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REPORT

Expression Profiles of *SIRT1* and *APP* Genes in Human Neuroblastoma SK-N-SH Cells Treated with Two Epigenetic Agents

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Abstract In our previous studies, significant hypermethylation of the sirtuin 1 (*SIRT1*) gene and demethylation of the β-amyloid precursor protein (*APP*) gene were found in patients with Alzheimer's disease (AD) compared with the normal population. Moreover, the expression of *SIRT1* was significantly decreased while that of *APP* was increased in AD patients. These results indicated a correlation of DNA methylation with gene expression levels in AD patients. To further investigate the epigenetic mechanism of gene modulation in AD, we used two epigenetic drugs, the DNA methylation inhibitor 5-aza-2′-deoxycytidine (DAC) and the histone deacetylase inhibitor trichostatin A (TSA), to treat human neuroblastoma SK-N-SH cells in the presence of amyloid β-peptide $A\beta_{25–35}$($A\beta_{25–35}$). We found that DAC and TSA had different effects on the expression trends of *SIRT1* and *APP* in the cell model of amyloid toxicity. Although other genes, such as microtubule-associated protein τ, presenilin 1, presenilin 2, and apolipoprotein E, were up-regulated after $A\beta_{25–35}$ treatment, no significant differences were found after DAC and/or TSA treatment. These results support the evidence in AD patients and reveal a strong correlation of *SIRT1*/*APP* expression with DNA methylation and/or histone modification, which may help understand the pathogenesis of AD.

Keywords *Sirtuin 1* · DNA methylation · Histone modification · Alzheimer's disease · Trichostatin A

Introduction

Epigenetics refers to the reversible regulation of various genomic functions occurring without a change in the DNA sequence. By modulating chromatin structure, gene transcription, and gene expression, epigenetic processes can result in long-term changes in cellular function across various pathways [1, 2]. The most frequently-studied epigenetic mechanisms are DNA methylation and histone modification. DNA methylation often occurs through the addition of a methyl radical to the cytosine base adjacent to a guanine (CpG dinucleotides). Usually, when DNA is methylated in the gene promoter region, the expression or function of the gene is changed [3]. Histone modification is another important epigenetic marker. Histone acetylation is linked with transcriptional activation, while deacetylation is associated with transcriptional repression [1, 2, 4]. An increasing number of studies have shown that epigenetic mechanisms are involved in the development of many diseases, including cancer and neurological disorders. During the progression of Alzheimer's disease (AD), changes in epigenetic mechanisms occur, such as DNA methylation and histone modification [5]. It is clear that

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mutations of AD-related genes are associated with AD, but some epigenetic dysfunctions are also involved in the pathological process of AD. For example, we previously showed the existence of relationships between the methylation profiles of silent information regulator two proteins 1 (*SIRT1*) and the severity of AD [6].

Silent information regulator two proteins (sirtuins or SIRTs), named after their yeast homologue (silent information regulator 2), are a group of histone deacetylases (HDACs) whose activities are dependent on and regulated by nicotinamide adenine dinucleotide (NAD^+). To date, seven sirtuins (SIRT1 to SIRT7) have been identified. Among them, SIRT1 is the best-characterized [7]. SIRT1 has been associated with metabolism, stress responses, cellular survival, transcription, aging, and various other processes [7, 8]. Moreover, accumulating evidence has suggested that SIRT1 plays an important role in neuronal functions, the aging process, AD, and other neuro-degenerative diseases. SIRT1 not only protects axons from degeneration [9], but also regulates memory and synaptic plasticity in the hippocampus [10]. Besides, loss of SIRT1 is closely associated with the accumulation of β-amyloid (Aβ) and microtubule-associated protein τ (Tau) in the cerebral cortex of AD patients [11]. Therefore, SIRT1 regulates memory and synaptic plasticity, providing insights into potential intervention against age-associated cognitive disorders. In addition, other genes such as β-amyloid precursor protein (*APP*), microtubule-associated protein τ (*Tau*), presenilin 1 (*PS1*), presenilin 2 (*PS2*), and apolipoprotein E4 (*ApoE4*) are known to be directly involved in the pathology/progression of AD. Expression changes or mutations in these genes may play important roles in the pathological process of AD [2, 12–14].

Our previous study with clinical samples showed, for the first time, significant hypermethylation of the *SIRT1* gene and demethylation of the *APP* gene in AD patients compared with the normal population. Meanwhile, significantly decreased expression of *SIRT1* and increased expression of *APP* have also been found in AD patients [6]. These results indicated that there may be relationships between DNA methylation behavior and the expression of these genes. However, how epigenetic modifications modulate the expression of these genes remains unclear. Therefore, in this study, we set out to further investigate the effects of epigenetic modifications on the expression profiles of these genes in a cell model.

Materials and Methods

Cell Culture

The human neuroblastoma cell line SK-N-SH was purchased from the American Type Cell Culture (ATCC, Rockville, MD). Cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 2 mmol/L glutamine, 100 μg/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified incubator containing an atmosphere of 5% CO_2.

Establishment of Amyloid Toxicity Cell Model and Drug Treatment

$A\beta_{25–35}$ (Sigma, St. Louis, MO) was diluted to 100 mmol/L stock solution with distilled water, incubated at 37 °C for 7 days, filter-packed, and stored at −20 °C. This stock solution was diluted in DMEM to different concentrations (5–30 μmol/L) and then added to SK-N-SH cells. To determine the suitable drug concentrations and incubation times, cell viability was tested. Finally, 20 μmol/L $A\beta_{25–35}$ for 48 h was chosen to establish the amyloid toxicity model.

The model cells were further treated with 5 μmol/L 5-aza-2′-deoxycytidine (DAC) (Sigma) and/or 200 nmol/L trichostatin A (TSA) (Sigma) for 12–72 h, and the medium with different drug concentrations was replaced every 24 h.

For viability tests, SK-N-SH cells were plated at 8×10^3 cells/well in 96-well plates, and incubated with 10% fetal calf serum and DMEM. The drug-treatment time was from 12 h to 6 days. During treatment, medium with different concentrations of $A\beta_{25–35}$, DAC, and/or TSA in each group was replaced every 24 h. After cells were treated for 24 h, the supernatant was replaced and 20 μL of 5 mg/mL MTT (Sigma) was added to each well and incubated for 4 h. Then 150 μL dimethyl sulfoxide was added, and after gentle vibration, the absorbance at 570 nm and 633 nm was measured on a Sunrise enzyme immunoassay instrument (Tecan, Mannedorf, Sweden). Cell viability = (average absorbance value of experimental group/average absorbance value of control group) × 100%.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from cultured SK-N-SH cells using the Trizol reagent protocol. The quality of total RNA was determined from the A_{260}/A_{280} ratio, which was 1.7–2.0 for all RNA preparations. A total of 2 μg RNA was reverse transcribed and synthesized into cDNA according to the reverse transcriptase kit instructions (Takara, Dalian, China) and the cDNA was stored at −80 °C.

Real-Time PCR

Taq-Man probe quantitative real-time PCR was performed using an Applied Biosystems 7500 Real-Time PCR detection system (Applied Biosystems, Foster City, CA), with the program running for 40 cycles at 95 °C for

15 min, 94 °C for 15 s, and 55 °C for 45 s. The target gene expression levels were normalized to *β-actin*. Primers and probes for different target genes and *β-actin* were designed using Beacon Designer 7.0 software (Table 1). Real-time PCR was repeated at least five times. Relative quantification of target mRNA was analyzed by the comparative threshold cycle (Ct) method [15].

Statistical Analysis

Data are expressed as mean ± SD and were analyzed by one-way ANOVA with post-hoc tests using SPSS ver. 13.0. $P < 0.05$ was considered to be statistically significant.

Results and Discussion

We used the human neuroblastoma cell line SK-N-SH for epigenetic drug treatment. SK-N-SH cells exhibit a neuronal phenotype with multiple neurochemical markers [16, 17] and are widely used in cell-mediated cytotoxicity assays. We showed that DAC and TSA treatment significantly reduced the viability of these cells and the cytotoxic effect was dependent on the drug concentration and treatment time (Fig. 1A, B). TSA was more toxic to SK-N-SH cells than DAC. Treatment with 400 nmol/L TSA for 96 h caused a cell inhibition rate >80% (Fig. 1B). To ensure cell growth and a demethylation/acetylation effect, 5.0 μmol/L DAC and 200 nmol/L TSA were used for further experiments.

Aβ accumulates to form a filamentous precipitate, the main component of senile plaques in the AD brain, and is a major cause of AD pathogenesis [18]. Among its complex mechanisms of action, its toxicity to neurons promotes AD progression and memory impairment [19]. $A\beta_{25–35}$, the toxic fragment of $A\beta_{42}$, can be used as an inducer of apoptosis to cause apoptosis in neurons with a dose-response and time-course similar to that of $A\beta_{42}$ [20]. In the present study, $A\beta_{25–35}$ significantly reduced the viability of SK-N-SH cells, and the level of inhibition was dose- and time-dependent (Fig. 1C). Based on previous reports [21–23] and the present study, 20 μmol/L $A\beta_{25–35}$ exposure for 48 h was used to establish the amyloid toxicity cell model.

mRNA Expression Levels of *SIRT1* and AD-Related Genes in Human Neuroblastoma SK-N-SH Cells Treated with DAC, TSA, or Both

Compared with the control group, the mRNA expression level of *SIRT1* in SK-N-SH cells was increased by 48- and 72-h treatment with DAC, TSA, or both ($P < 0.001$).

Table 1 Primers and probes for amplification of different target genes and the internal control gene (*β-actin*) by quantitative real-time PCR.

Gene	Primer and probe	Product size (bp)
β-actin	F: 5′ GACGACATGGAGAAAATCTG 3′	144
	R: 5′ GAAGGTCTCAAACATGATCTG 3′	
	P: 5′ Yellow ACCACACCTTCTACAATGAGCTGC Tamra 3′	
SIRT1	F: 5′ GTTGCTTTAGAAACATTAGTG 3′	123
	R: 5′ GCAGTTTAATACTTGTGGAA 3′	
	P: 5′ FAM CAATGCAAGCTCTACCACAGTGATAGG Tamra 3′	
APP	F: 5′ CGGTGTCCATTTATAGAATA 3′	143
	R: 5′ GAGAGATAGAATACATTACTGA 3′	
	P: 5′ FAM TCAGGCATCTACTTGTGTTACAGCA Tamra 3′	
Tau	F: 5′ AGGGACATGAAATCATCTTA 3′	129
	R: 5′ CAGAGTAATAACTTTATTTCCAAA 3′	
	P: 5′ FAM TCACTTTTACAGCAACAGTCAGTGT Tamra 3′	
PS1	F: 5′ GGATCATTTACTCTCACATG 3′	135
	R: 5′ CAGCTATCAAAATTATATCTTACC 3′	
	P: 5′ FAM TGTCTGCCTTCTGCTTCTGTGG Tamra 3′	
PS2	F: 5′ CCTTGTTATTTTATTGCCTTTAG 3′	102
	R: 5′ GGACTCATCTATTTATTGATATTACTA Tamra 3′	
	P: 5′ FAM CTGAGTCCTGTTCTTGTTACGGCA 3′	
ApoE4	F: 5′ GGACGAGGTGAAGGAGCA 3′	130
	R: 5′ CATGTCTTCCACCAGGGG 3′	
	P: 5′ FAM CTCCAGCTTGGCGCGCA Tamra 3′	

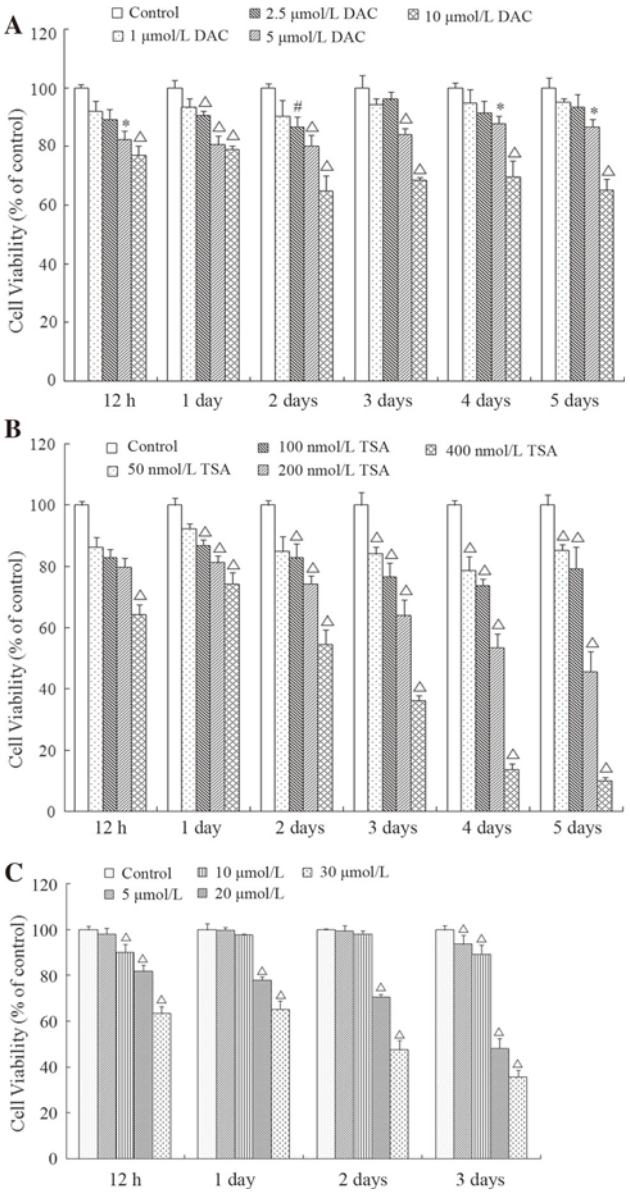

Fig. 1 Effects of various concentrations of DAC (**A**), TSA (**B**), and $A\beta_{25–35}$ (**C**) on the viability of human neuroblastoma SK-N-SH cells ($^{\#}P < 0.05$, $^{*}P < 0.01$, $^{\triangle}P < 0.001$, drug-treated group *vs* corresponding control group; one-way ANOVA with *post-hoc* tests).

However, at 24 and 96 h of treatment, *SIRT1* gene expression was lower in each drug group compared with the control group. In the TSA group, the *SIRT1* mRNA level at 72 h was >8.4-times higher than that in the control group (Fig. 2A).

In addition, the expression level of the *APP* gene was significantly up-regulated at 24 h of drug treatment (DAC, TAS, or both) (Fig. 2B). For the *Tau* gene, 72-h treatment with a combination of DAC and TSA significantly increased its mRNA expression level, which was about 3.3-times that of the control group (Fig. 2C). *PS1* gene expression was significantly higher than that in the control group at 72 h of TSA treatment and at 48 and 72 h of combined DAC/TSA treatment (Fig. 2D). The *PS2* gene expression level at 24 h of DAC treatment, as well as at 24, 48, and 72 h of TSA treatment was significantly higher than that in the control group (Fig. 2E). The *ApoE4* gene expression level at 48 h of treatment with a combination of DAC and TSA was 3.9-fold that in the control group. In the DAC or TSA treatment group, significant increases in the *ApoE4* gene expression level were also found at 48 h (Fig. 2F).

mRNA Expression Levels of *SIRT1* and AD-Related Genes in the Amyloid Toxicity Cell Model Treated with DAC, TSA, or Both

Compared with the control group, the expression level of *SIRT1* was down-regulated in the $A\beta_{25–35}$-treated group (Fig. 3A). This result is consistent with our previous finding in AD patients [6] and further confirmed successful establishment of the amyloid toxicity cell model. Interestingly, when DAC, TSA, or both was added to the $A\beta_{25–35}$-treated cells, a higher expression level of *SIRT1* was measured (Fig. 3A). After DAC treatment for 24 h, the *SIRT1* expression level was 7.7 times that of the control group. However, significant up-regulation of *SIRT1* was not found until 3 days of treatment (Fig. 3A). This could be due to a change in the methylation pattern that caused higher expression of *SIRT1*. Compared with the control group, though the *SIRT1* level was up-regulated at both 48 and 72 h, the changes in level were negatively correlated with treatment time. In the TSA group, the *SIRT1* level was significantly increased at 24, 48, and 72 h of treatment, with the greatest change at 24 h. Besides, combined DAC/TSA treatment also increased the *SIRT1* expression level, especially at 24 h. These results indicated that not only could DNA methylation and histone modification each affect gene transcription, but they may also interact to regulate the transcription of some genes [2]. SIRT1 is an NAD^{+}-dependent HDAC that can influence chromatin structure by histone acetylation or inhibit or silence gene transcription through DNA methylation [24]. Therefore, the results indicate that DNA methylation and histone modification may be important factors for the expression or function of SIRT1.

The *APP* gene expression level was up-regulated in the amyloid toxicity cell model compared with the control group. This is consistent with our previous studies on AD patients [6]. In addition, DAC significantly increased the *APP* expression level in this model at 24 and 48 h of treatment, and the level at 48 h of treatment in the DAC group was 2.3-fold that of the control group. TSA also increased the *APP* gene expression level at 24, 48, and 72 h of treatment. Similar results were also found for the

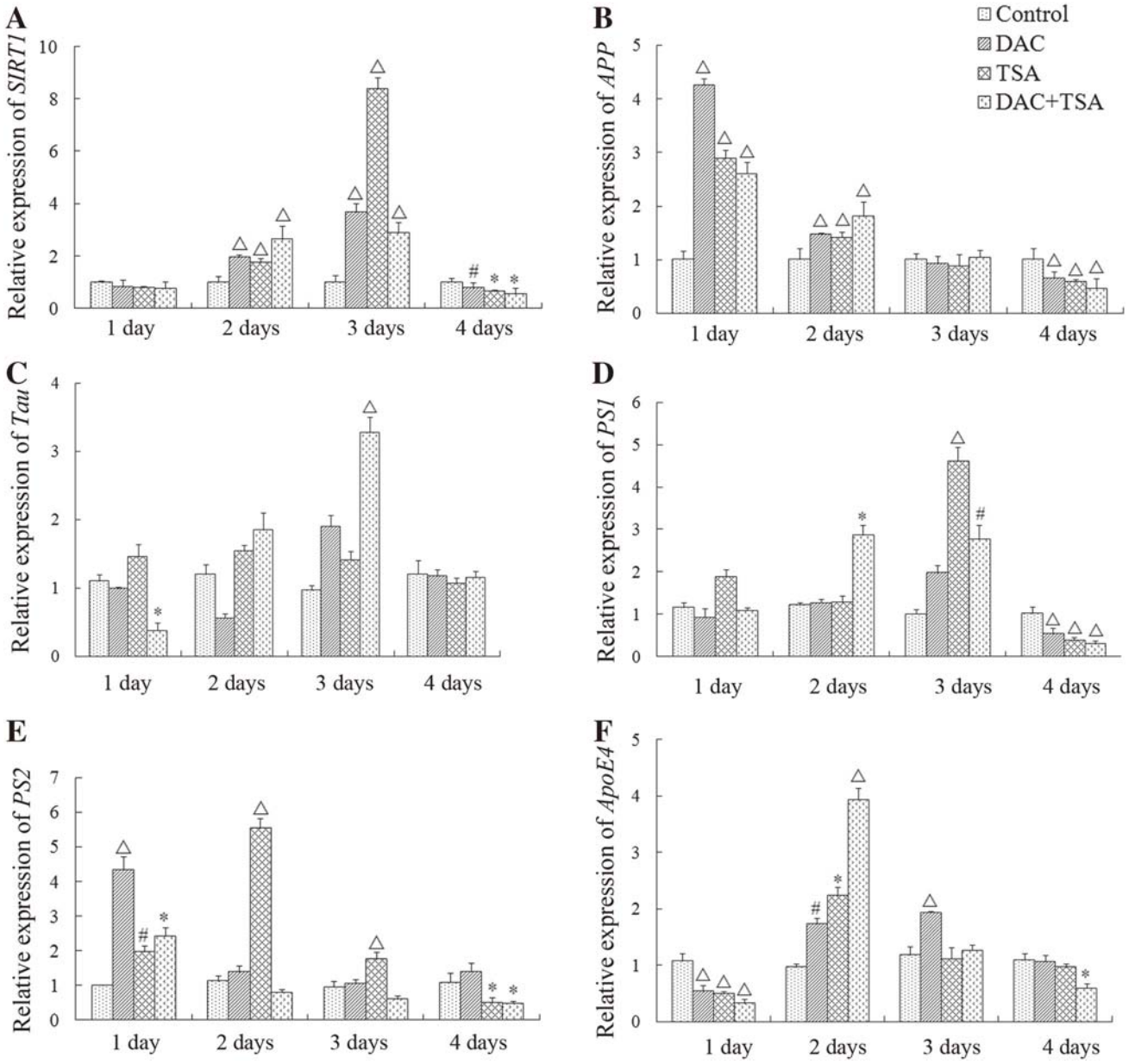

Fig. 2 mRNA expression levels of *SIRT1* (**A**) and AD-related genes *APP* (**B**), *Tau* (**C**), *PS1* (**D**), *PS2* (**E**), and *ApoE4* (**F**) in human neuroblastoma SK-N-SH cells treated with DAC, TSA, or both ($^{\#}P < 0.05$, $^{*}P < 0.01$, $^{\triangle}P < 0.001$, drug-treated group *vs* corresponding control group; one-way ANOVA with *post-hoc* tests).

combined DAC/TSA treatment. The *Tau* gene expression level was increased significantly in the TSA group at 24 h of treatment, and in the combined DAC/TSA treatment group at 24 and 48 h (Fig. 3C). Similarly, *PS1* expression was significantly increased by combined DAC/TSA treatment at 48 and 72 h, and by TSA treatment at 24 and 48 h compared with the control group. For *PS2*, the gene expression level was significantly increased by combined DAC/TSA at 24 and 48 h of treatment, and by DAC at 24 and 48 h (Fig. 3E). For *ApoE4* gene expression, significant increases were found after DAC, TSA, or combined DAC/TSA treatment for 48 and 72 h. Nonetheless, for these AD-related genes (*Tau*, *PS1*, *PS2*, and *ApoE4*), although the expression level was up-regulated after $A\beta_{25-35}$ treatment, no significant differences in the expression trend between the two cell models after DAC and TSA combination treatment were found. Further studies are needed to explore the underlying mechanisms of up-regulation of these genes after $A\beta_{25-35}$ treatment.

Epigenetics mainly involves changes in gene expression caused by mechanisms other than alterations in the DNA sequence. Among the epigenetic changes, DNA

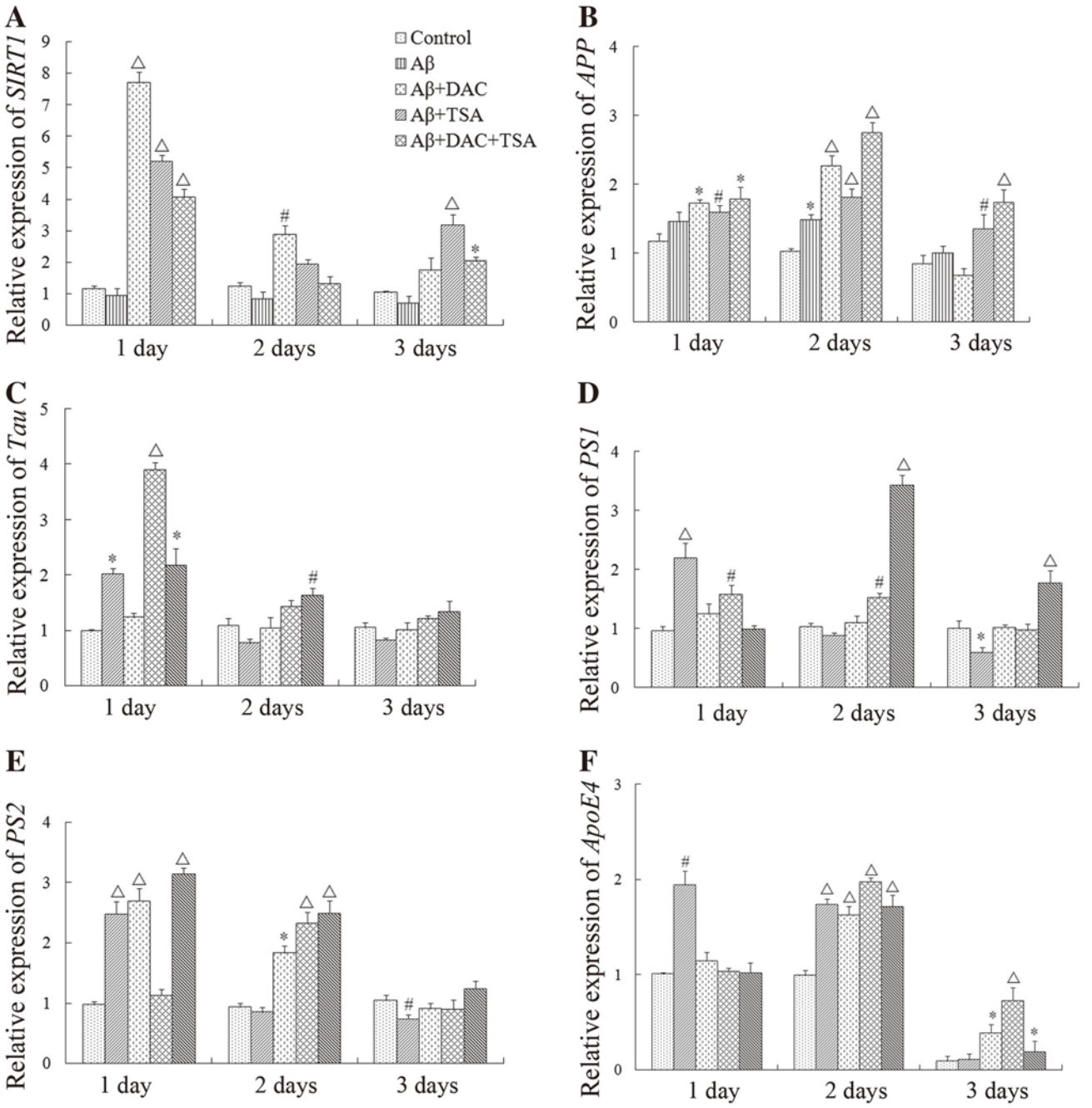

Fig. 3 mRNA expression levels of *SIRT1* (**A**) and AD-related genes *APP* (**B**), *Tau* (**C**), *PS1* (**D**), *PS2* (**E**), and *ApoE4* (**F**) in the amyloid toxicity cell model treated with DAC, TSA, or both ($^{\#}P < 0.05$, $^{*}P < 0.01$, $^{\triangle}P < 0.001$, drug-treated group *vs* corresponding control group; one-way ANOVA with *post-hoc* tests).

methylation and histone acetylation are the most widely studied. DNA methylation at the C5 position of cytosine in CpG dinucleotides is catalyzed by DNA methyltransferases. Histone acetyltransferases and HDACs are enzymes that catalyze the acetylation and deacetylation of histones as well as other proteins. Generally, the methylation of cytosines in and around genes results in gene silencing. DNA methylation alters chromosome structure and defines regions important for transcriptional regulation [25]. Moreover, the dynamic process of histone acetylation/deacetylation has been linked to gene transcription and chromatin remodeling. Our results suggested that the epigenetic drugs DAC and TSA affect *SIRT1* and *APP* gene expression in SK-N-SH cells and an amyloid toxic cell model, consistent with our previous finding that amyloid plays critical roles in the physiological processes of AD-

related genes. Moreover, DNA methylation and histone hyper-acetylation appear to be distinct but also have the potential to interact [26–28]. They have been implicated in the regulation of gene expression and chromatin structure, thus affecting a wide variety of biological processes including transposable element silencing, gene imprinting, and chromosomal inactivation. Studies have demonstrated the potential underlying processes: the patterns of initially established DNA methylation dictate the subsequent formation of local histone acetylation landscapes. And the activity of histone-modifying proteins (such as HDACs) has been found in multiprotein complexes that bind to methylated areas of DNA [29]. Moreover, local histone acetylation states can direct DNA demethylation activity, thus establishing DNA methylation patterns. Results have also shown that histone acetylation and DNA demethylation sequences can be established independent of the cell cycle and replication [30]. On the basis of previous studies, our results further suggest that DNA methylation and histone acetylation may interact to influence the transcription or the functions of the *SIRT1* and *APP* genes. Further investigation of the molecular mechanisms that regulate histone acetylation and DNA methylation will undoubtedly shed more light on potential hierarchical interactions between these two key mechanisms of epigenetic regulation and the role of such interactions in cognitive regulation which contains organization and emotional regulation and cognitive process.

Neuroblastoma SK-N-SH cells are a stable neuronal cell line that is widely and extensively used for studies of AD and other neurodegenerative diseases. The present study was preliminary and more studies are needed to further confirm the mechanism. While it is better to use AD patients, animal models, or even differentiated cells for this work, many difficulties have yet to be solved: epigenomic changes of neuroblastoma cells induced by retinoic-acid differentiation; drugs like TSA and DAC are too toxic to use in people, and also very difficult to use in animal models; and epigenetic drugs are not specific, limiting further studies. In conclusion, studies are still needed to explore the specific mechanisms underlying the effects of DNA methylation/histone acetylation on gene transcription and function. Overall, although various aspects still need further study, our results may contribute to the establishment of early diagnostic markers of AD.

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