

Knockdown of BACE1-AS by siRNA improves memory and learning behaviors in Alzheimer's disease animal model

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Abstract. Alzheimer's disease (AD) is a devastating neurodegenerative disease that causes progressive damage to neurons. Emerging evidence has demonstrated that long non-coding RNAs (lncRNAs) serve an important role in many neurological diseases, such as AD. β -secretase 1 (BACE1)-antisense transcript (BACE1-AS) was identified as a conserved non-coding antisense BACE1. Previous reports stated that BACE1-AS positively regulated BACE1 mRNA and subsequently BACE1 protein expression *in vitro* and *in vivo*. However, whether BACE1-AS is able to regulate memory and learning behaviors remains to be elucidated. In the present study, the role of lncRNA BACE1-AS on memory and learning was investigated. It was demonstrated that lncRNA BACE1-AS expression was highly expressed in blood samples from AD patients, and also upregulated in peripheral blood samples and hippocampi from an AD animal model. Knockdown of BACE1-AS by short interfering RNA increased the primary hippocampal neurons proliferation *in vitro*. Knockdown of BACE1-AS mediated by lentivirus *in vivo* improved the memory and learning behaviors of SAMP8 mice, inhibited BACE1 and amyloid precursor protein production, and phosphorylation of tau protein in hippocampi. Therefore, the present findings suggested that BACE1-AS may be a potential target for management of memory loss related diseases, such as AD.

Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disease that causes progressive damage to neurons (1). It is characterized by the impairment of cognition, memory and learning, and causes >80% of cases of dementia in the world's rapidly growing aging population (2). Currently, AD treatment is enormously expensive, and no curative treatment for AD is

available because the etiology of AD is poorly understood. Non-coding RNAs, including microRNAs (miRs) and long non-coding RNAs (lncRNAs), are regulatory molecules associated with a wide variety of biological processes and disease states (3). miR-339-5p levels are significantly reduced in brain specimens isolated from AD patients, and miR-339-5p regulates expression of β -secretase 1 (BACE1), a crucial enzyme in the pathophysiology of AD, in human brain cells (4). Emerging evidence has demonstrated that lncRNAs have an important role in many neurological diseases, such as AD, Parkinson's disease and Huntington's disease (5). Certain differentially expressed lncRNAs associated with AD have been identified (6,7). For example, gene set enrichment analysis identified a downregulated lncRNA n341006 in association with protein ubiquitination pathway, and significantly upregulated lncRNA n336934 associated with cholesterol homeostasis in AD patients (8). Massone *et al* (9) previously demonstrated that lncRNA 17A was upregulated in cerebral tissues derived from AD patients, and that it could enhance the secretion of amyloid β (A β) peptide and the A β 1-42/A β 1-40 peptide ratio.

The BACE1-antisense transcript (BACE1-AS) has been identified as a conserved non-coding antisense BACE1. BACE1-AS can positively regulate BACE1 mRNA and thus BACE1 protein expression *in vitro* and *in vivo* (10). In addition, silencing lncRNA BACE1-AS expression with short interfering RNA (siRNA) in senile plaque AD SH-SY5Y cells attenuates the ability of BACE1 to cleave amyloid precursor protein (APP) and reduce the production of A β 1-42 oligomers (11). However, whether BACE1-AS can regulate memory and learning behaviors remains unknown. The aim of the present study was to elucidate the role of lncRNA BACE1-AS in memory and learning.

Materials and methods

Blood samples. Peripheral blood samples of AD patients (n=30; male/female, 17/13; age range, 60-82 years) and age-matched normal subjects (n=36; male/female, 20/16; age range, 65-79 years) without notable illness, including diabetes, heart disease, stroke or cancer were collected at the Department of Neurology, Hefei Affiliated Hospital of Anhui Medical University (Hefei, China) between March 2015 and May 2016. Samples were stored at -80°C prior to further use. The present study was approved by the Ethics Committee of Hefei Affiliated

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Hospital of Anhui Medical University. All participants provided written informed consent.

Animals. Male SAMR1 (age, 6 months; weight range, 23–30 g; $n=8$) and male SAMP8 (age, 6 months; weight range, 22–32 g; $n=32$; 8 mice per group) mice were obtained from the Animal Center of Beijing University Medical Department (Beijing, China). SAMP8 is an AD animal model with age-related learning and memory deficits (12) and SAMR1 mice served as a healthy control. Mice were fed *ad libitum* and housed in a 12-h light/dark cycle at $25\pm 1^\circ\text{C}$ and 50% humidity. To knockdown BACE1-AS in hippocampus, SAMP8 mice anesthetized with chloral hydrate (40 mg/kg; cat. no. 47335-U; Merck KGaA, Darmstadt, Germany) were positioned in a stereotaxic apparatus with bregma and lambda at a horizontal level, and administered with $1.5\ \mu\text{l}$ 1×10^9 BACE1-AS siRNA lentivirus (2×10^9 titer units/ml diluted 10x with enhance infected solution) or an empty lentivirus (Shanghai GeneChem Co., Ltd., Shanghai, China) into bilateral hippocampi using the following coordinates: Anteroposterior -3.50 mm relative to bregma; lateral ± 1.50 mm; dorsoventral 3.5 mm from the skull, as previously described (13). The administration lasted 5 min, allowing slow diffusion. SAMP8 mice injected with empty lentivirus were used as negative control (NC). SAMP8 mice received an equal volume of vehicle were used as Control. Mice were allowed to survive for 3 weeks. Brains were harvested for further analysis following Y-maze and Morris water maze test behavioral tests (14,15). The experimental protocol was approved by the Animal Care and Use Committee of Hefei Affiliated Hospital of Anhui Medical University, in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Primary hippocampal neuron culture. A pregnant SAMP8 mouse (age, 3 months; weight 50 g; $n=1$; gestational age, 2 weeks) was obtained from the Animal Center of Beijing University Medical Department (Beijing, China) and was fed *ad libitum* and housed in a 12-h light/dark cycle at $25\pm 1^\circ\text{C}$ and 50% humidity prior to experiments. The mouse was anesthetized with chloral hydrate (40 mg/kg; cat. no. 47335-U; Merck KGaA, Darmstadt, Germany) and 8 embryos were harvested. Primary hippocampal neurons were obtained from embryonic day-15 hippocampi of SAMP8 mice. Briefly, the hippocampi were mechanically removed and cut into $1\ \text{mm}^3$ pieces and treated with trypsin and 0.05 mg/ml DNase (cat. no. AMPD1-1KT; Merck KGaA, Darmstadt, Germany) for 15 min at 37°C in serum-free Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Hippocampal cells were washed with DMEM containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and resuspended in completed culture medium [DMEM supplemented with 10% FBS, penicillin (50 U/ml), streptomycin (50 U/ml) and glutamine (0.5 mmol/l)]. The cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C . To knockdown BACE1-AS in primary hippocampal neurons, the cells were infected with BACE1-AS siRNA lentivirus (10×10^7 titer units/ml) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h at 37°C . After 72 h, cells were photographed using light microscopy (magnification, $\times 100$) to observe morphological alterations.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). An Ultrapure RNA kit (cat. no. CW0581M; CWBio, Beijing, China) was used to extract RNA from peripheral blood samples and hippocampi tissues from SAMR1 and SAMP8 mice peripheral blood samples from patients with AD and normal subjects or primary hippocampal neurons from embryos according to the manufacturer's instructions. Maxima First Strand cDNA Synthesis kit (cat. no. K1642; Thermo Fisher Scientific, Inc.) was used for reverse transcription according to the manufacturer's protocol. Expression of BACE1-AS was detected via UltraSYBR Mixture (cat. no. CW2602; CWBio). Expression of β -actin was used as an endogenous control. The following primers were used: BACE-AS1 forward, 5'-TCTGGGCAGTAGGGGGTTAC-3' and reverse, 5'-GACTACCTGCCACCCAAGA-3'; and β -actin forward, 5'-GCCCTATAA AACCCAGCGGC-3' and reverse, 5'-TCGATGGGGTACTT CAGGGT-3'. Amplification conditions were as follows: 95°C for 3 min, 40 cycles of denaturation at 95°C for 15 sec and annealing at 58°C for 45 sec. Data were quantified using the $2^{-\Delta\Delta\text{C}_q}$ method (16).

ELISA determination of $\text{A}\beta_{1-40}$ and 1-42. Hippocampi tissues were homogenized using a Tissue Protein Extraction kit containing protease inhibitor cocktail (cat. no. CW0891; CWBio) and centrifuged at $12,000\times g$ for 30 min at 4°C . Supernatants were used for ELISA quantification using a Mouse $\text{A}\beta_{1-40}$ ELISA kit (cat. no. CSB-E10787m; Cusabio Biotech Co., Ltd., Wuhan, China) and a Mouse $\text{A}\beta_{1-42}$ ELISA kit (cat. no. CSB-E08300m; Cusabio Biotech Co., Ltd.) according to the manufacturer's instructions.

Western blot analysis. Total protein was extracted from hippocampus tissues using a Cold Tissue Protein Extraction kit containing protease inhibitor cocktail (cat. no. CW0891; CWBio). A BCA Protein Assay kit (cat. no. CW0014S; CWBio) was used to determine the protein concentration. Equal protein samples (60 μg) were then separated by 12% SDS-PAGE and transferred to a $0.22\ \mu\text{m}$ nitrocellulose membrane (cat. no. CW2002S; CWBio). The membrane was blocked in 5% non-fat dried milk in TBS-Tween-20 for 2 h at room temperature and incubated with the following primary antibodies: Anti-BACE1 (cat. no. ab183612; dilution, 1:500), anti-APP (cat. no. ab12266; dilution, 1:500), anti-phosphorylated (p)-tau (cat. no. ab81268; dilution, 1:500), anti-tau (cat. no. ab64193; dilution, 1:500) and anti-GAPDH (cat. no. ab8245; dilution, 1:500; all Abcam, Cambridge, UK) overnight at 4°C . The membrane was washed and incubated with secondary antibodies: Goat anti-rabbit IgG (HRP; cat. no. ab6721; dilution, 1:3,000; Abcam, Cambridge, UK) or goat anti-mouse IgG (HRP; cat. no. ab205719; dilution, 1:3,000; Abcam) for 2 h at room temperature. The signal on the membrane was visualized using enhanced chemiluminescence reagent (EMD Millipore, Billerica, MA, USA) and densitometry analysis was performed using Image-Pro plus software 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Cell counting kit (CCK)-8 assay. At 24 h prior to the experiment, cells were plated in 96-well plates at a density of 1,000 cells in 100 μl medium per well at 37°C . The cell

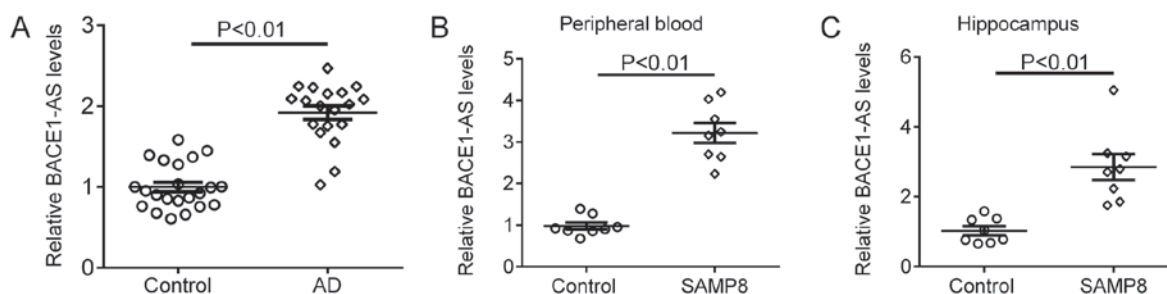


Figure 1. The expression of BACE1-AS in peripheral blood samples from AD patients. (A) RT-qPCR analysis for BACE1-AS in peripheral blood samples from AD patients (n=30) and age-matched normal subjects (n=36). (B) RT-qPCR analysis for BACE1-AS in peripheral blood samples from SAMR1 control (n=8) and SAMP8 mice (n=8). (C) RT-qPCR analysis for BACE1-AS in hippocampus tissues from SAMR1 control (n=8) and SAMP8 mice (n=8). BACE1-AS, β -secretase 1-antisense transcript; AD, Alzheimer's disease; RT-qPCR, reverse transcriptase-quantitative polymerase chain reaction.

viability was assessed via CCK-8 assay (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturers' instructions. The assay was repeated three times in triplicate wells.

Y-maze test. The Y-maze based on place or object exploration is used to assess spatial recognition memory (17). The Y-maze has been used to study learning and memory under certain conditions, such as chronic stress (18). Shin *et al* (15) previously measured spatial learning and memory using the Y-maze and Morris water maze in rats stimulated with Neuropep-1. The Y-maze test was performed as previously described (19). Briefly, mice were initially placed at the end of one arm and allowed to move freely for 10 min. The series of arm entries was recorded by a video camera. Spontaneous alternation was defined as successive entries into the three arms in overlapping triplet sets. The alternation percentage was determined as the ratio of actual alternations to maximum alternations.

Morris water maze test. The Morris water maze test was performed as described previously (14). Briefly, a circular pool divided into four quadrants with fixed visual cues was filled with opaque water at a constant temperature (22°C), and was monitored by a video camera. Each mouse was trained via four visible platform (10 cm in diameter) tests prior to the behavioral experiment. Each mouse was placed into the water facing the pool wall (back to platform) and given 60 sec to swim freely and climb onto the visible platform, once daily for 4 days to observe and record the time needed to find and climb onto the platform (escape latency). On day 5, hidden platform trials were performed four times per day for 6 days. The next day, a spatial probe trial was performed. The number of times of crossing the original platform location in the pool within 90 sec was recorded using a Morris water maze image automatic monitoring system (Gene and I Co., Ltd., Beijing, China). Following the experiment, mice were sacrificed and the brains were removed.

Statistical analyses. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA), and data were presented as the mean + or \pm standard error of the mean. Unpaired two-tailed Student's t-test was used to analyze differences between two groups, and one-way analysis of variance with a post hoc Bonferroni test was used

to analyze differences between three or more groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

BACE1-AS levels are increased in blood of patients with AD. The expression level of BACE1-AS was detected via RT-qPCR in AD patients (n=30) and age-matched normal controls (n=36). The present results demonstrated that BACE1-AS was significantly increased in peripheral blood of AD patients compared with controls (Fig. 1A). In addition, BACE1-AS expression was measured in the peripheral blood and hippocampus from SAMR1 (control) and SAMP8 mice. Compared with controls, the expression of BACE1-AS was significantly increased in peripheral blood and hippocampus tissues of SAMP8 mice, suggesting that BACE1-AS may be associated with age-related cognitive decline in AD.

Knockdown of BACE1-AS by siRNA promotes the survival of primary neurons. To test if BACE1-AS regulates hippocampal neurons proliferation, BACE1-AS was knocked down in hippocampal neurons and an CCK-8 assay was performed. BACE1-AS expression was significantly reduced in hippocampal neurons infected with BACE1-AS siRNA lentivirus compared with negative controls (Fig. 2A). No significant changes in the morphology of hippocampal neurons between the three groups were observed (Fig. 2B, upper panel). Compared with the negative control group, BACE1-AS siRNA transfection exhibited a significant promotion on cell proliferation (Fig. 2B, lower panel). These results suggested that BACE1-AS downregulation promotes hippocampal neurons proliferation.

Knockdown of BACE1-AS in hippocampi improves learning and memory behaviors of SAMP8 mice. To evaluate the functions of BACE1-AS on learning and memory behaviors *in vivo*, BACE1-AS siRNA lentivirus was administered to the hippocampi of SAMP8 mice. SAMR1 mice, which received an equal volume of vehicle, were used as controls and mice injected with empty lentivirus were used as negative controls. The expression of BACE1-AS in mice injected with BACE1-AS siRNA lentivirus was significantly decreased compared with negative control (Fig. 3A). Following 3 weeks of BACE1-AS siRNA lentivirus infection, downregulation of BACE1-AS in hippocampi significantly increased successive entries in

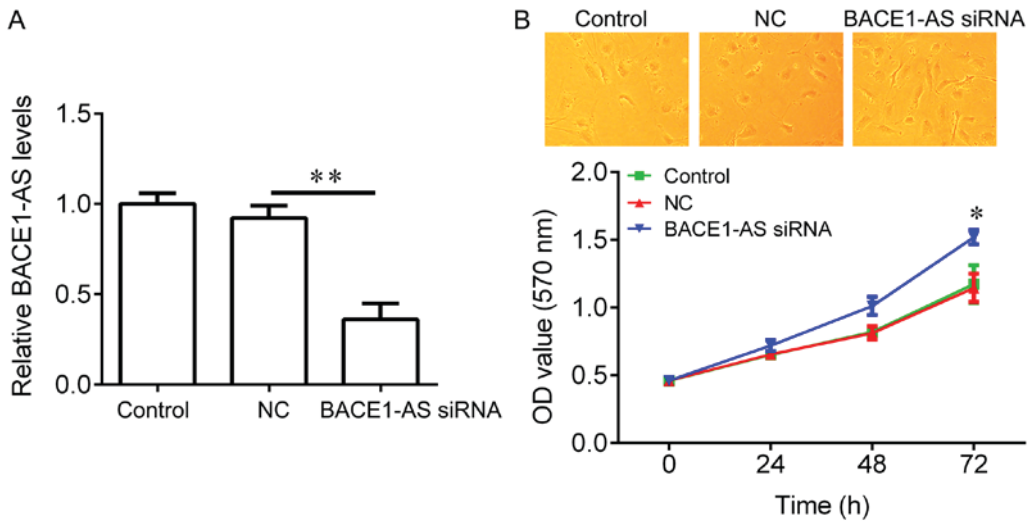


Figure 2. Knockdown of BACE1-AS promotes hippocampal neurons growth. (A) Reverse transcriptase-quantitative polymerase chain reaction analysis for BACE1-AS in primary hippocampal neurons following lentivirus infection. Untreated cells were used as controls, and the cells infected with empty lentivirus were used as NC. **P<0.01. (B) Upper panel, the representative images of neurons at 72 h following treatment (magnification, x100). Lower panel, cell counting kit-8 assay was performed to measure the cell growth of primary hippocampal neurons following lentivirus infection. *P<0.05 vs. NC. BACE1-AS, β -secretase 1-antisense transcript; NC, negative control; siRNA, short interfering RNA; OD, optical density.

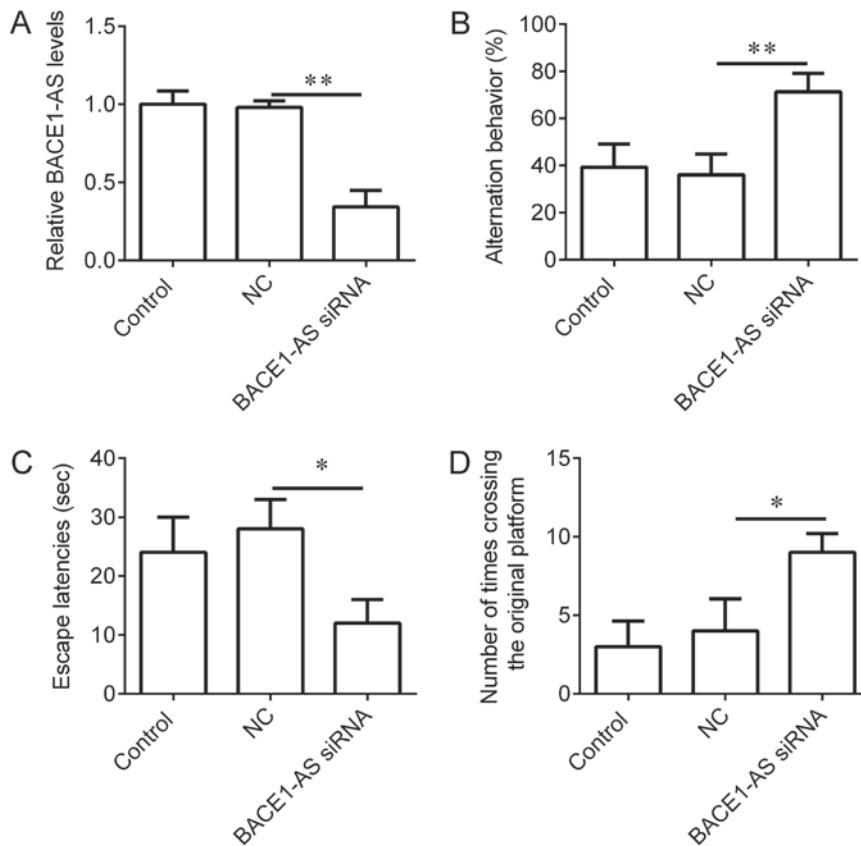


Figure 3. Knockdown of BACE1-AS improves memory and learning behaviors in SAMP8 mice. (A) Reverse transcriptase-quantitative polymerase chain reaction analysis for BACE1-AS in hippocampal tissues from SAMP8 mice following 3 weeks lentivirus injection. Mice subjected to sham operation were used as control, and mice injected with empty lentivirus were used as NC. (B) Y-maze test was performed to evaluate the memory behaviors in SAMP8 mice following indicated treatment. (C) Morris water maze test was performed to test spatial learning and memory of mice. The changes in the escape latencies of mice in different groups following training period are presented. The escape latency of mice with BACE1-AS knockdown was shorter than that of control and negative control mice. (D) The number of platform crossings was increased in mice with BACE1-AS knockdown. *P<0.05, **P<0.01 vs. NC. BACE1-AS, β -secretase 1-antisense transcript; NC, negative control; siRNA, short interfering RNA.

the Y-maze test (Fig. 3B), reduced the escape latencies in the Morris water maze (Fig. 3C) and increased instances

of crossing the original platform in the Morris water maze (Fig. 3D) in comparison with negative controls, indicating

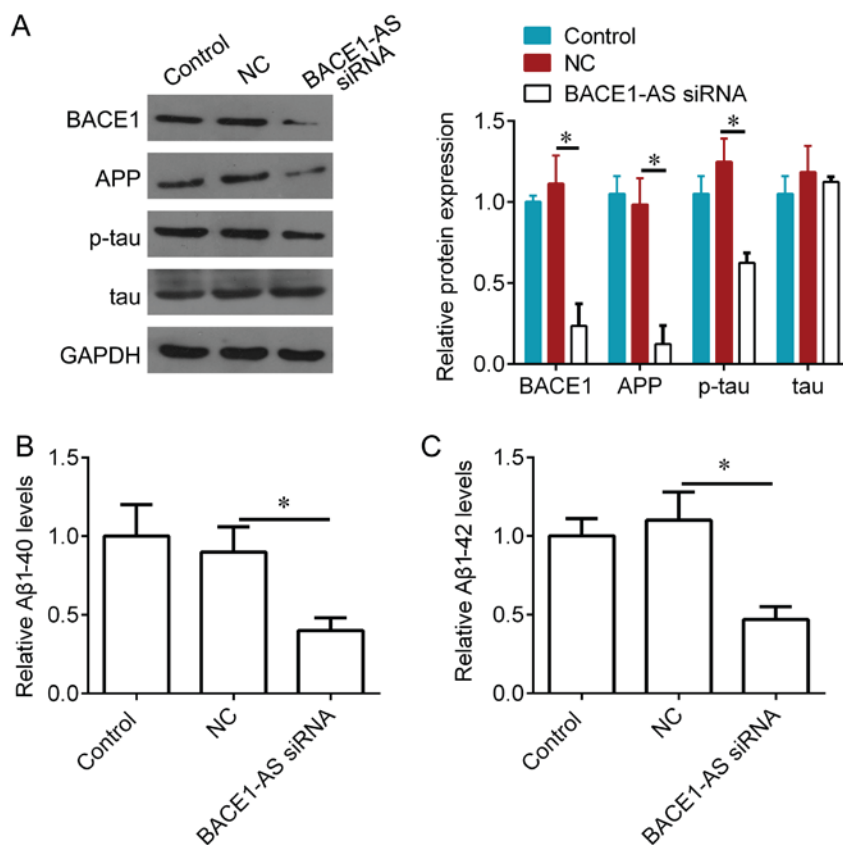


Figure 4. Knockdown of BACE1-AS decreases BACE1 and APP accumulation, and phosphorylation of tau protein in hippocampus of SAMP8 mice. (A) Western blot analysis for BACE1, APP, p-tau and total tau protein in hippocampal tissues from SAMP8 mice following 3 weeks lentivirus injection, and quantification of the bands. (B) ELISA analysis for Aβ1-40 in hippocampal tissues from SAMP8 mice following 3 weeks lentivirus injection. (C) ELISA analysis for Aβ1-42 in hippocampal tissues from SAMP8 mice following 3 weeks lentivirus injection. *P<0.05 vs. NC. BACE1-AS, β-secretase 1-antisense transcript; APP, amyloid precursor protein; p, phosphorylated; Aβ, amyloid β; NC, negative control; siRNA, short interfering RNA.

that BACE1-AS downregulation improves the learning and memory behaviors of SAMP8 mice.

Knockdown of BACE1-AS decreases BACE1 and Aβ levels *in vivo*. It was examined whether BACE1-AS could regulate several important proteins for AD, including BACE1, APP, tau and Aβ. Aβ 1-40 and Aβ 1-42 levels were measured by ELISA, and BACE1, APP, p-tau and tau expression was measured via western blotting. It was demonstrated that, compared with negative controls, BACE1-AS knockdown significantly inhibited BACE1, APP and p-tau expression (Fig. 4A), and also reduced the concentration of Aβ 1-40 and Aβ 1-42 in hippocampi treated with BACE1-AS siRNA (Fig. 4B and C).

Discussion

In the present study, it was demonstrated that lncRNA BACE1-AS expression was highly expressed in blood samples from AD patients, and also upregulated in peripheral blood samples and hippocampi from an AD animal model. Knockdown of BACE1-AS by siRNA increased the primary hippocampal neurons proliferation *in vitro*, and improved the memory and learning behaviors in SAMP8 mice by inhibiting BACE1 and APP production, and phosphorylation of tau protein.

Aβ peptide recurrently is accepted as the culprit in the pathogenesis of AD (20). BACE1 is required for the

production of Aβ peptide (21). This suggests that the inhibition of BACE1 and subsequent reduction of Aβ may cure or prevent AD. Although the precise mechanisms that trigger Aβ accumulation remain unclear, much effort has focused on screening BACE1 inhibitors (22). Recent studies in which BACE1 activity is specifically inhibited in animal models with knockout technology, virus-delivered siRNAs and bioavailable small-molecule agents support the use of therapeutic BACE1 inhibition (23,24). Genetic BACE1 inhibition may be a promising treatment strategy for AD (25). Non-coding RNAs were demonstrated to control BACE1 expression and Aβ production (26). Kim *et al* demonstrated that a reduction in miR-186 levels during aging may lead to the upregulation of BACE1 in the brain, thus increasing the risk of AD in elderly individuals (27). miR-195 negatively regulated by nuclear factor-κB-mediated Aβ aggregation and tau hyperphosphorylation in chronic brain hypoperfusion (28). In addition, lncRNAs also have critical roles in progression of AD by regulating BACE1 (29). Neuroblastoma differentiation marker 29 (NDM29) is a non-coding RNA that is dose-dependently induced by inflammatory stimulation (30). NDM29 can promote the cleavage activities of BACE to increase Aβ formation and the Aβx-42/Aβx-40 ratio (30).

BACE1-AS is a crucial enzyme in AD pathophysiology that was originally identified as a conserved non-coding antisense transcript for BACE1 (10). BACE1-AS transcript was increased in the parietal lobes and cerebellum from

postmortem brains of AD patients. BACE1-AS can regulate BACE1 mRNA and protein expression *in vitro* and *in vivo*, and A β 1-42 stimulation also can elevate the expression of BACE1-AS, increasing BACE1 mRNA stability and generating additional A β 1-42 through a post-transcriptional feed-forward mechanism (10). In addition, downregulation of lncRNA BACE1-AS expression in SH-SY5Y cells by siRNA silencing attenuates the ability of BACE1 to cleave APP and delays the induction of senile plaque formation in a senile plaque AD cell model (11). BACE1-AS levels were associated with HuD, a primarily neuronal RNA-binding protein that is implicated in learning and memory. BACE1-AS level was higher in the brain of HuD-overexpressing mice (31). HuD can interact with the 3'untranslated regions of BACE1 mRNA to increase the half-life of this mRNA (31). In addition, dysregulation of the BACE1/BACE1-AS/ β -amyloid axis was also relevant in heart failure pathogenesis (32). BACE1-AS also has a role in cancer (33). BACE1-AS was significantly increased in anisomycin-treated ovarian cancer stem cells. Elevation of lncRNA BACE1-AS expression can suppress human ovarian cancer stem cells proliferation and invasion (34).

The present study suggests that BACE1-AS levels are significantly upregulated in peripheral blood samples of patients with AD, suggesting that BACE1-AS might be an indicator for progression of AD. Knockdown of BACE1-AS by siRNA improves memory and learning behaviors, possibly via increasing the hippocampal neurons growth, and decreasing BACE1 and A β accumulation, and phosphorylation of tau protein in hippocampus of SAMP8 mice. Therefore, BACE1-AS may be a potential target for management of memory loss related disease, such as AD.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

WX and MX designed the study. WZ and HZ collected the patient data and samples. QW, WZ and HZ performed cell biological experiments. QW, WX and MX performed qPCR, ELISA and western blot. WX and MX performed the animal experiments. All authors contributed to writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animal experiments were approved by the Animal Care and Use Committee of Hefei Affiliated Hospital of Anhui Medical University in compliance with the National Institutes

of Health Guide for the Care and Use of Laboratory Animals. Experiments using human tissue were approved by the Ethics Committee of Hefei Affiliated Hospital of Anhui Medical University. All participants provided written informed consent.

Patient consent for publication

All participants provided written informed consent.

Competing interests

The authors declare that they have no competing interests.

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