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Dnmt3a Regulates Both Proliferation and Differentiation of Mouse Neural Stem Cells

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DNA methylation is known to regulate cell differentiation and neuronal function in vivo. Here we examined whether deficiency of a *de novo* DNA methyltransferase, Dnmt3a, affects in vitro differentiation of mouse embryonic stem cells (mESCs) to neuronal and glial cell lineages. Early-passage neural stem cells (NSCs) derived from Dnmt3a-deficient ESCs exhibited a moderate phenotype in precocious glial differentiation compared with wild-type counterparts. However, successive passaging to passage 6 (P6), when wild-type NSCs become gliogenic, revealed a robust phenotype of precocious astrocyte and oligodendrocyte differentiation in Dnmt3a$^{-/-}$ NSCs, consistent with our previous findings in the more severely hypomethylated Dnmt1$^{-/-}$ NSCs. Mass spectrometric analysis revealed that total levels of methylcytosine in Dnmt3a$^{-/-}$ NSCs at P6 were globally hypomethylated. Moreover, the Dnmt3a$^{-/-}$ NSC proliferation rate was significantly increased compared with control from P6 onward. Thus, our work revealed a novel role for Dnmt3a in regulating both the timing of neural cell differentiation and the cell proliferation in the paradigm of mESC-derived-NSCs.

Key words: DNA methylation; cell differentiation; cell proliferation; glial cells; neural stem cells; p53

DNA cytosine methylation is involved in multiple developmental mechanisms, such as gene regulation, genomic imprinting, and X-chromosome inactivation (Jaenisch and Bird, 2003). The DNA methylome is established and maintained by a family of DNA (cytosine-5) methyltransferases (Dnmts), including Dnmt1, Dnmt3a, and Dnmt3b (Bestor, 2000). Dnmt1 is essential for maintaining methylation patterns during DNA replication, whereas Dnmt3a and Dnmt3b are primarily responsible for de novo methylation in embryonic and postnatal tissues (Leonhardt et al., 1992; Okano et al., 1999; Chen et al., 2003). Targeted deletion of Dnmt1 (Li et al., 1992) or either Dnmt3a or −3b (Okano et al., 1999) in mice results in demethylation and embryonic death, indicating an essential role for DNA methylation and Dnmts in animal development.

Previous work has shown that DNA methylation is a major regulator of spatiotemporal development of the central nervous system in mice (Fan et al., 2001, 2005; Takizawa et al., 2001; Martinowich et al., 2003; Hutnick et al., 2009). On the other hand, human genetic disease studies revealed that abnormal DNA methylation pattern and mutation of Dnmts genes are associated with mental retardation disorders, such as immunodeficiency, centromere instability, and facial anomaly (ICF) syndrome, fragile X, and alpha-thalassemia retardation X-linked (ATRX) syndrome (Robertson and Wolffe, 2000).

Dnmt3a is considered to play a critical role in CNS development and neuronal maturation. Through histological examination, we have shown that Dnmt3a is expressed predominantly in embryonic neural precursor cells (NPCs) within the ventricular zone and in postnatal postmitotic neurons (Feng et al., 2005). CNS-specific conditional mutation of Dnmt3a demonstrated that Dnmt3a is involved in motor neuronal survival and

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methylation of glial genes in postnatal animals (Nguyen et al., 2007). More recently, it was demonstrated that Dnmt3a regulates adult neurogenesis in both subventricular zone (SVZ) and hippocampal dentate gyrus region. Dnmt3a deficiency in postnatal neural stem cells (NSCs) leads to impaired neuronal production, which is coupled with increased astrogliogenesis and oligodendrogenesis (Wu et al., 2010). In addition, we have previously shown that synapse plasticity and learning and memory behaviors were impaired in conditional mutant mice that were deficient for both Dnmt1 and Dnmt3a in forebrain postmitotic neuron during early postnatal development (Feng et al., 2010). These results argue that Dnmt3a might be essential for neural lineage differentiation and neuronal maturation.

Recent advances in stem cell biology hold the promise of deriving neuronal and glial cells from both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) for neural repair. We therefore examined whether Dnmt3a can play a role in regulating neurogenesis and gliogenesis during in vitro differentiation of mouse ESCs into neurons and glial cells. Consistently with previous findings in Dnmt1$^{-/-}$ NSCs, Dnmt3a$^{-/-}$ NSCs derived from mESCs produced more glial cells and at an earlier time point. However, unlike Dnmt1$^{-/-}$ NSCs, Dnmt3a$^{-/-}$ NSCs are viable over extended passages. Furthermore, Dnmt3a$^{-/-}$ NSCs exhibit a significant increase in cell proliferation compared with WT NSCs. Microarray analysis identified deregulated genes associated with cell proliferation and cell death, particularly in the p53 signaling pathway, in Dnmt3a$^{-/-}$ mNSCs. Together, these findings indicate that Dnmt3a is essential for terminal neural differentiation timing and cell proliferation of mNSCs.

MATERIALS AND METHODS

Mouse ESC Cultures

Both wild-type (WT) and Dnmt3a$^{-/-}$ ES cells were cultured on a layer of irradiated mouse embryonic fibroblasts in DMEM containing 15% fetal bovine serum, leukemia-inhibiting factor (LIF), penicillin/streptomycin, L-glutamine, 0.1 mM β-mercaptoethanol, and nonessential amino acids. Medium was changed every day, and cells were trypsinized to passage every 3–4 days. All feeder cells were depleted for two passages on 0.2% gelatin before extracting DNA and RNA.

Generation of Dnmt3a Rescue mESCs (TD3a)

To rescue Dnmt3a expression in Dnmt3a$^{-/-}$ ESCs, the Dnmt3a$^{-/-}$ ESCs were transfected with Dnmt3a expression plasmid containing the blasticidin selection via electroporation. The Dnmt3a expression plasmid was described previously (Chen et al., 2003). The cells were then plated at low density and grown in culture media containing blastcytidine for 10 days. Single-cell colonies were picked and expanded under continuing blastcytidine selection.

Conversion of Mouse ESCs Into NSCs

mNSCs were derived from WT, Dnmt3a$^{-/-}$, and TDnmt3a ES cells as previously described (Ying et al., 2003). Briefly, mESCs were cultured on 0.2% gelatin-coating plate in normal mESCs medium for one passage to exclude MEF feeder cells. When the plate was confluent, mESCs colonies were completely trypsinated to single cells, followed by washing with DMEM:F12 medium three times to wash off all serum, and then were passaged to a new 0.1% gelatin-coating plate. These cells were maintained for 7 days in serum-free N2B27 medium supplemented with epidermal growth factor (EGF; 10 ng/ml) and basic fibroblast growth factor (bFGF; 10 ng/ml). Neurosphere formation was carried out when the plate was confluent and colonies had begun showing partial differentiation morphology. Neurospheres were maintained in an ultralow-attachment plate with suspension culture in N2B27 medium supplemented with bFGF and EGF for 1 week. At the fourth day after formation of neurospheres, cells were transferred to a poly-L-ornithine (PO)/fibronectin (FN) coating plate and grown in N2B27 medium supplemented with bFGF and EGF. Neurospheres attached to the plate after 3–5 days, and bipolar cells could be found around the attached neurospheres. These bipolar cells were termed *mNSCs P0* and could be passaged with 0.025% trypsin/EDTA, followed by addition of trypsin inhibitor.

mNSCs Terminal Differentiation

For neuronal differentiation, mNSCs were plated on a PO/FN coating plate in DMEM:F12 medium with 2% B27 supplement and 1% penicillin/streptomycin. Neurotrophin 3 (NT3; 20 ng/ml) and brain-derived neurotrophic factor (BDNF; 10 ng/ml) were added to the neuronal differentiation medium to enhance the differentiation efficiency. Glial differentiation medium was composed of DMEM:F12 with 5% serum without bFGF and EGF. Also, 20 ng/ml bone morphogenic protein 4 (BMP4) and 50 ng/ml LIF could be added for the same purpose. For spontaneous differentiation, mNSCs were incubated in N2B27 medium without bFGF and EGF. No additional cytokines should be supplemented.

Immunocytochemistry was performed to identify the lineage-specific markers of differentiated cells on day 2 and day 6 of differentiation. We used polyclonal rabbit anti-Dnmt3a (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal rabbit antinestin (1:200; Abcam, Cambridge, MA), monoclonal mouse anti-Pax6 (1:50; DSHB, Iowa City, IA), monoclonal mouse anti-Tuj1 (1:1,000; Abcam), polyclonal rabbit anti-Mbp (1:500; Millipore, Bedford, MA), monoclonal mouse anti-Gfap (1:50; Sigma, St. Louis, MO), and monoclonal anti-BrdU (1:150; Millipore).

Cell Proliferation Rate Analysis

For cell proliferation assay, 1×10^4 cells were seeded in 0.1% gelatin-coated six-well plates containing N2B27 medium supplemented with EGF and bFGF. The cell number was counted every day to estimate the growth curve, and the population doubling time was calculated according to the exponential function of the growth curve. The cell cycle distribution was determined by propidium iodide (PI) staining

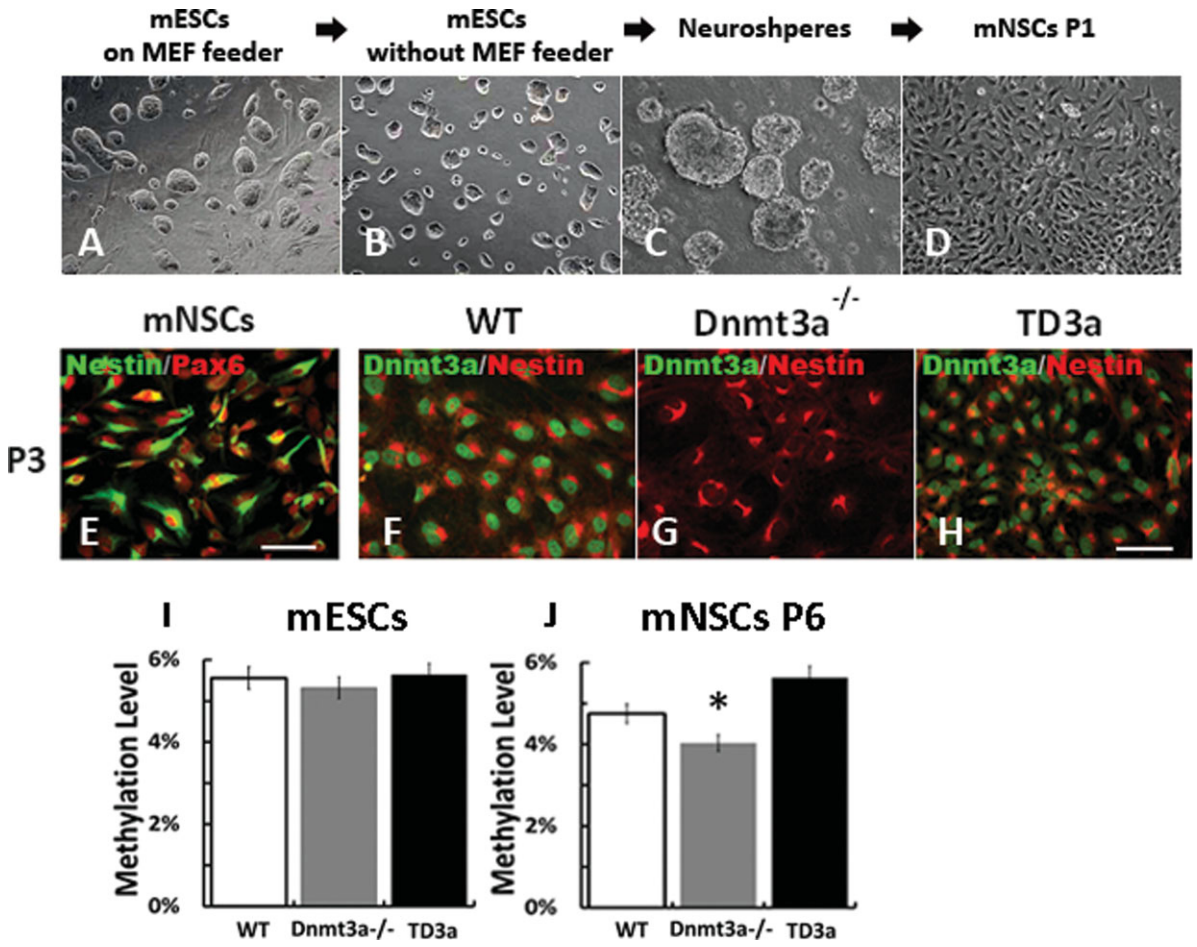

Fig. 1. Identification of mNSCs derived from WT, Dnmt3a$^{-/-}$, and TD3a mESCs. **A–D:** Experimental scheme describing the process of deriving mNSCs from mESCs. **E:** mNSCs derived from mESCs with the NSC-specific markers nestin (red) and Pax6 (green). **F–H:** Immunostaining confirms Dnmt3a expression deficiency in Dnmt3a$^{-/-}$ mNSCs and re-expression in TD3a rescued line. **I,J:** Mass spectrometry shows percentages of global 5mC in mESCs and mNSCs genomic DNA. Data are mean ± standard deviations from triplicate analyses. *$P < 0.05$ by Student's *t*-test. Scale bars = 40 μm in E (applies to A–E); 20 μm in H (applies to F–H).

and flow cytometric analysis. Bromodeoxyuridine (BrdU) incorporation assay and Ki67 staining were performed to measure DNA replication.

Microarray Hybridization

Gene expression microarrays were performed with Agilent Whole Genome microarrays (G4122A) using the suggested protocol. Briefly, RNA was isolated using Trizol (Invitrogen, Carlsbad, CA). We converted the RNA into cDNA and then the cDNA into cRNA using the Agilent Low RNA Input Linear Amplification Kit (Agilent, Santa Clara, CA). We used Nanodrop to quantify the labeled cRNA and used 0.75 μg of each sample for hybridization. Probes were fragmented in a mix of labeled probes, 10× blocking reagent, and 25× fragmentation buffer. The reaction was stopped with the addition of 2× hybridization buffer. We used Agilent Whole Genome microarrays for expression studies. Slides were hybridized at 65°C for 17 hr at 4 rpm and then washed once in Agilent Gene Expression wash buffer 1 and once in Agilent Gene Expression wash buffer 2 before a quick wash in acetonitrile. Slides were scanned immediately after washing to prevent ozone degradation. Arrays were performed in triplicate. Probe intensities were quantile-normalized and log2-transformed across all samples.

Accession Number

The data generated for this work have been deposited in the NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE38035.

RESULTS

Derivation of Dnmt3a$^{-/-}$ NSCs From Dnmt3a$^{-/-}$ mESCs

To understand better the role of Dnmt3a in neural differentiation, both Dnmt3a$^{-/-}$ and wild-type (WT) mESCs were converted into NSCs and then induced to terminally differentiated neural cell types (Fig. 1A–E). We found no visible morphological differences between

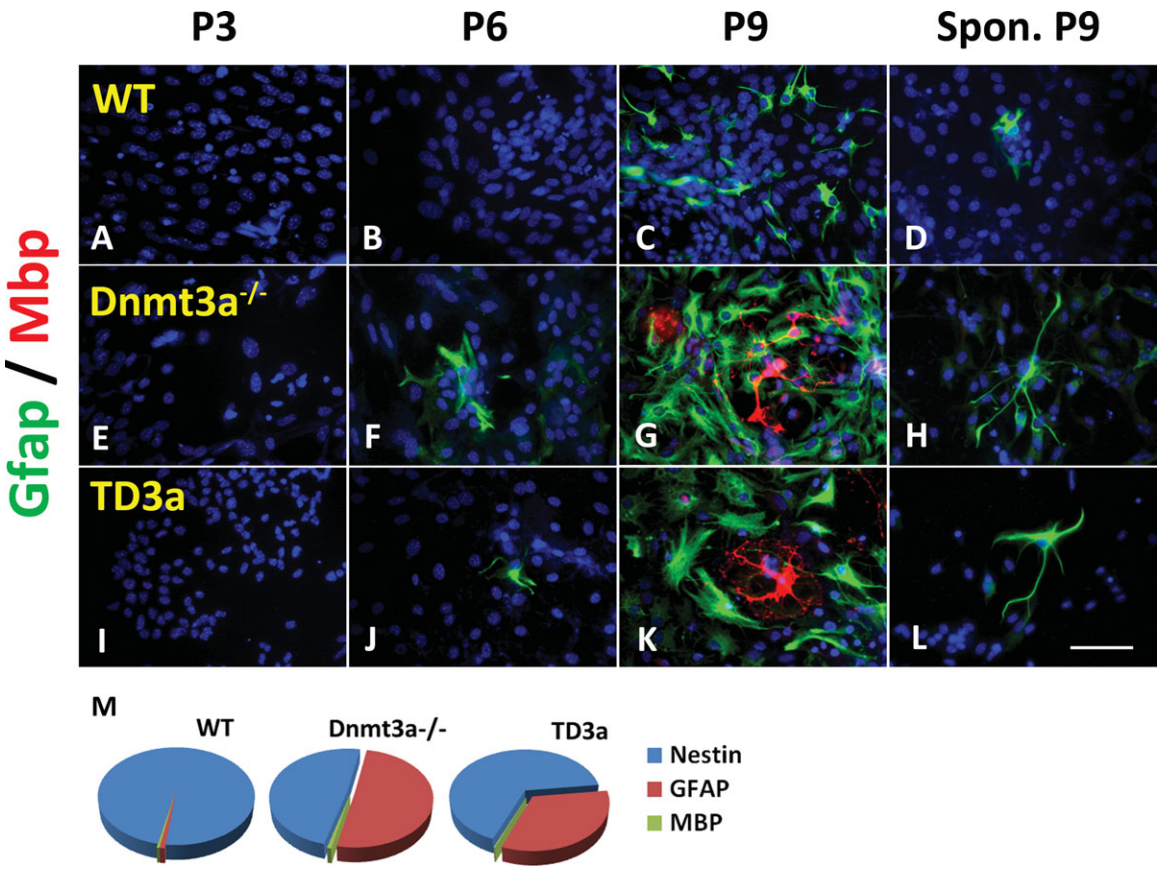

Fig. 2. Glial differentiation of mNSCs derived from WT (**A–D**), Dnmt3a$^{-/-}$ (**E–H**), and TD3a (**I–L**) mESCs. Immunostaining for Gfap (green) and Mbp (red) shows glial cell differentiation of three different passage stages in each mNSCs line, P3 (A,E,I), P6 (B,F,J), P9 (C,G,K), and spontaneous differentiation at P9 (D,H,L). **M:** Cell counting shows percentage of neural cell types after glial differentiation at P9 of all three mNSCs lines. Scale bar = 20 μm.

Dnmt3a$^{-/-}$ and WT NSCs, although immunostaining confirmed the lack of Dnmt3a in knockout NSCs (Fig. 1F–H). To detect alternation of methylation levels in the absence of Dnmt3a expression, mass spectrometry of genomic DNA was carried out to show the global methylcytosine level (Le et al., 2011). Whereas WT mESCs and mNSCs contain 4.7% and 5.5% 5-methylcytosine (5mC), the mutant mESCs and mNSCs contain 4.0% and 5.3% 5mC (Fig. 1I,J), confirming hypomethylation in Dnmt3a-deficient cells lines.

Dnmt3a Deficiency in NSCs Leads to Precocious Gliogenesis

Through consecutive trypsinization passages, early (P3)-, middle (P6)-, and late (P9)-passage homogenous mNSCs were generated. These mNSCs were further cultured in differentiation medium for up to 6 days. We found that loss of Dnmt3a expression in NSCs resulted in precocious differentiation of both astrocyte and oligodendrocyte lineages, but the timing and magnitude of neuronal differentiation were not affected. In the early passage (P3), both Dnmt3a$^{-/-}$ and WT cells revealed small number of differentiated glial cells. By P6 stage, precocious Gfap^{+} astrocytes could be seen only in Dnmt3a$^{-/-}$ mNSCs. By contrast, Mbp^{+} oligodendrocytes did not appear in either group. In the late passage (P9), more than 50% of Dnmt3a$^{-/-}$ mNSCs differentiated into astrocytes as well as a small population (0.79% ± 0.02%) of oligodendrocytes (Fig. 2). Furthermore, Gfap^{+} cells in the mutant group showed a more mature morphology (Fig. 2H). In contrast, very few Gfap^{+} astrocytes and no oligodendrocyte were found in the WT group (Fig. 2M). For neuronal differentiation, the percentages and morphology of Tuj1-positive cells in Dnmt3a$^{-/-}$ and WT cells were similar (Fig. 3). To determine more precisely the timing of neuronal maturation and gliogenesis, we carried out RT-PCR to detect expression of several neural markers, including the NPC marker Nestin, the neuronal marker Tuj1, the astrocyte marker Gfap, and the oligodendrocyte marker Mbp. In the absence of Dnmt3a, Gfap, and Mbp expression levels were dramatic higher than WT in the late passage (Fig. 4). To evaluate our differentiation system further, we compared the morphology of NSC-derived astrocytes with that of primary mouse fetal glial cells. With

Gfap staining (data not shown), we see similar cellular morphology between fetal astrocytes and NSC-derived astrocytes. Overall, we found that loss of Dnmt3a expression resulted in precocious gliogenesis but not impaired neuronal maturation.

Rescued Expression of Dnmt3a Could Partially Rescue the Differentiation Phenotype in Dnmt3a$^{-/-}$ mNSCs

To investigate whether this mutant phenotype could be rescued, we generated stable Dnmt3a$^{-/-}$ mES cell lines expressing Dnmt3a (referred to as *TD3a*). Immunostaining confirmed Dnmt3a re-expression in mutant cells (Fig. 1H), and mass spectrometry showed that global methylation was increased in both TD3a ESCs and TD3a NSCs (Fig. 1M). In addition, TD3a NSCs showed partial rescue of precocious glial cell maturation. As shown in Figure 2, TD3a NSCs had an ability to differentiation into glial cells similar to that of Dnmt3a$^{-/-}$ mNSCs at P6. However, TD3a NSCs showed reduced precocious glial cells differentiation compared with Dnmt3a$^{-/-}$ mNSCs. In late-passage mNSCs (P9), astrocytes and oligodendrocytes still could be found in the TD3a NSC differentiation process, but the percentage of Gfap^{+} cells in TD3a mNSCs (32.85% ± 0.52%) was significant lower than that in Dnmt3a$^{-/-}$ mNSCs (50.66% ± 0.61%; Fig. 2M). Thus, re-expression of Dnmt3a in Dnmt3a$^{-/-}$ mESCs could partially rescue defects in neural differentiation.

To clarify whether differentiation defects begin in ESCs or NSCs, we performed transient rescue experiments by transducing late-passage Dnmt3a$^{-/-}$ mNSCs with Dnmt3a expression constructs via lentivirus infection (termed *Dnmt3a-Res mNSCs*). Four days after infection, Dnmt3a-Res NSCs (P10) were transferred to a 0.1% gelatin coating plate in glial differentiation medium for 7 days. Medium supplied with BMP4 and LIF was changed every other day. Dnmt3a immunostaining showed that about 40% of total cells had Dnmt3a expression (Fig. 5A–D). We found among the Dnmt3a^{+} cells a reduced number of Gfap^{+} cells (54.0%) compared with noninfected Dnmt3a$^{-/-}$ mNSCs (75.3%), suggesting that Dnmt3a^{+} mNSCs had a lower glial differentiation efficiency (Fig. 5). The levels of gliogenic activity in Dnmt3a-Res NSCs were close to TD3a (42.8%) levels, indicating that reintroduction of Dnmt3a in the NSC stage can partially inhibit glial differentiation (Fig. 5E,F). Collectively, our results provide a direct link between Dnmt3a deficiency and high gliogenic activity.

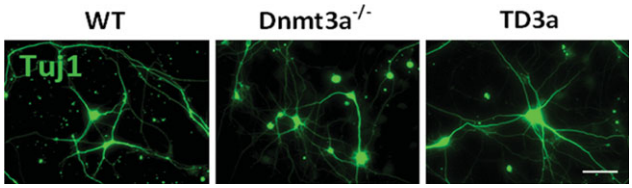

Fig. 3. Neuronal differentiation of mNSCs (P3) derived from WT, Dnmt3a$^{-/-}$, and TD3a mESCs. Tuj1 immunostaining shows neuron morphology cells. Scale bar = 10 μm.

Dnmt3a Deficiency Increases Cell Proliferation Rate in NSCs

During our cell culture work, we found that the passage time of Dnmt3a$^{-/-}$ mNSCs was noticeably shorter than that of WT mNSCs. To examine whether Dnmt3a regulated cell proliferation, we generated cell growth curves of both Dnmt3a$^{-/-}$ and WT mNSCs (see Materials and Methods) to compare their cell prolif-

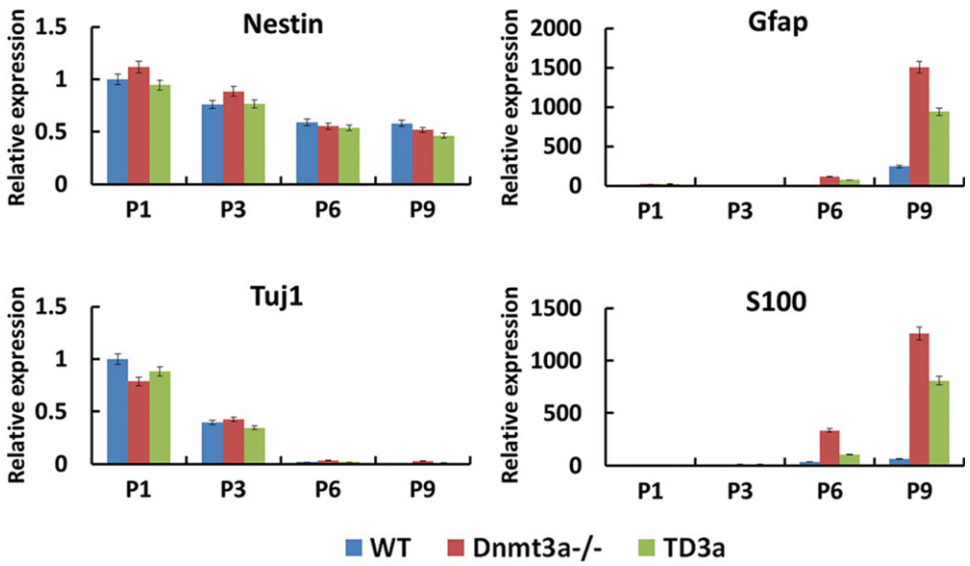

Fig. 4. RT-PCR analysis demonstrates nestin, Tuj1, Gfap, and S100 expression level changes during the differentiation time course. Y-axis is normalized to P1 WT gene expression.

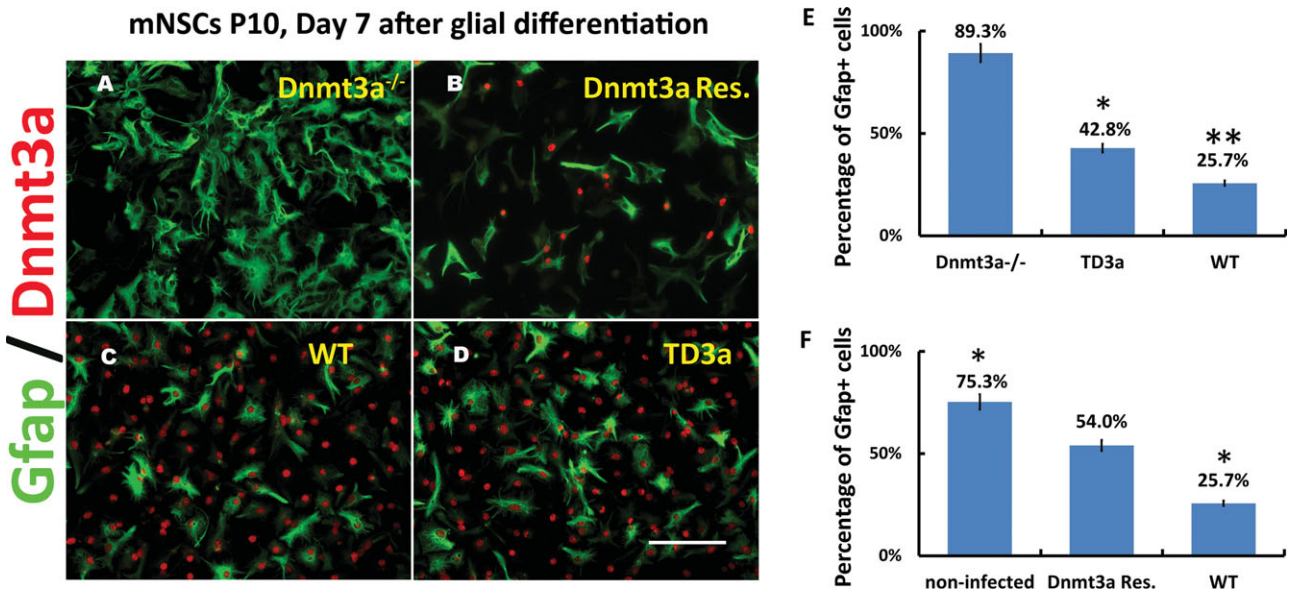

Fig. 5. Glial differentiation of Dnmt3a$^{-/-}$ (**A**), Dnmt3a-Res (**B**), WT (**C**), and TD3a (**D**) in late-stage mNSCs. Immunostaining for Gfap (green) and Dnmt3a (red) shows that glial differentiation efficiency is decreased as a result of both stable and transient Dnmt3a rescue. **E,F:** In counting Gfap-positive cells at P10, Dnmt3a transient rescue shows a lower gliogenic ability compared with noninfected Dnmt3a$^{-/-}$ mNSCs. *$P < 0.01$, **$P < 0.05$ (Student's *t*-test). Scale bar = 20 μm.

eration rates. After 5 days of counting, the total number of Dnmt3a$^{-/-}$ mNSCs was significantly higher than WT despite both starting with the same number of plated cells at day 0 (Fig. 6A). The cell doubling times (the time needed for total cell number to increase two-fold) of Dnmt3a$^{-/-}$ mNSCs and WT mNSCs were 25.88 ± 0.73 hr and 32.88 ± 2.02 hr, respectively. Furthermore, we employed flow cytometric analysis and BrdU staining to find the number of cells undergoing cell division in Dnmt3a$^{-/-}$ and WT cell lines. As shown in Figure 6B, flow cytometry revealed that 30.69% of Dnmt3a$^{-/-}$ mNSCs were in S phase, compared with 18.47% of WT mNSCs. By analyzing early-, middle-, and late-passage NSCs, we found an increasing number of dividing cells in Dnmt3a$^{-/-}$ (P3: 22.74%, P6: 27.28%, P9: 30.71%) but not in WT (P3: 16.76%, P6: 19.37%, P9: 19.45%; Fig. 6B). This result likely indicates that Dnmt3a deficiency stimulated proliferation in subpopulations of cells. Thus, it appears that our Dnmt3a$^{-/-}$ culture inherently selected for cells with greater proliferative capacity over prolonged passaging. Nevertheless, Dnmt3a$^{-/-}$ NSCs consistently showed more cells undergoing mitosis compared with WT. By contrast, lack of Dnmt3a expression in embryonic stem cells led to only a slightly higher cell proliferation rate (63.52%) compared with WT (57.24%). To complement our flow cytometry analysis, BrdU staining and Ki67 staining showed 1.83-fold more BrdU^{+} and 1.65-fold more Ki67^{+} cells in Dnmt3a$^{-/-}$ mNSCs compared with WT NSCs, respectively (Fig. 6C,D). Overall, Dnmt3a deficiency promotes increased cell cycle in subpopulations of differentiated mNSCs.

Analysis of Gene Expression Changes in Dnmt3a$^{-/-}$ mNSCs

To examine the molecular changes in WT and Dnmt3a$^{-/-}$ mNSCs, we generated gene expression profiles in technical triplicates using the Agilent two-color gene expression arrays with 44,000 probes. By using a <5% false-discovery rate and >1.5-fold cutoff to find differentially expressed genes, we identified 611 upregulated and 676 downregulated genes in Dnmt3a$^{-/-}$ mNSCs compared with WT cell via DAVID functional annotation analysis (Huang et al., 2009). Consistently with Dnmt3a$^{-/-}$ morphology, gene ontology (GO) analysis revealed that upregulated genes are associated with neuronal development and neuronal morphogenesis, suggesting that Dnmt3a has significant roles in regulating neuronal differentiation and maturation (Fig. 7A). Genes downregulated in Dnmt3a$^{-/-}$ cells were generally associated with cell proliferation and cell death (Fig. 7B). Furthermore, pathway analysis revealed that downregulated genes are involved in p53 signaling (Fig. 7C). p53 is a known cell-cycle-arrest protein and is also associated with apoptosis (Vousden and Prives, 2009). Downregulation of the p53 signaling pathway is consistent with the increased cell proliferation observed in Dnmt3a$^{-/-}$ mNSCs.

DISCUSSION

Dnmt3a is one of the major *de novo* methylation enzymes required for proper mammalian embryogenesis and brain development (Okano et al., 1999; Chen et al., 2003). During neurogenesis, Dnmt3a protein is strongly

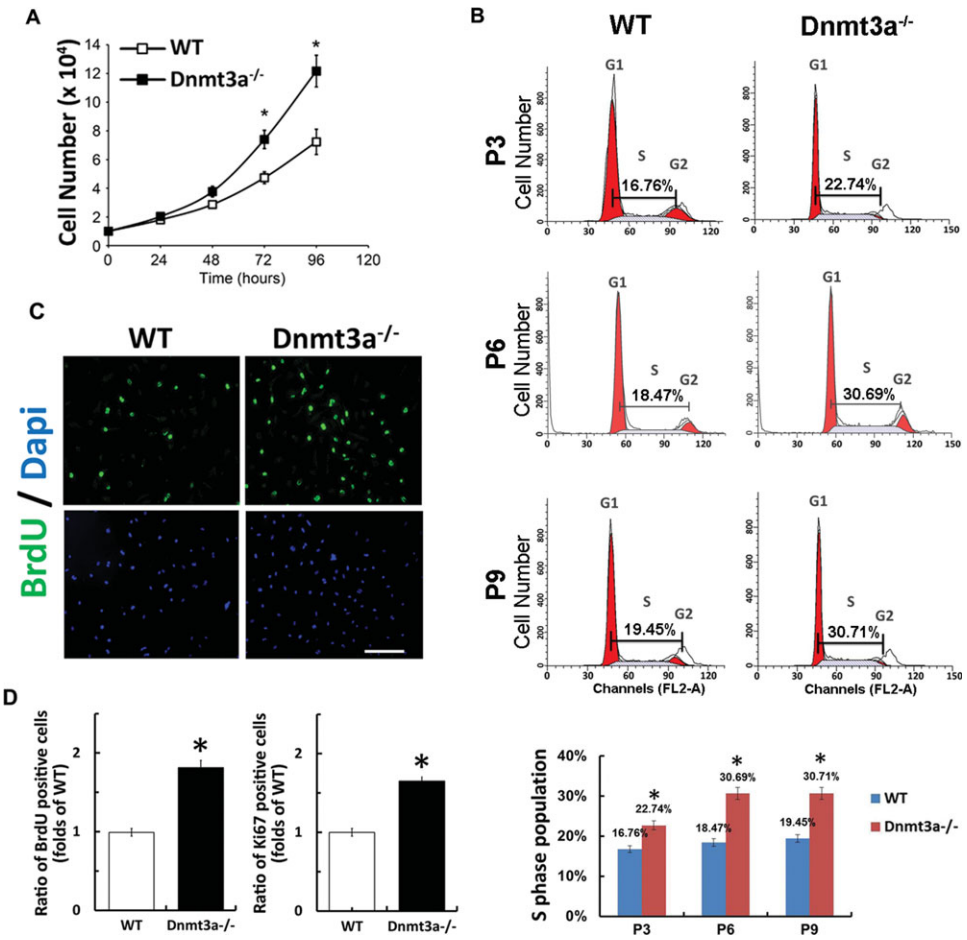

Fig. 6. Cell proliferation analysis of WT and Dnmt3a$^{-/-}$ mNSCs. **A:** Cell growth curve describes cell doubling speed. **B:** Flow cytometric analysis shows cell cycle distribution by propidium iodide (PI) staining. **C:** Colocalization of BrdU with DAPI. **D:** Amount of BrdU- and Ki67-positive cells in Dnmt3a$^{-/-}$ mNSCs (fold of WT mNSCs). A,C,D were assayed at P6. Scale bar = 20 μm.

expressed in neural precursor cells, postmitotic CNS neurons, and oligodendrocytes (Feng et al., 2005). Previous studies with Dnmt3a$^{-/-}$ mouse brain showed impaired postnatal neurogenesis at two neurogenic zones, including subependymal/subventricular zones (SEZ/SVZ) in the hippocampal dentate gyrus. Furthermore, Dnmt3a mutant mice had fewer Tuj1^{+} neurons and more glial cells compared with WT mice (Wu et al., 2010). These findings indicate that Dnmt3a is an important regulator in neurogenesis and gliogenesis. In this study, we were interested in whether Dnmt3a-deficient neural differentiation can be modeled in vitro. We found that Dnmt3a-deficient ESC-derived mNSCs showed a substantially greater number of both astrocytes and oligodendrocytes compared with WT cells, suggesting that loss of Dnmt3a results in precocious glial cells maturation. Precocious differentiation in Dnmt3a-deficient NSCs appeared to be more robust in the P6 passage, coincident with the onset of gliogenic activity in wild-type NSCs. Thus, Dnmt3a deficiency in early-passage NSCs shows more attenuated differentiation and proliferation phenotypes, raising the possibility that other epigenetic events must occur to facilitate more robust precocious differentiation in NSCs in the absence of Dnmt3a. However, neuronal differentiation was not impaired, although it is still unknown whether these Dnmt3a deficient neurons have impaired or altered function. Furthermore, we were able to rescue the Dnmt3a expression in both Dnmt3a$^{-/-}$ ESC and NSC stages. However, we did notice that the effects of our transient rescue in the NSC stage were less pronounced compared with the stable rescue in the ESC stage. One possibility is that the Dnmt3a virus is too toxic for non-infected Dnmt3a$^{-/-}$ mNSCs. Another possibility is that, because we performed transient rescue in late-passage NSCs, the hypomethylated DNA methylation patterns established in early-passage NSCs might already have been permissive for glial differentiation.

Our current study demonstrates that DNA methylation is required for proper neural differentiation. Unlike Dnmt1 deficiency, Dnmt3a deficiency results in mild genomewide hypomethylation and can produce more precocious glial cells. This may be due to increased survival of Dnmt3a-deficient NSCs compared with Dnmt1-

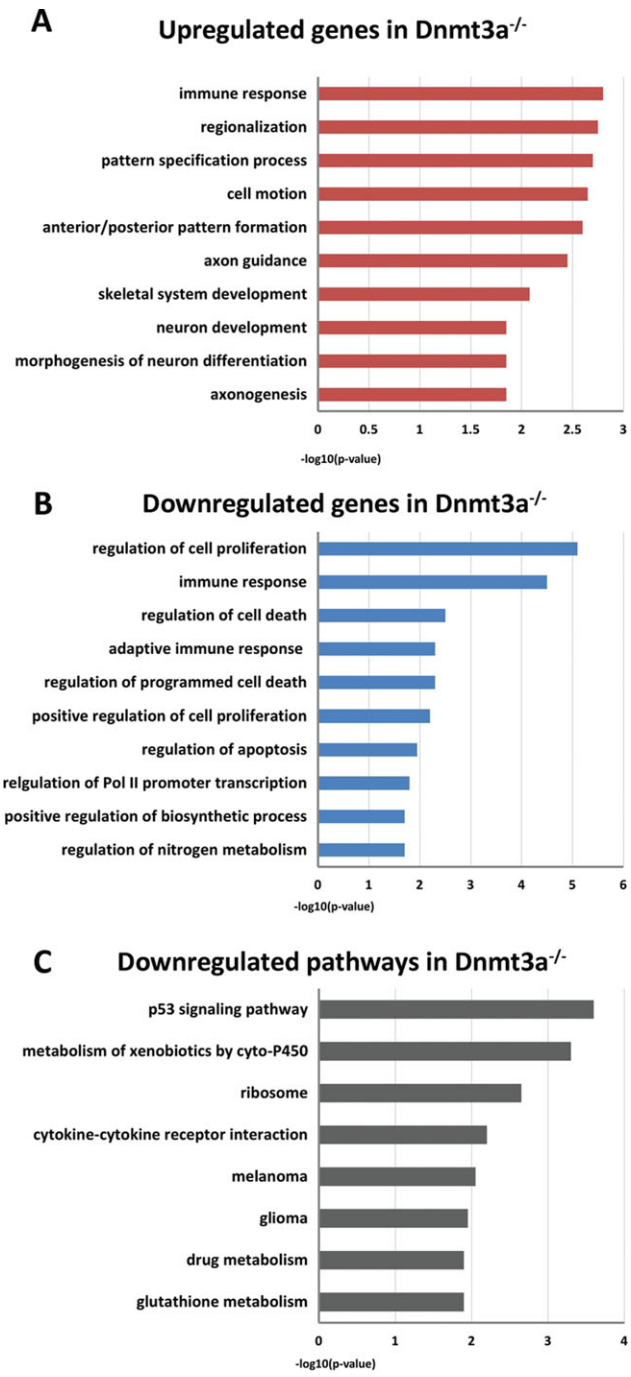

Fig. 7. Gene ontology analysis via DAVID annotation shows top 10 GO terms of upregulated/downregulated genes and downregulated KEGG pathways in Dnmt3a$^{-/-}$ mNSCs compared with WT mNSCs. Gene ontology analysis reveals 611 upregulated (**A**) and 676 downregulated (**B**) genes in Dnmt3a$^{-/-}$ mNSCs compared with WT mNSCs. (**C**) KEGG pathways associated with the downregulated genes.

deficient NSCs. Our previous study showed that conditional Dnmt1 deletion in NSCs results in precocious astrocyte differentiation (Fan et al., 2005), although most cells cannot be maintained over continued passage. Loss of Dnmt1 results in severe global hypomethylation, which dramatically reduces cell survival in culture (Fan et al., 2001). Dnmt1 conditional knockout mouse showed visibly smaller olfactory bulbs (OB) than WT mice (Fan et al., 2001). Interestingly, Dnmt3a$^{-/-}$ mouse OB size and the number of newborn neurons resembled those of Dnmt1-KO mice (Wu et al., 2010). Finally, in the most extreme case, triple-knockout (TKO) mESCs lines lacking all three of DNA methyltransferases cannot be induced toward neural-lineage cells and undergo apoptosis upon differentiation (Tsumura et al., 2006).

The role of Dnmt3a in cell proliferation can be seen in different human cancers. For example, Dnmt3a mutations in acute myeloid leukemia (AML) have recently been reported by three independent groups (Ley et al., 2010; Yamashita et al., 2010; Yan et al., 2011). These mutations led to select genome hypomethylation (Ley et al., 2010) and gene deregulation (Yan et al., 2011). In addition, other components of the DNA methylation pathway are found in AML cancers. For example, somatic mutation of TET2, which converts 5mC to 5-hydroxymethycytosine, was also found in AML individuals (Figueroa et al., 2010). However, unlike the case in the hematopoietic stem cell system, in which DNA methyltransferases were found to be essential for self-renewal but not differentiation (Tadokoro et al., 2007), we demonstrated that Dnmt3a regulated both cell self-renewal and differentiation activities in the neural lineage. Interestingly, Dnmt3a deficiency did not impact cell proliferation in the embryonic stem cell stage, perhaps because of compensation from the highly expressed Dnmt3b. Together, these data suggest that Dnmt3a behaves differently and has distinct roles in different cell lineages.

Mutations in Dnmt3a have been identified in other cancer types. Most relevant are studies in glioblastoma cell lines showing an association with decreased Dnmt3a expression and hypomethylation of satellite repeats at pericentromeric regions (Caprodossi et al., 2007). Intriguingly, ectopic expression of Dnmt3a in glioblastoma cell lines can partially rescue repeat hypomethylation. Overall, these results are consistent with the cell proliferation results in our mNSCs differentiation. Our studies indicate a role for the p53 tumor suppressor pathway that contributes to altered cell proliferation. Previous studies have shown that DNA hypomethylation led to chromosomal instability and tumorigenesis (Eden et al., 2003). Thus, the p53 pathway may be downregulated as a consequence of hypomethylation in Dnmt3a-deficient cells. Moreover, the effect of cell proliferation might be achieved by the cooperation of multiple factors, including abnormal gene mutations (*NPM1*, *FLT3*, and *UHRF1/2*, etc.), epigenetic modifications (Dnmt1/3b, histone methylation/deacetylation, and micro-RNA regulation, etc.), and induction of cytokines during differentiation. However, how Dnmt3a regulates cell proliferation and apoptosis still must be addressed in future work.

In many neural trauma and neural degenerative diseases, neural cell transplantation is becoming an

increasingly attractive alternative therapy for treatment. However, one major hurdle to overcome is our inability to control the cellular properties once the cells are transplanted into the human body; these properties include incorporation of neural cells into the neural network and cell proliferation. Dnmt3a may be a critical regulator of cell activities after transplantation in light of the results of this study. Epigenetic modification methylation is considered to play an important role in the graft survival process. For example, recent histone deacetylase inhibitor drug therapy introduced in a spinal injury mouse model showed enhanced improvement limb function (Abematsu et al., 2010). These findings are encouraging and suggest the use of other epigenetic drugs for enhanced transplantation therapy. Our studies will pave the way for clinical application of cell transplantation, such as spinal cord injury, stroke, and other CNS trauma.

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