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DNA methylation of the 5′-untranslated region at +298 and +351 represses BACE1 expression in mouse BV-2 microglial cells

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ABSTRACT

BACE1, which cleaves the amyloid precursor protein, is the rate-limiting enzyme for β-amyloid peptide production, leading to the pathogenesis of Alzheimer's disease (AD). A high plasma level of homocysteine, acting as a potent methyltransferase inhibitor, is assumed to be a risk factor for AD onset. Using the demethylating drug 5-aza-2′-deoxycytidine (5-Aza), we tested whether and how BACE1 expression is regulated in mouse BV-2 microglial cells. 5-Aza increased both BACE1 mRNA and protein levels in a dose-dependent manner. Bisulfite-sequencing analysis revealed that two CpG sites at positions +298 and +351 in the 5′-untranslated region (5′-UTR) of the BACE1 gene were specifically demethylated in BV-2 cells treated with 5-Aza. *In silico* analysis showed that the +351 site is the STAT3/CTCF-binding site; the function of the +298 site has not been identified. To assess whether these two CpG sites play an important role in 5-Aza-induced transcriptional activation of BACE1, we constructed a BACE1 gene promoter including the 5′-UTR (−1136 to +500) fused to a CpG-free luciferase gene (pCpGL-BACE1) and its mutant pCpGL-BACE1-AA, which has substituted CG dinucleotides at the two CpG sites of pCpGL-BACE1 to AA. Promoter analysis showed a significant decrease (~30%) in the activity of pCpGL-BACE1-AA compared with that of pCpGL-BACE1. Furthermore, *in vitro* methylation of these two reporter constructs showed a complete silencing of their promoter activities. Our data demonstrate that BACE1 gene expression is regulated by DNA methylation of at least two CpG sites at positions +298 and +351 in the 5′-UTR in BV-2 microglial cells.

1. Introduction

Alzheimer's disease (AD) is a progressive and irreversible neurodegenerative disorder that results in memory loss, dementia, and finally death. AD pathology is characterized by senile plaques and neurofibrillary tangles, which are associated with the massive loss of neurons and synapses mainly in the hippocampus and neocortex association regions. The major constituents of senile plaques are β-amyloid peptides (Aβ) of 39–43 amino acids, cleaved from amyloid precursor protein (APP). It is well-known that APP is processed by a group of secretases including α-, β-, and γ-secretase. β- and γ-Secretase generate Aβ from APP, whereas α-secretase generates a soluble product [1,2].

Two β-secretases, BACE1 and BACE2, are involved in the development of Alzheimer's disease by producing Aβ. It was reported that BACE1 was the major β-secretase for Aβ generation in neurons [3] by showing that the secretion of $A\beta_{1-40/42}$ and $A\beta_{11-40/42}$ was abolished in BACE1-knockout neurons. Several studies also demonstrated that increases in BACE1 levels and activities were detected in postmortem AD brains [4,5]. Recently, BACE1 is reported to be significantly augmented in the cerebrospinal fluid of patients with mild cognitive impairment (MCI) and may be an early biomarker of

Abbreviations: AD, Alzheimer's disease; Aβ, β-amyloid peptides; APP, amyloid precursor protein; MCI, mild cognitive impairment; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; 5-Aza, 5-aza-2′-deoxycytidine; 5′-UTR, 5′-untranslated region; pCpGL-BACE1, BACE1 gene promoter including the 5′-UTR (−1136 to +500) fused to a CpG-free luciferase gene; pCpGL-BACE1-AA, mutant pCpGL-BACE1 which has substituted CG dinucleotides at the two CpG sites of pCpGL-BACE1 to AA; pCpGL-unmetBACE1, unmethylated pCpGL-BACE1; pCpGL-metBACE1, methylated pCpGL-BACE1.

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AD [6]. Therefore, the regulation of BACE1 expression is regarded as an important initiating factor in senile plaque formation and AD development. Indeed, several BACE1 inhibitors have proceeded to preclinical or clinical trials [7].

An elevated plasma homocysteine level is generally assumed to be a risk factor for the onset of AD [8]. Using the Korean elderly population, we also reported that plasma homocysteine concentrations were higher in subjects with MCI than in normal elderly patients [9]. S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH), the substrate and product of essential cellular methyltransferase reactions, respectively, are important metabolic indicators of cellular methylation status. The accumulation of homocysteine causes the accumulation of SAH, a strong DNA methyltransferase inhibitor, which reinforces DNA hypomethylation [10]. From this phenomenon, we hypothesized that hyperhomocysteinemia in MCI and AD patients might lead to DNA hypomethylation of genes associated with AD, which act as one of the main causes for sporadic AD.

In order to verify this hypothesis, we investigated whether and how DNA methylation regulated the expression of BACE1 associated with the formation of senile plaques in mouse BV-2 microglial cells by using the DNA demethylating agent, 5-aza-2′-deoxycitidine (5-Aza). Our study identified for the first time the two CpG sites in the 5′-untranslated region (5′-UTR) as the epigenetic regulatory sites responsible for BACE1 expression.

2. Materials and methods

2.1. Cell culture and drug treatments

The mouse BV-2 microglial cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL, Gaithersberg, MD) supplemented with 10% fetal bovine serum (FBS), 0.37% $NaHCO_3$, penicillin (100,000 unit/l) and streptomycin (100 mg/l) in attachment factor-coated culture dishes at 37 °C in 5% CO_2. When the cells reached 80% confluence, they were incubated with the indicated concentrations of 5-Aza (Sigma, St. Louis, MO) in DMEM supplemented with 5% FBS for 24 h.

2.2. Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from the cells with or without treatment of 5-Aza using TRIzol Reagent (Invitrogen, Carlsbad, CA) as described [11], and was used for reverse transcription (RT) reactions. RT reaction was performed using 200 units of SuperScript™ III reverse transcriptase (Invitrogen) in a 20 μl reaction mixture containing 1 μg RNA for 1.5 h at 42 °C. PCR was subsequently performed in a volume of 20 μl, containing 0.5 units of Taq polymerase (Solgent, Seoul, Korea), 10 pmol of each primer, 0.25 mM dNTPs, and 2 μl of the RT sample. The amplified products were separated using a 1.5% agarose gel in TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA). The band of the target gene was visualized using 1 mg/ml ethidium bromide under UV. The following PCR primer pairs were designed to detect each gene: BACE1-F, 5-CAC CAT CCT TCC TCA GCA AT-3; BACE1-R, 5-AAC AAA CGG ACC TTC CAC TG-3; β-actin-F, 5-ATT GCT GAC AGG ATG CAG AA-3; and β-actin-R, 5-CCG ATC CAC ACA GAG TAC TT-3.

2.3. Western blot analysis

For Western blot analysis, cells were treated with indicated concentrations of 5-Aza, washed with ice-cold DPBS, and then lysed in lysis buffer 1 [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 mM β-glycerophosphate, 1 mM NaF, and 1 mM Na_3VO_4, and Protease Inhibitor Cocktail™ (Roche Molecular Biochemicals, Indianapolis, IN)]. The protein concentrations were then determined with a BCA protein assay kit (Sigma). Equal quantities of protein (20 μg) were electrophoresed on a sodium dodecyl sulfate polyacrylamide gel under reducing conditions and then electrophoretically transferred onto a nitrocellulose membrane. The blots were then probed with anti-BACE1 antibody (1:1000, AbCam, Cambridge, UK) followed by the corresponding secondary antibody and finally developed using enhanced chemiluminescence reagents (GE Healthcare, Piscataway, NJ).

2.4. Bisulfite sequencing

Genomic DNA was extracted from BV-2 cells with or without treatment of 5-Aza using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The genomic DNA (2 μg) was sheared by passage through a 30 g needle 15 times, denatured by 0.3 M NaOH for 30 min at 39 °C, and subjected to bisulfite sequencing as described [12]. Briefly, bisulfite solution (3.9 M sodium bisulfite, 0.66 mM hydroquinone, pH 5.1) was added to the denatured DNA, which was incubated at 55 °C for 16 h. The DNA was desalted using a Qiagen Quickspin column, and modification was completed by 0.3 M NaOH at 37 °C for 16 min. After the bisulfite-treated DNA was purified by a Qiagen Quickspin column, the promoter and 5′-UTR region (+68 to +446) of the BACE1 gene was amplified from the bisulfite-treated DNA by PCR and cloned into a TOPO pCR2.1 plasmid (Invitrogen) using the manufacturer's instructions. Four to seven clones obtained from each of the PCR products were randomly selected and sequenced. The primers used for PCR of bisulfite-treated DNA are as follows: BACE1-F, 5-TTT TTT TAG TTT GTT TAG GTG TTG G-3; and BACE1-R, 5-CTA AAT CTA AAT AAT AAT AAC TTC T-3.

2.5. Cloning of the BACE1 promoter

CpG-free pCpGL-basic vector (pCpGL), whose basal luciferase activity is unchanged by methylation [13], was used for cloning the BACE1 promoter region. PCR was conducted using the BACE1-F-1136 primer, the BACE1-R-ATG primer, Ex Taq polymerase (Takara, Osaka, Japan) and genomic DNA from BV-2 cells as a template. The amplified PCR product, which included the promoter region and the 5′-UTR of BACE1 gene located from −1136 to +500, was cloned into the pCpGL to generate pCpGL-BACE1. The primers used for cloning are as follows: BACE1-F, 5-GCT AAC TCG AGT AGC TGG GGC AGG TTA AAT G-3; and BACE1-R-ATG, 5-ATC GAA AGC TTA GTG AGC CCG GGC CTT GTG-3.

2.6. Mutagenesis of the BACE1 promoter

In order to substitute CG dinucleotides at positions +298 and +351 in pCpGL-BACE1 with AA dinucleotides (pCpGL-BACE1-AA), site-directed mutagenesis was performed twice for mutation of both CG sites using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The corrections were determined by DNA sequencing analyses (Macrogen Co. Ltd, Seoul, South Korea). The first primers used for mutagenesis at position +293 are as follows: BACE1-F, 5-GCT GGG TCC CCT GGA TAA CCA TCG TCG TCT C-3; and BACE1-R, 5-GAG ACG ACG ATG GTT ATC CAG GGG ACC CAG C-3. The second set of primers used for mutagenesis at position +351 are as follows: BACE1-F, 5-GCC CAC TCT CCG CAA CCTGGA CCG GGA A-3; and BACE1-R, 5-TTC CCG GTC CAG GTT GCG GAG AGT GGG C-3.

2.7. In vitro methylation of reporter constructs

Ten micrograms of pCpGL-BACE1 and pCpGL-BACE1-AA were methylated *in vitro* by incubation with SssI methyltransferase (2.5 U/mg DNA) in the presence of 160 mM SAM for 4 h at 37 °C, with another 160 mM SAM being added after the first 2 h incubation (pCpGL-metBACE1 and pCpGL-metBACE1-AA, respectively). Unmethylated reporter constructs were prepared as described above but without an enzyme (pCpGL-unmetBACE1 and pCpGL-unmetBACE1-AA, respectively). Unmethylated and methylated reporter constructs were then purified by phenol/chloroform extraction and ethanol precipitation.

2.8. Transfection and luciferase assays

HEK293T cells were transiently transfected using lipofectamine reagent (Invitrogen) with 2.5 μg of unmethylated or methylated reporter construct and 0.5 μg of pcDNA 3.1-Renilla (Promega, Madison, WI) to normalize the extent of transfection. Luciferase activity was measured in transfectants with a Veritas luminometer (Turner Biosystems, Sunnyvale, CA) using the Dual Luciferase Reporter Assay Kit (Promega) according to the manufacturer's instructions.

2.9. Statistical analysis

All results are expressed as means ± SD, with n indicating the number of experiments. Differences between the two groups were compared by Student's *t*-test. Comparison among the five groups was analyzed by ANOVA, followed by a Bonferroni-Dunn post-hoc test. Statistical analyses were performed using StatView software (SAS Institute), and $P < 0.05$ was considered to be significant.

3. Results

3.1. 5-Aza increases mRNA and protein expression of BACE1 in BV-2 cells

To investigate the effect of DNA methylation on BACE1 expression in mouse BV-2 microglial cells, cells were treated with various amounts (0, 5, 10, and 20 μM) of 5-Aza for 24 h. 5-Aza significantly increased mRNA (Fig. 1A) and protein (Fig. 1B) expression of BACE1 in a dose-dependent manner, suggesting the involvement of DNA methylation in BACE1 expression. Very low BACE1 mRNA and protein basal expression levels were found in untreated BV-2 cells.

3.2. Two CpG sites in the 5′-UTR of the BACE1 gene are specifically demethylated by 5-Aza treatment

In an attempt to identify putative DNA methylation sites responsible for BACE1 expression, bisulfite sequencing was performed on DNA extracted from cells with or without 5-Aza treatment. The CpG islands in the BACE1 gene were identified *in silico* using the primer design program called MethPrimer, which is freely available at the website (http://www.urogene.org/methprimer/index1) [14]. The islands are mainly located in the 5′-UTR at positions from +68 to +446 (Fig. 2). No significant CpG islands in the promoter up to ~1 kb upstream of the BACE1 gene were identified by MethPrimer program, and therefore our studies were focused on the 5′-UTR. In basal BV-2 cells (without 5-Aza treatment), we found that most of the CpG sites were highly (>80%) methylated except the positions at +127, +222, +257, and +371. These four sites were methylated by ~50 %. In cells treated with 5-Aza, however, significant demethylations were found only at two CpG dinucleotides at positions +298 and +351, while most of the other CpG sites were unaltered. These results suggest a correlation between demethylation of these two CpG sites and increased BACE1 expression. We also found that 5-Aza treatment hypermethylated four CpG sites at positions of +127, +222, +257, and +371, which is unlikely due to the inhibition of DNA methyltransferase.

3.3. Demethylations of two CpG sites in the 5′-UTR are associated with increased BACE1 expression by 5-Aza treatment

To confirm whether the increase in BACE1 expression by 5-Aza treatment is directly attributable to demethylations of the two CpG sites in the 5′-UTR, we conducted a promoter assay using several constructs as described in Section 2. These include pCpGL, pCpGL-unmetBACE1, pCpGL-metBACE1, pCpGL-unmetBACE1-AA, and pCpGL-metBACE1-AA; the latter four constructs are shown in Fig. 3A. In this study, we used HEK293T cells because very low transfection efficiency was obtained in BV-2 cells transfected with all the constructs under our experimental conditions. A significantly higher (~330-fold) luciferase activity was shown in HEK293T cells transfected with pCpGL-unmetBACE1 than in those with pCpGL, suggesting that the promoter (including the 5′-UTR) of the BACE1 gene located from −1089 to +446 has transcriptional activity (Fig. 3B). However, when all CpG sites were methylated *in vitro* (in case of pCpGL-metBACE1), its transcriptional activity was silenced to the level of basic vector (pCpGL), suggesting that DNA methylation in the promoter and 5′-UTR of the BACE1 gene tested in this study directly affects the transcriptional activity of the BACE1 gene. We also found that mutation of the two CpG sites in the 5′-UTR identified in this study (pCpGL-ummetBACE1-AA) conferred a significant reduction (~30%) in luciferase activity relative to pCpGL-ummetBACE1, indicating an important role for these sites in exhibiting the full transcriptional activity of pCpGL-ummetBACE1. Methylation of pCpGL-ummetBACE1-AA (pCpGL-metBACE1-AA) also showed almost no transcriptional activity of the BACE1 gene.

4. Discussion

Our study demonstrates that BACE1 expression is regulated by DNA methylation in mouse microglial cells. 5-Aza treatment led to a dose-dependent increase in the mRNA and protein expression of the BACE1 gene. DNA methylation in the promoter region (up to ~−1.0 kb) and the 5′-UTR of the BACE1 gene silenced its transcriptional activity as evidenced by the luciferase assay. Furthermore, 5-Aza specifically demethylated two CpG sites in the 5′-UTR of the BACE1 gene, and mutations of these sites decreased BACE1 transcriptional activity. Our results suggest that two CpG sites in the 5′-UTR may play an important role in BACE1 expression via an epigenetic regulation of DNA methylation.

The most important finding in this study is the identification of two CpG sites responsible for 5-Aza-mediated increased BACE1 levels in mouse BV-2 microglial cells. These two sites are located at positions +298 and +351 in the 5′-UTR. Although transcription factor binding specifically to the +298 site has yet to be identified by *in silico* promoter analysis using the Genomatix web site (www.genomatix.de/en/index.html), the CpG site at position +351 is known to contain the STAT3 and CTCF/BORIS binding sites. STAT3 was previously reported to be one of the important transcription factors that regulate BACE1 expression in human SH-SY5Y neuroblastoma cells [15]. Based on our data, it is likely that the demethylation of the CpG site within the STAT3 binding site (+348 to +369) might induce BACE1 transcription by increasing accessibility of STAT3 to the site. For example, the demethylation of a cytosine residue within a STAT3 binding site in the astrocyte-specific marker glial fibrillary acidic protein gene promoter

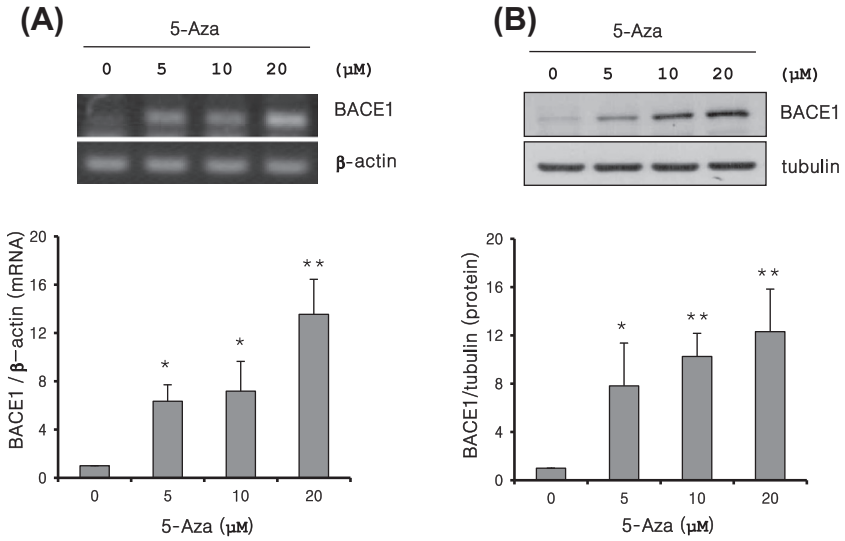

Fig. 1. 5-Aza treatment increases the mRNA and protein levels of BACE1 in a dose-dependent manner in BV-2 cells. Mouse BV-2 microglial cells were treated with 0, 5, 10, and 20 μM of 5-Aza for 24 h. The expressions of BACE1 and β-actin mRNAs were measured by RT-PCR using total RNA from BV-2 cells (A). Gel bands of BACE1 mRNA expression from 3 independent experiments were quantified, normalized to β-actin expression, and expressed relative to the BACE1 level in DMSO-treated control cells (0 μM 5-Aza treatment). The protein expressions of BACE1 and tubulin were measured by Western blot analysis using lysates from BV-2 cells (B). Gel bands of BACE1 protein expression from 3 independent experiments were quantified, normalized to tubulin expression, and expressed relative to the BACE1 level in DMSO-treated cells. Each bar shown is the mean fold increase above control ± SD ($n = 4$). Results are considered to be statistically significant at $^{*}P < 0.05$ and $^{**}P < 0.01$.

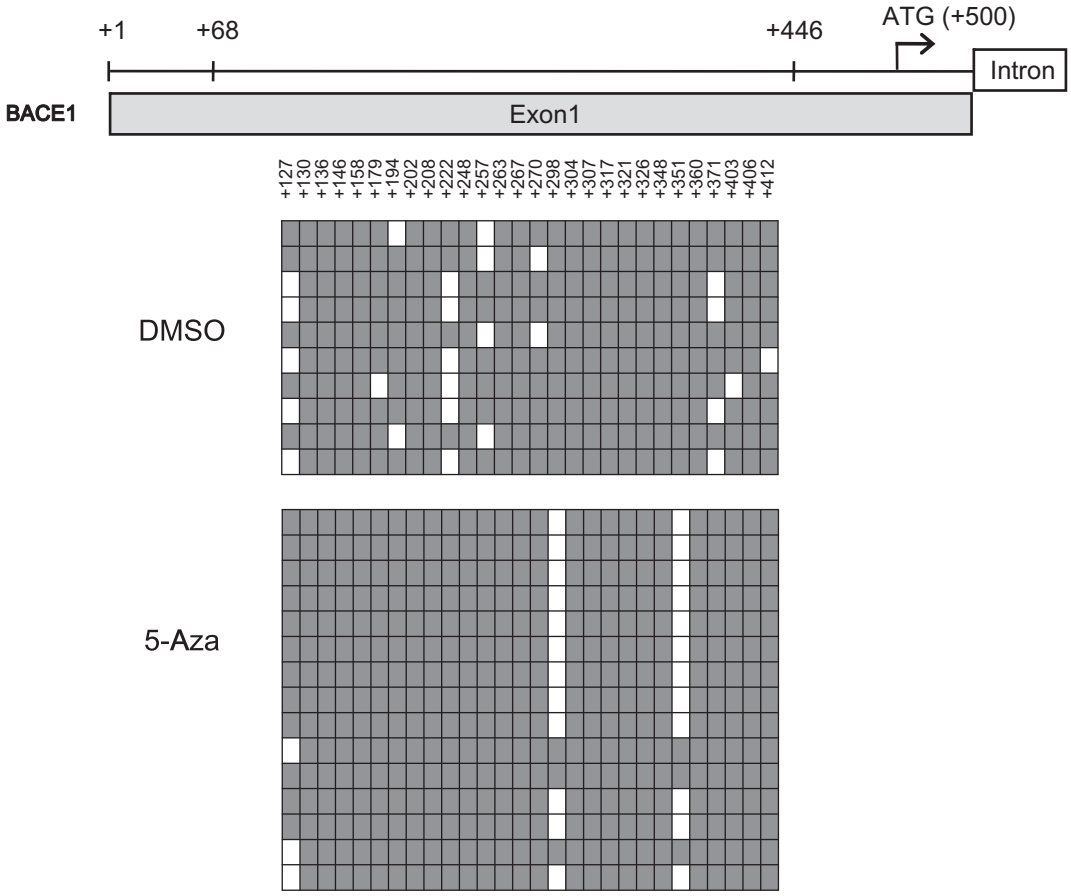

Fig. 2. CpG sites at positions +298 and +351 in the 5′-UTR were specifically demethylated by 5-Aza treatment. Schematic representation of the BACE1 5′-UTR (top). +1 Represents a transcriptional start site, and ATG (+500) represents a translational start site. The genomic DNA was extracted from BV-2 cells treated without (DMSO) and with 20 μM of 5-Aza for 24 h (5-Aza), and treated with bisulfite to convert unmethylated cytosines into uracils. Bisulfite-treated DNA was amplified by PCR and then cloned. Next, 10 and 15 clones obtained from each of the PCR products were selected at random and sequenced. The CpG sites depicted across the first row are numbered relative to the main transcriptional start site (+1) (bottom). Each subsequent row depicts the methylation status across the CpG sites in a single DNA molecule isolated by cloning. Filled and unfilled squares represent methylated and unmethylated CpG sites, respectively.

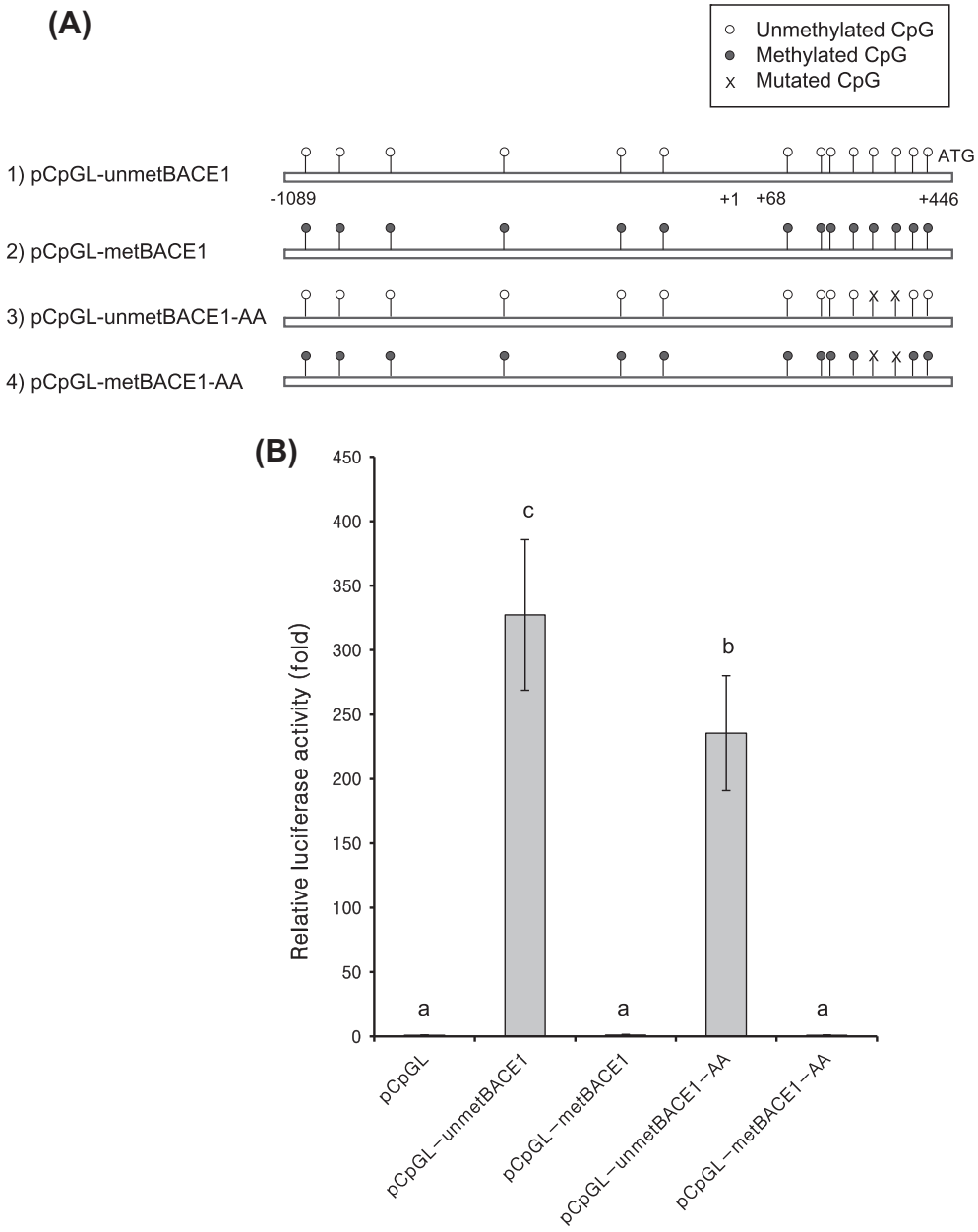

Fig. 3. DNA methylation of two CpG sites at +298 and +351 in the 5′-UTR is associated with BACE1 gene expression. Schematic representation of the 4 constructs used in this study are shown (A); CpG-free pCpGL-basic vector (pCpGL), whose basal luciferase activity is unchanged by methylation [13], was used for cloning the BACE1 promoter region. (1) pCpGL-unmetBACE1, cloned the promoter region and 5′-UTR of BACE1 gene into the pCpGL. (2) pCpGL-metBACE1, *in vitro* methylated pCpGL-BACE1. (3) pCpGL-unmetBACE1-AA, substituted GC dinucleotides at positions +298 and +351 in pCpGL-BACE1 with AA dinucleotides. (4) pCpGL-metBACE1-AA, *in vitro* methylated pCpGL-BACE1-AA. Filled and unfilled circles represent methylated and unmethylated CpG sites, respectively. An X mark, instead of a circle, represents the mutation of CG dinucleotides into AA dinucleotides. HEK293T cells were transfected with pCpGL and the 4 constructs described in (A), and luciferase activity was measured using the Dual Luciferase Reporter Assay Kit, normalized to Renilla expression, and expressed relative to that of pCpGL. The results shown are mean fold increases above control ± SD ($n = 3$). Statistical significance was evaluated by ANOVA, followed by a Bonferroni-Dunn post-hoc test and set at $P < 0.0083$.

was reported to be associated tightly with the onset of astrocyte differentiation from neuroepithelial cells [16] or embryonic stem cell-derived neuroectoderm-like cells [17]. CTCF can also bind to the position +331 to +357 in the BACE1 5′-UTR. CTCF, a zinc finger protein with multiple functions, binds to the APBβ site, an essential activator domain in the proximal promoter of the APP gene, and activates APP transcription [18]. Furthermore, methylation-sensitive CTCF binding was also known to control imprinted insulin-like growth factor 2 expression [19,20]. Together with previous data showing higher levels of plasma homocysteine in MCI and AD patients [8,9], the present study suggests that homocysteine, a demethylating agent similar to 5-Aza, may also specifically demethylate a cytosine residue within the STAT3 or CTCF binding site at position +351 in the BACE1 5′-UTR, leading to an increase in the BACE1 level and subsequent AD progression. Further detailed experiments are needed to clarify this issue.

Lin et al. demonstrated previously that SAH, another inhibitor of cellular methyltransferases, increased Aββ formation in BV-2 microglial cells through demethylation in the promoter region of presenilin 1 (PSEN1), a major catalytic subunit of γ-secretase [21]. In this study, however, treatment with SAH had a smaller effect on the alteration in BACE1 expression; only a very high

concentration (500 μM) of SAH slightly increased BACE1 expression. Whether 5-Aza increased BACE1 levels was not investigated in this previous study. Nonetheless, this data supported the hypothesis that AD development is closely associated with increased expression of AD-related genes such as PSEN1 via epigenetic regulation of DNA methylation. Concurrently, Scarpa et al. showed that treatment of SAM, a potent methyl donor, decreased PSEN1 gene expression and Aβ production in human neuroblastoma SK-N-SH cells [22]. The same research group also reported that in the human neuroblastoma cell lines, SK-N-SH and SK-N-BE, PSEN1 levels increased in culture medium deprived of folate and vitamin B_{12}, which returned to basal levels by SAM treatment [23]. Folate and vitamin B_{12} are known to be essential for the transformation of homocysteine to SAM in the homocysteine/SAM cycle. Furthermore, these authors demonstrated that the alteration in PSEN1 gene expression was mediated via the methylation of the PSEN1 gene promoter. Although the deprivation of folate and vitamin B_{12} and/or replenishment with SAM regulate BACE1 expression in this previous study, which methylation site(s) of the BACE1 promoter was associated with the regulation of BACE1 levels has not been investigated. To the best of our knowledge, this is the first study to provide evidence that BACE1 expression is epigenetically regulated at least by DNA methylation at two CpG sites in the 5′-UTR of the BACE1 gene and may be associated with AD development.

In conclusion, our study provides the first solid evidence that DNA methylation at two CpG sites in the 5′-UTR is associated with the regulation of BACE1 expression in mouse microglial cells. Although further detailed experiments are necessary, the accessibility of potential transcription factor(s), STAT3 and CTCF, to these identified CpG sites may confer the molecular mechanism by which demethylating agents such as 5-Aza and homocysteine increase BACE1 expression in mouse microglial cells.

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