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Dnmt3a regulates both cell proliferation and differentiation of mouse neural stem cells

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Abstract

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 to wild-type counterparts. However, successive passaging to passage six (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-spectrometry analysis revealed total levels of methylcytosine in Dnmt3a^{-/-} NSCs at P6 were globally hypomethylated. Moreover, Dnmt3a^{-/-} NSC proliferation rate was significantly increased when compared to control from P6 on. Thus, our work revealed a novel role for Dnmt3a in regulating both the timing of neural cell differentiation and cell proliferation in the paradigm of mESC-derived-NSCs.

Keywords

DNA methylation; Cell differentiation; Cell proliferation; Glial cells; Neural stem cells; p53

INTRODUCTION

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 (Chen et al. 2003; Leonhardt et al. 1992; Okano et al. 1999). Targeted deletion of

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Dnmt1 (Li et al. 1992) or either Dnmt3a/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 works have shown that DNA methylation is a major regulator of spatiotemporal development of central nervous system in mice (Fan et al. 2001; Fan et al. 2005; Hutnick et al. 2009; Martinowich et al. 2003; Takizawa et al. 2001). On the other hand, human genetic disease studies revealed that abnormal DNA methylation pattern and/or mutation of Dnmts genes are associated with mental retardation disorders, such as ICF (immunodeficiency, centromere instability and facial anomaly) syndrome, Fragile X, and ATRX (Alpha-Thalassemia Retardation X-linked) syndrome (Robertson and Wolffe 2000).

Dnmt3a is considered to play a critical role in CNS development and neuronal maturation. By using histological examination we have shown that Dnmt3a is predominantly expressed 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 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 as well as learning and memory behaviors were impaired in conditional mutant mice that are deficient of 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. Consistent 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^{-/-} are viable over extended passages. Furthermore, Dnmt3a^{-/-} NSCs exhibit a significant increase in cell proliferation compared to 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 implicate that Dnmt3a is essential for terminal neural differentiation timing and cell proliferation of mNSCs.

MATERIAL 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, penicillin/streptomycin, L-glutamine, 0.1mM beta-mercaptoethanol and non-essential 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 in previous work (Chen et al. 2003). The cells were then plated at low density and grown in culture media containing blastocytidine for ten days. Single-cell colonies were picked and expanded under continuing blastocytidine 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 get rid of MEF feeder cells. When the plate was confluent, mESCs colonies were completely trypsinized to single cells followed by washing with DMEM: F12 medium three times to wash off all serum, and then passaged to a new 0.1% gelatin-coating plate. These cells were maintained for 7 days in serum-free N2B27 medium supplemented with EGF (10 ng/ml) and bFGF (10 ng/ml). Neurosphere formation was carried out when the plate was confluent and colonies began showing partial differentiation morphology. Neurospheres were maintained in an ultra-low attachment plate with suspension culture in N2B27 medium supplemented with bFGF and EGF for a week. At the fourth day after neurospheres formation, cells were transferred to 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 adding trypsin inhibitor.

mNSCs terminal differentiation

For neuronal differentiation, mNSCs were plated on PO/FN coating plate in DMEM: F12 medium with 2% B27 supplement and 1% penicillin/streptomycin. 20ng/ml NT3 and 10ng/ml BDNF 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, 20ng/ml BMP4 and 50ng/ml LIF could be added with 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), polyclonal rabbit anti-Nestin (1:200, Abcam), monoclonal mouse anti-Pax6 (1: 50, DSHB), monoclonal mouse anti-Tuj1 (1:1000, Abcam), polyclonal rabbit anti-Mbp (1:500, Millipore), monoclonal mouse anti-Gfap (1:50, Sigma), 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 6-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 and flow cytometric analysis. Bromodeoxyuridine (BrdU) incorporation assay and Ki67 staining were performed to measure DNA replication.

Microarray Hybridization

Gene expression microarrays were done with Agilent Whole Genome microarrays (G4122A) using the suggested protocol. Briefly, RNA was isolated using Trizol (Invitrogen). We converted the RNA into cDNA and then the cDNA into cRNA using the

Agilent Low RNA Input Linear Amplification Kit (Agilent). We used Nanodrop to quantify the labeled cRNA and used 0.75µg of each sample for hybridization. Probes were fragmented in a mix of labeled probes, 10× blocking reagent, and 25× Fragmentation buffer. Reaction was stopped with the addition of 2× Hybridization buffer. We used Agilent Whole Genome microarrays for expression studies. Slides were hybridized at 65° for 17 hours at 4 RPMs 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

Data has been submitted to the Gene Expression Omnibus (GEO) database and will be made publically available upon publication.

RESULTS

Derivation of *Dnmt3a*^{-/-} NSCs from *Dnmt3a*^{-/-} mESCs

In order to better understand the role of *Dnmt3a* in neural differentiation, both *Dnmt3a*^{-/-} and WT mESCs were converted into NSCs then induced to terminally differentiated neural cell types (Fig. 1A–E). We found no visible morphological differences between *Dnmt3a*^{-/-} and WT NSCs, though immunostaining confirmed 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% 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 trypsinizational passages, early (P3), middle (P6), and late (P9) passage of 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 was not affected. In the early passage (P3), both *Dnmt3a*^{-/-} and WT cells revealed small number of differentiated glial cells. By P6 stage, precocious GFAP positive astrocytes could only be seen in *Dnmt3a*^{-/-} mNSCs. By contrast, MBP positive oligodendrocytes did not appear in both groups. In the late passage (P9), more than 50% *Dnmt3a*^{-/-} mNSCs differentiated into astrocytes as well as a small population (0.79% ± 0.02%) of oligodendrocytes (Fig. 2). Furthermore, Gfap+ cells in mutant group showed more mature morphology (Fig. 2H). In contrast, very few Gfap+ astrocytes and no oligodendrocyte were found in WT group (Fig. 2M). For neuronal differentiation, the percentages and morphology of Tuj1 positives cells in *Dnmt3a*^{-/-} and WT cells were similar (Fig. 3). In order to more precisely determine the timing of neuronal maturation and gliogenesis, we carried out RT-PCR to detect expression of several neural markers, including NPC marker Nestin, neuronal marker Tuj1, astrocyte marker Gfap, and oligodendrocyte marker Mbp. In the absence of *Dnmt3a*, Gfap and Mbp expressions level were dramatic higher than WT in the late passage (Fig. 4). To further evaluate our differentiation system, we compared the morphology of NSC-derived astrocytes to primary mouse fetal glial cells. Based on 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

In order to investigate if 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 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 similar ability to differentiation into glial cells as Dnmt3a^{-/-} mNSCs at P6. However, TD3a NSCs showed reduced precocious glial cells differentiation compared to Dnmt3a^{-/-} mNSCs. In late passage of mNSCs (P9), astrocytes and oligodendrocytes still could be found in the TD3a NSCs differentiation process, but the percentage of Gfap positive cells in TD3a mNSCs (32.85% ± 0.52%) was significant lower than 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 lenti-virus infection (termed Dnmt3a-Res mNSCs). Four days after infection, Dnmt3a-Res NSCs (P10) were transferred to 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). Of the Dnmt3a positive cells, we found a reduced number of Gfap positive cells (54.0%) compared to non-infected Dnmt3a^{-/-} mNSCs (75.3%), suggesting Dnmt3a positive 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 re-introduction 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.

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 WT mNSCs. In order to examine whether Dnmt3a regulated cell proliferation, we generated cell growth curves of both Dnmt3a^{-/-} and WT mNSCs (Material & Methods) to compare their cell proliferation rates. After five 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 time (the time needed for total cell number to increase two folds) of Dnmt3a^{-/-} mNSCs and WT mNSCs were 25.88±0.73 hours and 32.88±2.02 hours, respectively. Furthermore, we employed flow cytometry 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 to 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 Dnmt3a deficiency stimulated proliferation in subpopulations of cells. Thus, it appears our Dnmt3a^{-/-} culture inherently selected for cells with greater proliferative capacity over prolonged passaging. Nevertheless, Dnmt3a^{-/-} NSCs consistently showed more cells undergoing mitosis compared to WT. By contrast, lack of Dnmt3a expression in embryonic stem cells only led to slightly higher cell proliferation rate (63.52%) compared to than WT (57.24%). To further complement our flow cytometry analysis, BrdU staining and Ki67 staining showed 1.83 folds more BrdU positive and 1.65 fold more Ki67 positive cells in Dnmt3a^{-/-} mNSCs compared to WT NSCs,

respectively (Fig. 6C,D). Taken together, Dnmt3a deficiency promotes increased cell cycle in subpopulations of differentiated mouse neural stem cells.

Analysis of gene expression changes in Dnmt3a^{-/-} mNSCs cells

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 44k probes. Using < 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 to WT cell via DAVID functional annotation analysis (Huang da et al. 2009). Consistent with Dnmt3a^{-/-} morphology, gene ontology (GO) analysis reveals upregulated genes are associated with neuronal development and neuronal morphogenesis, suggesting Dnmt3a has significant roles 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 downregulated genes are involved in p53 signaling (Fig. 7C). P53 is a known cell cycle arrest protein and 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 (Chen et al. 2003; Okano et al. 1999). During neurogenesis, Dnmt3a protein is strongly expressed in neural precursor cells, postmitotic CNS neurons, and oligodendrocytes (Feng et al. 2005). Previous studies in Dnmt3a^{-/-} mice brain showed impaired postnatal neurogenesis at two neurogenic zones, including subependymal/subventricular zones (SEZ/SVZ) in the hippocampal dentate gyrus. Further, Dnmt3a mutant mice had fewer Tuj1 positive neurons and more glial cells compared to WT mice (Wu et al. 2010). These evidences indicate 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*. Our results found that Dnmt3a-deficient embryonic stem cells derived mNSCs showed a substantially greater number of both astrocytes and oligodendrocytes compared to WT cells, suggesting 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 show 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, though it is still unknown whether these Dnmt3a 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 the effects of our transient rescue in the NSC stage was less pronounced compared to the stable rescue in the ESC stage. One possibility is that Dnmt3a virus is too toxic for non-infected Dnmt3a^{-/-} mNSCs. Another possibility is that since we performed transient rescue in late passage NSCs, the hypomethylated DNA methylation patterns established in early passage NSCs may already have been permissive for glial differentiation.

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

(Fan et al. 2005), though 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^{-/-} mice OB size and the number of newborn neurons resembled 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) has been recently 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 de-regulation (Yan et al. 2011). In addition, other components of the DNA methylation pathway are also found in AML cancers. For example, somatic mutation of TET2, which convert 5-methylcytosine to 5-hydroxymethylcytosine, was also found in AML individuals (Figueroa et al. 2010). However, unlike the hematopoietic stem cell system, where 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 embryonic stem cells stage, perhaps due to compensation from the highly expressed Dnmt3b. Together, these data suggests Dnmt3a behave differently and have distinct roles in different cell lineages.

Mutations in Dnmt3a have been identified in other cancer types. Most relevant, studies in glioblastoma cell lines showed 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 implicate 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. Science. 2003). Thus, p53 pathway may be downregulated as a consequence of hypomethylation in Dnmt3a-deficient cells. Moreover, the effect of cell proliferation might be achieved by cooperation of multiple factors, including abnormal genes mutations (*NPM1*, *FLT3*, and *UHRF1/2*, etc.), epigenetic modifications (Dnmt1/3b, histone methylation/deacetylation and microRNA regulation. etc), and cytokines induction during differentiation. However, how Dnmt3a regulates cell proliferation and apoptosis still need to be addressed in future work.

In many neural trauma and neural degenerative diseases, neural cell transplantation is becoming an increasingly attractive alternative therapy for patients' treatment. However, one major hurdle to overcome is our inability to control cellular properties of cells once transplanted into 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. DNA methylation is considered to play an important role in graft survival process. For example, recent histone deacetylase inhibitor drug therapy introduced in spinal injury mouse model showed enhanced improvement limb function (Abematsu et al. 2010). These findings are encouraging and implicate the use of other epigenetic drugs for enhanced transplantation therapy. Our studies will pave the way for clinical application of cell transplantation, like spinal cord injury, stroke, and other CNS trauma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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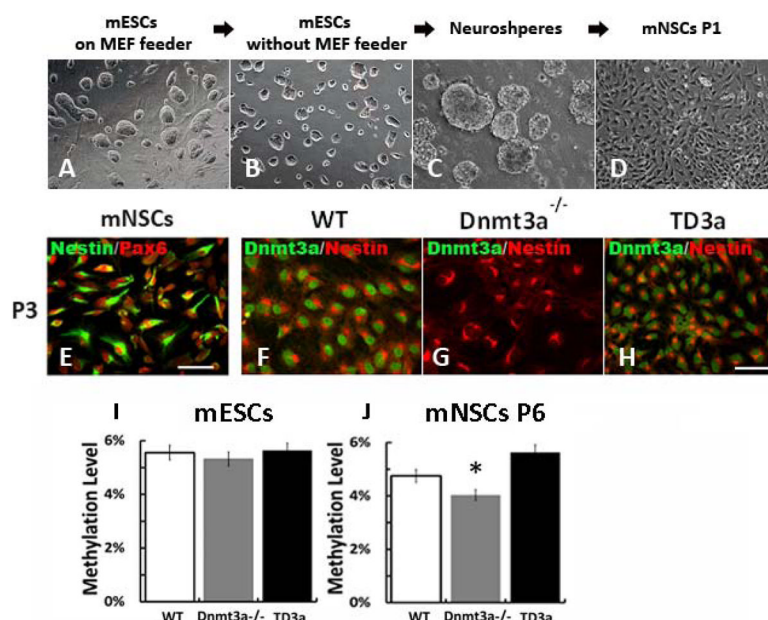


Figure 1. Identification of mNSCs derived from WT, Dnmt3a^{-/-}, and TD3a mouse embryonic stem cells lines. **A–D:** Experimental scheme describing the process of deriving mNSCs from mESCs. **E:** mNSCs derived from mESCs with neural stem cells specific marker Nestin (red) and Pax6 (green). **F–H:** Immunostaining confirms Dnmt3a expression deficiency in Dnmt3a^{-/-} cell 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 the means \pm standard deviations from triplicate analyses. * indicate significance level of $P < 0.05$ by Student-t test. Scale bars = 40 μ m in A–D; 20 μ m in E–H.

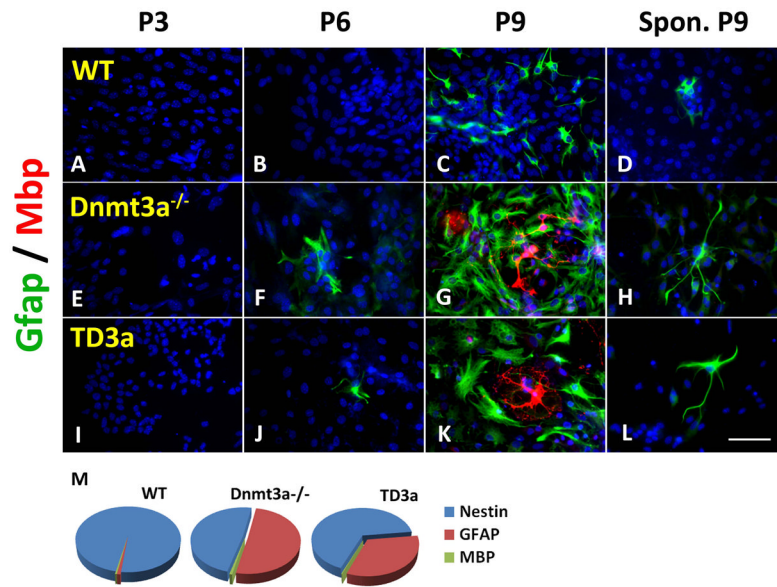


Figure 2. Glial differentiation of mNSCs derived from WT (A–D), *Dnmt3a*^{-/-} (E–H) and TD3a (I–L) mESCs. Immunostaining for Gfap (green) and Mbp (red) shows glial cells differentiation of three different passage stages in each mNSCs line: P3 (A, E and I); P6 (B, F and J); P9 (C, G and K); and spontaneously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

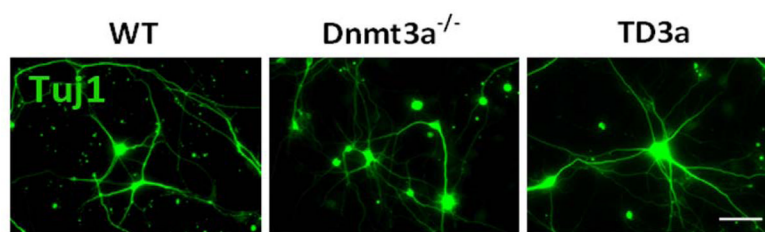


Figure 3. Neuronal differentiation of mNSCs (P3) derived from WT, Dnmt3a^{-/-} and TD3a mESCs. TuJ1 immunostaining shows neuron morphology cells. Scale bars = 10 μ m.

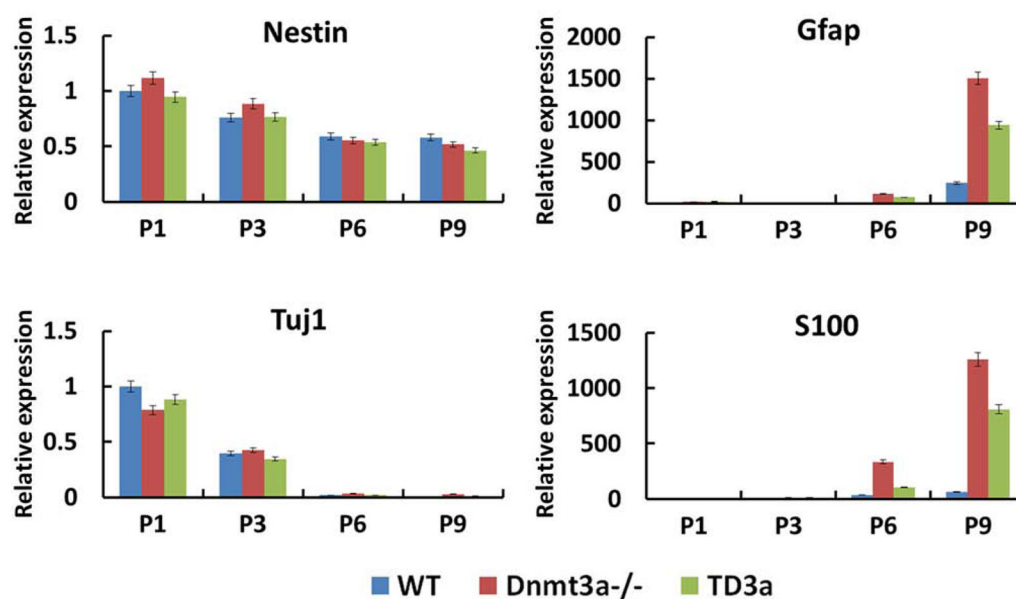


Figure 4. RT-PCR analysis demonstrates Nestin, Tuj1, Gfap, and S100 expression level changes during differentiation time course. Y-axis is normalized to P1 WT gene expression.

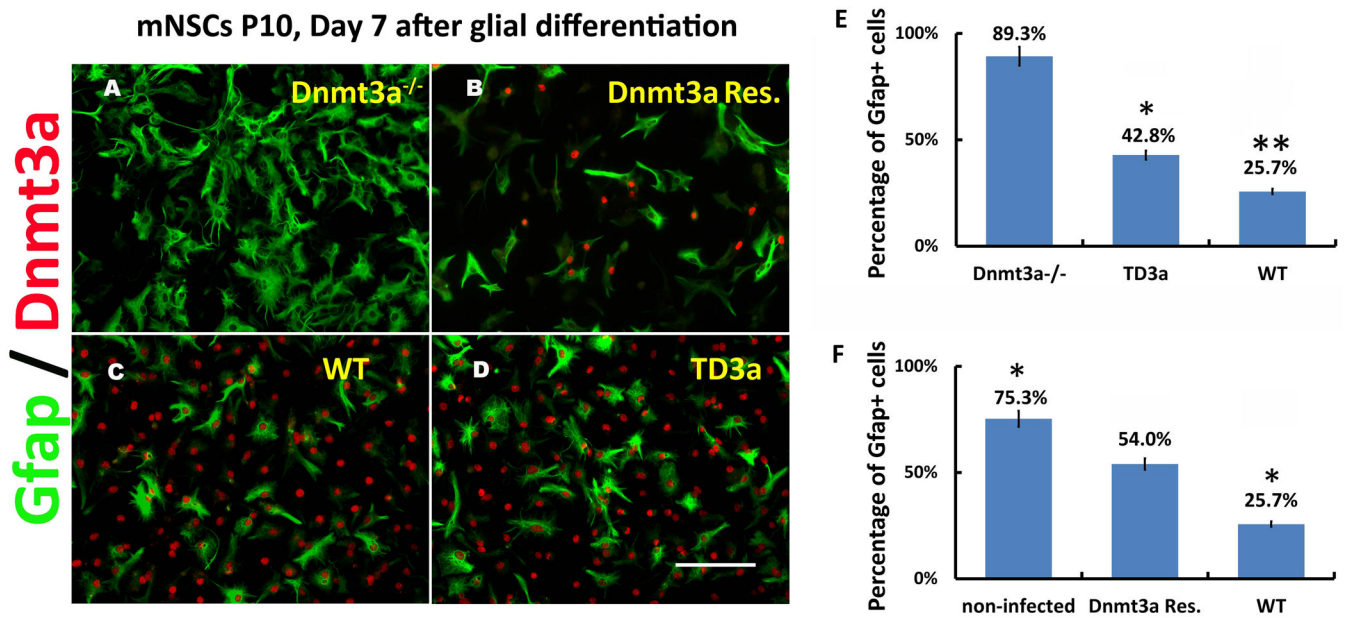


Figure 5. Glial differentiation of Dnmt3a^{-/-} (A), Dnmt3a-Res (B), WT (C), and TD3a (D) in late stage mNSCs. Immunostaining for Gfap (green) and Dnmt3a (red) shows glial differentiation efficiency is decreased due to both stable and transient Dnmt3a rescue. **E–F:** By counting Gfap positive cells at P10, Dnmt3a transient rescue shows lower gliogenic ability compared to non-infected Dnmt3a^{-/-} mNSCs. * $p < 0.01$ and ** $p < 0.05$ (Student-t test). Scale bar = 20 μ m.

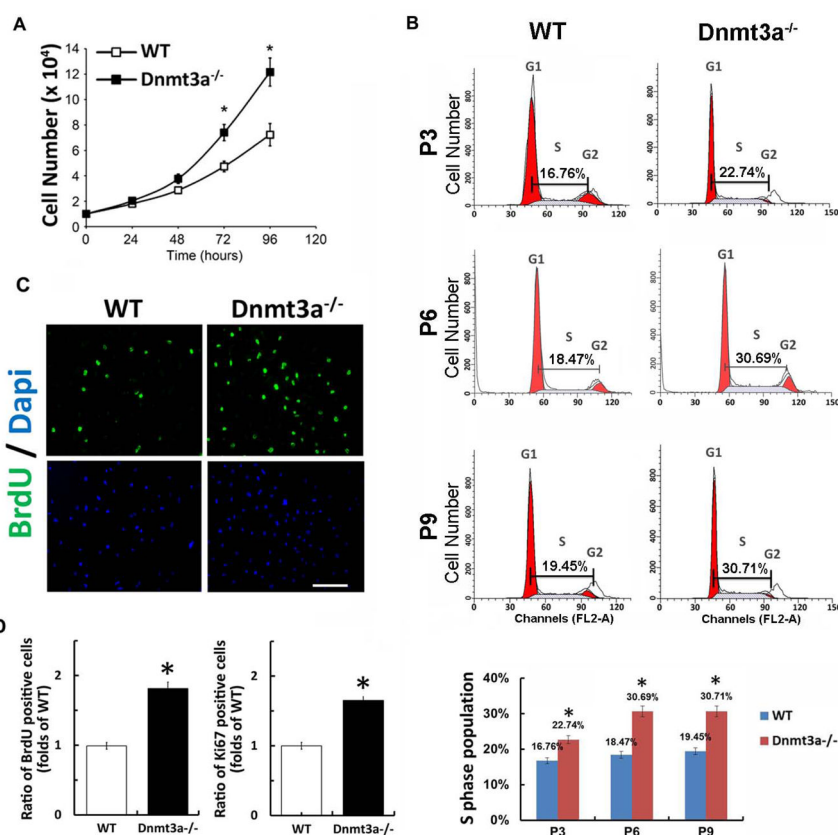


Figure 6. Cell Proliferation analysis of WT and Dnmt3a^{-/-} mNSCs. **A:** Cell growth curve describes cell doubling speed. **B:** Flow cytometric analysis shows cell cycle distribution by propidium Iodide (PI) staining. **C:** Colocalization of Bromodeoxyuridine (BrdU) with Dapi. **D:** Amount of BrdU and Ki67 positive cells in Dnmt3a^{-/-} mNSCs folds of WT mNSCs. Scale bars = 20 μ m in C. Panels A,C, and D were assayed at P6.

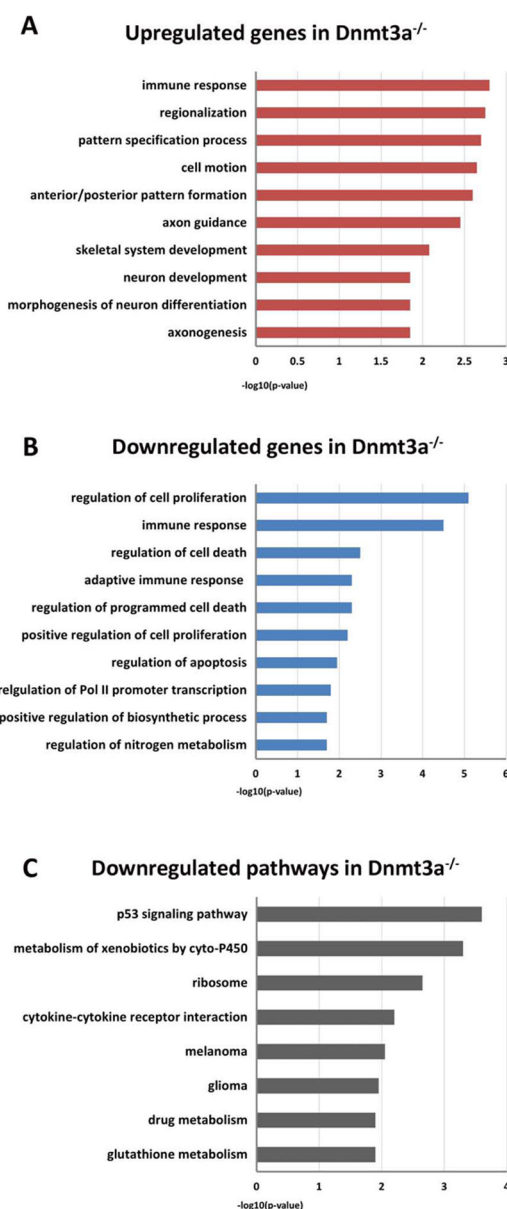


Figure 7.

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