

# Folic Acid Alters Methylation Profile of JAK-STAT and Long-Term Depression Signaling Pathways in Alzheimer's Disease Models

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**Abstract** Dementia has emerged as a major societal issue because of the worldwide aging population and the absence of any effective treatment. DNA methylation is an epigenetic mechanism that evidently plays a role in Alzheimer's disease (AD). Folate acts through one-carbon metabolism to support the methylation of multiple substrates including DNA. We aimed to test the hypothesis that folic acid supplementation alters DNA methylation profiles in AD models. Mouse Neuro-2a cells expressing human APP695 (N2a-APP cells) were incubated with folic acid (2.8–20 μmol/L). AD transgenic mice were fed either folate-deficient or control diets and gavaged daily with water or folic acid (600 μg/kg). Gene methylation profiles were determined by methylated DNA immunoprecipitation-DNA microarray (MeDIP-chip). Differentially methylated regions (DMRs) were determined by Quantitative Differentially Methylated Regions analysis, and differentially methylated genes (DMGs) carrying at least three DMRs were selected for pathway analysis. Folic acid up-regulated DNA methylation levels in N2a-APP cells and AD transgenic mouse brains. Functional network analysis of folic

acid-induced DMGs in these AD models revealed subnetworks composed of 24 focus genes in the janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway and 12 focus genes in the long-term depression (LTD) signaling pathway. In conclusion, these results revealed a role for folic acid in the JAK-STAT and LTD signaling pathways which may be relevant to AD pathogenesis. This novel finding may stimulate reinvestigation of folic acid supplementation as a prophylactic or therapeutic treatment for AD.

**Keywords** Folic acid · Alzheimer's disease · DNA methylation profile · JAK-STAT signaling pathway · Long-term depression signaling pathway

## Abbreviations

AD	Alzheimer's disease
APP	Amyloid precursor protein
APP/PS1 mice	The mice with APP <sup>swe</sup> /PS1 <sup>ΔE9</sup> mutations
BATMAN	Bayesian tool for methylation analysis
CREB	cAMP response element binding protein
DMEM	Dulbecco's modified Eagle's medium
DMG	Differentially methylated gene
DMR	Differentially methylated region
DMROI	Differentially methylated region of interest
FA	Folic acid
JAK-STAT	Janus kinase-signal transducer and activator of transcription
LTD	Long-term depression
MeDIP-chip	Methylated DNA immunoprecipitation-DNA microarray
N2a-APP	N2a neuroblastoma cells overexpressing APP695

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ROI	Region of interest
TSS	Transcription start site
TTS	Transcription termination site
UMROI	Undifferentially methylated region of interest

## Introduction

Dementia has emerged as a major societal issue because of the worldwide aging population and the absence of any effective treatment [1]. Assuming that age-specific prevalence rates remain stable, the number of cases of dementia worldwide is projected to more than triple between 2010 and 2050 [2]. Alzheimer's disease (AD) accounts for approximately 60 % of dementia cases overall [3]. China had more cases of AD in 2010 than any other country in the world. The absolute numbers of 5.69 million cases of AD and 9.19 million cases of dementia in China in 2010 may pose the single largest challenge to health and social care systems in terms of finding effective and affordable responses [4].

AD is a progressive brain amyloidosis that injures brain regions involved in memory consolidation and other cognitive functions [5]. However, AD has a non-Mendelian etiology that may be due to epigenetic mechanisms mediating the disease's onset and progression [6]. DNA methylation is an epigenetic mechanism that evidently plays a role in AD [7–9]. For instance, DNA methylation levels differ between late-onset AD and cognitively normal controls in human frontal cortex [7].

Folate metabolism (one-carbon metabolism) has been associated with AD risk [10]. In particular, folate deficiency is a risk factor for cognitive decline and AD [11]. Also, decreased folate levels in serum and cerebrospinal fluid were observed in AD patients [12–14]. The underlying mechanisms may include folate deficiency-induced hypomethylation of DNA [15] as well as oxidative stress, calcium influx into cells, and accumulation of hyperphosphorylated tau and  $\beta$ -amyloid [11].

In the present study, the effects of folic acid on gene methylation profiles in AD models were assessed by methylated DNA immunoprecipitation-DNA microarray (MeDIP-chip). We discovered that the methylation profiles of the janus kinase-signal transducer and activator of transcription (JAK-STAT) and long-term depression (LTD) signaling pathways were altered by folate deficiency and folic acid supplementation in AD models. This discovery provides insight into the mechanism by which folate metabolism may influence AD.

## Results

### DNA Methylation Levels in N2a-APP Cell Cultures and APP/PS1 Mouse Brains

Bayesian tool for methylation analysis (BATMAN) of MeDIP-chip assay data found 23,282 region of interests (ROIs) in nine N2a-APP cell cultures and nine APP/PS1 mouse brains. DNA methylation levels were higher in the cell cultures than those in the brains (Fig. 1). For this reason, the data from each of the two AD models are presented separately below.

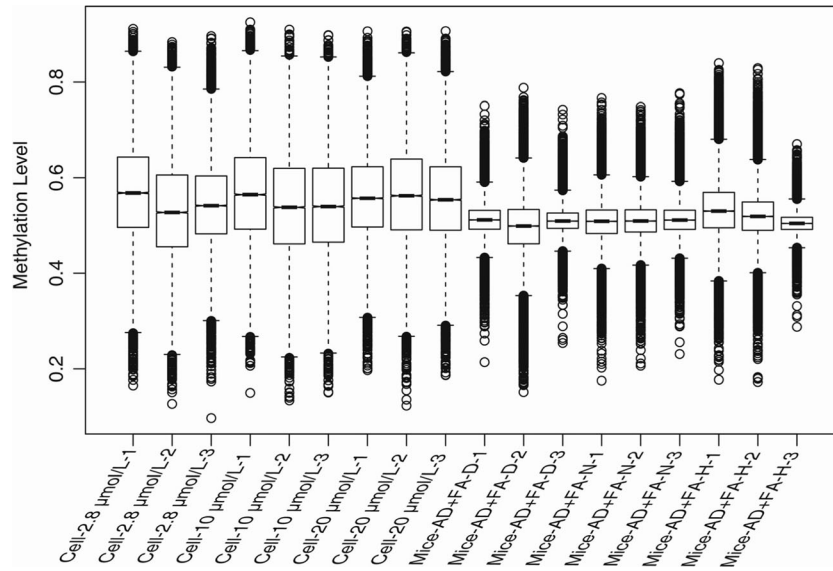
In N2a-APP cells, methylation rates rose with folic acid dose and were elevated significantly by the highest folic acid dose compared to the folic acid-deficient condition (i.e., folic acid concentrations of 20 and 2.8  $\mu\text{mol/L}$ , respectively) (Fig. 2a). Cluster analysis found that folic acid increased methylation levels in differentially methylated region of interest (DMROI) and undifferentially methylated region of interest (UMROI), with significantly higher methylation rates occurring at 20  $\mu\text{mol/L}$  than at lower folic acid concentrations (Fig. 3a). Genome distribution did not differ between DMROI and UMROI in N2a-APP cells (Fig. 3b).

In APP/PS1 mouse brains, methylation rates were decreased significantly by the folic acid-deficient condition (AD+FA-D) and increased significantly by the highest folic acid dose (AD+FA-H), compared to the control condition (AD+FA-N) (Fig. 2b). Cluster analysis found that folic acid increased methylation levels in DMROI in a significantly dose-dependent manner (Fig. 4a). Genome distribution did not differ between DMROI and UMROI in APP/PS1 mouse brains (Fig. 4b).

### Pathway Analysis

There were 2328 DMROI in N2a-APP cells and 2328 DMROI in APP/PS1 mouse brains. Folic acid up-regulated 546 DMROIs in both of these AD models. KEGG pathway mapping of these genes revealed that they were enriched in JAK-STAT, LTD, neuroactive ligand-receptor interaction, Fc epsilon RI signaling pathway, pantothenate and CoA biosynthesis, cyanoamino acid metabolism, basal cell carcinoma, galactose metabolism, GnRH signaling pathway, phenylalanine metabolism, phenylalanine metabolism, arrhythmogenic right ventricular cardiomyopathy, endometrial cancer, taurine and hypotaurine metabolism, selenoamino acid metabolism, and histidine metabolism signaling pathways.

Functional network analysis of differentially methylated genes (DMGs) revealed subnetworks based on their functional annotation and known molecular interactions. The subnetworks were composed of 24 focus genes in the JAK-STAT signaling pathway (Fig. 5, marked with stars) and 12 focus genes in the LTD signaling pathway (Fig. 6, marked with



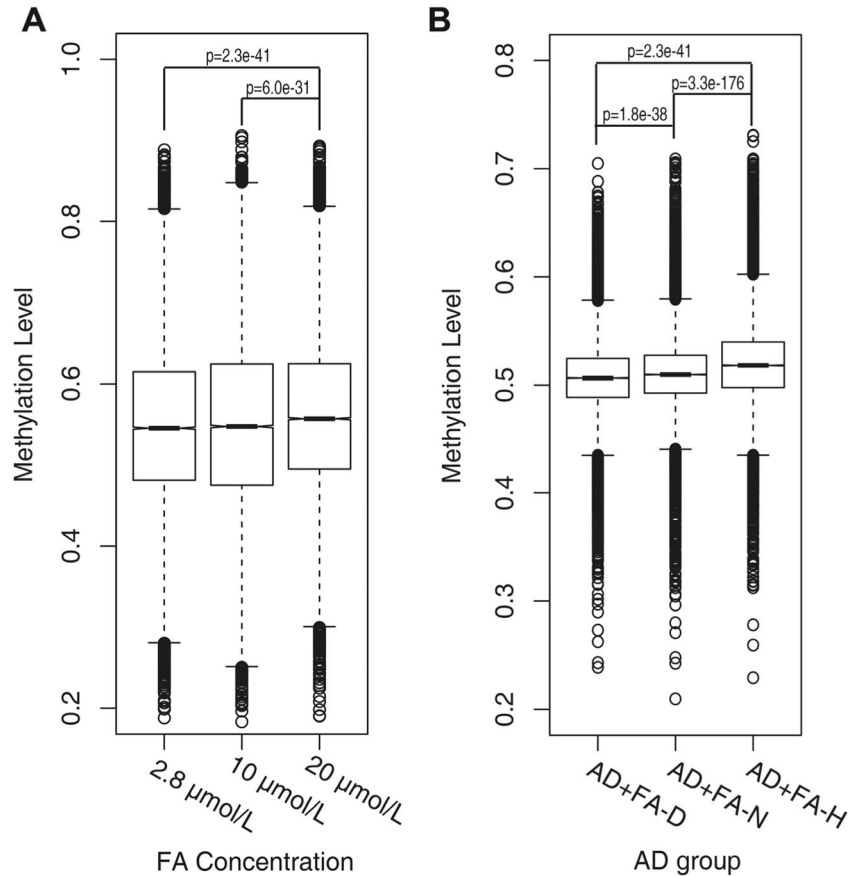
**Fig. 1** The distribution of DNA methylation in N2a-APP cells samples and APP-PS1 mouse brains. Cultures of N2a-APP cells were incubated with 2.8, 10, or 20  $\mu\text{mol/L}$  folic acid for 96 h (three cell cultures per treatment group). APP/PS1 mice were treated with folate-deficient diet plus daily 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  $\mu\text{g/kg}$  folic acid (AD+FA-H) for 60 days (three mice per treatment group). The *line* in each box represents the median methylation level

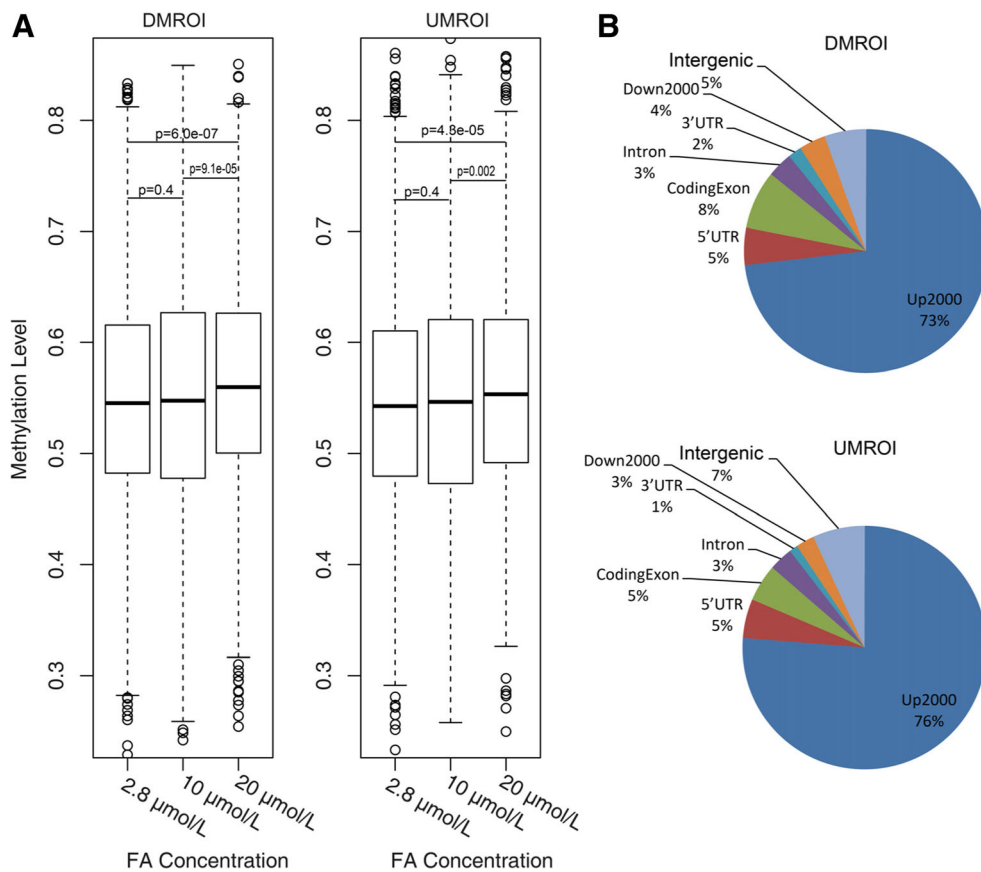
stars). The focus genes for JAK-STAT signaling pathway included colony stimulating factor (CSF) 2, growth hormone, growth factor receptor-bound protein 2 (Grb2), interferon

gamma (IFNG), interferon gamma receptor 1 (IFNGR1), interleukin (IL) 3, IL4, IL5, IL12B, IL13, IL 20 receptor alpha chain (IL20RA), IL22RA2, IL22, IL22b, leukemia inhibitory

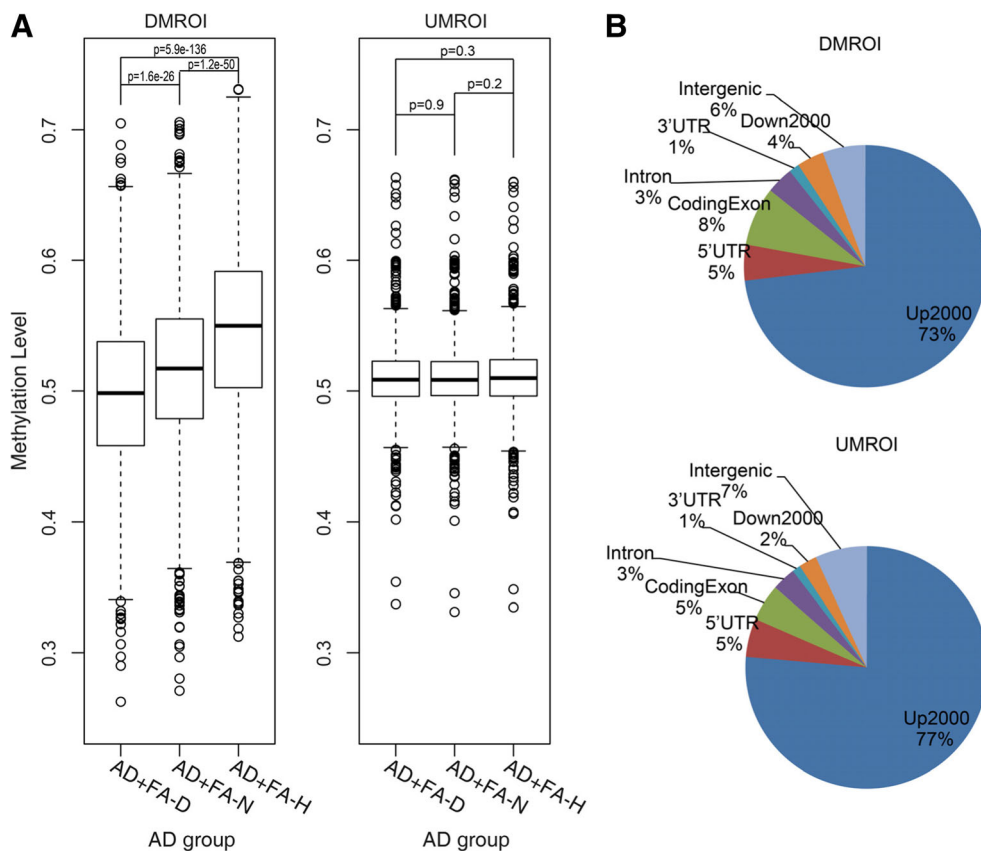
**Fig. 2** Folic acid increases DNA methylation level in N2a-APP cells and APP/PS1 mice. The cells and mice were treated as described in Fig. 1. Shown are data for three cell cultures or mice per treatment group. The *line* in each box represents the median methylation level. *t* tests were performed to determine the *p* values shown

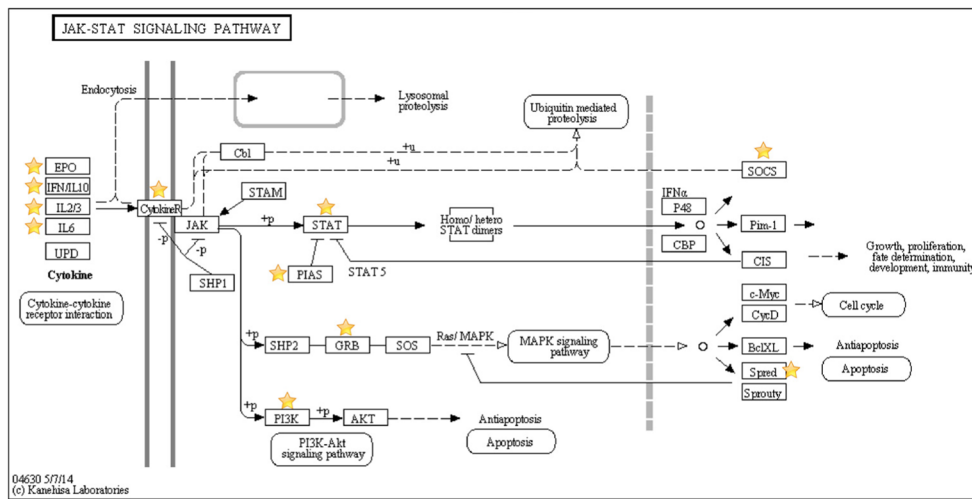


**Fig. 3** Cluster analysis of the effects of folic acid on DMRs in N2a-APP cells. The cells were treated as described in Fig. 1. The *line* in each box represents the median methylation level. **a** The DNA methylation level for DMROIs and UMROIs. Shown are data for three cell cultures per treatment group. **b** The genome distribution for DMROI and UMROI



**Fig. 4** Cluster analysis of the effects of folic acid on DMRs in APP/PS1 mice. The mice were treated as described in Fig. 1. The *line* in each box represents the median methylation level. **a** The DNA methylation level for DMROIs and UMROIs. Shown are data for three cell mice per treatment group. **b** The genome distribution for DMROI and UMROI





**Fig. 5** DMGs in JAK-STAT signaling pathway. Stars indicate the folic acid-induced DMGs in the JAK-STAT signaling pathway. The focus genes for JAK-STAT signaling pathway included colony stimulating factor (CSF) 2, growth hormone, growth factor receptor-bound protein 2, interferon gamma (IFNG), interferon gamma receptor 1 (IFNGR1),

interleukin (IL) 3, IL4, IL5, IL12B, IL13, IL 20 receptor alpha chain (IL20RA), IL22RA2, IL22, IL22b, leukemia inhibitory factor, oncostatin M, protein inhibitor of activated STAT 4, phosphoinositide-3-kinase 5, suppressor of cytokine signaling 3, sprouty-related EVH1 domain containing 2, STAT 3, STAT 5A, STAT 5B, and STAT 6

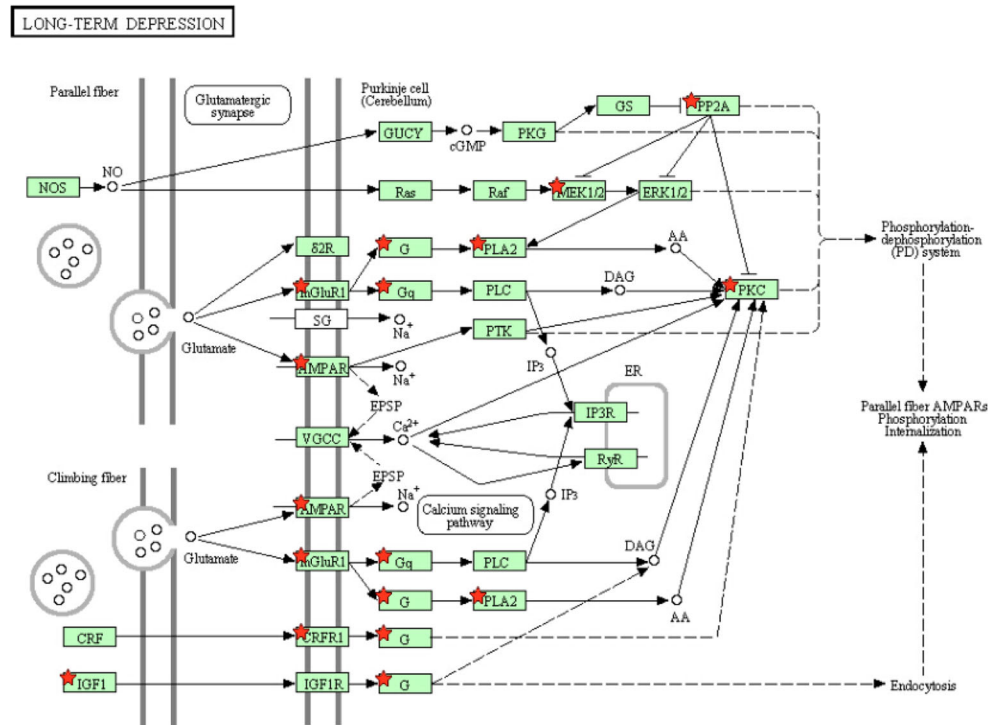
factor, oncostatin M, protein inhibitor of activated STAT 4, phosphoinositide-3-kinase, regulatory subunit 5 (Pik3r5), suppressor of cytokine signaling 3, sprouty-related EVH1 domain containing 2, STAT 3, STAT 5A, STAT 5B, and STAT 6 (Fig. 5). The focus genes for LTD signaling pathway included corticotropin releasing hormone receptor (CRHR) 1, insulin-like growth factor (IGF) 1, guanine nucleotide binding protein (GNA) 11, GNA 13, GNAZ, glutamate receptor (GR) IA1, GRM1, mitogen-activated protein kinase kinase 2 (Map2k2),

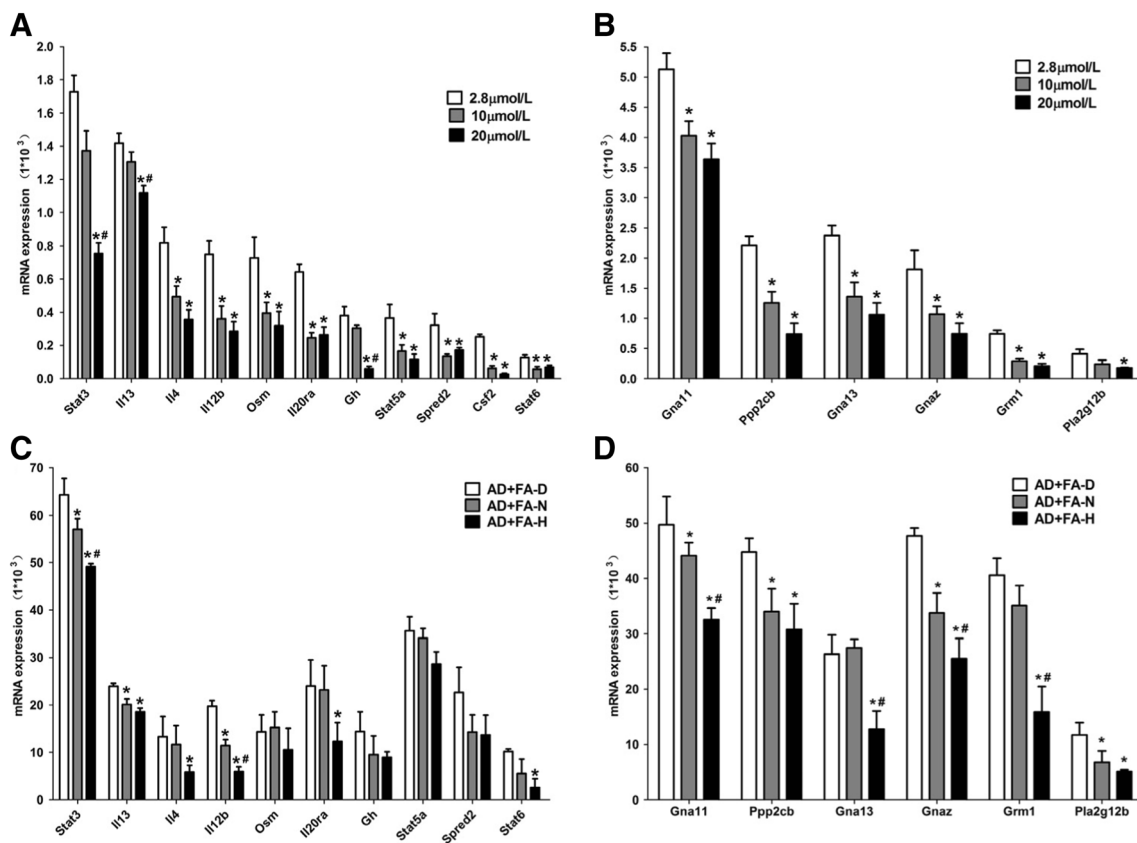
phospholipase A2 (PLA) G12B, PLA2G3, serine/threonine protein phosphatase 2Ac (PP2Ac), and protein kinase C  $\alpha$  (Prkca) (Fig. 6). Thus, folic acid modifies the methylation profiles of the JAK-STAT and LTD signaling pathways.

**Validation of Differentially Methylated Genes**

Real-time PCR detected, respectively, 24 genes of AKT-STAT and 12 genes of LTD signaling pathways (Fig. 7).

**Fig. 6** DMGs in long-term depression signaling pathway. Stars indicate the folic acid-induced DMGs in the LTD signaling pathway. The focus genes for LTD signaling pathway included corticotropin releasing hormone receptor (CRHR) 1, insulin-like growth factor (IGF) 1, guanine nucleotide binding protein (GNA) 11, GNA 13, GNAZ, glutamate receptor (GR) IA1, GRM1, mitogen-activated protein kinase kinase 2 (Map2k2), phospholipase A2 (PLA) G12B, PLA2G3, serine/threonine protein phosphatase 2Ac, and protein kinase C  $\alpha$





**Fig. 7** Twenty-four genes of AKT-STAT and 12 genes of LTD signaling pathways respectively were detected by real-time PCR. Cultures of N2a-APP cells were incubated with 2.8, 10, or 20  $\mu\text{mol/L}$  folic acid for 96 h (three cell cultures per treatment group). APP/PS1 mice were treated with 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  $\mu\text{g/kg}$  folic acid (AD+FA-H) for 60 days (five mice per treatment group). Shown are the gene expression levels that were significantly changed by folic acid. **a** Expression of the STAT3, IL13, IL4, IL12b, Osm, IL20ra, Gh, STAT5a,

Spred2, Csf2, and Stat6 genes of the AKT-STAT signaling pathway in N2a-APP cells. **b** Expression of the GNA11, Ppp2cb, Gna13, Gnaz, Grm1, and Pla2g12b genes of the LTD signaling pathway in N2a-APP cells. **c** Expression of the genes of the AKT-STAT signaling pathway in APP/PS1 mice. **d** Expression of the genes of the LTD signaling pathway in APP/PS1 mice. The plotted values are mean  $\pm$  SD values for three experiments for cell or five mice. \* $P < 0.05$  compared with the lowest concentration of folic acid. # $P < 0.05$  compared with middle concentration of folic acid

Folic acid significantly decreased the expression of STAT3, IL13, IL4, IL12b, Osm, IL20ra, Gh, STAT5a, Spred2, Csf2, Stat6, GNA11, Ppp2cb, Gna13, Gnaz, Grm1, and Pla2g12b in N2a-APP cells. Also, folic acid significantly decreased the expression of STAT3, IL13, IL4, IL12b, IL20ra, Stat6, GNA11, Ppp2cb, Gna13, Gnaz, Grm1, and Pla2g12b in APP/PS1 mice. Therefore, folic acid both decreased the expression of STAT3, IL13, IL4, IL12b, IL20ra, Stat6, GNA11, Ppp2cb, Gna13, Gnaz, Grm1, and Pla2g12b in both AD models.

## Discussion

The present study found that DNA methylation levels were higher in N2a-APP cell cultures than those in APP/PS1 mouse brains. Despite this difference in baseline levels, folic acid dose-dependently increased DNA methylation and up-

regulated 546 DMROIs in both AD models. The present finding that folic acid stimulated DNA methylation level is consistent with previous observations in other AD models [15]. This effect of folic acid may be important clinically because DNA methylation is altered in the brains of AD patients [7–9].

In the present study of AD models, functional network analysis of folic acid-induced DMGs revealed subnetworks composed of focus genes in the JAK-STAT and LTD signaling pathways. Previously, an effect of folic acid on the JAK-STAT signaling pathway was found by functional network analysis of DMGs in neural stem cells [16]. A growing body of evidence suggests that this pathway mediates gene expression in response to cell stress and also plays a key role in the pathogenesis of dementia [17–20]. The observations that folic acid up-regulated the methylation of JAK-STAT signaling pathways and decreased the expression of the stress markers IL13 and IFNGR1 in AD models suggest that folic acid may

decrease cell responses to stress through increased methylation of JAK-STAT signaling pathways in AD models.

The LTD signaling pathway also may be involved in the pathogenesis of AD. LTD is an electrophysiological correlate of memory formation, and it is impaired in APP/PS1 mouse brain [21, 22]. Serine/threonine protein phosphatase 2A can target the transcription factor cAMP response element binding protein (CREB), whose phosphorylation at Ser133 is down-regulated during LTD [23]. Folate metabolism alters the methylation of protein phosphatase 2Ac and PI3K/Akt/CREB [16, 24, 25]. This may explain our observation that LTD signaling pathways were altered by folate deficiency and folic acid supplementation in AD models.

In conclusion, the present study of AD models found that folic acid increases DNA methylation level both in vitro and in vivo. These results revealed a role for folic acid in the JAK-STAT and LTD signaling pathways which may be relevant to AD pathogenesis. This novel finding may stimulate reinvestigation of folic acid supplementation as a prophylactic or therapeutic treatment for AD.

## Methods

### Cell Cultures

Mouse neuroblastoma N2a cells stably expressing human APP695 (N2a-APP cells) were obtained from Professor Huaxi Xu (Institute for Biomedical Research, Xiamen University, Xiamen, China). Cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM)/Opti-MEM (1:1, *v/v*), with 200 µg/ml G418, 10 % fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin, at 37 °C in humidified 5 % CO<sub>2</sub>/95 % air, as described previously [26]. The cells were passaged every 3 days when growing to 80 % confluence. Folic acid-free DMEM powder was purchased from Gibco-BRL (Paisley, UK) and combined with predetermined amounts of folic acid to make culture media for the experiment. Cell cultures were exposed to folic acid (2.8–20 µmol/L) for 96 h immediately prior to the end of the experiment.

### Mice and Diets

The Tianjin Medical University Animal Ethics Committee approved the experimental protocols in this study (study number TMUaMEC 2012016). Male mice with APP<sup>swe</sup>/PS1<sup>ΔE9</sup> mutations (APP/PS1 mice), backcrossed to C57Bl6/J, were obtained from Chinese Academy of Medical Sciences Institute of Laboratory Animal Sciences. These double transgenic mice are models of AD because they express a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695<sup>swe</sup>) and a mutant human presenilin 1 (PS1-ΔE9), both directed to central nervous system neurons under

the control of independent mouse prion protein promoter elements [27]; consequently, young adult APP/PS1 mice deposit amyloid β-peptide in neuritic plaques that are absent in age-matched wild-type mice. After genotyping, the APP/PS1 mice were maintained on the control diet until age 7 months and then were assigned in equal numbers to three groups for 60 days. Those groups were 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 folic acid 0.2 mg/kg diet) and the control diet (folic acid 2.1 mg/kg diet) were purchased from TestDiet (St. Louis, USA). All mice received food and drinking water ad libitum. At the conclusion of the experiment, the mice were anesthetized by intraperitoneal injection of 7 % chloral hydrate (5 ml/kg) and perfused transcardially with PBS. Brains were removed, bisected in the sagittal plane, and stored at -80 °C.

### MeDIP-Chip Assay

Genomic DNA was extracted from N2a-APP cells and APP/PS1 mouse brains (whole brain) using the DNeasy Blood & Tissue Kit (QIAGEN, Fremont, CA). The DNA was sheared to approximately 200–1000 bp with a Bioruptor sonicator (Diagenode, Ougrée, Belgium) on “Low” mode for 10 cycles of 30 s “on” and 30 s “off”. Immunoprecipitation of methylated DNA was performed using Biomag<sup>TM</sup> magnetic beads coupled to mouse monoclonal anti-5-methylcytidine antibody (Diagenode, Ougrée, Belgium). DNA (1 µg) was heat-denatured at 94 °C for 10 min, rapidly cooled on ice, and immunoprecipitated overnight with 1 µL primary antibody in 400 µL immunoprecipitation buffer (0.5 % BSA in PBS, 4 °C). To recover the immunoprecipitated DNA fragments, 200 µL of anti-mouse IgG magnetic beads were added and incubated for an additional 2 h at 4 °C with agitation. After immunoprecipitation, a total of five washes were performed with ice-cold immunoprecipitation buffer. Washed beads were resuspended in TE buffer with 0.25 % SDS and 0.25 mg/mL proteinase K for 2 h at 65 °C and then cooled to room temperature. MeDIP DNA was purified using Qiagen MinElute columns (QIAGEN, Fremont, CA) and amplified using the GenomePlex<sup>®</sup> Complete Whole Genome Amplification kit (Sigma-Aldrich). The amplified DNA samples were then purified with the QIAquick PCR purification kit (QIAGEN, Fremont, CA) and quantified with the NanoDrop ND-1000. For DNA labeling, the NimbleGen Dual-Color DNA Labeling Kit was used according to the NimbleGen MeDIP-chip protocol (Nimblegen Systems, Inc., Madison, WI, USA). DNA (1 µg) from each sample was incubated for 10 min at 98 °C with 1 OD of Cy5-9mer primer (IP sample) or Cy3-9mer primer (input sample). Then 100 pmol of deoxynucleoside

triphosphates and 100 U of the Klenow fragment (New England Biolabs, USA) were added and the mix incubated at 37 °C for 2 h. The reaction was stopped by adding 0.1 volume of 0.5 mol/L EDTA and the labeled DNA was purified by isopropanol/ethanol precipitation. The total input and immunoprecipitated DNA were labeled with Cy3- and Cy5-labeled random nonamers, respectively, and hybridized to the NimbleGen Rat CpG Promoter array, which is a single array design containing 15,809 CpG islands and all well-characterized RefSeq promoter regions (from about -1300 to +500 bp of the transcription start site (TSS)) totally covered by ~385,000 probes. Scanning was performed with the Axon GenePix 4000B microarray scanner. Raw data were extracted as pair files by NimbleScan software. Median centering, quantile normalization, and linear smoothing were performed by Bioconductor packages Ringo, limma, and MEDME. From the normalized log<sub>2</sub>-ratio data (immunoprecipitated/input), a sliding-window peak-finding algorithm provided by NimbleScan.v2.5 (Roche-NimbleGen) was applied to find the enriched peaks with specified parameters (sliding window width, 750 bp; mini probes per peak, 2; *P* value minimum cutoff, 2; maximum spacing between nearby probes within peak, 500 bp). Then the log<sub>2</sub>-ratio methylation level was expressed in a range from 0 to 1 (full methylation) using BATMAN software developed by Down et al. [28]. The BATMAN was used to calculate enrichment from the MeDIP-chip data.

Quantitative Differentially Methylated Regions analysis was used to quantify DNA methylation difference and identify by entropy the differentially methylated regions (DMRs) from genome-wide methylation profiles of cell cultures and mice in the various folic acid treatment groups [28, 29]. A lower entropy indicates a larger DNA methylation difference. Therefore, for cluster analysis of dose-dependent effects of folic acid, DMROI were defined as DMRs with entropy values in the lowest 10 % and undifferentially methylated ROI (UMROI) were defined as DMRs with entropy values in the highest 10 %.

### Genome Categories of ROIs

For each ROI, the nearest gene were searched and classified into seven genome categories in Up2kb, 5'UTR, CodingExon, Intron, 3'UTR, Down2kb, and Intergenic as described in Zhang et al. [29]. The promoter ROIs were those localized in Up2kb and 5'UTR.

### Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis

The number of DMRs was calculated for each gene region, which was defined as the 1300 bp upstream of the TSS to the transcription termination site (TTS). Each gene having at least

three DMRs was considered to be a differentially methylated gene (DMG) and was selected for pathway analysis.

Functional network analyses of DMROIs and DMGs were performed by KEGG pathway mapping (<http://www.genome.jp/kegg/>). The KEGG database was used to reveal the gene network of DMGs (20, 21). The KEGG pathway map is a molecular interaction network diagram represented in terms of the KEGG ontology groups, so that experimental evidence from a specific organism can be generalized to other organisms through genomic information [30, 31]. Enrichment was calculated as (ng/na)/(Ng/Na), where ng is the number of differential genes within the particular pathway, na is the total number of genes within the same pathway, Ng is the number of differential genes which have at least one pathway annotation, and Na is the number of genes which have at least one pathway annotation in the entire microarray. A Fisher exact test was used to find significant enrichment pathways. The resulting *P* values were adjusted using the BH FDR algorithm [32] and pathways with FDR < 0.05 were reported.

### Validation of Differentially Methylated Genes by Real-Time PCR

Real-time PCR was used to quantify the relevant genes expression of Akt-STAT and LTD signaling pathway. Total RNA was extracted using Trizol according to the instructions of the manufacturer. RNA quality was good, with 260/280 ratios slightly higher than 2.0 and 260/230 ratios slightly higher than 1.8. First-strand cDNA was synthesized from 2 µg total RNA using MMLV reverse transcriptase. The 20-µl reaction volume was incubated for 50 min at 42 °C, 5 min at 90 °C, 5 min at 5 °C, and then held at -20 °C. cDNA was then added to the RT<sup>2</sup> SYBR Green qPCR Master Mix (QIAGEN, Fremont, CA). Next, each sample was aliquoted on RT<sup>2</sup> profiler PCR arrays which contain dried assays (Custom Catalog Number: CAPM13059). All steps were done according to the manufacturer's protocol for the Roche 480 Sequence Detection System. Data normalization was based on correcting all Ct values for the average Ct values of several constantly expressed housekeeping genes (HKGs) present on the array. PCR array analysis results were evaluated and also compared to the outcome of the MeDIP-chip assay.

### Statistical Analysis

Differences between mean values of MeDIP-chip assay were compared using the *t* test or Fisher exact test. Real-time PCR data were expressed as mean ± 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.



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### Compliance with Ethical Standards

**Conflict of Interest** The authors state that they have nothing to disclose and that there are no potential conflicts of interest.

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