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ORIGINAL CONTRIBUTION

Folic acid attenuates the effects of amyloid β oligomers on DNA methylation in neuronal cells

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Abstract

Purpose Alzheimer's disease (AD) is a highly prevalent type of dementia. The epigenetic mechanism of gene methylation provides a putative link between nutrition, one-carbon metabolism, and disease progression because folate deficiency may cause hypomethylation of promoter regions in AD-relevant genes. We hypothesized that folic acid supplementation may protect neuron cells from amyloid β (Aβ) oligomer-induced toxicity by modulating DNA methylation of *APP* and *PS1* in AD models.

Methods Primary hippocampal neuronal cells and hippocampal HT-22 cells were incubated for 24 h with a combination of folic acid and either Aβ oligomers or vehicle and were then incubated for 72 h with various concentrations of folic acid. AD transgenic mice were fed either folate-deficient or control diets and gavaged daily with various doses of folic acid (0 or 600 µg/kg). DNA methyltransferase (DNMT) activity, cell viability, methylation potential of cells, *APP* and *PS1* expression, and the methylation of the respective promoters were determined.

Results Aβ oligomers lowered DNMT activity, increased *PS1* and *APP* expression, and decreased cell viability. Folic acid dose-dependently stimulated methylation potential and DNMT activity, altered *PS1* and *APP* promoter methylation, decreased *PS1* and *APP* expression, and partially preserved cell viability. Folic acid increased *PS1* and *APP* promoter methylation in AD transgenic mice.

Conclusion These results suggest a mechanism by which folic acid may prevent Aβ oligomer-induced neuronal toxicity.

Keywords Aβ oligomer · Alzheimer's disease · *APP* · Folic acid · Methylation · *PS1*

Abbreviations

Aβ	Amyloid β-peptide
AD	Alzheimer's disease
APP	Amyloid precursor protein
CBS	Cystathionine-beta-synthase
DMEM	Dulbecco's Modified Eagle's Medium
DNMT	DNA methyltransferase
FBS	Fetal bovine serum
OD	Optical density
PBS	Phosphate-buffered saline
PS	Presenilin
SAH	*S*-adenosylhomocysteine
SAM	*S*-adenosylmethionine

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Introduction

Alzheimer's disease (AD) is a highly prevalent type of dementia [1, 2]. A hallmark of AD is the appearance of neuritic plaques containing neurotoxic fibrillar amyloid β (Aβ) in the brain, which is accompanied by loss of neurons [2–4]. Also associated with AD is the increased expression of presenilin 1 (PS1) and amyloid precursor protein (APP) [5, 6]. These proteins are relevant to plaque formation because PS1 (as a part of the

γ-secretase complex) catalyzes the production of Aβ from APP [5].

Environmental factors that induce methylation of gene promoters may regulate gene expression [7]. For instance, in neuroblastoma cells, increased methylation of the *PS1* promoter silences the gene and decreases both *PS1* expression and Aβ production [8, 9]. The epigenetic mechanism of gene methylation provides a putative link between nutrition, one-carbon metabolism, and disease progression because dietary deficiency in transmethylation micronutrients (i.e., folate and other B vitamins) may cause hypomethylation of promoter regions in AD-relevant genes. This effect is observed in AD models; for example, in transgenic TgCRND8 mice (carrying mutant *APP*) fed a diet deficient in folate, vitamin B12, and vitamin B6 [7, 10, 11]. Gene hypomethylation may be due, at least partially, to depletion of *S*-adenosylmethionine (SAM) and elevation of intracellular *S*-adenosylhomocysteine (SAH), resulting in an overall decrease in methylation potential (i.e., the SAM:SAH ratio). In particular, the SAH concentration may rise to levels that competitively inhibit the SAM-dependent methylation of genes by DNA methyltransferases (DNMT) [12].

The folate/B6/B12-dependent epigenetic mechanisms described above may be relevant to clinical AD [8]. Previous studies paid close attention to how a deficiency in folate and vitamin B12 would decrease the methylation of *PS1* and whether SAM supplementation could alter this phenomenon [10, 13]. Because folic acid supplementation is a potential therapeutic intervention, it is important to determine the effects of manipulating the levels of exogenous folic acid independently of those of vitamin B6 and B12. Therefore, the aim of the present study was to elucidate the dose-dependent effects of folic acid on *PS1* and *APP* promoter methylation, PS1 and APP protein expression, and cell viability in neurons exposed to Aβ oligomers.

Materials and methods

Cell cultures

The Tianjin Medical University Animal Ethics Committee approved all experimental protocols in this study. For primary hippocampal neuronal cultures, hippocampal tissue was isolated from neonatal Sprague–Dawley rats (postnatal less than 24 h, P0) and washed 3 times with Dulbecco's Modified Eagle's Medium (DMEM). The tissue was cut into small pieces, dissociated by incubation with 0.25 % parenzyme and 0.02 % EDTA, and centrifuged. The resulting cells were resuspended and cultured in DMEM/nutrient mixture F-12 Ham (F12) (1:1, v/v) medium containing 5 % FBS and 2 % N2, at 37 °C in a humidified atmosphere containing 5 % CO_2/95 % air. Cells were cultured for 7 days and grown on coverslips to identify neurons by staining for β-tubulin-III (anti β-tubulin, 1:30, Abcam, Cambridge, MA).

Mouse hippocampal HT-22 cells (supplied by the National Academy of Sciences of China; Shanghai, China) were grown in DMEM in medium containing 10 % fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5 % CO_2/95 % air. The HT-22 cells were passaged every 3 days when they grew to 80 % confluence.

In this study, folic acid-free DMEM powder was purchased from Gibco-BRL (Paisley, UK) as was F12 medium containing folic acid at 2.4 μmol/L. Thus, the medium used to culture primary neuronal cells contained folic acid at 1.2 μmol/L, and the medium used to culture HT-22 cells contained no folic acid. Other groups used DMEM/F12 medium or DMEM medium combined with predetermined amounts of folic acid to prepare culture media for the experiment. FBS contained folic acid at 81.6 nmol/L. In this study, FBS was added to the culture medium at concentrations of 5 and 10 %; thus, the folic acid contributed by the addition of FBS to the culture medium is minimal and can be ignored. However, FBS was necessary for neuronal cell growth; hence, FBS was used only for maintenance of the cultures.

Experimental procedures

Folic acid-free DMEM powder was purchased from Gibco-BRL (Paisley, UK) and combined with predetermined amounts of folic acid to prepare culture media for the experiments. Aβ oligomers were prepared by incubating Aβ1–40 (100 μmol/L in distilled water) at 37 °C for 7 days, followed by centrifugation at 10,000×g for 10 min [14]. Then, freshly prepared Aβ oligomers were detected using Tris/Tricine SDS-polyacrylamide gel (2 μg of protein) and were then immunoblotted and probed with the anti-Aβ antibody. We found that the molecular weight of the prepared Aβ oligomers exceeded 55 kDa. Primary neuronal cells and HT-22 cells were incubated for 24 h with a combination of folic acid and either Aβ oligomers or vehicle and were then incubated for 72 h with various concentrations of folic acid. For primary neuronal cells, the folic acid concentration was in the range of 1.2–40 μmol/L, and the Aβ oligomer concentration was 2 μmol/L. For HT-22 cells, the folic acid concentration was in the range of 0–40 μmol/L and the Aβ oligomer concentration was 3 μmol/L.

Mice and diets

The Tianjin Medical University Animal Ethics Committee approved the experimental protocols in this study (Study number: TMUaMEC 2012016). Male mice with

APPswe/PS1ΔE9 mutations (APP/PS1), backcrossed to C57Bl6/J, were obtained from the Chinese Academy of Medical Sciences Institute of Laboratory. These double transgenic mice are models of AD because they express a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and a mutant human presenilin 1 (PS1-dE9), both directed to central nervous system neurons under the control of independent mouse prion protein promoter elements. Consequently, young adult APP/PS1 mice have Aβ-containing, neuritic plaques that are absent in age-matched wild-type mice. After genotyping, the APP/PS1 mice were maintained on the control diet until the age of 7 months and were then assigned in equal numbers to four groups for 60 days. The groups were as follows: folate-deficient diet plus daily gavage with water (AD+FA-D); control diet (normal folic acid content) plus daily gavage with water (AD+FA-N); and control diet plus daily gavage with 600 µg/kg folic acid (AD+FA-H).

The folate-deficient diet (containing 0.2 mg folic acid/kg diet food) and the control diet (2.1 mg folic acid/kg diet food) were purchased from TestDiet (St. Louis, MO). All mice received food and drinking water ad libitum. At the conclusion of the experiment, the mice were anesthetized with an intraperitoneal injection of 7 % chloral hydrate (5 mL/kg). Their brains were removed, bisected in the sagittal plane, and stored at −80 °C until further use.

Pyrosequencing

Methylation levels of CpG sites of *APP* and *PS1* promoter regions were determined by pyrosequencing using the PyroMark Q24 Reagent (QIAGEN Ltd., Crawley, UK), according to the manufacturer's protocol. Mice brain hippocampal tissues were homogenized using a motor-driven tissue homogenizer (PT1200E, Kinematica, Luzern, Switzerland). HT-22 cells and mice brain tissue homogenates were digested with 100 µg/mL proteinase K at 50 °C overnight and subjected to phenol/chloroform extraction and ethanol precipitation of DNA. Then, incubation of the DNA with sodium bisulfite converted unmethylated cytosine residues into uracil while leaving methylated cytosine residues unchanged. Real-time PCR was performed using the LightCycler 480 SYBR Green I Master Kit (Roche, Mannheim, Germany). The 25-µL PCR mixture included 12.5 µL of PyroMark PCR Master, 2.5 µL of CoralLoad Concentrate, 1 µL of DNA, 2.5 µL of forward primer, 2.5 µL of reverse primer, and 4 µL of water (PCR-grade). The reaction mixtures were incubated at 95 °C for 15 min, which was followed by 45 amplification cycles (94 °C for 30 s; 56 °C for 30 s; and 72 °C for 30 s). Primers specific for *APP* (forward,

5′-TGATTTGGGTTAGGGAGAGG-3′; reverse, 5′-AAC CTTAACTCCTCAACCA CATTTA-3′) and PS1 (forward, 5′-GGGTTGGAGTTGGTTTAA-3′; reverse, 5′-CAAACCCATCCTTTCCTACAA-3′) were used. As negative controls of bisulfite modifications, we used unmethylated purified PCR products of *PS1* promoter, obtained with the same primers; the same purified PCR products where methylated in vitro with *Sss*I methylase, which methylates only cytosine residues in the CpG dinucleotide, and these were used as positive controls.

Gene expression

Real-time PCR was used to quantify *APP*, *PS1*, *DNMT1*, and *DNMT3a* expressions. Total RNA was extracted using TRIzol, according to the instructions of the manufacturer. First-strand cDNA was synthesized from 2 µg total RNA by using MMLV reverse transcriptase. The 20 µL reaction volume was incubated for 50 min at 42 °C, 5 min at 90 °C, and 5 min at 5 °C and was then stored at −20 °C. Real-time PCR was performed using the LightCycler 480 SYBR Green I Master Kit (Roche, Mannheim, Germany). The 20 µL PCR mixture included 10 µL of PCR Master mix, 5 µL of cDNA, 1 µL of forward primer, 1 µL of reverse primer, and 3 µL of water (PCR grade). The reaction mixtures were incubated at 95 °C for 5 min, which was followed by 45 amplification cycles (denaturation, 95 °C for 10 s; annealing, primer-dependent temperature (Table 1) for 10 s; extension, 72 °C for 10 s). The assay was performed using the Roche 480 sequence detector. The expression of each gene was normalized to that of β-actin.

Western blot analysis

Protein expression of APP, PS1, and DNMT isoforms in cultured cells was assessed by western blot analysis. The cells were washed with ice-cold PBS and lysed with TNE-NP40 buffer. Proteins were separated on 12 % sodium dodecyl sulfate-polyacrylamide gels by electrophoresis and transferred to PVDF membranes. The membranes were blocked with 5 % non-fat milk and incubated with primary antibodies (anti-APP [for detecting APP695, APP770, and APP751], 1:1000, CST (Danvers, MA); anti-PS1, 1:1000, Abcam (Cambridge, UK); anti-DNMT1, 1:1000, CST; anti-DNMT3a, 1:1000, CST) overnight at 4 °C, which was followed by incubation with the appropriate secondary antibodies (IgG-horseradish peroxidase, Zhongshan Golden Bridge Biotechnology, Beijing, China) for 1 h at room temperature. Proteins were detected by chemiluminescence assay. Protein levels were quantified by densitometric analysis by using NIH Image software (version 1.61). The

Table 1 Nucleotide sequence of the forward and reverse primers for real-time PCR

Gene name	Primer	Annealing temperature (°C)
Mouse-Actin	Forward: AATGTGTCCGTCGTGGATCT Reverse: GGTCCTCAGTGTAGCCCAAG	60
Mouse-APP	Forward: GGCAACAGGAACTTTGA Reverse: CTGCTGTCGGGGAAGTTTA	59
Mouse-PS1	Forward: GCCCCAGAGTAACTCAAGACA Reverse: CCGGGTATAGAAGCTGACTGA	63
Mouse-DNMT1	Forward: CCTAGTTCCGTGGCTACGAGGAGAA Reverse: TCTCTCCTCTGCAGCCGACTCA	59
Mouse-DNMT3a	Forward: GCCGAATTGTGTCTTGGTGGATGACA Reverse: CCTGGTGGAATGCACTGCAGAAGGA	59
Rat-Actin	Forward: ATCACCATCTTCCAGGAGCGA Reverse: AGCCTTCTCCATGGTGGTGAA	60
Rat-APP	Forward: AAGTGCGCCCCATTCTTTTA Reverse: AAAGTTGTTCCTGTTGCGC	60
Rat-PS1	Forward: GCAGCAGGCGTATCTCATTA Reverse: ATACGAAGTGGACCTTTGGG	60
Rat-DNMT1	Forward: CAATCCCAAAGCTCAACCCG Reverse: CATTGGTCAGCATCTGGGGT	62
Rat-DNMT3a	Forward: AGCTATTTACAGAGCTTCGGGC Reverse: TTGGCTTTCTTCTCAGCCCAT	58

intensity of each protein band was normalized to that of the respective actin band (anti-β-actin, 1:5000, Abcam).

Cell viability

Cell viability was measured using the Alamar Blue (resazurin) assay. The cells were incubated for 3 h with 0.1 mg/mL Alamar blue reagent (Gibco-BRL, Paisley, UK) in cell culture medium, and then, the optical density (OD) at 575 nm was read in a microplate reader (Bio-Tek ELX800uv, Bio-Tek Instrument Inc., Winooski, VT).

DNMT activity

Nuclear extracts were isolated from neuronal cells and HT-22 cells by using a nuclear extraction kit (Merck KGaA, Darmstadt, Germany). DNMT activity was measured in these extracts by the Active Motif DNA Methyltransferase Activity/Inhibition Assay Kit (Active Motif, Carlsbad, CA), according to the manufacturer's instructions. A lot-specific standard curve was created with the DNMT1 provided in the kit. Optical density (OD) was measured on a microplate reader at 450 nm, and DNMT activity [OD/(h mg)] was calculated according to the following formula:

DNMT activity [OD/(h mg)]

$$= \frac{(\text{Average sample OD} - \text{Average blank OD})}{\text{Protein amount (μg)}^* \times \text{Hour}^{**}} \times 1000$$

*Protein amount added into the reaction; **incubation time used for the reaction.

Methylation potential

Cells (2×10^7) were centrifuged and washed with cold phosphate-buffered saline (PBS) twice. Subsequently, the cells were homogenized in cold perchloric acid (0.4 mol/L) and centrifuged at $20,000 \times g$ for 10 min at 4 °C. The resulting supernatant was filtered (0.45 μm; Millipore, Billerica, MA) and injected into a high-performance liquid chromatography (HPLC) system (Waters, Milford, MA), containing a Venusil MP-C18 column (250 mm × 4.6 mm, 5-μm particles; Agela Technologies, Wilmington, DE). A 2-buffer elution system was used: mobile phase A and B both contained 10 mmol/L ammonium formate and 4 mmol/L L-heptanesulfonic acid (pH 4), whereas mobile phase B also contained 50 % acetonitrile by volume. Elution of SAM and SAH was achieved at a flow rate of 1 mL/min with the following parameters: 0–0.5 min, 100 % A; 0.5–20 min, linear gradient to 75 % A and 25 % B; 20–30 min, 25 % B; and 30–45 min, 100 % A. Eluted compounds were monitored at 254 nm by an ultraviolet detector connected to a Hewlett-Packard HP3394 integrator with automatic peak area integration. SAM and SAH standards were used to identify the elution peaks [15]. SAM and SAH concentrations were normalized to the cellular protein content that was determined by using the BCA protein assay kit (Boster; Wuhan, China).
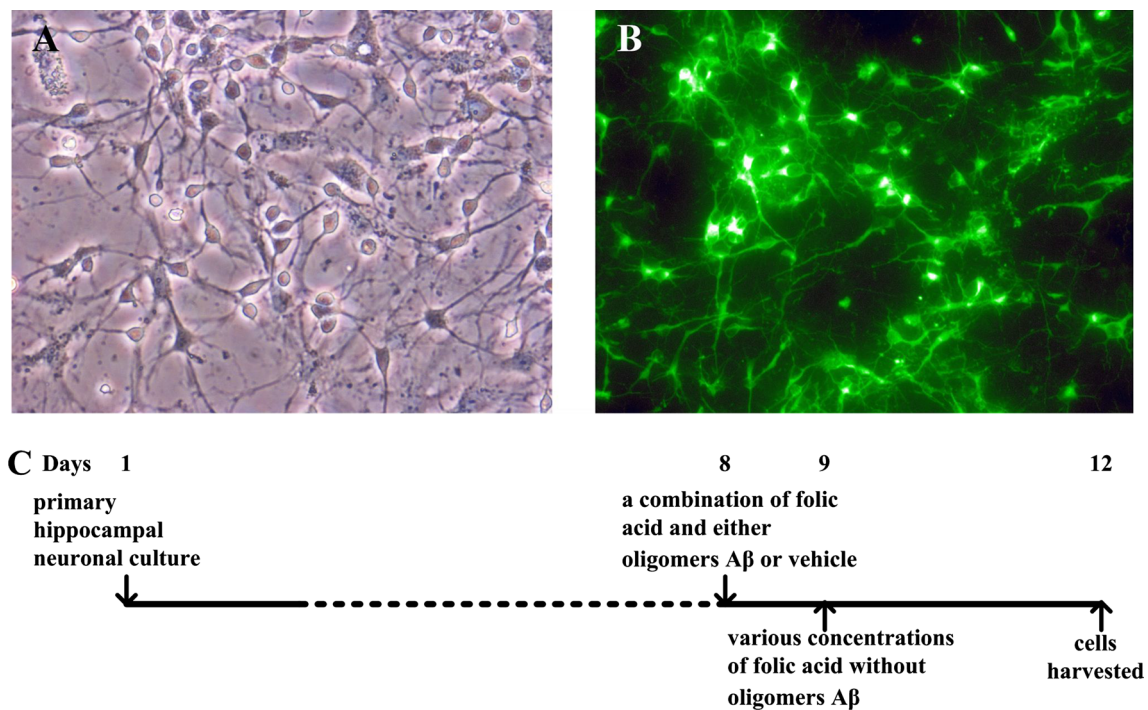
Fig. 1 Identification of neuronal cells in primary hippocampal cell cultures and the time course of the experiment. **a** Photomicrographs showing the neuronal appearance of cells (×200). **b** Cells stained for the neuronal marker β-tubulin-III (*green*, ×200). **c** Time course of the experiment

Statistical analysis

Data were expressed as mean ± SEM or SD. The statistical software package SPSS 16.0 was used to evaluate differences between treatment groups by one-way ANOVA and either Tukey's HSD test or Dunnett's test. $P < 0.05$ was considered significant.

Results

Cell identification, DNMT activity, and viability

Rat primary neuronal cultures and the mouse HT-22 cell line were used in the study; these represent different species, making our results more widely applicable.

More than 95 % cells in the primary cultures displayed a neuronal appearance and were immunoreactive for the neuronal marker β-tubulin-III after 1 week in culture (Fig. 1). Exposure to Aβ oligomers decreased DNMT activity in cells incubated with 10 μmol/L folic acid (2.18 ± 0.41 and 1.73 ± 0.27, respectively), but DNMT activity was protected fully by increasing the folic acid concentration even more; the highest DNMT activity was 2.35 ± 0.23 at a folic acid concentration of 20 μmol/L (Fig. 2a). Aβ oligomers decreased cell viability in cells incubated with 10 μmol/L folic acid (0.22 ± 0.02 and 0.18 ± 0.01, respectively),

and higher folic acid concentrations (20–40 μmol/L) conferred partial protection, with the highest cell viability of 0.19 ± 0.01 at a folic acid concentration of 40 μmol/L (Fig. 2b). Folic acid thus raises DNMT activity and cell viability in primary neuronal cells incubated with Aβ oligomers.

Aβ oligomers also decreased DNMT activity (2.56 ± 0.17 decreased to 2.08 ± 0.32) and cell viability (0.40 ± 0.02 decreased to 0.30 ± 0.01) in HT-22 cells (Fig. 2c, d). Folic acid dose-dependently protected against all the effects of Aβ oligomers on DNMT activity and partly against the effect on cell viability; the greatest effect occurred with exposure to 40 μmol/L folic acid (2.74 ± 0.30 and 0.30 ± 0.02, respectively; Fig. 2c, d). Folic acid therefore increases DNMT activity and cell viability in mouse HT-22 cells incubated with Aβ oligomers.

DNA methylation

Pyrosequencing assay data were evaluated by dividing the *APP* and *PS1* promoters into 13 and 10 CpG sites, respectively (Fig. 3a, d). Folic acid supplementation of HT-22 cells increased DNA methylation levels at CpG sites 2, 3, and 13 in the *APP* promoter (the highest methylation levels were observed with exposure to 40 μmol/L folic acid and were 12.0 ± 3.61, 8.3 ± 2.08, and 62.0 ± 2.65, respectively; Fig. 3b). Folic acid also increased DNA
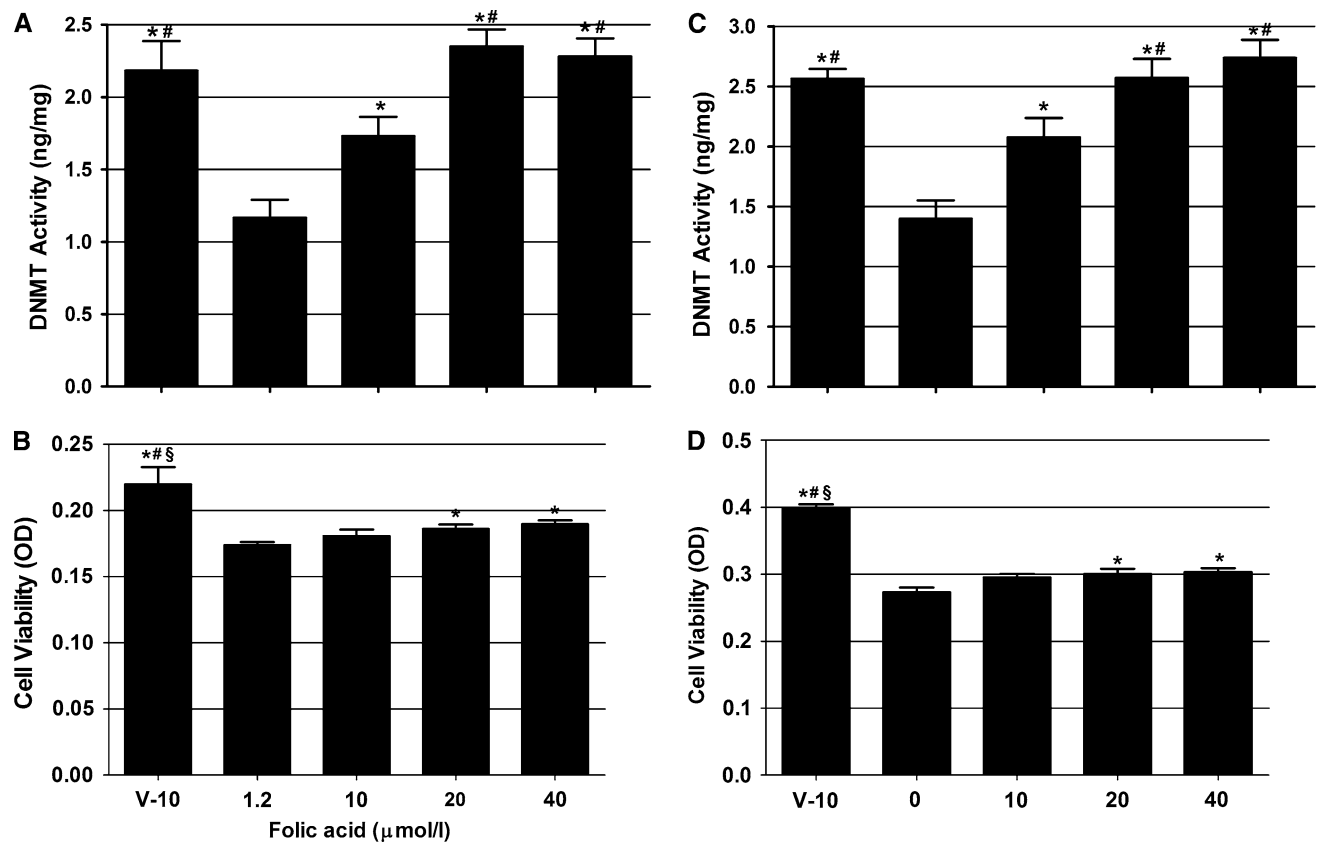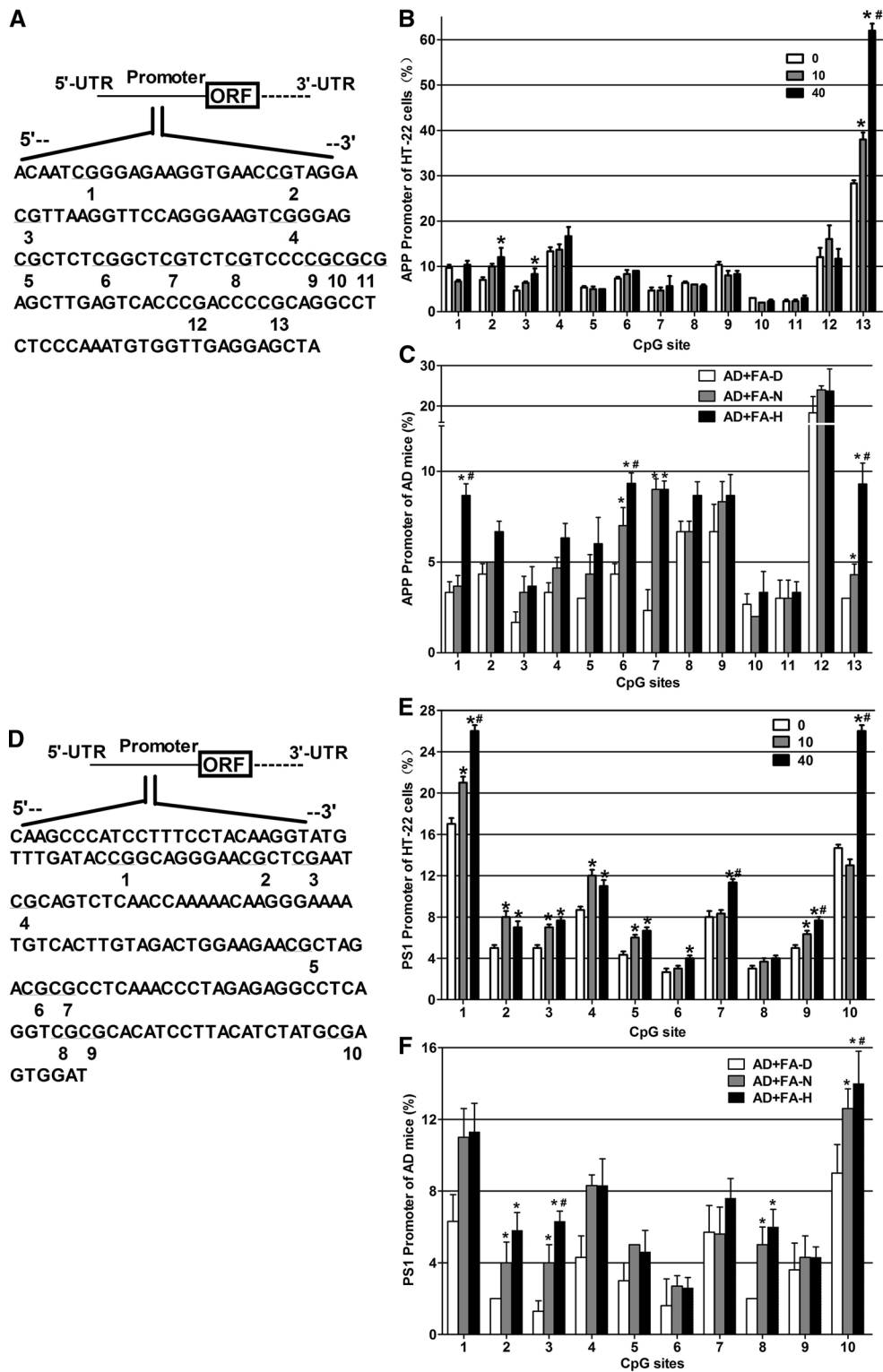
Fig. 2 Folic acid increases DNMT activity and cell viability. **a**, **b** Primary hippocampal neuronal cells were incubated for 24 h with a combination of folic acid and either 2 μmol/L Aβ oligomers or vehicle, and were then incubated for 72 h with 1.2–40 μmol/L folic acid. Subsequently, DNMT activity was measured in nuclear extracts and cell viability was assessed using the Alamar blue assay. **c**, **d** HT-22 cells were incubated for 24 h with a combination of folic acid and either 3 μmol/L Aβ oligomers or vehicle, and were then incubated for 72 h with 0–40 μmol/L folic acid. Thereafter, DNMT activity and cell viability were measured. V-10 signifies cells that were incubated with a combination of 10 μmol/L folic acid and vehicle for 24 h, which was followed by incubation with 10 μmol/L folic acid for 72 h. Similarly, 0, 1.2, 10, 20, and 40 signify cells that were incubated for 24 h with a combination of folic acid and Aβ oligomers, and were then incubated for 72 h with various concentrations of folic acid (1.2–40 or 0–40 μmol/L). The plotted values represent the mean ± SEM values of three experiments. *$P < 0.05$ compared with the lowest concentration of folic acid; #$P < 0.05$ compared with the 10 μmol/L concentration of folic acid; §$P < 0.05$ compared with 20 μmol/L

methylation levels at CpG sites 1–7 and 9–10 in the *PS1* promoter (the highest methylation levels occurred with exposure to 40 μmol/L folic acid at CpG sites 1, 3, 5–7, and 9–10, and were 26.0 ± 1.00, 7.7 ± 0.58, 6.7 ± 0.58, 4.0 ± 0.50, 11.3 ± 0.58, 7.7 ± 0.58, and 26.0 ± 1.00, respectively. At CpG sites 2 and 4, the highest methylation levels occurred with exposure to 10 μmol/L folic acid, and were 8.0 ± 1.00 and 12.0 ± 1.00, respectively, Fig. 3e). Clearly, folic acid modulates the methylation patterns in the *APP* and *PS1* promoters in HT-22 cells after treatment with Aβ oligomers.

For APP/PS1 mice, folic acid supplementation increased DNA methylation levels at CpG sites 1, 6, 7, and 13 in the *APP* promoter (the highest methylation levels were observed with exposure to 40 μmol/L folic acid and were 8.7 ± 0.56, 9.3 ± 0.58, 9.0 ± 0.47, and 9.3 ± 1.15, respectively; Fig. 3c). Folic acid also increased DNA methylation levels at CpG sites 2, 3,

Fig. 3 Folic acid modulates methylation patterns in *APP* and *PS1*.▶ HT-22 cells were incubated for 24 h with a combination of folic acid and 3 μmol/L Aβ oligomers and were then incubated for 72 h with 0, 20, or 40 μmol/L folic acid. The treatments for APP/PS1 mice were as follows: folate-deficient diet plus daily gavage with water (AD+FA-D); control diet (normal folic acid content) plus daily gavage with water (AD+FA-N); and control diet plus daily gavage with 600 μg/kg folic acid (AD+FA-H). The methylation levels at CpG sites of gene promoter regions were measured by dividing the *APP* and *PS1* promoters into 13 and 10 CpG sites, respectively. **a** Schematic diagram of *APP*. **b** Mean methylation levels of CpG sites in *APP* in HT-22 cells ($n = 3$ experiments). **c** Mean methylation levels of CpG sites in *APP* in brains of APP/PS1 mice ($n = 3$ mice/group). **d** Schematic diagram of *PS1*. **e** Mean methylation levels of CpG sites in PS1 in HT-22 cells ($n = 3$ experiments). **f** Mean methylation levels of CpG sites in *PS1* in brains of APP/PS1 mice ($n = 3$ mice/group). The plotted values represent mean ± SEM or SD values. The white column represents 0 μmol/L folic acid or the AD+FA-D group, the gray column represents 10 μmol/L folic acid or the AD+FA-N group, and the black column represents 40 μmol/L folic acid or the AD+FA-H group. *$P < 0.05$ compared with 0 μmol/L folic acid or AD+FA-D group; #$P < 0.05$ compared with 10 μmol/L folic acid or AD+FA-N group

A

5'-UTR Promoter ORF 3'-UTR

5'-- --3'
ACAATCGGGAGAAGGTGAACCGTAGGA
1 2
CGTTAAGGTTCCAGGGAAGTCGGGAG
3 4
CGCTCTCGGCTCGTCTCGTCCCCGCGCG
5 6 7 8 9 10 11
AGCTTGAGTCACCCGACCCCGCAGGCCT
12 13
CTCCCAAATGTGGTTGAGGAGCTA

D

5'-UTR Promoter ORF 3'-UTR

5'-- --3'
CAAGCCCATCCTTTCCTACAAGGTATG
TTTGATACCGGCAGGGAACGCTCGAAT
1 2 3
CGCAGTCTCAACCAAAAACAAGGGAAAA
4
TGTCACTTGTAGACTGGAAGAACGCTAG
5
ACGCGCCTCAAACCCTAGAGAGGCCTCA
6 7
GGTCGCGCACATCCTTACATCTATGCGA
8 9 10
GTGGAT

8, and 10 in the *PS1* promoter (the highest methylation levels were observed with exposure to 40 μmol/L folic acid and were 5.8 ± 1.00, 6.3 ± 0.58, 6.0 ± 0.98, and 14.0 ± 1.80, respectively; Fig. 3f). Folic acid, therefore, modulates methylation patterns in *APP* and *PS1* promoters in APP/PS1 mouse brains.

APP and *PS1* expression

Exposure to Aβ oligomers increased *APP* gene and protein expression in primary neuronal cells incubated with 10 μmol/L folic acid (11.60 ± 2.86 increased to 63.00 ± 4.41; 0.51 ± 0.14 increased to 0.94 ± 0.06,
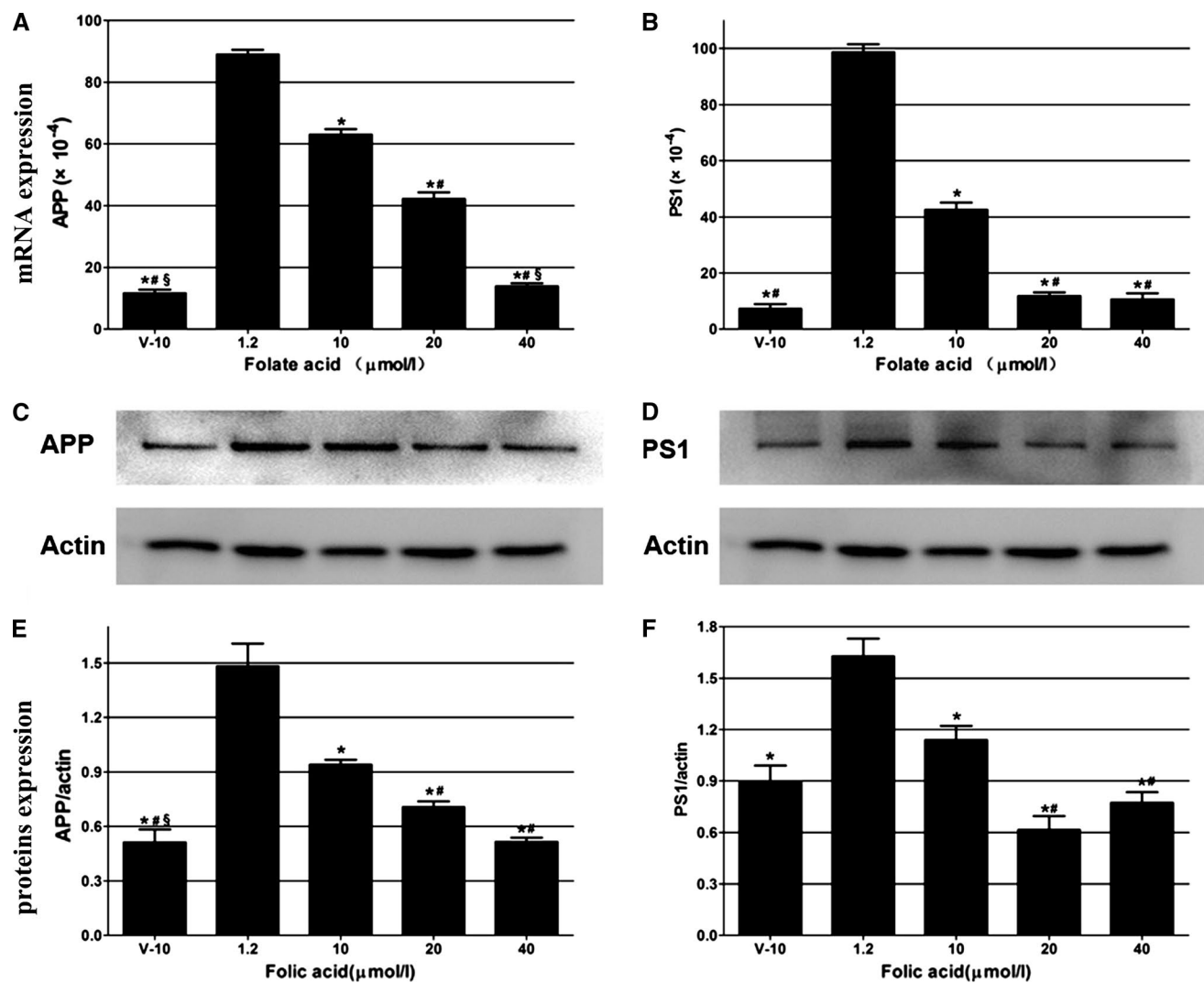
Fig. 4 Folic acid modulates the expression of *APP* and *PS1* in primary neuronal cells. The cells were incubated as described in Fig. 2. **a**, **b** Gene expression levels of *APP* and *PS1* as measured by real-time PCR (*the upper histograms*). **c**, **d** Representative western blots of APP, PS1, and actin proteins. **e**, **f** Summaries of densitometric analyses of western blots of APP, PS1, and actin proteins (*the lower histograms*). The plotted values are mean ± SEM values for three experiments. $*P < 0.05$ compared with 1.2 μmol/L; $^{\#}P < 0.05$ compared with 10 μmol/L; $^{§}P < 0.05$ compared with 20 μmol/L

respectively; Fig. 4a, c, e). These effects of the Aβ oligomers were exacerbated at a lower folic acid concentration (1.2 μmol/L: 88.91 ± 3.87 and 1.48 ± 0.25, respectively) but were completely inhibited by a higher folic acid concentration (40 μmol/L: 13.84 ± 2.59 and 0.51 ± 0.04, respectively; Fig. 4a, c, e). Folic acid decreased the expression of *APP* gene and protein in primary neuronal cells after cells were treated with Aβ oligomers.

Furthermore, in primary neuronal cells, Aβ oligomers increased *PS1* mRNA expression (7.26 ± 4.20 increased to 42.48 ± 6.28), but not PS1 protein expression when the folic acid concentration was 10 μmol/L (Fig. 4b, d, f). Compared to 10 μmol/L folic acid, a lower folic acid concentration enhanced (98.60 ± 7.14 and 1.62 ± 0.23,

respectively), while higher folic acid concentrations reduced (10.59 ± 5.21 and 0.77 ± 0.14, respectively), the *PS1* mRNA and protein expression in neuronal cells exposed to Aβ oligomers (Fig. 4b, d, f). Thus, folic acid decreased the expression of *PS1* mRNA and protein levels in primary neuronal cells after treatment of cells with Aβ oligomers.

Exposure of HT-22 cells to Aβ oligomers increased the gene and protein expression of both *APP* and *PS1* when the folic acid concentration was 10 μmol/L (Fig. 5). However, this stimulation of *APP* and *PS1* expression by Aβ oligomers was inhibited dose-dependently by folic acid (Fig. 5). Folic acid decreased the expression of *APP* and *PS1* genes and proteins in mouse HT-22 cells after cells were treated with Aβ oligomers.
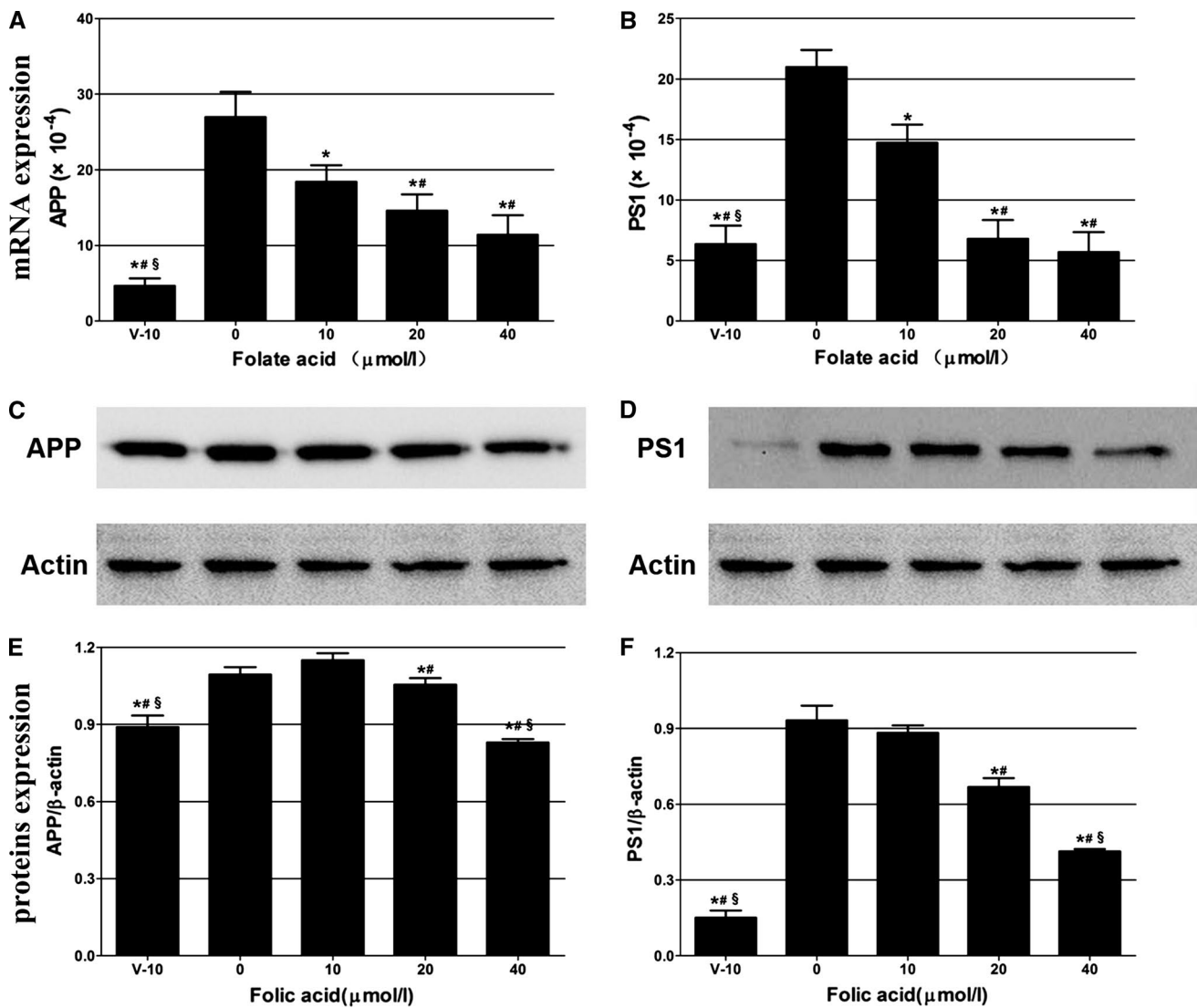
Fig. 5 Folic acid modulates the expression of *APP* and *PS1* in HT-22 cells. The cells were incubated as described in Fig. 2. **a**, **b** Gene expression levels of *APP* and *PS1* as measured by real-time PCR (*the upper histograms*). **c**, **d** Representative western blots of APP, PS1, and actin proteins. **e**, **f** Summaries of densitometric analyses of western blots of APP, PS1, and actin proteins (*the lower histograms*). The plotted values are mean ± SEM values for three experiments. *$P < 0.05$ compared with 0 μmol/L; #$P < 0.05$ compared with 10 μmol/L; §$P < 0.05$ compared with 20 μmol/L

DNMT expression

Aβ oligomers did not change *DNMT1* gene and protein expression in primary neuronal cells incubated with 10 μmol/L folic acid (Fig. 6a, c, e). Incubation with the highest folic acid concentration (40 μmol/L) elevated *DNMT1* gene expression (28.46 ± 8.20), but not DNMT1 protein expression (Fig. 6a, c, e). Aβ oligomers decreased *DNMT3a* mRNA expression in the presence of 10 μmol/L folic acid (3.97 ± 0.31 decreased to 3.26 ± 0.24), but 40 μmol/L folic acid prevented this effect (4.16 ± 0.31; Fig. 6b). DNMT3a protein expression was reduced in the presence of 10 μmol/L folic acid (1.13 ± 0.03 decreased

to 0.93 ± 0.09), and this inhibitory effect was exacerbated in the presence of 1.2 μmol/L (0.58 ± 0.14) but was not prevented by supplementation with 20–40 μmol/L folic acid (Fig. 6d, f). Folic acid increased DNMT3a expression in primary neuronal cells after cells were treated with Aβ oligomers.

Exposure of HT-22 cells to Aβ oligomers in the presence of 10 μmol/L folic acid decreased *DNMT1* mRNA expression (15.48 ± 3.94 decreased to 10.35 ± 3.50), but it did not change that of *DNMT3a* or the expression of DNMT1 and DNMT3a proteins (Fig. 7). *DNMT1* and *DNMT3a* gene and protein expression were higher in the presence than in absence of 1.2 μmol/L folic acid, with the most
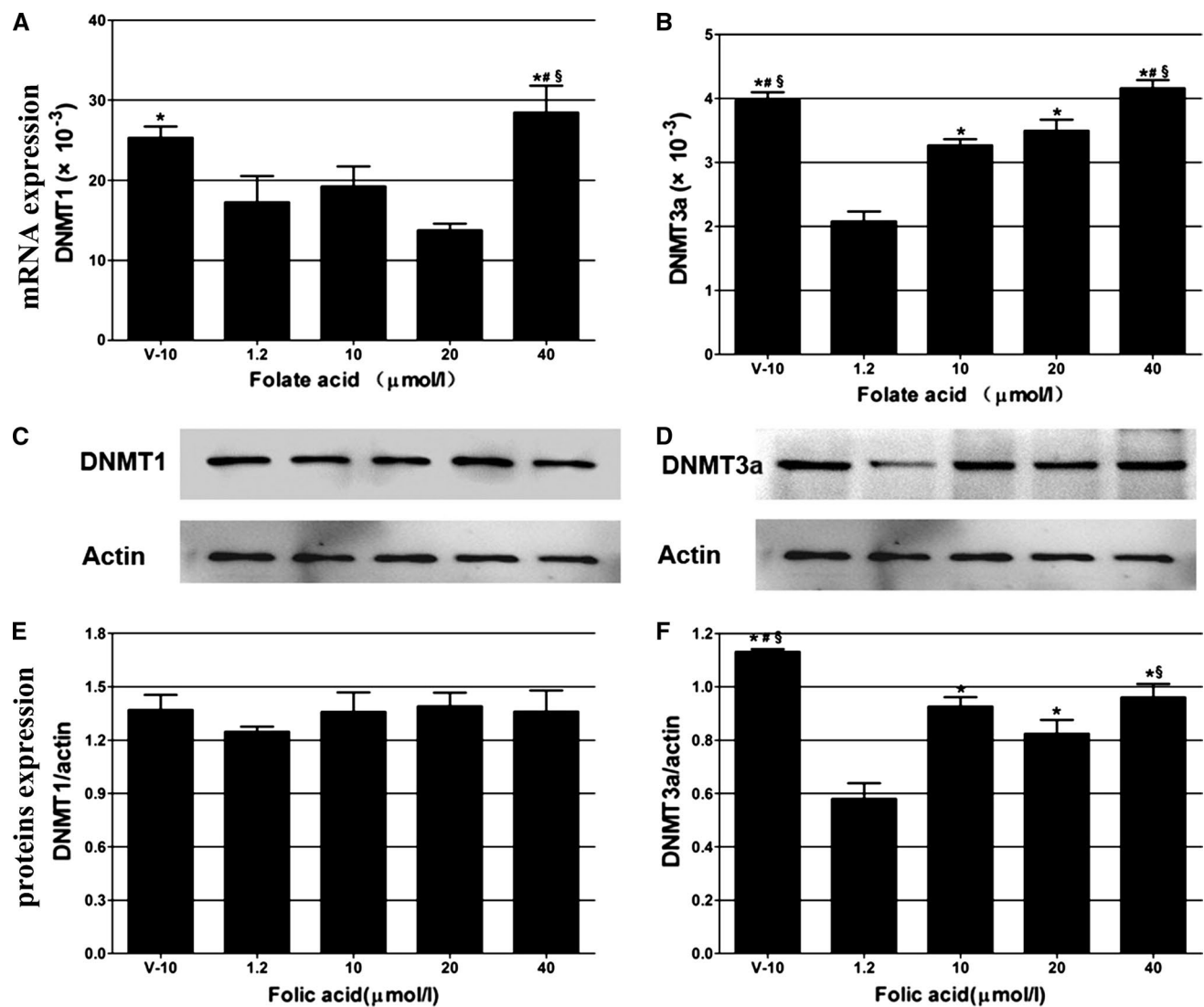
Fig. 6 DNMT expression in primary neuronal cells. The cells were incubated as described in Fig. 2. **a**, **b** Gene expression levels of *DNMT1* and *DNMT3a* as measured by real-time PCR (*the upper histograms*). **c**, **d** Representative western blots of DNMT, DNMT3a, and actin proteins. **e**, **f** Summaries of densitometric analyses of western blots of DNMT1, DNMT3a, and actin proteins (*the lower histograms*). The plotted values are mean ± SEM values for three experiments. *$P < 0.05$ compared with 1.2 µmol/L; #$P < 0.05$ compared with 10 µmol/L; §$P < 0.05$ compared with 20 µmol/L

effective folic acid concentrations being 40 µmol/L for *DNMT1* mRNA (16.14 ± 2.11), 10–40 for DNMT1 protein, 40 µmol/L for *DNMT3a* mRNA, and 20–40 µmol/L for DNMT3a protein (Fig. 7). Taken together, folic acid increased *DNMT1* and *DNMT3a* expression in mouse HT-22 cells treated with Aβ oligomers.

Methylation potential

In primary neuronal cells and HT-22 cells, folic acid dose-dependently increased SAM concentration and lowered SAH concentration, stimulating the methylation potential (i.e., SAM:SAH ratio). The highest methylation potential

was achieved with 40 µmol/L folic acid (180.29 ± 13.10 and 149.49 ± 11.28, respectively; Fig. 8). Folic acid increased methylation potential in neuronal cells after cells were treated with Aβ oligomers.

Discussion

The present study established that gene methylation links nutrition to neuronal viability in an experimental model of AD by using folic acid supplementation as an intervention in primary neurons and HT-22 cells exposed to Aβ oligomers. We observed that Aβ oligomers lowered
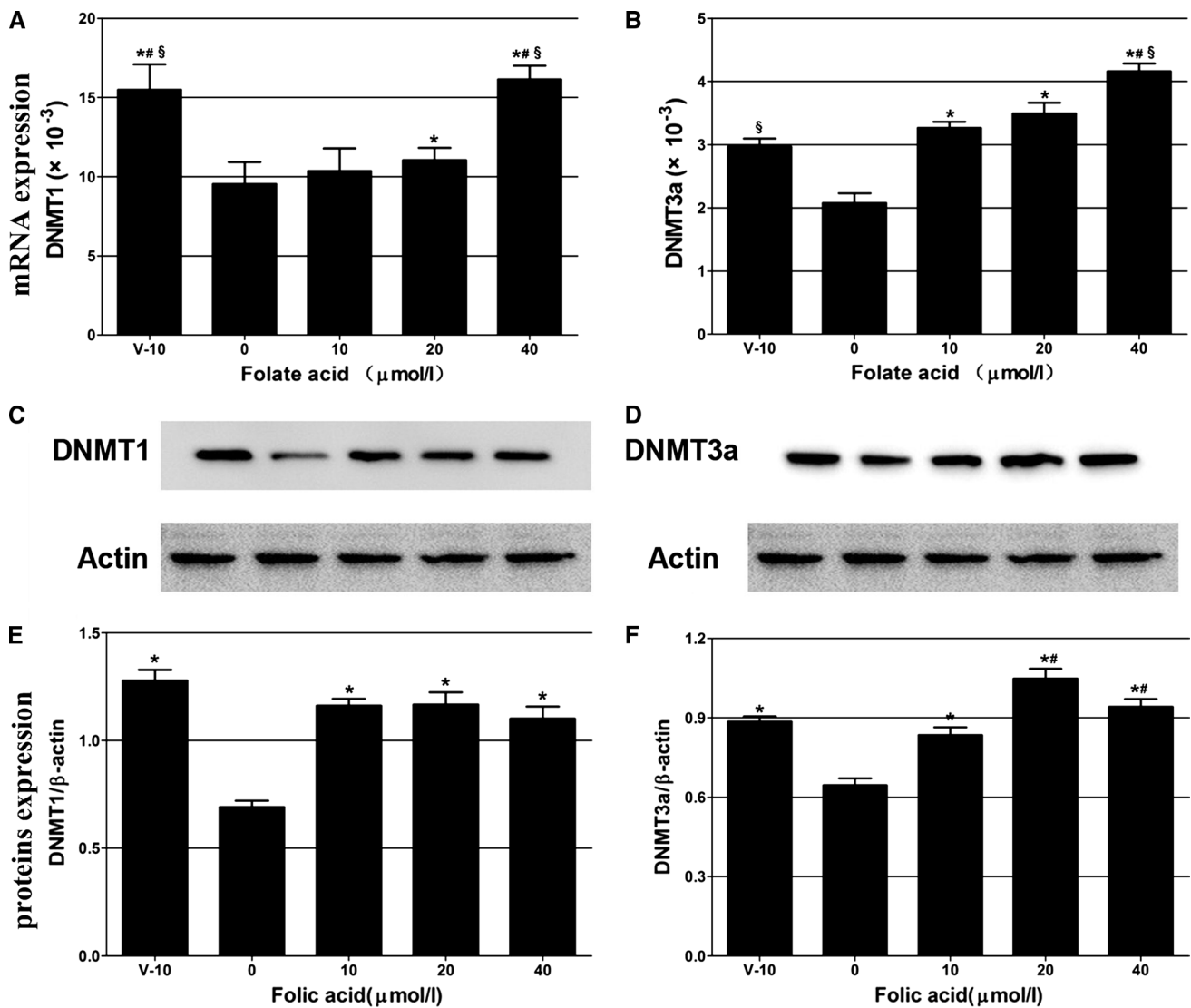
Fig. 7 DNMT expression in HT-22 cells. The cells were incubated as described in Fig. 2. **a**, **b** Gene expression levels of *DNMT1* and *DNMT3a* as measured by real-time PCR (*the upper histograms*). **c**, **d** Representative western blots of DNMT1, DNMT3a, and actin proteins. **e**, **f** Summaries of densitometric analyses of western blots of DNMT1, DNMT3a, and actin proteins (*the lower histograms*). The plotted values are mean ± SEM values for three experiments. *$P < 0.05$ compared with 0 µmol/L; #$P < 0.05$ compared with 10 µmol/L; §$P < 0.05$ compared with 20 µmol/L

DNMT activity, increased *PS1* and *APP* expression, and decreased cell viability. Folic acid increased the methylation of *PS1* and *APP* promoters in AD transgenic mice. Moreover, folic acid dose-dependently stimulated methylation potential and DNMT activity, altered *PS1* and *APP* promoter methylation, decreased *PS1* and *APP* expression, and partially preserved cell viability in the presence of Aβ oligomers. High-dose folic acid stimulated the expression of *DNMT1* and *DNMT3a* in primary neurons and HT-22 cells after the cells were treated with Aβ oligomers. Folic acid also increased the protein expression of DNMT3a in both primary neurons and HT-22 cells after treatment with Aβ oligomers. Taken together, these results indicated that

folic acid induced methylation potential-dependent DNMT activity, which then methylated and silenced *APP* and *PS1*. It is likely that these effects of folic acid decreased endogenous Aβ synthesis by PS1 and APP and may have resulted in the increased cell viability observed in neurons exposed to exogenous Aβ oligomers (Fig. 9).

AD is characterized by continuous accumulation of amyloid β-peptide fibrils into plaques. The toxic effects of these on synaptic connections and neurons are explained by the amyloid cascade hypothesis. The accumulation of intraneuronal Aβ has been speculated to affect intracellular trafficking, which disrupts fast axonal transport [16]. A recent study in APP/Aβ mouse AD models indicated that
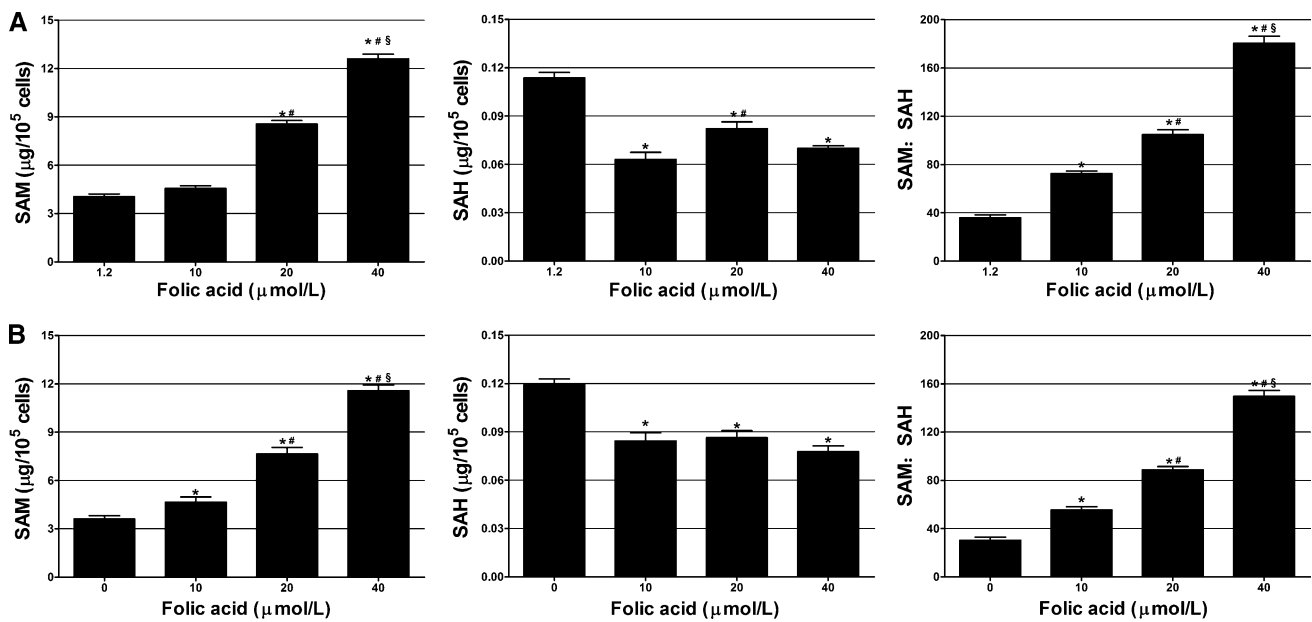
Fig. 8 Folic acid increases methylation potential in neuronal cells. **a** Primary hippocampal neuronal cells were incubated for 24 h with a combination of folic acid and 2 μmol/L Aβ oligomers and were then incubated for 72 h with 1.2–40 μmol/L folic acid. SAM concentration, SAH concentration, and methylation potential (i.e., SAM:SAH ratio) in primary neuronal cells. **b** HT-22 cells were incubated for 24 h with a combination of folic acid and 3 μmol/L Aβ oligomers and were then incubated for 72 h with 0–40 μmol/L folic acid. SAM concentration, SAH concentration, and methylation potential in HT-22 cells. The plotted values are mean ± SEM values for three experiments. *$P < 0.05$ compared with the lowest concentration of folic acid; #$P < 0.05$ compared with 10 μmol/L folic acid; §$P < 0.05$ compared with 20 μmol/L folic acid
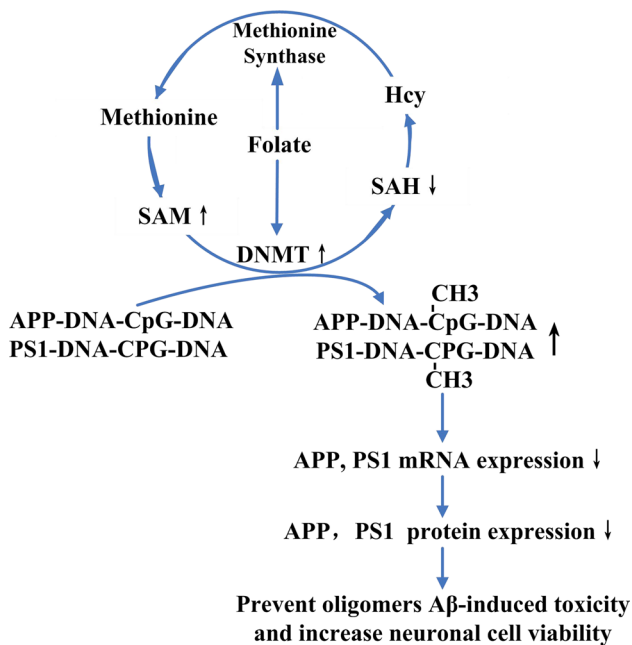

Fig. 9 The mechanism by which folic acid prevents Aβ oligomer-induced neuronal toxicity

extracellular Aβ can cause early synaptic deficits, cholinergic neuron loss, and hippocampus atrophy [17]. Exogenous fibrillar Aβ could mediate AD-like damage in neuron cells.

In this study, neurons incubated in 10 μmol/L folic acid (V-10) were used as a control group. Folate is necessary for cell growth; in this study, neurons were cultured in vitro, under conditions differing from those in vivo, and the cells had greater nutritional requirement for survival. DMEM or neurobasal medium is recommended in the protocol for culturing neuronal cells [18–20], and both DMEM and neurobasal medium contain folic acid at 4 mg/mL (ca. 10 μmol/L) as a standard constituent. Therefore, we used the V-10 group (neuronal cell medium without Aβ oligomers, containing 10 μmol/L folic acid) as the control group representing the baseline conditions of the cells. Consequently, we considered 20 and 40 μmol/L folic acid (i.e., two- and fourfold more concentrated than control conditions) as supplement concentrations in vitro. However, the level used in this study is very high. We also need to study the effect of folic acid on physiology level. The folic acid-free DMEM used in this study contains 30 mg/L (0.2 mmol/L) methionine, which is the same as the concentration in standard DMEM. Folate functions as an enzyme cofactor that carries one-carbon units in a network of anabolic pathways known as one-carbon metabolism (OCM). OCM is essential for de novo purine and thymidylate synthesis and for remethylation of homocysteine to methionine, which can then be adenosinylated to form the universal methyl donor SAM. Thus, under folate-deficient

conditions, SAM cannot be synthesized, even when methionine levels are sufficient [21].

Our previous study showed the effect of folic acid treatment for 6 days on the differentiation of neural stem cells (NSCs), which mostly differentiate into neurons and astrocytes. Compared with the folic acid-deficient group, cells grown in the presence of 8 μg/mL (20 μmol/L) and 32 μg/mL (80 μmol/L) folic acid showed increased DNMT activity in differential NSCs; however, use of 32 μg/mL folic acid could not increase DNMT activity further. A similar phenomenon was observed for the methylation potential (i.e., the SAM:SAH ratio) [22]; thus, the dose-dependent effect seen with folic acid occurs only after cells were exposed to Aβ oligomers.

Some effects of folic acid supplementation in the present study are reminiscent of those of SAM supplementation in AD models. SAM has even more far-reaching effects on AD risk factors. For example, in 3xTg-AD mice that harbor mutant genes for human APP, PS1, and tau, SAM supplementation decreased hippocampal Aβ levels [23]. Furthermore, when TgCRND8 mice are fed a folate/B6/B12-deficient diet, SAM supplementation prevented the inhibition of DNA methylation, PS1 expression, and Aβ production, which are consequences of this diet [7, 10]. Both a deficiency in SAM and an accumulation of SAH may cause DNA hypomethylation and affect malignancy.

Rogaev's study report that sequences upstream from −500 bp in the APP promoter display complex, tissue-specific patterns of methylation in human [24]. However, to our knowledge, similar observations of APP methylation have not previously been reported by others in an in vitro model. Our results also demonstrated that folic acid deficiency causes APP overexpression through DNA demethylation and that folic acid supplementation reverses such overexpression, preventing demethylation.

Additionally, some studies have reported the methylation pattern of PS1 gene. Fuso and colleagues have described the methylation pattern of the PS1 promoter in SK-N-BE neuroblastoma cells and in TgCRND8 mice, in a vitamin B (folate, B12, and B6)-deficiency paradigm [7, 10]. Vitamin B deficiency induces hypomethylation of specific CpG moieties in the 5′-flanking region of PS1, but when SAM is supplemented as a methyl donor, this effect is reversed. However, those studies only described the methylation pattern of PS1 under physiological conditions; the effect of Aβ oligomers was not considered. Moreover, the methylation pattern of PS1 was only described in a vitamin B deficiency and SAM supplementation paradigm in previous studies, while we designed this study to show whether folic acid supplementation could protect against the neuronal toxicity of Aβ oligomers. We showed that folic acid

supplementation appeared to protect against the neuronal toxicity of Aβ oligomers by inducing methylation potential-dependent DNMT activity and methylation and silencing of APP and PS1, at least when present at a concentration of less than 40 μmol/L.

In conclusion, folic acid decreased endogenous Aβ synthesis by PS1 and APP and may lead to the increased cell viability observed in treated neurons after exposure to exogenous Aβ oligomers. Therefore, the novel findings of the present study in AD cell models may stimulate further investigation of folic acid supplementation as a treatment for AD patients.

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