Identification of the biological affection of long noncoding RNA BC200 in Alzheimer's disease

Huanyin Li*, Lan Zheng*, Aihua Jiang, Yankqing Mo and Qi Gong

BC200 is a long noncoding RNA expressed at high levels in the Alzheimer's disease (AD), and blocking of BC200 by siRNA is assumed to be an effective method for various disease therapy. We have established an AD cell model overexpressing amyloid β-peptide (Aβ)1-42 to observe the effects of BC200 on the cell viability and apoptosis, and to investigate the associated underlying mechanisms. Efficient knockdown and overexpression of BC200 were established using BC200 siRNA and BC200 mimics, respectively. Cell viability following BC200 knockdown and overexpression was assessed by 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide assay, and cell apoptosis was monitored by flow cytometry. We successfully established an AD cell model overexpressing Aβ1-42 gene, and reported the results of change of BC200 on Aβ1-42 levels. Knockdown of BC200 significantly suppressed b-site amyloid precursor protein-cleaving enzyme 1 (BACE1) expression, and overexpression of BC200 increased BACE1 expression. Besides, inhibition of BC200 significantly increased cell viability and reduced cell apoptosis in the AD model via directly targeting BACE1, which can be increased by overexpression of BC200. BC200 regulated AD cell viability and apoptosis via targeting BACE1, and it may be one of the putative target in AD development and provides potential new insights into genetic therapy against AD. *NeuroReport* **29:1061–1067**

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Keyword: Alzheimer's disease, amyloid β-peptide, b-site amyloid precursor protein-cleaving enzyme 1, BC200, long noncoding RNAs

Department of Neurology, Central Hospital of Minhang District, Shanghai, China

Correspondence to Huanyin Li, MD, Department of Neurology, Central Hospital of Minhang District, 170 XinSong Road, Shanghai, 201199, China
Tel/fax: +86 216 492 3400; e-mail: lihuanyinsh@163.com

*Huanyin Li and Lan Zheng contributed equally to the writing of this article.

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Background

Alzheimer's disease (AD) is the most common form of dementia, which is characterized pathologically by accumulation of insoluble senile plaques consisting primarily of amyloid β-peptide (Aβ) deposits and numerous neurofibrillary tangles formed from filaments of microtubule [1,2]. There were more than five million people in the USA alone and 44 million worldwide with AD in 2015, and this number is expected to increase to 13.8 million by 2050 [3]. In 2010, 5.69 million people experienced AD in China, and it seems to be increasing faster than generally assumed [4]. Currently, details of AD pathogenesis still remain elusive, and there is no cure with drugs and methods, and the only treatment of AD is by medications that are used to treat the symptoms of the disease. Therefore, novel therapeutic strategies are actively being pursued.

Aβ, a cleavage product of the amyloid precursor protein (APP), is generated by b-site APP-cleaving enzyme 1 (BACE1) and γ-secretase complex, and is strongly implicated in the pathogenesis of AD [5]. Aβ-mediated neurotoxicity plays a central role, and abnormal accumulation of Aβ in the brain is considered to be the rate-limiting step of AD [6,7]. Thus, decreases in Aβ accumulation are actively being pursued as novel therapeutic targets.

Long noncoding RNAs (lncRNAs), a subclass of ncRNAs, are most commonly defined as transcripts more than 200 nucleotides in length that lack an open reading frame of significant length [8]. By definition, lncRNAs should not be translated into protein, but this can be difficult to ascertain using informatics alone because many lncRNAs still contain short open reading frames that could be translated and some are likely to express functional proteins [9]. Accumulating evidences reveal that lncRNAs play important role in multiple biological phenomena, such as genomic loci imprinting, chromosome conformation, cell regulatory activity, and even cancer pathogenesis [10–12]. The role of lncRNAs in AD has attracted considerable attention, and numerous AD-associated lncRNAs have been discovered [13,14]. Brain cytoplasmic RNA 1 (BCYRN1 or BC200), a long noncoding RNA, has been reported with substantially higher levels and plays important role in human breast, lung, skin, esophagus, and cervix tumors [15,16]. Several recent studies have demonstrated BC200 RNA was significantly upregulated in AD brains, and this upregulation in AD was specific [17]. However, studies on the role of BC200 in AD pathogenesis have only recently been initiated; the precise biological role and mechanism of BC200 remain largely unclear and need to be investigated.

Here, we establish an AD cell model overexpressing Aβ1-42 to observe the effects of BC200 on the cell viability

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and apoptosis and to investigate the associated underlying mechanisms. We found that BC200 and BACE1 were increased upon treatment with Aβ1-42, and inhibition of BC200 rescued this Aβ1-42-mediated dysfunction, as indicated by the interaction of BC200 directly targeting BACE1. Moreover, inhibition of BC200 increased AD cell growth and reduced cells apoptosis. Taken together, our data demonstrate that BC200 is a potent positive regulator of BACE1 in AD cells.

Materials and methods

Cell culture and establishment of an Alzheimer's disease cell model

The SH-SY5Y human neuroblastoma cell line was purchased from American Type Culture Collection (ATCC, Manassas, Virginia, USA). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco, California, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, New York, USA) and 1% penicillin-streptomycin (Gibco, New York, USA) in a humidified incubator in 95% air–5% CO_2 at 37°C. The AD cell model was established as previously described [18]. In summary, SH-SY5Y cells were transfected with overexpression plasmid Aβ1-42 (Sigma, Ronkonkoma, New York, USA) using the Lipofectamine 2000 reagent (Thermo Fisher Scientific, Cleveland, Ohio, USA) according to the manufacturer's instructions at 70% confluence. The cells were separated into the following groups: a nontransfected control group (control), a transfection reagent-treated group (Mock) [19], and a pcDNA31-Aβ1-42-transfected group (Aβ1-42). The cells were collected after 48 h of transfection for subsequent experiments.

Amyloid β-peptide1-42 insult and miRNA transfection

SH-SY5Y cells (ATCC) were seeded into 24-well and 96-well plates at 1×10^6 cells/ml; BC200 siRNA (GenePharma Co. Ltd, Shanghai, China), BC200 mimics (GenePharma Co. Ltd) and/or Aβ1-42 were transfected into cultured cell, respectively. The cells were separated into the following groups: control group, BC200 siRNA group, Aβ1-42 group, and Aβ1-42 + BC200 siRNA group. The cells were collected after 48 h of transfection for subsequent experiments.

Real-time quantitative PCR

The amount of mRNA in AD cell model was evaluated with quantitative real-time PCR (qRT-PCR). Total cellular RNA was extracted by using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Complementary DNA (cDNA) was reverse transcribed using AccuPower Rocketscript Cycle RT Premix (Bioneer, Daejeon, Korea). qRT-PCR was performed by using SYBR Green PCR Master mix (Thermo Fisher Scientific) on an ABI 7300 machine (Applied Biosystems Inc., Carlsbad, USA). The reaction mixtures were incubated at 95°C for 30 s, followed by 40 amplification cycles of 95°C for 5 s, primer annealing by 58°C for 32 s, and elongation at 72°C for 10 s. The primers were obtained from Shenggong (Shanghai, China), and the sequences were as follows. Aβ1-42 – forward: 5′-GGACGATGAGGATGGTGATGAG-3′; reverse: 5'-GGTACTGGCTGCTGTTGTAGGA-3'; BC200 – forward: 5′-TGGCTCACGCCTGTAATCC-3′; reverse: 5′-CCCAGGCAGGTCTCGAACT-3′; BACE1 – forward: 5′-ACCGACGAAGAGTCGGAGGAG-3′ and reverse: 5′-CACAATGCTCTTGTCATAG-3′; results were normalized to β-actin using the $2^{-\Delta\Delta C_t}$ method – forward: 5′-GAAGTGTGACGTGGACATCC-3′; reverse: 5′-CCGATCCACACGGGTACTT-3′. All experiments were triplicated.

Western blot analysis

Following 48 h of transfection, total protein from each group was extracted using ice-cold radioimmunoprecipitation assay buffer (Sigma-Aldrich). Cell lysates were centrifuged at 12 000 *g* for 30 min at 4°C to produce whole-cell extracts. Protein concentration was determined using a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology, Haimen, China). Proteins (30 μg) were separated by SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane (EMD Millipore Corporation, Billerica, Massachusetts, USA). The membrane was blocked with skim milk prepared in TBS-T (20 nM Tris pH: 7.2, 150 mM sodium chloride, 0.1% Tween 20) for 90 min at room temperature, followed by incubation with primary antibody against APP (Aβ42, BC200, BACE1, 1 : 1000; GenePharma Co. Ltd) or β-actin (1 : 1000; Millipore) for 18 h at 4°C. The membrane was then probed with a secondary antibody (Abcam, Shanghai, China) for 90 min at room temperature and visualized using enhanced chemiluminescence solution (Millipore).

Cell proliferation assay

Following transfection for 48 h, cell proliferation was detected using 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's protocol. In summary, MTT solution (final concentration, 0.2 mg/ml; Sigma) was added to each well, and incubated for 2 h at 37°C in a humidified, 5% CO_2 atmosphere. Formed formazan crystals were dissolved in dimethyl sulfoxide, and optical density values were measured at 490 nm using an absorption spectrophotometer (Olympus, Tokyo, Japan). Data were collected from three independent experiments.

Apoptosis assay

A total of 1.0×10^6 cells were collected after 48 h of transfection and stained with both Annexin V-FITC and PI (Thermo Fisher Scientific) according to the manufacturer's instructions. The FITC Annexin V/Dead Cell Apoptosis Kit contains recombinant Annexin V conjugated to fluorescein (FITC Annexin V), as well as a ready-to-use solution of the red fluorescent propidium iodide (PI) nucleic acid-binding dye. PI is impermeant in live cells and apoptotic cells but stains dead cells with red fluorescence, binding tightly to the nucleic acids in the cell. After staining a cell population with FITC

Annexin V and PI in the provided binding buffer, apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence. These populations can easily be distinguished using a flow cytometer. In summary, 10^6 cells were washed twice with 1× binding buffer (Thermo Fisher Scientific) and re-suspended in 100 µl 1× binding buffer. Then, 10 µl Annexin V-FITC per 10^6 cells was added, mixed well, and incubated for 15 min in the dark at room temperature immediately before analysis by flow cytometry (FACScan; BD Biosciences, San Jose, California, USA) equipped with CellQuest software (BD Biosciences). Cells were categorized as early apoptotic cells, late apoptotic cells, dead cells, or viable cells. The ratio of early apoptotic cells to late apoptotic cells was compared with that for controls from each experiment. A total of five parallel experiments were performed for each group. The experiments were performed three times.

Statistical analysis

All values are expressed as the mean ± SD. The data were analyzed using Graphpad Prism 5.0 (GraphPad Software Inc., San Diego, California, USA). One-way analysis of variance with post-hoc Tukey *t*-tests was used to determine statistical significance. *P* value less than 0.05 was considered statistically significant.

Results

Establishment and identification of an Alzheimer's disease cell model overexpressing amyloid β-peptide1-42 gene

To establish an AD cell model, a pcDNA3.1 Aβ1-42 plasmid overexpressing Aβ1-42 gene was transfected into SH-SY5Y cells, and the expression levels of Aβ1-42 in the cells were detected using RT-qPCR and western blot analyses. The mRNA and protein expression levels of Aβ1-42 in the Aβ1-42 transfection group were 4.2-fold (Fig. 1a; $P < 0.01$) and 2.2-fold (Fig. 1b; $P < 0.01$) higher, respectively, as compared with those in mock group. These results suggested that the AD cell model was successfully established.

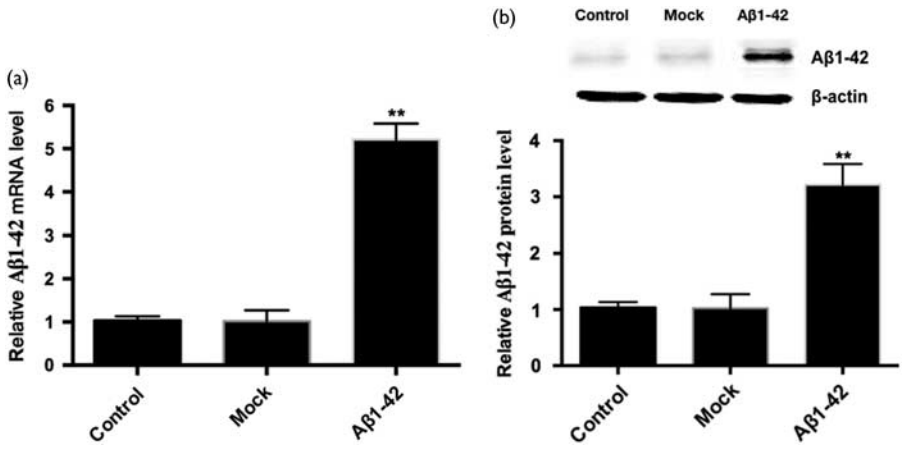
Transfection of BC200 siRNA downregulated BC200 expression in Alzheimer's disease cell

To determine the roles of BC200 in AD, the expressions of BC200 at mRNA and protein level in BC200 siRNA treated or untreated AD cells were detected by qRT-PCR and western blot assay. BC200 is efficiently knocked down by BC200 siRNA in SH-SY5Y cells (Fig. 2; $P < 0.01$). The transfection of Aβ1-42 induced an upregulation of BC200 level, which implicated a high expression of BC200 in AD cell, whereas this can be blocked by BC200 siRNA effectively (Fig. 2; $P < 0.01$).

The inhibition of BC200 increased Alzheimer's disease cell viability

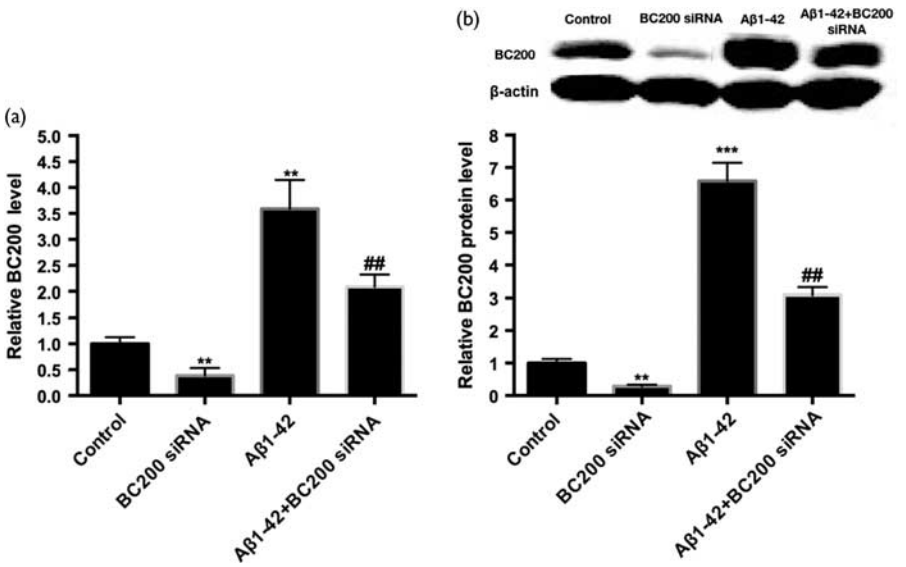
The effects of BC200 on SH-SY5Y cell viability in the AD model were detected using an MTT assay. The results showed that cell viability was significantly decreased in the Aβ1-42 group at 48 h (Fig. 3; $P < 0.01$), as compared with those without Aβ1-42 treatment. However, BC200 knockdown reversed the inhibition effect caused by Aβ1-42 overexpression. Cell viability was significantly decreased following Aβ1-42 plus BC200 siRNA transfection, compared with BC200 siRNA treatment alone (Fig. 3; $P < 0.05$). BC200 mimics reduced cell viability in consistent with Aβ1-42; all of these indicated that BC200 may play as a negative regulator of human AD disease.

Fig. 1



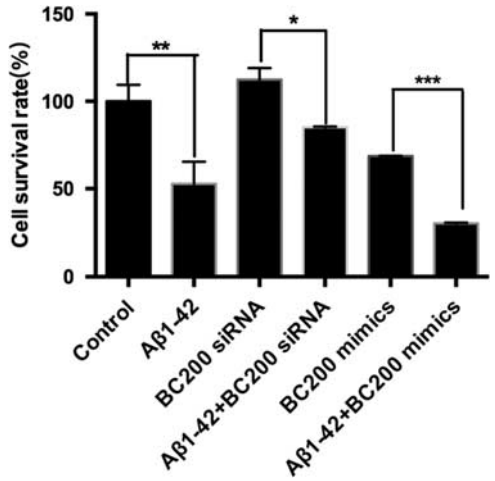
Establishment of an Alzheimer's disease cell model that overexpresses Aβ1-42. (a) Detection of Aβ1-42 mRNA expression levels using qRT-PCR assay. (b) Detection of Aβ1-42 protein expression levels using western blot assay. β-actin was used as an internal control. All experiments were repeated three times. Data were represented as mean ± SD. Groups: control, untreated cells; mock, cells treated with transfection reagent only; Aβ1-42, cells transfected with Aβ1-42 vector. Aβ1-42, amyloid β-peptide 1-42; qRT-PCR, quantitative real-time PCR. $^{**}P < 0.01$.

Fig. 2



Relative BC200 level evaluated in cellular AD model with BC200 knockdown. (a) BC200 mRNA level in each group revealed by qRT-PCR assay in cellular AD model. (b) BC200 protein level in each group revealed by qRT-PCR assay in cellular AD model. All experiments were repeated three times. Data were represented as mean ± SD. Groups: control, untreated cells; BC200 siRNA, cells treated with BC200 siRNA; Aβ1-42, cells transfected with Aβ1-42 vector; Aβ1-42 + BC200 siRNA, cells transfected with both Aβ1-42 and BC200 siRNA. For BC200 siRNA (Aβ1-42) group versus control group; ##for Aβ1-42 and BC200 siRNA group versus Aβ1-42 group. Aβ1-42, amyloid β-peptide 1-42; AD, Alzheimer's disease; qRT-PCR, quantitative real-time PCR. $^{**}P < 0.01$; $^{***}P < 0.001$.

Fig. 3



Inhibition of BC200 decreased the cell viability. Detection of cell viability using MTT assay. Cells were inoculated onto 96-well plates and treated with Aβ1-42, BC200 siRNA, BC200 mimic alone or together. The absorbance values (optical density, 490 nm) of each group were measured at 48 h. Data were represented as mean ± SD. Aβ1-42, amyloid β-peptide 1-42; MTT, 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide; qRT-PCR, quantitative real-time PCR. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

The inhibition of BC200 expression decreased the apoptotic cells

To determine the effects of BC200 depletion on apoptosis of SH-SY5Y cell, Annexin V/PI double staining was performed. Our research revealed that Aβ1-42 insult markedly aggravated the cell late apoptosis (Fig. 4; $P < 0.001$). Statistical analysis revealed that approximately fourfold increase of late apoptotic populations was detected in Aβ1-42 treatment cells (45.1%), as compared with control cells (11.1%, $P < 0.001$). Meanwhile, BC200 inhibitor plus Aβ1-42 treatment induced less cell apoptosis compared with the cultures of Aβ treatment alone (21.1%, $P < 0.01$). These results indicated downregulation of BC200 could decrease cell apoptosis in AD cells.

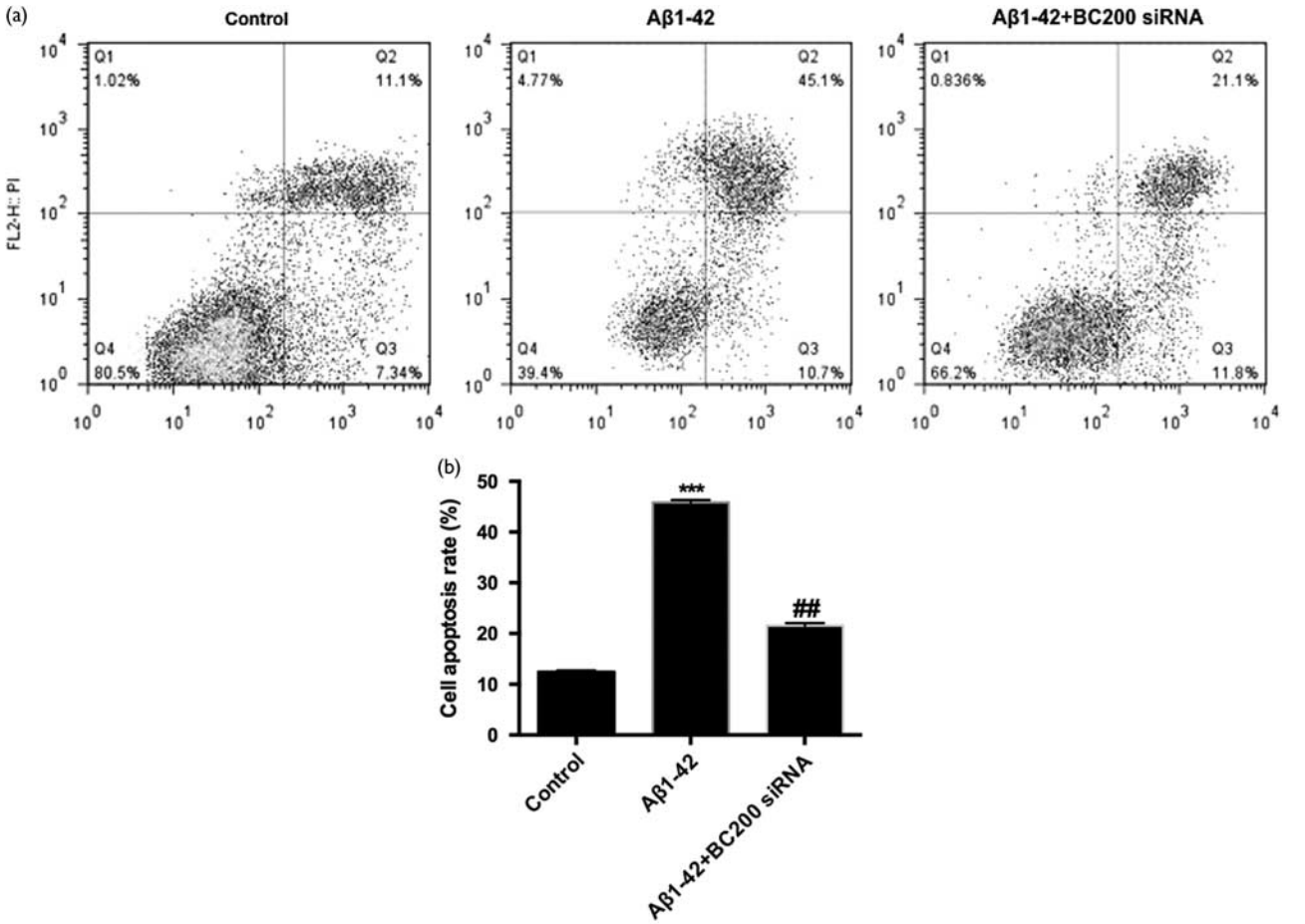
BC200 regulates b-site amyloid precursor protein-cleaving enzyme 1 expression in Alzheimer's disease

As shown before, BC200 functioned in AD development and BACE1 is correlated with AD progression, we wondered how BACE1 works in AD cell. As shown in Fig. 5, a marked upregulation of BACE1 expression was observed in AD cells. On the contrary, BACE1 expression was downregulated after BC200 siRNA transfection ($P < 0.01$). These results suggest that BC200 can regulate BACE1 expression to promote progression in AD.

Discussion

The study of lncRNAs in the regulation of gene expression is a rapidly expanding field with significant implications for human health and disease. Nowadays, more than 60 000 lncRNAs have been demonstrated to exhibit multiple biological functions in various cancer cell

Fig. 4



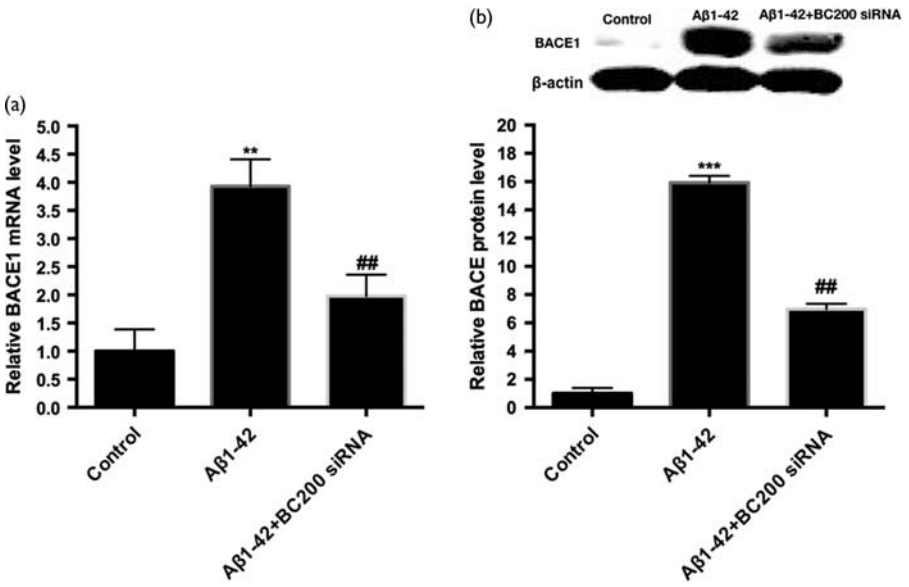
Inhibition of BC200 suppressed cells apoptosis. (a) Detection of cell apoptosis using flow cytometry. The cells were seeded onto 12-well plates and treated with Aβ1-42, BC200 siRNA alone or together for 48 h. Then cells were analyzed using flow cytometry. (b) Statistical analysis of the apoptosis assay. Data were represented as mean ± SD. For BC200 siRNA (Aβ1-42) group versus control group; $^{\#\#}$for Aβ1-42 and BC200 siRNA group versus Aβ1-42 group. Aβ1-42, amyloid β-peptide 1-42; qRT-PCR, quantitative real-time PCR. $^{***}P < 0.001$.

biology [20]. Zhou and Xu [21] identified AD-associated lncRNAs based on post-mortem tissue samples of patients with AD. Magistri *et al.* identified several annotated and nonannotated lincRNAs that are differentially expressed in the hippocampus in late-onset AD [22]. However, the roles of lncRNAs in AD remain largely unknown, and analysis of lncRNAs may provide new insights into the pathophysiology of AD.

BC200, a long noncoding RNA expressed at high levels in the brain and a variety of tumor types, is reported aberrantly expressed in AD [23]. Blocking of BC200 by siRNA assumed to be an effective method for the treatment of AD. Here, we established an AD cell model that overexpressed Aβ1-42. Our results demonstrated that knockdown of BC200 increased cell viability and reduced cell apoptosis in the AD model via mediating BACE1, which can be improved by overexpression of BC200. The increased expression of BACE1 was observed in patients with sporadic AD [24], and it is indeed an important risk factor and critical for the development of AD. Besides, lower Aβ secretion could be achieved by decreasing BACE1 activity [25]. In fact, several studies unequivocally demonstrated that expression and activity of BACE1 are increased during aging in the brains mice, rats, and humans, which raises the possibility of AD risk [26–28], and RNAi-mediated knockdown or genetic deletion of BACE1 ameliorates Aβ-associated pathologies [29,30]. Therefore, identifying the regulatory mechanisms of BACE1 expression may provide new therapeutic opportunities for AD.

In summary, our findings confirmed that inhibition of BC200 significantly suppressed BACE1 expression. BC200 may act as a basic regulating factor to induce cell apoptosis in the process of AD through mediating BACE1. Taken together, BC200 may be one of the putative target in AD development, providing potential

Fig. 5



BC200 regulates BACE1 expression in AD. (a) BACE1 mRNA level in each group revealed by qRT-PCR assay in cellular AD model. (b) BACE1 protein level in each group in cellular AD model. All experiments were repeated three times. Data were represented as mean ± SD. Groups: control, untreated cells; Aβ1-42, cells transfected with Aβ1-42 vector; Aβ1-42 + BC200 siRNA, cells transfected with both Aβ1-42 and BC200 siRNA. For Aβ1-42 group versus Control group; ##for Aβ1-42 and BC200 siRNA group versus Aβ1-42 group. Aβ1-42, amyloid β-peptide 1-42; AD, Alzheimer's disease; BACE1, b-site amyloid precursor protein-cleaving enzyme 1; qRT-PCR, quantitative real-time PCR. $^{**}P < 0.01$; $^{***}P < 0.001$.

new insights into genetic therapy against AD. Based on in-vitro research, further in-vivo studies should be performed on the rodent AD model.

Conclusion

Long noncoding RNA BC200 facilitates AD pathogenesis by upregulating Aβ through BACE1.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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