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Original Article

Effect of miR-506 on the biological behavior of PC12 cells by regulating BACE1 gene

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Abstract: Background and purpose: The amyloid β-protein (Aβ) aggregation is widely recognized as the main molecular mechanisms of Alzheimer's disease. However, regulation of BACE1 (β-site amyloid precursor protein-cleaving enzyme 1), the rate-limiting enzyme of Aβ, remains to be fully elucidated. In this study, we observed the effect of miR-506 on the function of rat adrenal gland PC12 cells by targeting the predicted gene BACE1. Methods: miRNA-506 mimics, miRNA-506 inhibitor and NC group was respectively transfected with Lipofectamine™2000 in Rat PC12 cells to influence the BACE1 expression. The effect of miR-506 on cell proliferation was assessed by MTT assay and colony formation assay. Cell cycle and apoptosis assay were also used to explore the potential function on cell cycle and apoptosis. Quantitative real time-Polymerase chain reaction (qRT-PCR) and Western blot analysis were carried out to confirm the successful suppression of BACE1 gene and protein by miR-506. Finally, luciferase reporter assays were performed to validate the targeted binding of miR-506 to BACE1 gene. Results: As MTT assay and colony formation assay showed, the proliferation of PC12 cells was successfully enhanced by miR-506 mimics group compared with negative control while the miR-506 inhibitor group was not significantly changed. Besides, we found that percentage of cells in miR-506 mimics group at G0/G1 phase decreased than other two groups. Luciferase assays revealed that miR-506 directly bound to the 3'-untranslated region (3'-UTR) of BACE1. Western blot analysis verified that miR-506 regulated expression of BACE1 at protein level. Conclusion: miR-506 can promote cellular growth and regulate cell cycle by targeting BACE1.

Keywords: Alzheimer's disease, miR-506, BACE1, cellular function

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease with progressive cognitive dysfunction and behavioral impairment. To date, the most generally acknowledged pathology of AD consists of amyloid-β deposition and neurofibrillary tangles. Still no efficient therapeutic strategies can reverse the cognitive dysfunction. The exact mechanisms underlying AD has not been fully elucidated.

β-site amyloid precursor protein-cleaving enzyme 1 (BACE1), known as a membrane-bound aspartate protease, catalyses the initial step in the amyloidogenic metabolism, releasing a soluble amyloid precursor protein (sAPPβ) and C-terminal fragment consisting of 99 amino acids (CTF99). Then is further processed by γ-secretase enzyme which generates AT peptide [1, 2]. The identification of Aβ, as basis of neurofibrillary tangles and progressive insoluble senile plaques, is strongly implicated in the pathogenesis of AD. Thus, interruption of 'amyloid cascade' at BACE1 site potentially becomes a promising target to alleviate the amyloid burden. However, the regulation mechanisms of BACE1 expression have not been established firmly.

MicroRNAs (miRNAs), as endogenous sets of RNAs, are non-protein-coding and regulators of gene expression. It inhibits the post-transcriptional process through base pairing with short regions 3'-untranslated region (3'-UTRs) of target mRNAs, and results in translational repression or mRNA destabilization. It is known that miRNAs play an important role in the physiologi-

cal and pathological processes as apoptosis, proliferation, differentiation. What's more, abnormal miRNA expression has been generally observed in the development of neurodegenerative diseases including AD [3]. The presumption that variation in miRNAs networks in the brain lead to neurodegenerative disease is attractive. Studies are as follows: elevated miR-146a targets complement factor H (CFH) and IL-1 receptor-associated kinase-1 (IRAK1) [4]; miR-103a targets actin-binding protein cofilin [5]; miR-29a targets neuronnavigator 3 (NAV3), which has been recognized to correlate with AD [6]. Moreover, several miR-clusters such as miR-29a/b [7], miR-107 [8], miR-298, miR-328 [9], miR-485-5P [10], miR-124 [11], miR-195, miR-135a, etc. have been reported to significantly decrease in AD patients, with a correlation with aberrantly high BACE1 protein. Therefore, miRNAs has shown an emerging new look of regulating BACE1 levels. Deregulated miRNAs might play important roles in the pathogenesis of AD. However, the mechanism for miRNAs in the progression of AD has not been fully elucidated.

In present study, we investigated the effect of miR-506 on the function of rat PC12 cells. Then we elucidated the expression alteration of one of its potential downstream targets, BACE1, and the profiles of cell death and survival following treatments with mimics or inhibitor of miR-506.

Materials and methods

Cell culture and extraction

The rat PC12 cell line was purchased from Chinese of Sciences in Shanghai. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco), penicillin (100 units/ml) and streptomycin (100 μg/ml) (Enpromise, China). Cells were incubated at 37°C in a humidified chamber supplemented with 5% CO_2.

Firstly, execute the pregnant SD rats on embryonic day 15 by cervical dislocation. Dissociate the brain cortex and wash it in Hank's buffered saline solution. Then cut it into small pieces, digest with trypsin, isolate with pipette, and centrifuge to separate un-dissociated tissue. Make sure the extracted cells were single with the 150-200 mesh filter. At last, the neurons were resuspended in serum-free neurobasal medium and plated into 6-well plates at a culture density of 1×10^6 cells/mL at 37°C supplemented with 5% CO_2. Cytosine arabinoside was added at 48 h to inhibit the excessive growth of non neuronal cells such as Glial cells and few fibroblasts.

miRNA transfection

Select the cells of exponential growth phase; add cells (1×10^5) into each well of a 6-well plate and culture them with DMEM medium off with serum and antibiotics. Culture the confluency of rat adrenal gland PC12 cells to 30-50%. Meanwhile miR-506 mimics, miRNA-506 inhibitor and lipofect were respectively diluted to 250 μl at the ratio of 1 μg: 1 μg: 3 μl by DMEM medium, and incubated for 5 min at room temperature. MiR-506 mimics and miRNA-506 inhibitor respectively were softly mixed with lipofect and incubated for 25 min. Then 500 μl of 50 nM were added to each well. After 4-6 h of incubation, replace the DMEM medium with DMEM with 10% FBS, and incubate these cells at 37°C in a CO_2 incubator for 48 h before further tests.

RNA extraction and quantitative reverse-transcription PCR (qRT-PCR)

Total RNA was extracted from the PC12 cells using TRIzol (Invitrogen, Carlsbad, CA, USA) as to the manufacturer's protocol, then analyzed by ultraviolet spectrophotometer. To detect the miR-506 expression, primer design and qRT-PCR were experimented as to the manufacturer's directions. The primers were as followed: miR-506 forward, 5'-TAAGGCACCCTTCTGAGTAGA-3', reverse, 5'-GCGAGCACAGAATTAATACGAC-3'; U6 forward, 5'-AGAGCCTGTGGTGTCCG-3', reverse, 5'-CATCTTCAAAGCACTTCCCT-3'; BACE1 forward, 5'-AGCTGGATTATGGTGGCCTGAG-3', reverse, 5'-CCTGCAGCTTTCAGGGTCTTC-3'; β-actin forward, 5'-CGTCTTCCCCTCCATCGT-3', reverse, 5'-GAAGGTGTGGTGCCAGATTT-3'.

cDNA was produced by reverse transcription using the PrimeScript™ RT-PCR kit according to the manufacturer's directions (Takara, Tokyo, Japan). With the 7900HT Fast RT-PCR instrument (Applied Biosystems, Singapore), real-time PCR was carried out as follows: 5 min at 95°C, followed by 35 cycles of 30 sec at 95°C,

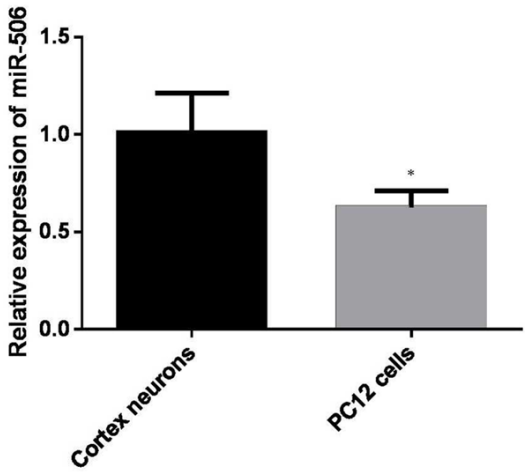

Figure 1. Expression of miR-506 in PC12 cells. Expression level of miR-506 was down-regulated in PC12 cells compared to cortical neurons.

45 sec at 65°C and 45 sec at 72°C . The fold-change of mRNA expression was calculated by the relative quantification equation, 2-ΔΔCT. Each sample was experimented in triplicate.

Western blot analysis

Cells were extracted after 48 h transfection. Total cell protein was extracted by RIPA lysis buffer. The concentration was quantified by bicinchoninic acid assay (Pierce, USA). Then, denature 20 μg protein samples with 5X sodium dodecyl sulfate (SDS) loading buffer at 95°C for 5 min. Next, separate the total cell protein by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transfer onto 0.45-μm nitrocellulose membranes (Beyotime). Afterwards, block membranes in 5% fat-free milk and incubate with the BACE1 antibody (1:1,000) and the β-actin antibody (1:1,000) (both from Cell Signaling Technology, USA) overnight at 4°C. Wash the protein blots and then incubate for 1 h with specific secondary antibodies. Washed by PBST for 3 times again, immune-reactive protein bands were detected using the odyssey scanning system (LI-COR, Lincoln, NE, USA). The experiment was performed three times in triplicate. Representative photographs are shown.

Cell viability assay

Select the PC12 cells of Logarithmic growth phase; add cells (2×10^3) into each well of a 96-well plate (BD Biosciences, USA) and culture them with DMEM medium. Transfect PC12 cells with 50 nM miR-506 mimics, miR-506 inhibitor and NC control. We evaluated the cell multiplication at 24, 48, 72 and 96 h post-transfection by the MTT assay. In short, add 20 μl (5 mg/ml) MTT (Sigma, USA) solution to each well. After a 4-h incubation at 37°C, the supernatant was replaced with 100 μl dimethylsulfoxide (DMSO; Sigma) and vortexed for 10 min. At last, the optical density (OD) of each well was analyzed using a microplate spectrophotometer at 490 nm. Data was collected from three independent experiments.

Colony formation assay

Five hundred cells of each group (miR-506 mimics, miR-506 inhibitor and NC) were added in a 6-well plate in complete medium. After 8-10 days, or when the colonies were visible by the naked eye, the culture was terminated. Then wash the plates twice in phosphate buffered saline (PBS) after removing the complete medium. The colonies were fixed in 95% ethanol for 15 min, dried and stained with 0.1% crystal violet solution for 15 min. Finally, each plate was washed twice with flowing water. Colonies less than 2 mm in diameter and faintly stained were neglected, the number of colonies was counted under a microscope in 10 random view fields. The experiment was repeated for three times.

Cell cycle analysis

To investigate if miR-506 can lead to apoptosis of PC12 cells, PC12 cells were transfected with 50 nM of miR-506 mimics, inhibitor or NC for 48 h. Post-transfected PC12 cells were trypsinized and centrifuged at 1,000 rpm for 5 min, then subsequently washed by precooled PBS twice. Add 3.0 ml ice-cold ethanol and fix the cells for two hours. 250 μl 0.05 g/L Propidium iodide (PI) staining solution was added into each sample and incubated for 30 min in dark at room temperature. Finally, analyze the cells on a flow cytometer (FACSCanto™ II, BD Biosciences). The experiment was performed three times in triplicate.

Cell apoptosis analysis

Post-transfected PC12 cells till 48 h were trypsinized and centrifuged at 1,000 rpm for 5 min, then subsequently washed by precooled PBS twice. Double-stained with fluorescein (FITC)-conjugated Annexin V, cells were added with propidium iodide (FITC-Annexin V/PI) (BD

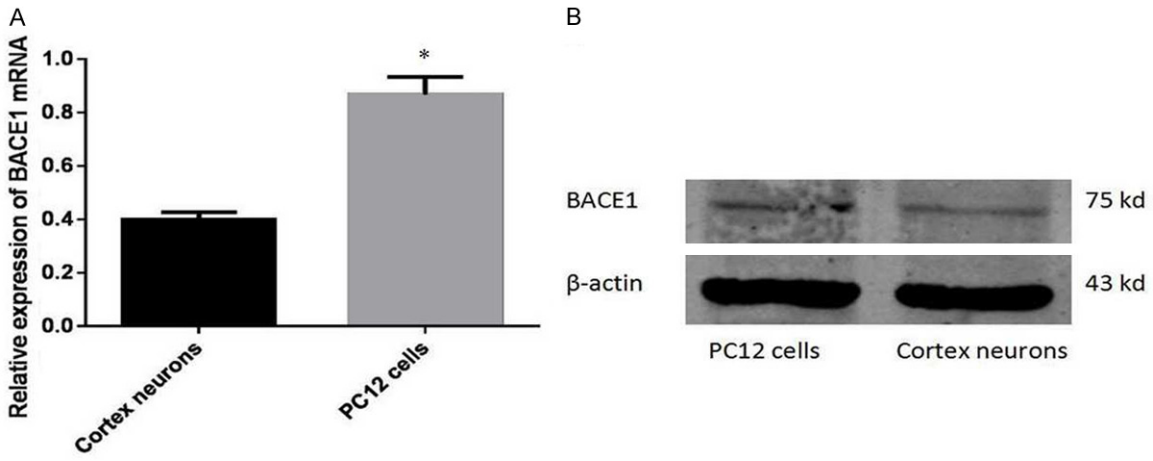

Figure 2. Expression of BACE1 in PC12 cells. A. Contrary to the low level of miR-506, endogenous BACE1 mRNA was up-regulated in PC12 cells compared with primary cortical neurons. Data represent the 2-ΔΔCt values ± SD, ($P < 0.05$). B. Western blot indicated that the expression level of BACE1 protein in PC12 cells is higher than that in cortex neurons ($P < 0.05$).

Biosciences, San Diego, CA, USA) and then analyzed on a FACSCalibur flow cytometer (BD Biosciences) to assess rate of apoptosis. The experiment was repeated for three times.

Luciferase assays

Amplifie the 3'-untranslated region (3'UTR) of mRNA sequence of BACE1 containing predicted miR-506 binding site by PCR following the direction of Primer star kit (Takara). ClonePCR products into the Xho I site in the 3'-UTR of Renilla luciferase of psiCHECK-2 reporter vector (Promega, USA). Reporter plasmids (200 ng psiCHECK-2 reporter vector containing BACE1 3'UTR) and 100 nM miR-506 mimics were cotransfected into 293T cells (80-90% confluence) using Lipofectamine 2000 (Invi-trogen, USA), control groups include cells cotransfected with psiCHECK-2/BACE1 3'-UTR (200 ng) and NC (100 nM), psiCHECK-2/BACE1 3'-UTR mutant (200 ng) and miR-506 mimics (100 nM) as well as psiCHECK-2/BACE1 3'-UTR mutant (200 ng) and NC (100 nM). After 48 h, lyse the cells and detect the reporter activity using Dual-luciferase report assay system (Promega, USA). Firefly luciferase values were normalized to Renilla, and the ratio of firefly/renilla was shown. All experiments were performed three times.

Statistical analysis

Each vitro experiment was performed in triplicate. Data were expressed as the means ± standard deviation (SD). One-way ANOVA was used for comparisons. *P* values < 0.05 were defined statistically significant between groups.

Results

Expression levels of miR-506 and BACE1 in PC12 cells

In order to determine the expression level of miR-506 and BACE1 in PC12 cell, we compared it with primary cortical neurons at both mRNA and protein level. As shown in **Figure 1**, PC12 cells had less expression of miR-506 in comparison with cortical neurons ($P < 0.05$). Contrary to the low level of miR-506, endogenous BACE1 was up-regulated in PC12 cells (**Figure 2**).

Over-expression of miR-506 promotes proliferation of PC12 cells

To examine the effect of miR-506 on the proliferation of PC12 cells, miR-506 mimics, miR-506 inhibitor and NC were transfected into PC12 cells at the final concentrations of 50 nM. We evaluated cell proliferation at 24, 48, 72 and 96 h post-transfection by MTT assays. In short, inhibition rate was calculated as following: inhibition rate (%) = (OD value of the control group-OD value of experimental group)/OD value of control group iR-506. In comparison with the NC group, miR-506 mimics group was promoted significantly ($P < 0.05$, **Figure 3B**) in time dependent manner. Conversely, cell prolif-

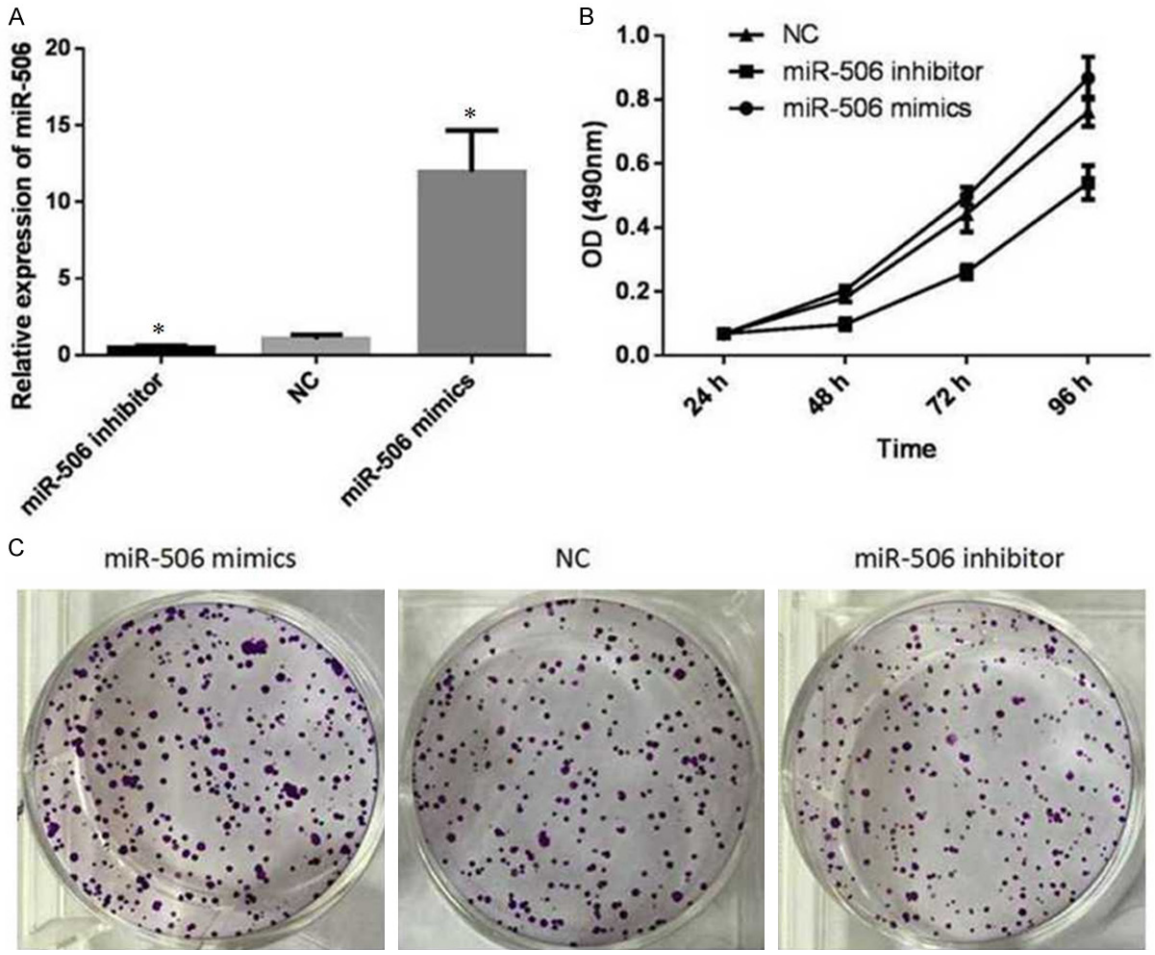

Figure 3. Over-expression of miR-506 promotes the proliferation of PC12 cells. A. The transfection efficiency was observed by the levels of miR-506 after transfection with miR-NC, miR-506 inhibitor or miR-506 mimics. Relative miR-506 expression of NC group was lower than miR-506 mimics group and higher than miR-506 inhibitor group. B. The MTT assay was performed to monitor the proliferation level of post-transfected PC12 cells. The optical density of each well was analyzed using a microplate spectrophotometer at 490 nm. Up-regulation of miR-506 significantly promoted the growth rate of PC12 cells, while the inhibitor suppressed the growth rate ($P < 0.05$). Graph represents OD 490 nm ± SD. C. Colony formation assays showed much more colony formation in the group transfected with miR-506 mimics group compared with the NC group and miR-506 inhibitor ($P < 0.05$).

eration was strongest inhibited when cells were interfered with 50 nM miR-506 inhibitor for 96 h ($P < 0.05$, **Figure 3B**). Colony formation assays also showed much more colony formation in the group transfected with miR-506 mimics. The colony number decreased when transfected with miR-506 inhibitors ($P < 0.05$, **Figure 3C**). On the whole, these data suggested that over-expression of miR-506 promoted proliferation of PC12 cells.

Over-expression of miR-506 can change the PC12 cells cycle distribution

Forty-eight hours after the transfection, flow cytometry analysis indicated that the percentage of G0/G1 phase (57.44%) dramatically decreased in the miR-506 mimics group, when compared with that of the NC group (64.19%) and miR-506 inhibitor group (65.19%) ($P < 0.05$, **Figure 4**), while there were no statistical difference between the NC group and miR-506 inhibitor group ($P > 0.05$). These findings revealed that miR-506 can reduce G0/G1 phase arrest. Upregulation of miR-506 expression could lead to increase of S-phase and G2/M phase cells (**Figure 4**).

Over-expression of miR-506 has no significant effect on apoptosis of PC12 cells

Apoptotic cell death was assessed using flow cytometric analysis of Annexin V-FITC/PI staining. As presented in **Figure 5**, the percentage of

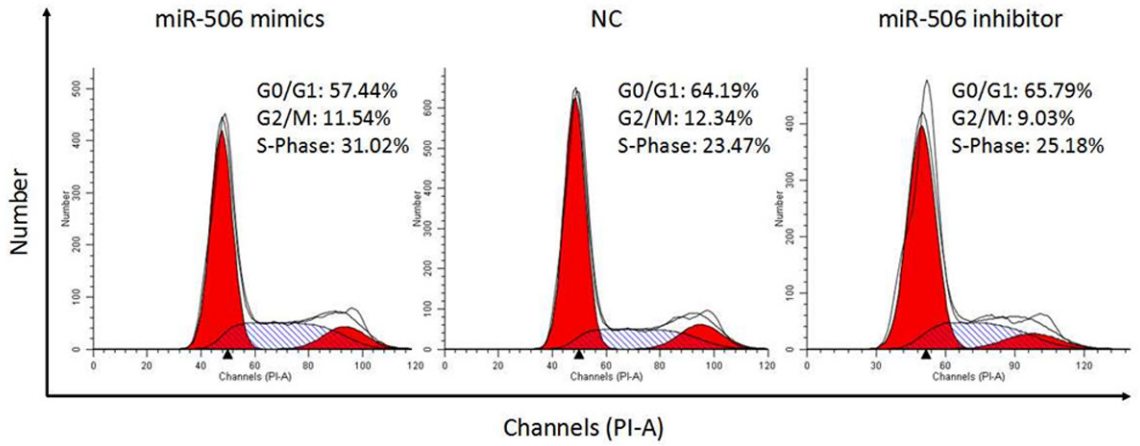

Figure 4. Cell cycle distribution was analyzed by flow cytometry 48 h after transfection of PC12 cells with 50 nM miR-506 mimics or inhibitor or NC. Flow cytometry analysis indicated that the percentage of G0/G1 phase cells (57.44%) dramatically decreased in the miR-506 mimics group, when compared with that of the NC group (64.19%) and miR-506 inhibitor group (65.79%) ($P < 0.05$).

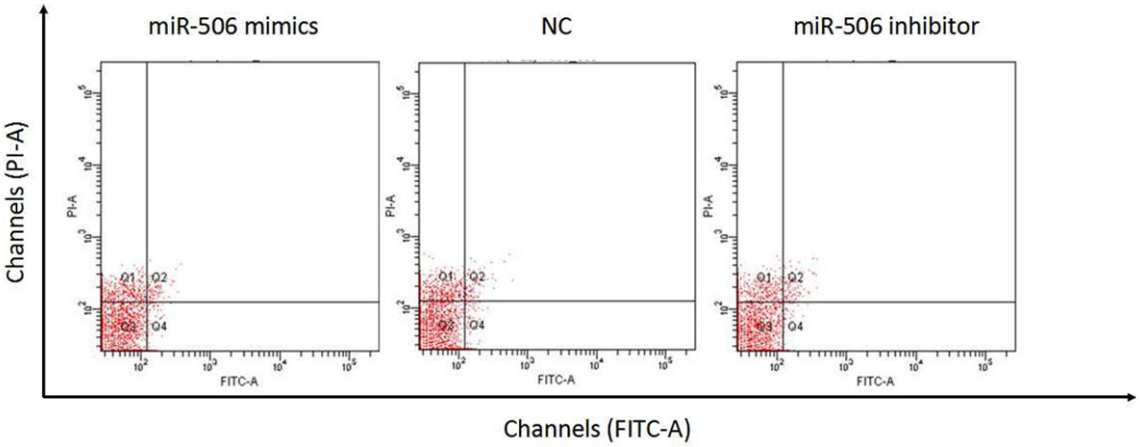

Figure 5. Cells transfected with NC, miR-506 inhibitor or miR-506 mimics were stained with PI and analyzed by flow cytometry. As shown above, the percentage of the early and late apoptotic cells was a little higher in the miR-506 inhibitor group in comparison with the miR-506 mimics group and NC group, but no significant difference had been found between the three ($P > 0.05$).

the early and late apoptotic cells was a little higher in the miR-506 inhibitor group compared with the miRNA mimics group. But we found no statistical difference between these three groups ($P > 0.05$). These results indicated that miR-506 had no significant effect on PC12 cell apoptosis in vitro.

miR-506 can specifically target BACE1 gene and inhibit BACE1 at protein levels

In the preliminary experiment, we found that contrary to the low expression of miR-506, endogenous BACE1 mRNA and protein expression were up-regulated in PC12 cells compared with primary cortical neurons. These results supported our previous hypothesis.

To validate that miR-506 can bind to the predicted site BACE1, we performed a luciferase reporter assay in the 293T cell line. As presented in **Figure 6**, the luciferase activity significantly decreased after co-transfection with psiCHECK-2/BACE1 3'-UTR and miR-506 mimics in comparison with NC. However, this significant action of miR-506 was abolished following co-transfection of psiCHECK-2/BACE1 3'-UTR mutant and miR-506 mimics. Briefly, this result

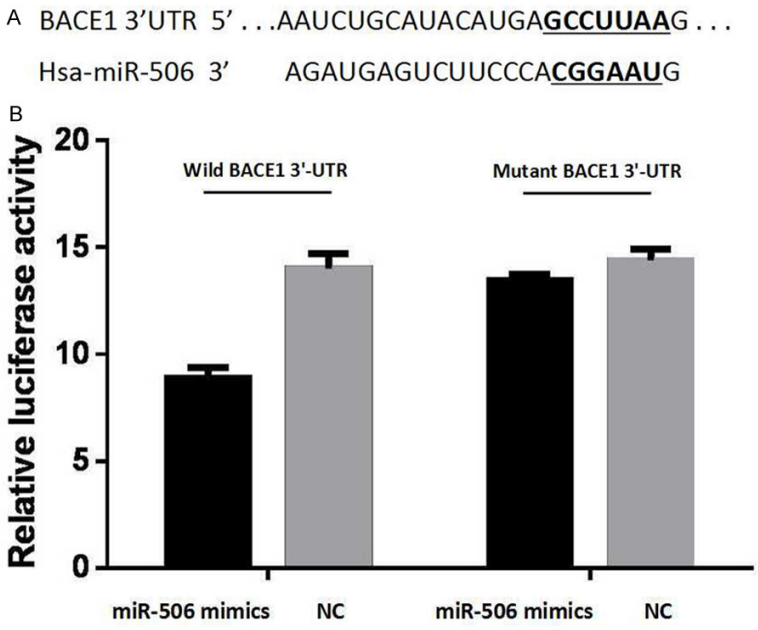

Figure 6. MiR-506 can specifically target BACE1 gene. A. The binding site for miR-506 in the 3'-UTR of BACE1 mRNA. B. The relative luciferase activity was analyzed in PC12 cells after co-transfection of the BACE1 3'-UTR or BACE1 3'-UTR mutant luciferase construct with either miR-506 mimics or NC. As shown above, the relative BACE1 expression was significantly down-regulated by BACE1 3'-UTR luciferase construct with miR-506 mimics compared with NC group ($P < 0.05$). However, no significant difference was found between BACE1 3'-UTR mutant luciferase construct with miR-506 mimics and NC control.

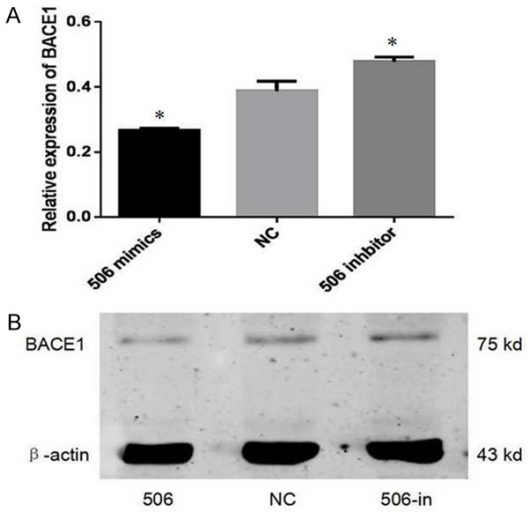

Figure 7. miRNA-506 can down-regulate the BACE1 expression at protein levels. A. The grey level of BACE1 in miR-506 inhibitors group is much more than that in miR-506 mimics group ($P < 0.05$). B. Western blot analysis was used to detect BACE1 protein expression levels. BACE1 decreased when transfected with miR-506 mimics but increased when transfected with miR-506 inhibitors at protein levels. Data represent means ± SD.

indicated that miR-506 specifically binds to the 3'-UTR of BACE1 mRNA.

To discover whether miR-506 regulates BACE1, we transfected miR-506 inhibitors or mimics (50 nM) into PC12 cells, and the levels of BACE1 protein were detected.

As shown in **Figure 7**, we found that at the protein levels, BACE1 expression clearly decreased in the miR-506 mimics group in comparison with NC group and miR-506 inhibitor group. Together, these results validate our previous hypothesis that BACE1 is a novel target of miR-506.

Discussion

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and its most common characteristic is defined as cognitive dysfunction. To date, no existing therapeutic agents can effectively reverse its progression. In search for non-invasive biomarkers for Alzheimer's disease (AD) diagnosis and prognosis, circulating miRNAs are emerging as a promising candidates these decades. What's more, increasing evidence indicates that miRNAs may affect cancer pathogenesis. Dysregulation of miRNAs is related to initiation and progression of AD since they may affect the amyloid production as well as neurofibrillary tangles. For example, miR-29 expression contrarily correlated with BACE1 (miR-29) whereas it had been presented to increase amyloid production in vitro [7]; miR-34, expressed highly in the hippocampus of patients with AD, regulated the p53 associated to tau phosphorylation [12]; additionally, miR-107 [13], reported to be down-regulated in temporal cortex at an early stage of AD had a converse correlation with BACE1 and plaque density [14]. In the present study, we are interested in the role of miR-506 in AD. The role of miR-506 in cell differentiation and senescence was recently demonstrated in some tumour types, including neuroblastoma [15], lung [16], breast, ovarian [17] and cervical

cancer [18] etc. The previous studies showed that miR-506, effectively reduced the tumor burden and inhibited invasive growth and metastasis. Therefore, miR-506 has been conceived as a promising new therapeutic agent that can be carried to suppress cancer progression. But no connection has been discovered with the neurodegenerative disease.

We performed qRT-PCR to assess the expression level of miR-506 in primary cortical neurons and PC12 cell lines. We further investigated the biological impact of miR-506 and the molecular mechanisms by which miR-506 modulates the behavior of PC12 cells.

Firstly, we investigated the expression levels of miR-506 in primary cortical neurons and PC12 cell lines. Intriguingly, we found that the expression levels of miR-506 were obviously decreased in PC12 cells compared with cortical neurons extracted from SD fetal rats.

What's more, we further tested the gain-or-loss effects of miR-506. MiR-506 mimics, miR-506 inhibitor and NC were transfected into PC12 cells to adjust its own expression. The exogenous over-expression of miR-506 regulating by miR-506 mimics strikingly promoted proliferation and colony formation ability of PC12 cells as evaluated by MTT and colony formation assays, respectively. We also found that miR-506 distinctly decreased percentage of cells at the G0/G1 phase in the cell cycle assay.

To determine how miR-506 caused these effects on the cell function, we tested assumed targets of miR-506 and identified BACE1, which is the most critical protease in the amyloidogenic metabolism. In previous research, BACE1, levels of which have been shown to be increased in the common late-onset sporadic AD [19]. Considered as a potential target for therapies against AD, BACE1 is characterized by accumulation of plaques formed of amyloid precursor protein [20-22]. Furthermore, Willem M found BACE1 was important in the formation of myelin sheaths in peripheral nerve cells [23]. Recently, BACE1 level has shown to be regulated by microRNAs, such as miR-29a, miR-29b-1, miR-29c [24], miR-107 and miR-124 [25] etc. in vitro.

In our study, we performed a luciferase reporter assay to determine that miR-506 can bind to the predicted site BACE1. We identified BACE1 as a direct target of miR-506 in PC12 cells. As shown above, BACE1 decreased when transfected with miR-506 mimics but increased when transfected with miR-506 inhibitors. In a word, these results show that enforced expression of miR-506 in PC12 cells triggered an evident inhibitory effect on BACE1 expression. On the whole, this experiment is carried out in the transient transfection of miR-506 with Lipofectamine™2000, and the inhibition of gene is not stable enough. In addition, we have not clarified the role of miR-506 in vivo but only in PC12 cells. Related research shall be further expanded.

To conclude, our findings indicate that miR-506 is down-regulated in PC12 cells and over-expression of miR-506 is able to promote cellular proliferation as well as disrupt the cell cycle via direct regulation of BACE1. Furthermore, its expression has a negative correlation with BACE1, which is highly direct correlation with amyloid production, neuritic plaque density, as well as neurofibrillary tangles. Just as the emerging role for miRNA in AD, miR-506 may also act as a potential therapeutic target spot for AD.

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Disclosure of conflict of interest

None.

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