) but not in the VZ (Bin 1) or the upper IZ (Bin 3, Fig. 4D). By contrast, the majority of Prdm16 loss-of-function cells remained within the upper IZ (Fig. 4C and Fig. S3A) and had aberrant shapes, leading to inhibition of CP invasion (Fig. S3C). In further experiments, we labeled multipolar cells with a Cre-loxP clonal expression plasmid system comprising pCAG-FloxP-EGFP-N1 and pCAG-Cre (Shitamukai et al., 2011), and monitored morphological differences using IUE (Fig. 4E, Movies 1-3). The ensuing data showed that upregulation and downregulation of Prdm16 led to increased or decreased numbers of...
Prdm16 knockdown is not non-specific (Fig. S4A). In addition, we confirmed that Prdm16 gain of function increases in EGFP-positive cells, even in the VZ (Fig. S3D), and some multipolar cells were present even in the VZ of Prdm16 gain of function (Fig. 5A, arrows). However, Prdm16 loss of function decreased numbers of NeuroD1-positive cells, and whereas NeuroD1 promoter activity was significantly increased in Prdm16 overexpressing cells, it was significantly decreased in Prdm16 knockdown primary cultures (Fig. 5B). A similar phenotype was reproduced by the introduction of pCAG-Prdm16ΔPR and ΔZF, but not ΔΔZF, significantly induced NeuroD1 promoter activity in Neuro2a cells (Fig. 5D). Subsequently, we examined sh-Prdm16 using IUE with Prdm16 expression plasmids to determine whether migration defects could be rescued (Fig. S4F). IUE of full-length Prdm16, ΔPR or ΔZF, in the presence of sh-Prdm16 plasmid expression led to partial rescue of migration defects, suggesting that Prdm16 mediates induction of NeuroD1-positive multipolar cells and that Prdm16 function depends on the nZF domain, which is typically found in neocortical cells.

### NeuroD1 represses Prdm16 expression in multipolar cells

Overexpression of NeuroD1 in dissociated cortical cells dramatically suppressed Prdm16 expression (Fig. 5E). Taken with immunostaining patterns (Fig. 3B", C", G), these data indicate that Prdm16 expression was restricted to VZ (strongly) and MAZ (weakly) cells. In addition, following initial expression of Prdm16 in progenitors, NeuroD1 expression was induced in MAZ cells (Fig. 3C") and Prdm16 expression was not detected in NeuroD1-expressing multipolar cells. Moreover, Prdm16 immunostaining of NeuroD1 loss-of-function brains showed increased populations of Prdm16-expressing cells located ectopically above the VZ (Fig. S5A, arrows), indicating that Prdm16 induces the expression of NeuroD1, which then represses Prdm16.

To investigate NeuroD1 feedback regulation against Prdm16 at specific time points, we sorted cells using FACs at 24, 36 and 48 h after IUE of control or sh-NeuroD1 with GFP plasmids on E14.5, and compared Prdm16 mRNA expression levels. In control cells, Prdm16 expression levels were dramatically decreased from 24 to 48 h, whereas NeuroD1 loss-of-function cells showed maintenance of Prdm16 expression at 36 and 48 h after IUE (Fig. 5F). Taken together, these results suggest that negative-feedback regulation of Prdm16 expression by NeuroD1 plays a crucial role in the normal progression of multipolar cells.

### Analysis of time-lapse images of Prdm16 gain- and loss-of-function cells

Because Prdm16 function appears to be essential for multipolar migration, we examined the role of Prdm16 in neuronal migration and morphology using live imaging. To achieve this, we performed IUE at E14.5 and examined neuronal migration and morphology after 42 h. Subsequent time-lapse imaging analyses revealed striking differences in migration profiles between control and Prdm16 gain-of-function cells (Movie 4). In control experiments, GFP-positive neurons transformed from multipolar to bipolar shapes in the upper IZ, smoothly migrated into the CP and then moved to the pial surface (Fig. 6A). By contrast, some Prdm16 gain-of-function cells transformed into multipolar cells in the VZ; they were prevented from constructing the MAZ and remained stranded in the VZ, but showed multipolar morphology and characteristic irregular migration (Fig. 6B). Average migration distances were also decreased in Prdm16 gain-of-function cells (P<0.0001) owing to abnormal morphological changes and polarity (Fig. 6C).

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**Table 1. Microarray analysis of ROS<sup>high</sup> versus ROS<sup>low</sup> cells**

<table>
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<tr>
<th>Gene symbol</th>
<th>Fold change</th>
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<td>Sod1</td>
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<td>Superoxide dismutase 1, soluble</td>
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<td>Sod2</td>
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<td>Superoxide dismutase 2, mitochondrial</td>
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<td>Sod3</td>
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<td>Superoxide dismutase 3, extracellular</td>
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<td>Foxo3</td>
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<td>Forkhead box O3</td>
</tr>
<tr>
<td>Foxo4</td>
<td>1.329</td>
<td>Forkhead box O4</td>
</tr>
<tr>
<td>Foxo6</td>
<td>1.221</td>
<td>Forkhead box O6</td>
</tr>
<tr>
<td>Nrf1</td>
<td>1.212</td>
<td>Nuclear respiratory factor 1</td>
</tr>
<tr>
<td>Atm</td>
<td>0.972</td>
<td>Ataxia telangiectasia mutated homolog</td>
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<td>Prdm16</td>
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<td>PR domain containing 16</td>
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<td>Jnk1 (Mapk8)</td>
<td>0.718</td>
<td>Mitogen-activated protein kinase 8</td>
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<tr>
<td>Hif1a</td>
<td>1.101</td>
<td>Hypoxia inducible factor 1, alpha subunit</td>
</tr>
<tr>
<td>Hgf</td>
<td>1.341</td>
<td>Hepatocyte growth factor</td>
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Gene expression profiles of mtROShigh and mtROS<sup>low</sup> cells from E14.5 neocortical tissues were compared using microarray (two independent samples) analyses. Representative genes that were correlated with ROS homeostasis are listed.
In further studies, time-lapse imaging experiments were performed at a different time point (E13.5) to investigate morphological impairments of the same stages as in Fig. 5A in Prdm16 gain- and loss-of-function cells (Fig. 6D; Movie 5). Prdm16 gain-of-function cells possessed multipolar morphologies even in the VZ; this was consistent with the data presented in Fig. 5A,C. Moreover, both gain- and loss-of-function cells showed inhibition of CP invasion and aberrant migration profiles (Fig. 6E,F). These time-lapse images further suggest that Prdm16 is involved in the transition of progenitors into multipolar cells.

Prdm16-mediated regulation of multipolar cells plays a critical role in laminar formation

To further assess the roles of Prdm16 during neural differentiation, we examined control, gain- and loss-of-function cells at postnatal day of 5 (P5) after IUE at E12.5. In the presence
of the control vector, GFP-positive neurons were found in layers II-VI of the neocortex at P5 (Fig. 7A). By contrast, following transfection with the Prdm16 overexpression vector (Fig. 7B,D), GFP-positive neurons did not enter the CP correctly and were primarily localized at the boundary between CP and white matter. In addition, ectopic GFP-positive neurons maintained NeuroD1 expression until postnatal stages (Fig. 7E-E″). By contrast, in the presence of a Prdm16 knockdown vector, GFP-positive neurons

![Image](image_url)
were located primarily in the deep layer (Fig. 7C,D). However, these GFP-positive cells were positioned below the Sox5-positive layer VI (Fig. 7F) and formed abnormally in another layer (indicated by an asterisk in Fig. 7C,F). Moreover, GFP-positive cells below the layer VI included neurons that expressed upper layer markers such as Brn2 (data not shown), and some of...
Fig. 6. Analyses of time-lapse images of Prdm16 gain-of-function and loss-of-function cells. (A-C) IUE of control or Prdm16 gain-of-function vectors was performed at E14.5. Neocortical slices were prepared about 36 h after electroporation, cultured and time-lapse images recorded. (A) Time-lapse imaging observations of Prdm16 gain-of-function cells stranded around the MAZ of electroporated cortical slices; upper panels, control cells; lower panels, Prdm16 gain-of-function cells. Scale bar: 100 µm. (B) Tracing of control (left) and Prdm16 gain-of-function (right) cells using time-lapse imaging from E16. Migratory tracks are shown as colored lines with numbers. (C) Calculation of migration speeds of control cells (left) or Prdm16 gain-of-function cells (right) with abnormal morphological changes around the MAZ. Experiments were repeated three times independently with similar results. *P<0.01 (Student’s t-test); more than 600 cells/group were analyzed. (D-F) IUE of control, Prdm16 gain-of-function or Prdm16 loss-of-function vectors was performed at E13.5, and neocortical slices were prepared about 36 h after electroporation. Slices were then cultured and time-lapse images were recorded. (D) Time-lapse imaging observations of Prdm16 gain-of-function cells showed multipolar morphologies even in the VZ; both gain-of-function and loss-of-function cells showed inhibition of invasion into the CP. Scale bar: 100 µm. (E) Tracing of control, Prdm16 gain-of-function or loss-of-function cells using time-lapse imaging from E15. Migratory tracks are shown as colored lines. (F) Calculation of migration speeds of control cells, and Prdm16 gain- and loss-of-function cells with abnormal morphological changes around the VZ, the MAZ and the IZ; experiments were repeated twice independently with similar results. *P<0.01 (Tukey-Kramer test); more than 600 cells/group were analyzed.
these cells originated from late-born cells (Fig. 7G). Taken together, these results suggest that Prdm16-mediated regulation of multipolar cells plays a crucial role in laminar formation (Fig. S6A).

Prdm16 and NeuroD1 coordinate PGC1α activation through the insulin-responsive element DNA-binding motif

We performed gene expression profiling of sorted Prdm16 gain-of-function cells from dissociated neocortical cells at E14.5 (Table 2).
Table 2. Microarray analysis of control versus Prdm16 gain-of-function cells

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<td>Ebf2</td>
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<td>Early B-cell factor 2</td>
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<tr>
<td>Wnt5a</td>
<td>2.36</td>
<td>Wingless-related MMTV integration site 5A</td>
</tr>
<tr>
<td>Tubalin</td>
<td>2.07</td>
<td>Tubulin, alpha 4A</td>
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<tr>
<td>Igf2</td>
<td>1.91</td>
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<tr>
<td>Neurod1</td>
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Gene expression profiles of control versus Prdm16-overexpressing cells after GFP sorting of transfected 1-day-old cultures; genes with expression levels that were more than 1.4-fold higher in Prdm16-overexpressing cells than in control cells are listed.

Table 2. Microarray analysis of control versus Prdm16 gain-of-function cells

*Genes are associated with ROS regulation or were responsive to oxidative stress.

These experiments showed marked upregulation of Igf2, Prmt8, Mgst1, Pdk2 and PGClα (Ppargc1α), which are known regulators of mitochondrial metabolism and ROS balance (Handschin and Spiegelman, 2006; St-Pierre et al., 2006; Austin and St-Pierre, 2012) in Prdm16 gain-of-function cells, and were confirmed by qPCR in three independent samples (Fig. S6B).

Peroxosomal proliferative activated receptor 1 (PGC1α) transcriptional co-activators are the major regulators of several crucial aspects of energy metabolism. Specifically, PGClα controls aspects of oxidative metabolism, such as mitochondrial biogenesis and respiration, by co-activating various nuclear receptors and factors that are not members of the nuclear receptor family. Recent studies show that PGClα is a broad and powerful regulator of ROS metabolism (St-Pierre et al., 2006), and that Prdm16 activates brown fat cell characteristics by directly binding and activating PGClα (Seale et al., 2007). Therefore, we performed transient transfection experiments in Neuro2a cells using a plasmid containing the PGClα promoter region fused to a luciferase reporter gene (Fig. 8A). Subsequently, Prdm16 gain of function significantly increased PGClα promoter activity, whereas NeuroD1 gain of function significantly suppressed it. This suppression of promoter activity was also observed with Prdm16 loss of function (data not shown), and was abolished with the PGClα-AIRE promoter mutant (Fig. 8A). These data suggest that both Prdm16 and NeuroD1 act as transcription factors that coordinately regulate PGClα activity through the insulin responsive element (IRE).

In further studies, we examined PGClα gain of function using primary cultures with the sh-Prdm16 plasmid and determined whether mtROS levels could be rescued. These experiments confirmed partial rescue of mtROS levels following transfection with the PGClα plasmid (Fig. 8C). Although, Prdm16 gain-of-function cells had slightly decreased mtROS levels, co-transfection of sh-NeuroD1 with Prdm16 gain-of-function vectors significantly increased mtROS levels (Fig. 8C) and mtDNA contents (Fig. S6C,D), suggesting Prdm16 positively regulates PGClα activity and leads to the mtROShigh phenotype, whereas NeuroD1 negatively regulates PGClα activity and leads to the mROSlow phenotype. However, PGClα represses NeuroD1 to prevent premature expression in progenitors (Fig. 8D). This transitional balance and timing is important for progression to the normal multipolar stage. Accordingly, transfection with the PGClα plasmid partially rescued migration defects (Fig. 8B).

In summary, dynamic changes in mtROS levels occur during differentiation and the transition of progenitor cells into postmitotic multipolar cells. As a possible modulator of mtROS, Prdm16 is specifically expressed by these progenitors in the VZ. Hence, downregulation of Prdm16 just outside the VZ and complementary upregulation of NeuroD1 are crucial for the regulation of PGClα-mediated changes in cellular redox environments (Fig. 8E). This event is required for the transition of progenitors into normal multipolar cells and the acquisition of proper laminar construction.

DISCUSSION

Emerging data indicate that stem cells possess metabolic characteristics that differ from those of differentiated cells (McGraw and Mittal, 2010). ROS stimulation of undifferentiated cells promotes neural stem cell self-renewal, whereas the same levels of ROS that are stimulatory to proliferative cells are toxic when present during differentiation. The present data show that embryonic neural progenitor cells have higher cellular ROS levels, as shown previously (Le Belle et al., 2011). Moreover, dynamic changes in ROS levels during neural differentiation were closely related to the transition of progenitor cells from proliferation to neuronal differentiation. In agreement, recent studies show that redox regulation is highly correlated with cell migration (Hurd et al., 2012). Therefore, the effects of ROS may be dependent on cellular differentiation states, and drastic morphological or migration behavioral changes likely occur in response to changes in ROS signaling.

In hematopoietic stem cells, Prdm16 regulates cellular ROS levels by specifically altering mtROS but not NADPH oxidase-generated ROS (Chuikov et al., 2010). In the developing neocortex, we found that Prdm16 expression was restricted in progenitors and was transiently downregulated in postmitotic multipolar cells of the MAZ or in the lower region of the IZ. As a transcription factor, Ngn2 plays important roles in the specification of glutamatergic neuronal differentiation, in the regulation of neuronal migration and in the activation of the downstream effector gene NeuroD1 in postmitotic cells. In the present experiments, NeuroD1 was exclusively expressed during the multipolar phase, and this expression was initiated when Prdm16 expression was downregulated. We also demonstrated that upregulation of NeuroD1 at the beginning of the multipolar phase enables termination of Prdm16 expression and facilitates progression towards the multipolar phase. Cells that overexpressed Prdm16 had elevated NeuroD1 expression, even in the VZ, and aberrant maintenance of NeuroD1 expression until the postnatal stage. They subsequently stopped migrating to the boundary between CP and white matter. Consistent with recent findings, this layer did not form properly, suggesting that the timing and duration of the multipolar phase is essential for neocortical formation (LoTurco and Bai, 2006; Yamagishi et al., 2011; Miyoshi and Fishell, 2012; Ohtaka-Maruyama et al., 2013; Inoue et al., 2014).

Translational control of mitochondrial activity and energy production is essential for normal development. A recent study demonstrated that knockout mice lacking cytoplasmic
polyadenylation element-binding protein 1 have brain-specific dysfunctional mitochondria and reduced ATP levels due to defective translation of NADH dehydrogenase ubiquinone flavoprotein 2 (NDUFV2) mRNA (Oruganty-Das et al., 2012). Moreover in utero suppression of NDUFV2 reportedly arrested neuronal migration and impaired the multipolar-bipolar transition, suggesting that the regulation of mitochondrial function and metabolic state is crucial for multipolar migration (Chen et al., 2015).

Numerous transcription networks contribute to the regulation of cellular and mitochondrial metabolism; the PGC1 family of transcriptional co-activators have recently emerged as central regulators of metabolism (Austin and St-Pierre, 2012) and
positive regulators of mitochondrial biogenesis, gluconeogenesis and various other metabolic processes (Handschin and Spiegelman, 2006). Therefore, PGC1α-mediated control of global oxidative metabolism is an emerging concept. Accordingly, lowered oxidative metabolism was associated with decreased PGC1α activity in Prdm16 knockdown cells, and may be accompanied by decreased mtROS levels. PGC1α and Prdm16 are transcriptional co-activators that are involved in the control of energy metabolism, and their ectopic expression in white adipose tissue induces the acquisition of brown adipose tissue features (Hondares et al., 2011; Ohno et al., 2012; Ringham et al., 2013). A recent study suggested that Sirt1 deacetylates PPARγ during energy deprivation and deacetylated PPARγ then interacts with Prdm16 to alter the balance from energy storage to energy expenditure (Qi et al., 2012). Moreover, overexpression of PGC1α suppresses NeuroD1 mRNA expression in isolated rat and human islets (Kim et al., 2016). Similarly, we found that deletion of IRE abolished the regulation of PGC1α by Prdm16, suggesting that Prdm16 directly binds the PGC1α promoter region. We also demonstrated that PGC1α is positively or negatively regulated by both Prdm16 and NeuroD1, leading to altered mtROS levels. Furthermore, we found that PGC1α overexpression partially rescues mtROS levels in Prdm16 knockdown cells. Finally, the mtROS inhibitor and the PGC1α loss-of-function plasmid suppressed NeuroD1 activity, thus preventing premature expression of NeuroD1 in progenitors. Thus, we suggest that the transition from progenitors to multipolar cells involves dynamic changes in the cellular energy demands of the underlying mtROS environment, depending on changes in expression from Prdm16 to NeuroD1.

To our knowledge, the present data are the first to correlate mtROS regulation with neural differentiation during neocortical development. Our principal finding is that appropriate neural differentiation is highly correlated with changes in mtROS levels, which are partially regulated by the sequential expression of Prdm16 and NeuroD1. Our results suggest an important role of dynamic changes in the cellular redox environment during neural differentiation, which is essential for the proper assembly of the neocortex.

MATERIALS AND METHODS

Mice

In vivo experiments were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Doshisha University, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Doshisha University. ICR mice were obtained from Shimizu Laboratory Supplies (Kyoto, Japan) and Tg(NeuroD1::GFP) bacterial artificial chromosome (BAC) transgenic mice were obtained from the GENESAT project at the Rockefeller University (NY, USA).

Plasmid construction

Prdm16 shRNA plasmid (psh-Prdm16, supplementary Materials and Methods) was kindly provided by Dr S. Kajimura (UCSF) (Ohno et al., 2012). Full-length Prdm16 cDNA clones were purchased from Addgene (catalogue number 15503). For the generation of expression plasmids, amplified Prdm16 cDNA (pCAG-Prdm16) and deletion mutants of Prdm16 (pCAG-Prdm16ΔPR, ΔZF and ΔZF) were inserted into pCAG-FLAG-IRES. The shRNA plasmids were generated by inserting annealed oligonucleotides into pSuper.retro.Puro vector, as previously described (Mizutani and Saito, 2005; Mizutani et al., 2009). Electroporation was performed using an electroporator (CUY21E, Nepa Gene) as previously described (Mizutani and Saito, 2005; Mizutani et al., 2009, 2015). The following plasmids were used in this study: pCAG-EGFP, pCAG-mCherry, pCAG-Prdm16, psh-Prdm16, psh2-Prdm16, pNeuroD1p-mCherry, psh-NeuroD1, pCAG-PGC1α, pCAG-FloxP-EGFP-N1 and pCAG-Cre.

Immunohistochemistry

Embryos were dissected, and the brains were fixed in 4% paraformaldehyde (PFA) for 1-3.5 h. For the postnatal stage, brains were fixed in 4% PFA overnight. Following 30% sucrose replacement, fixed brains were embedded in OCT compound (Sakura Tissue-Tek). The antibodies used are listed in Table S1. Immunostained sections were imaged on Zeiss LSM 710 or Olympus IX81. For details, see supplementary Materials and Methods.

Microscopy and imaging analysis

Images were acquired on a confocal microscope (LSM 710, Zeiss) or a fluorescent microscope (IX81, Olympus). ZEN and Metamorph softwares were used to acquire all confocal and fluorescent microscope images, respectively. Images were finally processed using Adobe Photoshop. 3D image processing and analysis were performed with Imaris (Bitplane). For quantification of multipolar cells around MAZ and IZ, we used a combination of molecular markers and cellular morphology. In our previous studies (Tabata and Nakajima, 2003; Tabata et al., 2009, 2012, 2013), we found that multipolar cells in the MAZ/IZ frequently had a long ascending process and a retraction bulb, representing STL morphology. These cells tended to be tangentially aligned and assumed typical multipolar cell morphology with tangentially oriented thin multiple processes. For details, see supplementary Materials and Methods.

Quantitative real-time PCR (qPCR)

qPCR was performed using SYBR Green labeling (SYBR Premix Ex TaqII, Takara) and a TP850 Real-Time PCR System (Takara). For details, see Table S2 and supplementary Materials and Methods.

DNA microarray analysis

Procedures were performed as previously described (Inoue et al., 2014). For details, see supplementary Materials and Methods.

Cell culture, in vitro electroporation, and mtROS quantification

Embryonic neocortical cells were isolated from E14.5 wild-type mice, followed by TrypLE Express (Gibco) treatment and trituration to generate a single-cell suspension. Plasmid DNA was introduced into primary neocortical cells using Neon Transfection System (Life Technologies). Next, neocortical neurospheres were isolated from E14.5 wild-type mice and the lateral ventricles of the embryonic forebrain using a glass micropipette. Electroporation was performed using an electroporator (CUY21E, Nepa Gene) as previously described (Mizutani and Saito, 2005; Mizutani et al., 2007; Inoue et al., 2014, 2015; Yamanishi et al., 2015). The following plasmids were used in this study: pCAG-EGFP, pCAG-mCherry, pCAG-Prdm16, psh-Prdm16, psh2-Prdm16, pNeuroD1p-mCherry, psh-NeuroD1, pCAG-PGC1α, pCAG-FloxP-EGFP-N1 and pCAG-Cre.

Cell sorting

FACS analysis was performed using FACS.Aria II and analyzed using FACS.DIVA 6.1 software (Becton Dickinson). The sorted cells were collected in TRIzol (Life Technologies) for 15 min at 37°C and analyzed using FACS.

Luciferase assay

A luciferase reporter assay was performed using E14.5 primary neocortical culture or Neuro2a cells. For details, see supplementary Materials and Methods.
Time-lapse imaging
E13.5 or E14.5 embryos were electroporated with pSuper-Retro-Puro (control), CAF-Prdm16 or sh-Prdm16 (2.5 μg/μl) together with pCAG-EGFP (0.8 μg/μl). Organotypic coronal brain slices (250 μm) from the level of interventricular foramen were prepared 42 h after electroporation with a vibrating microtome (HM650 V; Thermo Fisher Scientific) in Hanks’ balanced salt solution (Wako), placed on an insert membrane (Millipore) and cultured in Neurobasal medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum and 2% B27 (Thermo Fisher Scientific) as described previously (Tabata and Nakajima, 2003). The dishes were then mounted in CO2 incubator chamber (Tokai Hit, 5% CO2, 40% O2) fitted onto an Olympus FV1000 confocal laser microscope. The dorsolateral region of the neocortex was observed. For details, see the supplementary Materials and Methods.

Statistical analysis
Statistical analysis was performed using Microsoft Excel. Student’s t-test as stated in the appropriate experiments was used to test the significance.

P<0.01 was considered to be statistically significant. Error bars indicate the s.e.m.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
K.M. designed this study and wrote the manuscript; M.I., R.I., H.T., D.K., M.K.-S., H.I., F.M., K.N. and K.M. performed this study and analyzed data; H.I., Y.M. and T. Inoue, M., Kuroda, T., Honda, A., Komabayashi-Suzuki, M., Komai, T., Shinkai, Y. and Mizutani, K. (2014). Prdm8 regulates the morphological transition at multipolar phase during neocortical development. PLoS ONE 9, e86356.


