

Folic Acid Acts Through DNA Methyltransferases to Induce the Differentiation of Neural Stem Cells into Neurons

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Abstract The present study investigated the roles of folic acid and DNA methyltransferases (DNMTs) in the differentiation of neural stem cells (NSCs). Neonatal rat NSCs were grown in suspended neurosphere cultures and identified by their expression of SOX2 protein and capacity for self-renewal. Then NSCs were assigned to five treatment groups for cell differentiation: control (folic acid-free differentiation medium), low folic acid (8 µg/mL), high folic acid (32 µg/mL), low folic acid and DNMT inhibitor zebularine (8 µg/mL folic acid and 150 nmol/mL zebularine), and high folic acid and zebularine (32 µg/mL folic acid and 150 nmol/mL zebularine). After 6 days of cell differentiation, immunocytochemistry and western blot analyses were performed to identify neurons by β -tubulin III protein expression and astrocytes by GFAP expression. We observed that folic acid increased DNMT activity which may be regulated by the cellular S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH), and the abundance of neurons but decreased the number of astrocytes. Zebularine blocked these effects of folic acid. In conclusion, folic acid acts through elevation of DNMT activity to increase neuronal differentiation and decrease astrocytic differentiation in NSCs.

Keywords Folic acid · Neural stem cells · DNA methyltransferases · Differentiation

Introduction

Folic acid supplementation has proven to be extremely effective in preventing neural tube defects and other congenital abnormalities in humans [1, 2]. There is also evidence of associations between either dietary or blood folic acid status and some adult neurodegenerative diseases [3–5]. Neural stem cells (NSCs) are undifferentiated brain cells that have the capacity to self-renew and to differentiate into neurons and astrocytes [6]. The idea that NSCs may be used therapeutically for treatment of neurodegenerative disorders is very appealing and has led to numerous studies of the mechanisms underlying NSC self-renewal and differentiation [7, 8]. In particular, folic acid supplementation has been shown to stimulate cell proliferation and decrease cell death in neurosphere cultures of rat NSCs [9, 10]. Additionally, folic acid dose-dependently induces NSC proliferation and stimulates hippocampal neurogenesis in a rat model of cerebral ischemia [11]. It remains to be determined if folic acid induces differentiation of NSCs into neurons.

Recent studies showed that epigenetic regulation may interact with transcription factors and environmental cues to modulate the differentiation of NSCs [12, 13]. DNA methylation, which is an example of epigenetic regulation, undergoes marked changes in the developing brain that suggest a role for DNMTs in brain cell differentiation [14]. Recent experiments have discovered that folic acid supplementation stimulates the regeneration of injured spinal neurons by a mechanism that depends on DNA methylation pathways [15]. Since folic acid is a methyl donor that

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stimulates DNA methylation and folate deficiency causes genomic hypomethylation [16, 17], we hypothesized that this nutrient affects NSC differentiation by altering DNMT activity.

An inhibitor of DNMTs was needed to test this hypothesis. Zebularine is a cytidine analog that binds DNMTs covalently and inhibits the enzymatic activity. Zebularine has advantages over previously studied DNMT inhibitors because it is stable in aqueous solution and less toxic [18]. Therefore, the present study's hypothesis was tested by incubating NSCs with folic acid and zebularine.

Materials and Methods

Reagents

Folic acid-free Dulbecco's modified Eagle's medium (DMEM), N2, B27 supplement, fetal bovine serum (FBS), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) were obtained from Gibco (Carlsbad, CA, USA). Folic acid and DAPI were purchased from Sigma (St Louis, CA, USA). Zebularine was purchased from Merck Chemicals (Darmstadt, Germany). DNA Methyltransferase Activity/Inhibition Assay kit was purchased from Active Motif (Carlsbad, CA, USA). Monoclonal anti- β tubulin III antibody, Polyclonal anti-GFAP antibody, and SOX2 were obtained from Abcam, Inc (Cambridge, MA, USA). Monoclonal anti-BrdU antibody and β -actin were obtained from Cell Signaling Technology, Inc (Boston, MA, USA). All fluorescent secondary antibodies were obtained from Zhongshan Goldbridge Biotechnology (Beijing, China). BCA protein assay kit was obtained from Boster Biological Technology (Wuhan, China).

Cell Culture

Brain tissue was isolated from neonatal rats and washed 3 times with DMEM. The tissue was cut into small pieces and then dissociated by incubation with 0.25 % pepsin and 0.02 % EDTA. This step was followed by agitation, centrifugation, and resuspension of the cells in DMEM containing 2 % B27 supplement, 20 ng/mL EGF, 20 ng/mL bFGF, 2 μ mol/mL L-glutamine, 100 U/mL penicillin and streptomycin. The resulting cell suspension was cultured at 37 °C in a humidified atmosphere containing 95 % air/5 % CO₂. The culture medium was changed every 2 d, while the suspended cells formed neurospheres.

Some neurospheres were incubated with the proliferation marker BrdU (10 μ g/mL) for 24 h before harvest on day 6. The remaining neurospheres were mechanically dissociated for subculture on day 6. The dissociated cells (NSCs) were plated at a density of 5×10^4 cell/mL and

incubated in differentiation medium containing 5 % FBS and 2 % N₂. These NSCs were assigned to 5 treatment groups: control (folic acid-free), low folic acid (8 μ g/mL folic acid), high folic acid (32 μ g/mL folic acid), low folic acid and DNMT inhibitor zebularine (8 μ g/mL folic acid and 150 nmol/mL zebularine), and high folic acid and zebularine (32 μ g/mL folic acid and 150 nmol/mL zebularine). The duration of the folic acid treatment was 6 d. In the cell cultures that received zebularine, the NSCs were exposed to zebularine for 48 h and then were incubated without the drug for 6 d, before being harvested for immunocytochemistry and western blot analysis.

Immunocytochemistry Analysis

The cells were fixed in 4 % paraformaldehyde for 20 min and incubated with blocking buffer for another 20 min. Subsequently, the cells were permeabilized with 0.1 % Triton X-100 in PBS for 15 min and then blocked for 1 h at room temperature with 1 % FBS. To detect NSCs and proliferating cells, respectively, the cells were incubated overnight at 4 °C with a mixture of 1:200 rabbit anti-rat monoclonal anti-SOX2 and mouse anti-rat monoclonal BrdU antibodies. The reason we identified SOX2 was because it is a persistent marker for multipotential NSCs [19, 20]. To detect neurons and astrocytes, cells were incubated overnight at 4 °C with a mixture of 1:100 rabbit anti-rat monoclonal anti- β -tubulin III and mouse anti-rat polyclonal GFAP antibodies. Subsequently, cells were incubated for 2 h at room temperature with TRITC (tetramethylrhodamine isothiocyanate)-conjugated goat anti-mouse IgG and FITC (fluorescein isothiocyanate)-conjugated goat anti-rabbit IgG. Cell nuclei were counterstained by incubating cells with 4'-6-diamidino-2-phenylindole (DAPI) for 5 min and then washing extensively with distilled water. Images were obtained using an IX71SIF-2 fluorescence microscope (Olympus, Tokyo) and displayed on a monitor, and at least six fields of view were selected randomly for cell counting at $\times 200$ magnification. Results were expressed as the percentages of DAPI-positive cells that were also β -tubulin III-positive or GFAP-positive cells.

Western Blot Analysis

Cells were washed with ice-cold PBS and lysates were centrifuged at 12,000 rpm for 10 min at 4 °C, and then the pellets were discarded. Protein concentrations in the supernatants were determined by BCA protein assay kit, using bovine serum albumin as a standard. Equal amounts of protein were loaded in each well for sodium dodecyl sulfate 12 % polyacrylamide gel electrophoresis and then the separated proteins were transferred to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline

containing Blotto solution for 2 h at room temperature. The membranes were then incubated with the primary antibodies (rabbit anti- β -tubulin III antibodies, 1:300; mouse anti-GFAP antibodies, 1:1,000) and β -actin (1:1,000) overnight at 4 °C in Tris-buffered saline containing 0.1 % Tween 20 (TBST). Membranes were rinsed three times with TBST before being incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000 in TBST) for 2 h and detected by chemiluminescence. Quantitation of proteins was performed by densitometry with NIH Image software (version 1.61).

DNMTs Activity

DNA methylation is accomplished by three enzymes: DNMT1, DNMT3a, and DNMT3b. DNMT1 may be important, because experiments with conditional mutants that lack DNMT1 showed that DNMT1 deficiency in NSCs resulted in DNA hypomethylation in daughter cells and the latter died quickly [21]. DNMT 3a and 3b are de novo methyltransferases, capable of methylating previously unmethylated CpG sequences, and have been detected in NSCs and postmitotic young neurons in vivo [22]. DNMTs activity was measured using Active Motif's DNA Methyltransferase Activity/Inhibition Assay kit, according to the manufacturer's instructions, and expressed as OD/(h.mg) nuclear protein.

HPLC Analysis

Cell cultures were rinsed twice with PBS and frozen at -80 °C. After thawing, cells were scraped into 1 mL of deionised water and sonified for 15 s in ice. The macromolecules were precipitated using 1.5 mmol/mL PCA solution at 4 °C for 1 h adjusting the pH to 4–5 with KOH and then centrifuged for 15 min at $9,000\times g$. The supernatant was freeze-dried and then subjected to HPLC analysis using a Waters HPLC system (Framingham, MA, USA) equipped with a 600E pump and a 2487 UV detector set to 254 nm. Remote control of the HPLC system, data acquisition, and calculation of peak areas were performed via computer-based data system (Waters, USA). The samples were injected through an injection valve with a 20 μ L sample loop. The mobile phase was pumped at a low-rate of 1.00 mL/min. The samples were hydrolyzed with perchloric acid and the hydrolysate was adjusted to centrifuged and filtrated with a 0.45 μ m membrane, the supernatants were separated on the reverse-phase column (Agela, Venusil MP-C18 ODS, 4.6×250 mm, 5 μ m).

The mobile phases were mixture of 50 μ mol/mL NaH_2PO_4 (pH 4.38), $\text{C}_7\text{H}_{15}\text{NaO}_3\text{S}$, and methanol. Standards of SAM and SAH, dissolved in 10^{-4} mmol/mL HCl, were run before the experimental samples.

Statistical Analysis

The data were expressed as mean \pm SD values and analyzed by statistical software SPSS13.0. One-way analysis of variance and the Student-Newman-Keuls test for multiple comparisons were used to determine significant differences among the experimental groups. The criterion for statistical significance was $P < 0.05$.

Results

Identification of NSCs

Undifferentiated NSCs formed round neurospheres that remained suspended in the culture medium (Fig. 1a). Almost all cells in the neurospheres expressed SOX2 protein, which was detected as intense green staining of nuclei in the immunofluorescence assay (Fig. 1b). Many of the cells in neurospheres also incorporated BrdU, as indicated by the red staining of nuclei in Fig. 1c. Double immunofluorescence staining of cells showed colocalization of SOX2 and BrdU (Fig. 1d). Taken together, these results demonstrated that NSCs capable of self-renewal were isolated from neonatal rat brains and grown successfully in suspended neurospheres.

Folic Acid Induces Differentiation of NSCs into Neurons

Next, we determined the effect of folic acid treatment for 6 d on the differentiation of NSCs that had been mechanically dissociated from neurospheres and subcultured. Neurons were identified by their expression of β -tubulin III protein and astrocytes by their expression of GFAP. Immunocytochemistry analysis showed that β -tubulin III and GFAP never co-localized (Fig. 2a–j), confirming that these detection of these proteins identified distinct cell phenotypes. Further, compared to control, folic acid increased the percentage of cells that were neurons ($P < 0.05$; Fig. 2k) and decreased the percentage that were astrocytes ($P < 0.05$; Fig. 2l). The effects of folic acid at 8 μ g/mL were the same as those at 32 μ g/mL (Fig. 2k, l). Immunocytochemistry analysis also showed that the DNMT inhibitor zebularine blocked the effects of both doses of folic acid (8 and 32 μ g/mL) on NSC differentiation into neurons and astrocytes ($P < 0.05$; Fig. 2k, l).

The results of immunocytochemistry were confirmed by western blot analysis. Representative western blots of β -tubulin III and GFAP are shown in Fig. 3. Densitometry data from western blots showed that, compared to control, folic acid increased the expression of the neuron-specific protein β -tubulin III and decreased the expression of the

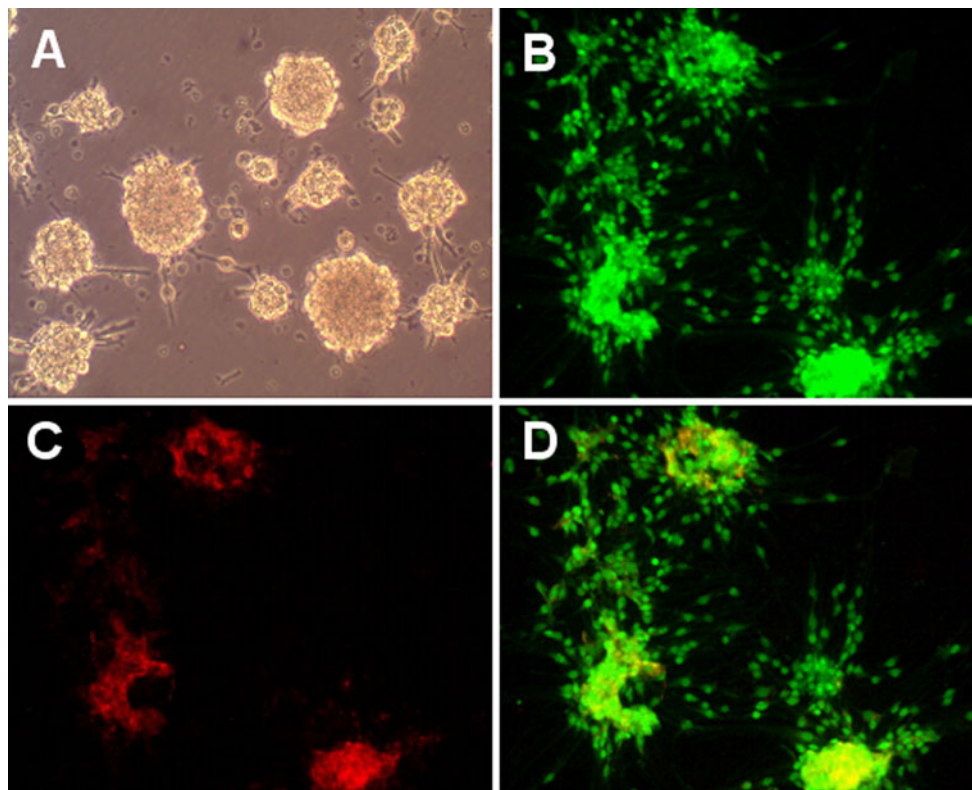


Fig. 1 Neurospheres contain proliferating neural stem cells (NSCs). **a** Photomicrographs of neurospheres ($\times 200$). **b** Immunofluorescence staining for SOX2 (*green*, $\times 200$). **c** Immunofluorescence staining for

BrdU (*red*, $\times 200$). **d** Merged view showing colocalization of SOX2 and BrdU (*yellow*, $\times 200$)

astrocyte-specific protein GFAP ($P < 0.05$; Fig. 3a, b). The effects of the 2 doses of folic acid (8 and 32 $\mu\text{g}/\text{mL}$) did not differ (Fig. 3a, b). Zebularine blocked the effects of both doses of folic acid on the expression levels of β -tubulin III and GFAP ($P < 0.05$; Fig. 3a, b).

Taken together, these data show that folic acid acts on NSCs to stimulate differentiation into neurons and inhibit differentiation into astrocytes. These data also indicate an important role for zebularine-sensitive DNMT activity in the responses of NSC to folic acid.

Effect of Folic Acid on DNMT Activity

Folic acid supplementation increased DNMT activity compared to control ($P < 0.05$; Fig. 4). The DNMT activity after incubation with folic acid at 8 $\mu\text{g}/\text{mL}$ was the same as that at 32 $\mu\text{g}/\text{mL}$. Zebularine decreased DNMT activity to lower levels in the cells that received the low dose of folic acid (8 $\mu\text{g}/\text{mL}$) than in those that received the high dose (32 $\mu\text{g}/\text{mL}$) ($P < 0.05$; Fig. 4). These results showed that the folic acid stimulation of NSC differentiation into neurons was accompanied by increased DNMT activity and that zebularine's effect on neuron production was associated with inhibition of DNMTs.

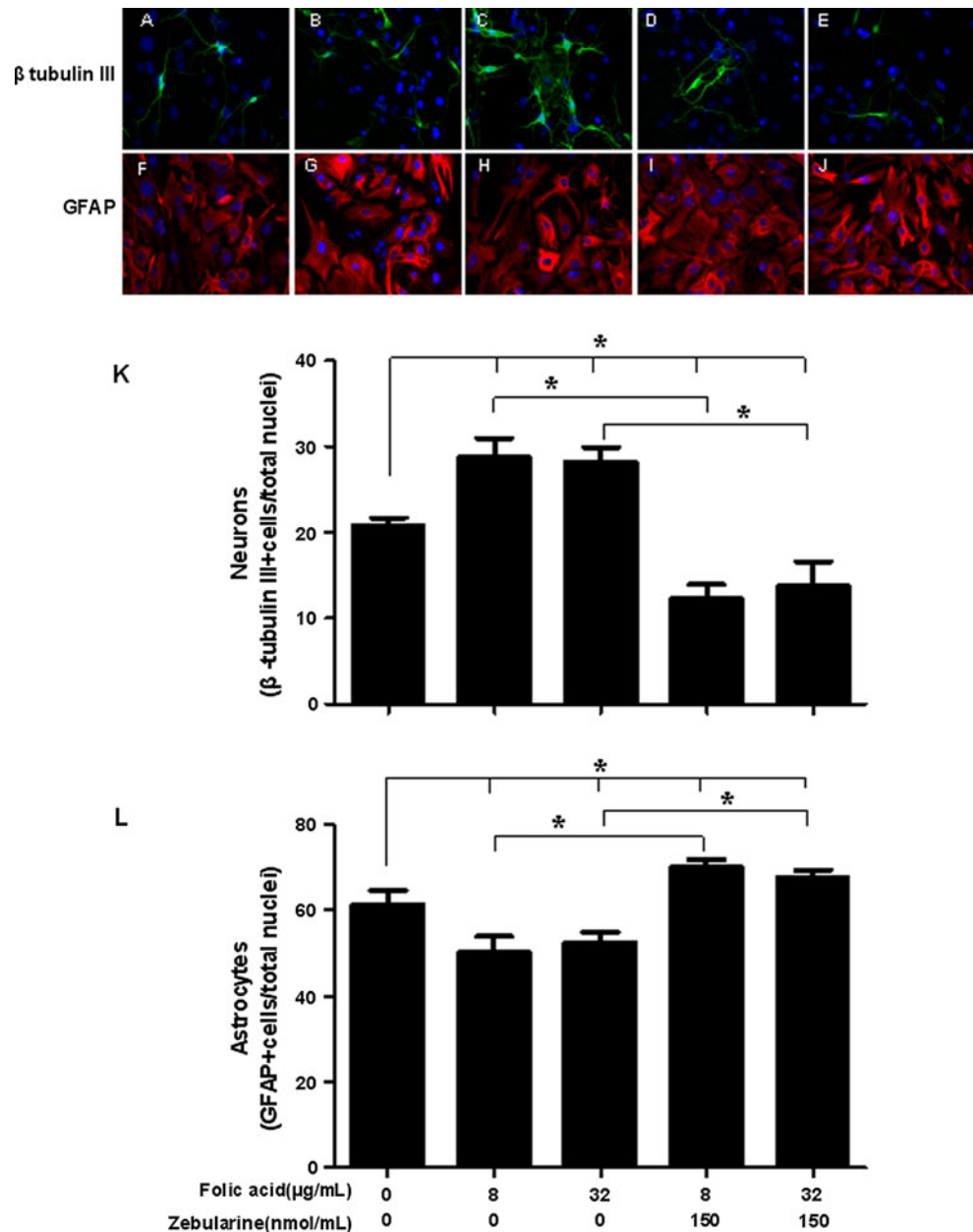
Intracellular Level of SAM, SAH, and the Ratio of SAM/SAH

To examine the physiological consequences of increased methyl donor availability after folic acid treatment, and accordingly increase of DNMT activity, the cellular concentrations of SAM and SAH were measured by HPLC method. We verified that the SAM levels were significantly increased in two treatment groups compared with the control ($P < 0.05$; Fig. 5a); in contrast, the SAH levels were significantly decreased ($P < 0.05$; Fig. 5b). Furthermore, the SAM-to-SAH ratios were significantly decreased in two treatment groups ($P < 0.05$; Fig. 5c).

Discussion

One of the main findings of the present study is that folic acid supplementation induces neuronal differentiation of NSCs at the expense of astrocytic differentiation. Immunocytochemistry analysis of BrdU labeling and SOX2 expression showed that the neurospheres contained proliferating NSCs. When the neurospheres were dissociated and the NSCs were incubated in differentiation medium, folic acid increased the

Fig. 2 Immunocytochemistry analysis showing that folic acid induces differentiation of NSCs into neurons instead of astrocytes. Differentiated cells were incubated with specific primary antibodies and fluorescent secondary antibodies to detect neuron marker β -tubulin III and astrocyte marker GFAP. **a–j** Fluorescence photomicrographs of cells expressing β -tubulin III (green, $\times 200$) and GFAP (red, $\times 200$) are shown. **k** The abundance of neurons (β -tubulin III-positive cells) expressed as a percentage of the total number of cells. The mean \pm SD values from 3 experiments are shown. **l** The abundance of astrocytes (GFAP-positive cells) expressed as a percentage of the total number of cells. The mean \pm SD values from 3 experiments are shown. * $P < 0.05$



abundance of β -tubulin III-positive neurons and decreased the number of GFAP-positive astrocytes. These immunocytochemistry results were confirmed by western blot analysis. As we have known, astrocytes are the most abundant cells of the human brain. They perform many functions, including biochemical support of endothelial cells that form the blood–brain barrier, provision of nutrients to the nervous tissue, maintenance of extracellular ion balance, and a role in the repair and scarring process of the brain and spinal cord following traumatic injuries [23]. However, neurons are still considered as the most important cells responsible for brain function. A new study showed that newly generated or newborn neurons in the adult hippocampus are critical for memory retrieval [24]. Finding ways to make new brain cells

are important steps in the search for treatments for brain-wasting diseases such as Alzheimer’s and Parkinson’s. In our report, folic acid facilitated the transformation of precursor cells into more neurons. Stimulatory effects of folic acid on NSC proliferation and neuronal differentiation (present experiments) may explain previous observations that folic acid enhances hippocampal neurogenesis and lessens the impairment of cognitive function that occurs after experimental stroke [11]. Stimulation of NSC proliferation and neuronal differentiation may also explain, at least in part, folic acid’s protective actions against neural tube defects and adult spinal nerve degeneration. Further, the effects of the nutrient on NSCs may account for the observed associations of folic acid status and adult

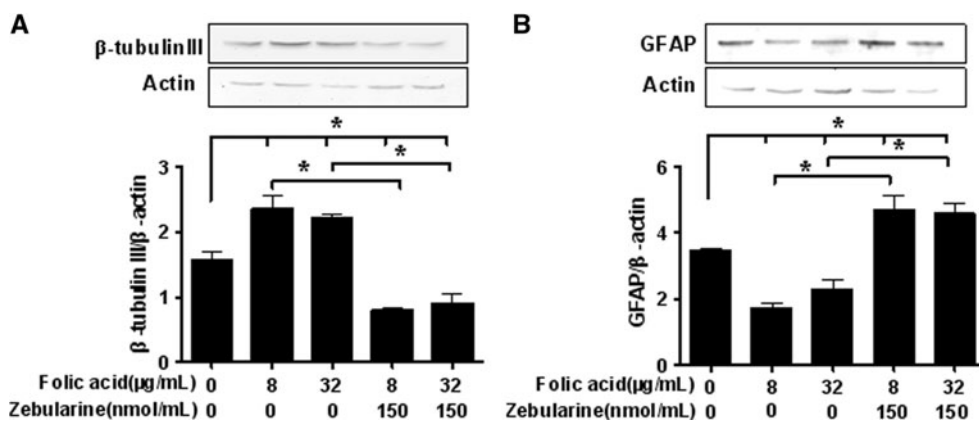


Fig. 3 Western blot analysis showing that folic acid induces differentiation of NSCs into neurons. β-tubulin III and GFAP were detected by western blot analysis and normalized to β-actin expression. **a** Representative western blots and bar graph of β-tubulin III

expression level (β-tubulin III: β-actin ratios) in differentiated NSCs. **b** Representative western blots and bar graphs of GFAP expression level (GFAP : β-actin ratios) in differentiated NSCs. Shown are mean ± SD values of 6 experiments. *P < 0.05

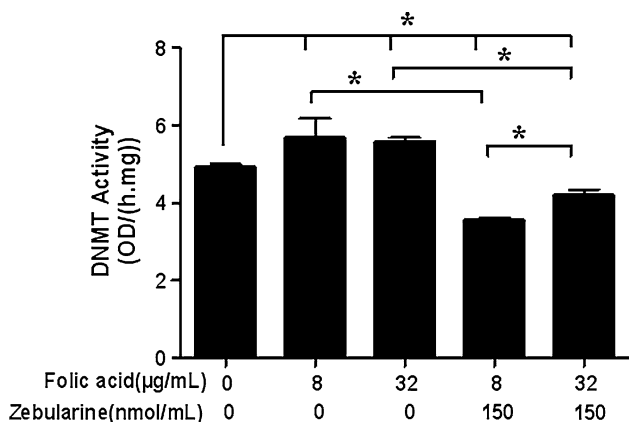


Fig. 4 Folic acid stimulates DNMT activity. Shown are the mean ± SD values from 3 experiments. *P < 0.05

zebularine treatment attenuated the effects of folic acid on NSC differentiation into neurons and astrocytes. These results are consistent with previous reports that DNMT activity is involved in initial specification of the neuronal cell phenotype as well as subsequent neuronal maturation and function [27, 28]. In contrast to the neuronal differentiation arising from increased DNMT activity in NSCs, hypermethylation of astrocytic gene promoters such as GFAP prevent NSCs from differentiating into astrocytes [29]. Thus folic acid-dependent DNMT activity may be a critical factor controlling NSC differentiation.

neurodegenerative disease [25, 26]. Thus the present work provides insight into potential therapies for brain and spinal cord diseases.

The DNMTs are a family of enzymes that catalyze the transfer of methyl groups from SAM to cytosine residue in DNA, thus producing 5-methylcytosine and SAH. SAH, as a product, can be recycled back to SAM as part of the methyl cycle. It has been reported that DNMT activity can be affected by SAM/SAH levels in the cell as SAH is a reversible DNMT inhibitor [30, 31]. A decrease in SAM or the accumulation of SAH could reduce DNMT activity [32]. In our study, to understand the mechanism underlying the change in DNMT activity caused by folic acid treatment, we measured the cellular concentration of SAM and SAH by HPLC method. The results showed that SAM and the ratio SAM/SAH were elevated in folic acid treated

Another main finding of the present study is that DNMTs mediates the stimulation by folic acid of neuronal differentiation of NSCs. The evidence is that, first, folic acid supplementation of NSC cultures stimulated DNMT activity. Second, partial inhibition of DNMT activity by

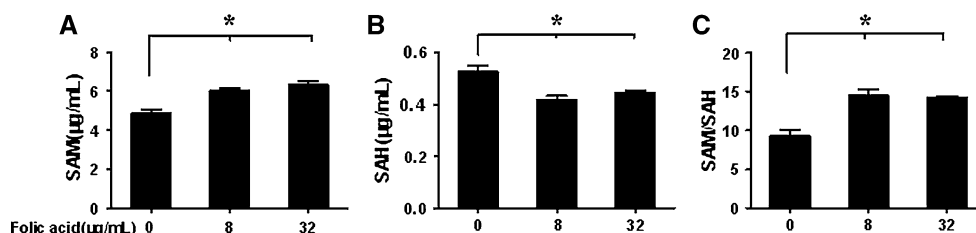


Fig. 5 The intracellular SAM and SAH concentrations were measured by HPLC. SAM levels, SAH levels, and SAM/SAH ratio were determined as described in Materials and Methods. Data represents mean ± SD (n = 3). *P < 0.05

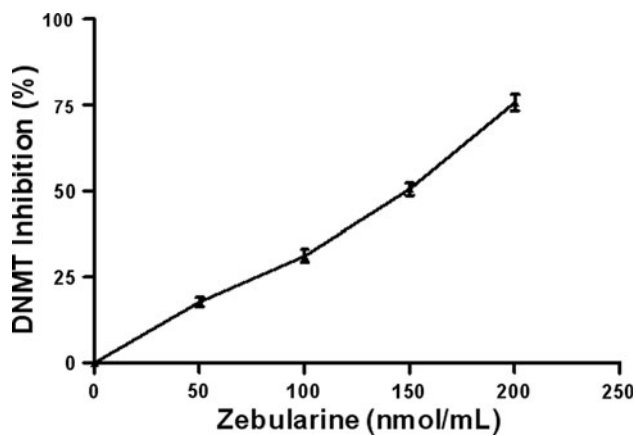


Fig. 6 Zebularine inhibited DNMT activity in a dose-dependent manner. Zebularine treatment was associated with a dose-dependent depletion of DNMT in all four doses. The NSCs were treated with various concentrations of Zebularine and DNMT inhibition was determined by Active Motif's DNA Methyltransferase Activity/Inhibition Assay kit. A dependent decrease in DNMT was seen with the application of Zebularine (50, 100, 150, and 200 nmol/mL; $P < 0.05$). A Zebularine concentration of 150 nmol/mL accounted for the inhibition of DNMT inhibition of 50 %

groups. Since folic acid, in the form of tetrahydrofolate coenzymes, is an essential carrier of methyl groups within cells, it is necessary in the conversion of homocysteine to methionine in the synthesis of SAM. Thus, there is no doubt that folic acid may alter SAM and SAH concentration by the methyl cycle. So we speculated that folic acid may indirectly increase DNMT activity by decreasing SAM and increasing SAH levels. Furthermore, DNA methylation is an important epigenetic modification that is catalyzed by specific DNMTs, including DNMT1, DNMT3a, and DNMT3b, and it has been proven to be an active component and an intrinsic program during differentiation of NSCs [33]. Intracellular SAM/SAH is considered as a more reliable biomarker for cellular methylation status [34]. Since folic acid can alter DNMT activity and SAM/SAH ratio, our results suggested that folic acid affects the differentiation of NSCs, at least in part, by interfacing with DNA methylation.

Folic acid concentrations used in this study were set on the analysis of many other *in vitro* experiments, where concentrations up to 400 $\mu\text{g/mL}$ [35–37]. In our previous study, NSCs were plated in complete growth media with no supplemental folic acid (0 $\mu\text{g/mL}$) or with addition of folic acid to final concentrations of 4, 8, 16, 32, 64 $\mu\text{g/mL}$. Cell proliferation was assessed 48 h later by MTT. The results showed that mild folic acid (8 $\mu\text{g/mL}$), which better promoted NSC survival (data not shown). In our present experiments, final concentration of 8 $\mu\text{g/mL}$ was also the optimal concentration for NSC differentiation into more neurons (Fig. 2). In addition, some *vivo* studies have shown to be administered continuously at a lower dose of

zebularine to maintain demethylation for a prolonged period [38, 39]. According to our present study, 0–200 nmol/mL zebularine treatment resulted in dose-dependent decreases of DNMT (Fig. 6; $P < 0.05$). A zebularine concentration of 150 nmol/mL which inhibited DNMT activity of 50 % was chosen to insure minimal cytotoxic *in vitro*, and showed a maximal effect on NSC differentiation into astrocytes (Fig. 21, 3b).

In conclusion, the present study showed that folic acid supplementation alters the DNMT activity and cell differentiation of NSCs. Folic acid acts through elevation of DNMT activity to increase neuronal differentiation and decrease astrocytic differentiation in NSCs.

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