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# MiR-16 regulates cell death in Alzheimer's disease by targeting amyloid precursor protein

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**Abstract. – OBJECTIVE: The aim of this study was to investigate the role of miR-16 in Alzheimer's disease (AD) and to explore its mechanism of action.**

**MATERIALS AND METHODS: A cellular AD model using PC12 cells and primary hippocampal neurons was established to evaluate the expression level of miR-16. Transfection of a miR-16 mimic and a miR-16 inhibitor were performed to explore its effect on cell apoptosis and cell viability. In addition, we carried out bioinformatics analysis, luciferase reporting gene assay, and gene expression analyses to identify the potential target of miR-16 and to verify the effect of the target gene on the cellular AD model.**

**RESULTS: Downregulation of miR-16 was confirmed in the cellular AD model with both PC12 cells ($p < 0.05$) and primary hippocampal neurons ($p < 0.05$). Overexpression and inhibition of miR-16 in the cellular AD model with primary hippocampal neurons decreased and increased apoptosis, respectively. The gene encoding amyloid precursor protein (APP) was identified as the target gene of miR-16. Knockdown of APP in primary hippocampal neurons decreased cell apoptosis and increased cell viability in the cellular AD model.**

**CONCLUSIONS: Our results demonstrate that downregulation of miR-16 in primary hippocampal neurons play an important role in the paracrine effect and might be involved in the development of AD.**

*Key Words:*
Alzheimer's disease, miR-16, Amyloid precursor protein, Primary hippocampal neurons, Apoptosis.

## Introduction

Alzheimer's disease (AD), a chronic neurodegenerative disease characterized by loss of mental ability that is severe enough to influence ordinary daily life, is the most common cause of dementia[1]. The occurrence of AD strongly correlates with increasing age, and the prevalence of AD in people over 65 years old is reported to range from 4.4% to 9.7%[2]. AD will be an even greater public health problem in the future, since the number of elderly (65+ years) is expected to reach 1 billion in 2030[3]. Therefore, there is an urgent need for new molecular targets, concepts, and approaches to treat this disease.

At the cellular level, AD is characterized by neurofibrillary tangles and progressive formation of insoluble senile plaques composed mainly of β-amyloid (Aβ) peptide, which is a cleavage product of the amyloid precursor protein (APP)[4,5]. Moreover, Aβ-mediated neurotoxicity plays a critical role in the disease and the accumulation of Aβ in the brain is strongly associated with the pathogenesis of AD[4,5].

A class of endogenous, highly conserved noncoding small RNAs, miRNAs, exert a role in post-transcriptional suppression of gene expression via target gene degradation and inhibition of translation[6]. There is growing evidence[7,8] that miRNAs participate in various biological events, and recent studies[9,10] have suggested that miRNAs are involved in the pathogenesis of AD. Lukiw et al[11] showed that some miRNAs, such as miR-9 and miR-125b, were elevated in AD patients. Hebert et al[12] proposed that miR-29a/b-1 can regulate beta-amyloid precursor protein-converting enzyme (BACE) 1/β-secretase, which is involved in APP cleavage into Aβ, in patients with sporadic AD.

In this study, we investigated the role of miR-16 in AD and explored its mechanism of action. Our results demonstrated the downregulation of miR-16 in a cellular AD model of primary neurons. We validated *APP* as the target gene of miR-16. Furthermore, we showed that overexpression of miR-16 in primary neurons protected the primary neurons from death. Our data suggest that miR-16 plays an important role in the pathogenesis of AD.



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## Materials and Methods

### Cell Culture and Treatment

The primary hippocampal neurons were isolated according to previously described methods[12]. Briefly, the brain cortex was isolated from Sprague-Dawley (SD) rat embryos on embryonic day 16. After rinsing with Hank's buffered saline, tissues were cut into small pieces, digested with trypsin, dissociated, and collected by centrifugation. Cells were resuspended and seeded in poly-D-lysine pre-coated cell culture plates at a density of $1\times10^6$ cells/ml, and cultured in serum-free neurobasal medium containing $1 \times B_{27}$ and 0.05 mM glutamine.

Rat PC12 cells were obtained from ATCC, plated in poly-D-lysine pre-coated cell culture plates at a density of $1 \times 10^6$ cells/ml, and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 5% fetal bovine serum (Hyclone, Logan, UT, USA), 10% horse serum, and 1% penicillin-streptomycin. PC12 cells were maintained at 37°C in 5% $CO_2$.

For the Aβ insult, an aggregated form of $A\beta_{1\text{-}42}$ was prepared according to previously described methods[12] and was used at a concentration of 10 μM.

### Cell Transfection

Transfection of neurons with amyloid precursor protein (APP) siRNA (Invitrogen, Carlsbad, CA, USA), miR-16 mimic (GenePharma Co.Ltd, Shanghai, China; 50 nM), miR-16 inhibitor (GenePharma Co. Ltd; 50 nM), Pre-miR Precursor Negative Control (Ambion, Austin, TX, USA; 10 nM), and pre-miR-16 (Ambion; 50 nM) were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

### Quantitative Real-time Reverse Transcription-Polymerase Chain Reaction (RT–PCR)

Total cellular RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was quantified using a Nanodrop 2000 (Thermo Scientific, San Jose, CA, USA). A High Capacity cDNA Reverse Transcription Kit and TaqMan MicroRNA Reverse Transcription Kit were used for mRNA and miRNA reverse transcription, respectively (Applied Biosystems, Foster City, CA, USA). RT-PCR was conducted using a One Step SYBR® Prime Script™ RT-PCR Kit (Takara, Dalian, China) to detect APP and GAPDH mRNA as well as using TaqMan microRNA assays to detect miRNA and U6 (Applied Biosystems, Foster City, CA, USA). Expression of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 genes were assessed simultaneously in all samples as internal controls. Relative gene expression was determined by the $2^{-\Delta\Delta CT}$ method[13].

### Quantification of Apoptosis by Hoechst/propidium Iodide Nuclear Staining

Double nuclear staining with fluorescent dyes Hoechst 33342 and propidium iodide (PI) (Molecular Probes, Eugene, OR, USA) was used to determine cell apoptosis according to a previously described method[14]. Briefly, after the cells were incubated with different treatments for 24 h, Hoechst 33342 and PI were added to the culture medium at concentrations of 8 and 1.5 μM, respectively. The cells were further incubated at 4°C for 30 min. Images were taken under a fluorescent microscope, cells in 5 randomly picked fields (400× magnification) were counted, and the percentage of apoptotic cells was calculated.

### MTT cell Proliferation

Primary cultured neurons ($1 \times 10^6$ cells/ml) were seeded in 96-well plates, transfected with either the miR-16 mimic or inhibitor, and incubated for 48 h before Aβ insult for 24 h. Cells were treated with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; 5 mg/mL) at 24 h intervals and proliferation was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Results were reported as percentages of the optical density (OD) values of the control group.

### Western Blotting

Primary hippocampal neurons with pre-miR-control or pre-miR-16 transfections were lysed in RIPA buffer, followed by high-speed centrifugation and quantification using bicinchoninic acid. Cellular proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. After blocking, membranes were incubated with *APP* (Sigma, St. Louis, MO, USA). GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the loading control. The appropriate horseradish peroxidase-conjugated secondary antibodies were applied, and the pro-

tein bands were detected with SuperSignal Ultra Chemiluminescent Substrate (Pierce, Rockford, IL, USA) on X-ray films (Kodak, Tokyo, Japan).

### Luciferase Assays

The pMIR-APP-3'UTR plasmid, with the putative binding site of the APP 3'UTR downstream of the firefly luciferase gene, was generated by cloning and inserting a 900-bp sequence of the 3' UTR downstream of the XhoI and XbaI sites in the pMIR-REPORT Luciferase Vector (Ambion, Foster City, CA, USA). For luciferase activity measurement, primary hippocampal neurons were grown in 24-well plates to 60-70% confluence, followed by the co-transfection of luciferase plasmid with Renilla plasmid (Ambion) (as a control for transfection efficiency) and pre-miR-16 (as a negative control) using Lipofectamine 2000 (Invitrogen). The activities of luciferase and Renilla were assessed after 48 h with the Dual-Luciferase Reporter 1000 Assay System (Promega, Madison, WI, USA).

### Statistical Analysis

All statistical analyses were carried out using SPSS v18 (SPSS Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation (SD). Student's *t*-test was used to examine differences between groups and $p < 0.05$ was considered significant.

## Results

### Downregulation of miR-16 in the Cellular AD Model

We employed cellular AD models of rat neuronal differentiated PC12 cells and hippocampal neurons from SD rats. In both models, we found a significant decrease in the level of miR-16 after Aβ treatment (both $p < 0.05$, Figure 1).

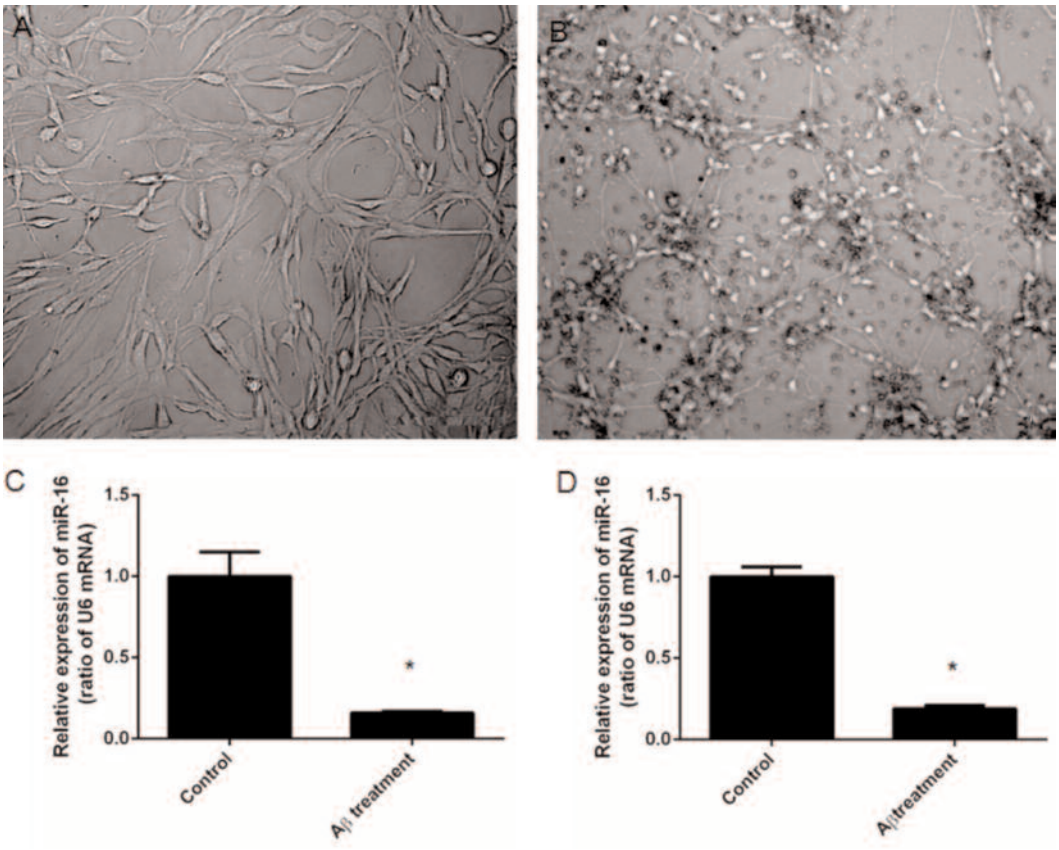


**Figure 1.** The expression of miR-16 was decreased in Aβ insult cellular Alzheimer's disease ***(AD)*** model. The morphology of rat neuronal differentiated PC12 cells ***(A)*** and hippocampal neurons from Sprague-Dawley rats. The expression of miR-16 was evaluated in cellular AD model of PC12 ***(C)*** and primary hippocampal neurons ***(D)*** by using realtime PCR. *$p < 0.05$ when compared to control cells.

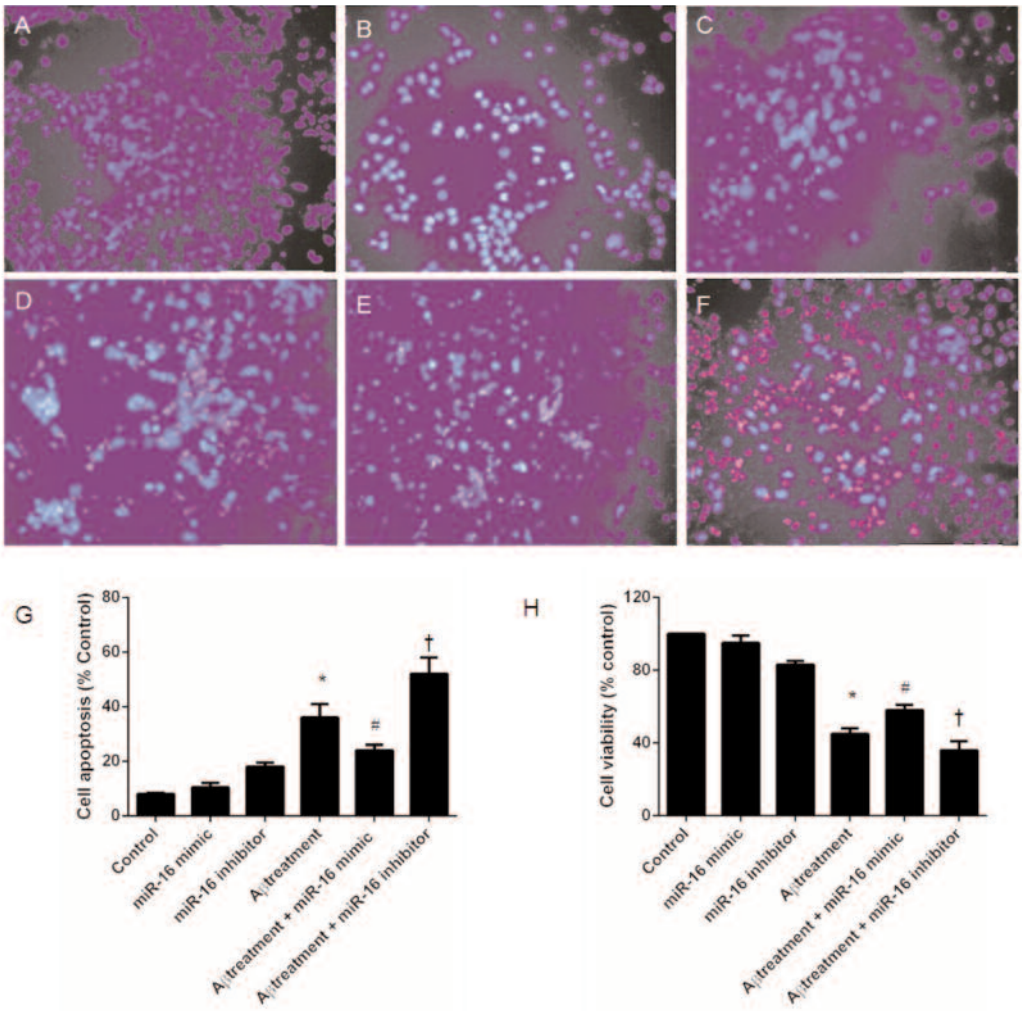


**Figure 2.** miR-16 regulates cell apoptosis and cell viability in cellular Alzheimer's disease (AD) model. Cell apoptosis was determined by Hoechst 33342 (blue)/PI (red) nuclear double staining ***(A)***. Normal primary hippocampal neurons cells; ***(B)***. Primary hippocampal neurons with miR-16 mimic treatment ***(C)***. Primary hippocampal neurons with miR-16 inhibitor treatment; ***(D)***. Primary hippocampal neurons with Aβ treatment; ***(E).*** Primary hippocampal neurons with Aβ and miR-16 mimic treatment; ***(F)***. Primary hippocampal neurons with Aβ and miR-16 mimic treatment. ***(G)*** Quantification of cell apoptosis; ***(H)*** Cell viability was determined by MTT assay. $^{*}p < 0.05$ when compared to control group; $^{\#}p < 0.05$ when compared to Aβ treatment group; $^{\dagger}p < 0.05$ when compared to Aβ treatment group

### *Overexpression of miR-16 Decreased Cell Apoptosis and Viability in Cellular AD Model*

Since primary neurons are more similar to neurons *in vivo*, we performed all the analyses in a cellular model of primary hippocampal neurons. After the miR-16 mimic and inhibitor transfections, we performed the Aβ treatment. The results showed a significant decrease and increase in cell apoptosis in the primary neurons with miR-16 mimic ($p < 0.05$) and inhibitor ($p < 0.05$) transfections, respectively (Figure 2A-G). Moreover, we found significantly decreased cell viability in the primary neurons with themiR-16 mimic transfection ($p < 0.05$), and decreased cell viability in the primary neurons with the transfected miR-16 inhibitor ($p < 0.05$) (Figure 2H).

### *miR-16 targets APP at the 3'UTR and Decreases its Expression in Primary Neurons*

We searched for miR-16 target genes using the Targetscan database, and the results predicted a putative miR-16 binding site within the 3' UTR of APP (Figure 3A). To explore the effect of miR-16 on the mRNA and protein levels of APP, primary hippocampal neurons transfected with pre-miR-16 were examined. The results suggested

that there was a decrease in APP mRNA and protein after transfection with pre-miR-16 (Figure 3C and D). A luciferase experiment was also employed to detect binding of miR-16 to the 3'UTR of APP. Luciferase activity decreased after a partial segment of the 3'UTR of APP was cloned into the luciferase reporter vector (Figure 3B).

### *APP Knockdown Exhibits a Similar Effect as miR-16A Overexpression*

We employed APP siRNA to verify the effect of APP knockdown in the cellular AD model. The results showed that cell apoptosis was decreased following Aβ insult in primary neurons after APP siRNA transfection ($p < 0.05$, Figure 4A–D), while cell viability was significantly increased following Aβ insult in primary neurons after siRNA APP transfection ($p < 0.05$, Figure 4E).

## Discussion

During the past few years, several agents have been approved that enhance the cognition and global function of AD patients, and recent advances in understanding AD pathogenesis have led to the development of numerous compounds that might modify the disease process. A wide array of anti-amyloid and neuroprotective therapeutic approaches is under investigation. These are based on the hypothesis that Aβ protein plays a pivotal role in disease onset and progression and those secondary consequences of Aβ generation and deposition, including tau hyperphosphorylation and neurofibrillary tangle formation, oxidation, inflammation, and excitotoxicity, contribute to the disease process. Interventions in these processes with agents that reduce amyloid production, or that remove, limit aggregations of, or immunize against Aβ, might block the cascade of events comprising AD pathogenesis. Reducing tau hyperphosphorylation, limiting oxidation and excitotoxicity, and controlling inflammation might be beneficial disease-modifying strategies[15,16].

In the present study, we found a significantly decreased level of miR-16 following Aβ insult in the cellular Alzheimer's disease (AD) models of rat neuronal differentiated PC12 cells and hippocampal neurons from SD rats. MiR-16 overex-

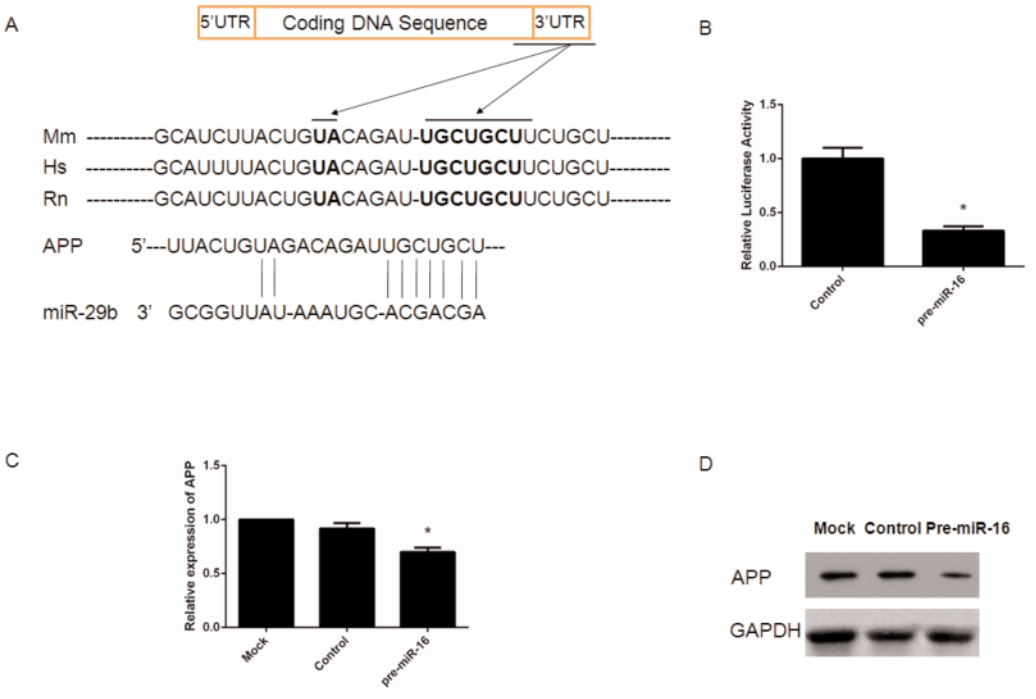


**Figure 3.** miR-16 targets amyloid precursor protein (APP) in primary hippocampal neurons and reduced APP expression. ***A,*** A putative target site of miR-16 in 3-UTR of APP mRNA predicted by TargetScan. Mm: mouse; Hs: Human; Rn: rat. ***B,*** Decreased luciferase activity after pre-miR-16 transfection. ***C,*** miR-16 overexpression decreased the gene expression of HDAC4. ***D,*** miR-16 overexpression reduced the protein level of HDAC4. *$p < 0.05$ when compared to control miRNA.

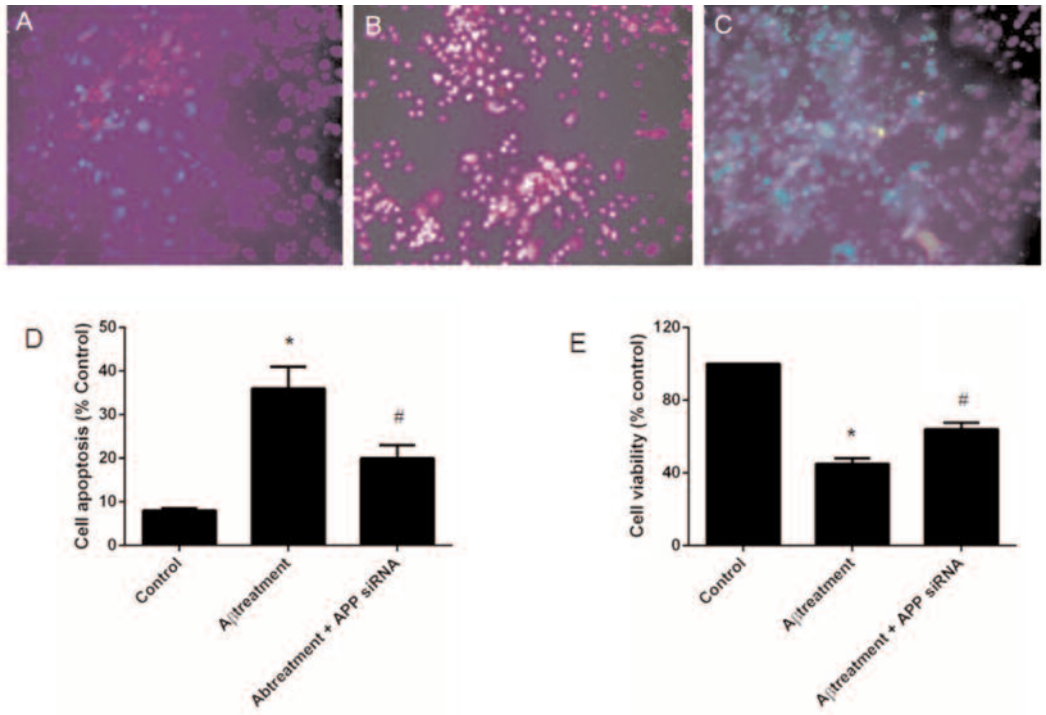


**Figure 4.** Amyloid precursor protein (APP) knockdown exhibited a similar effect on cell apoptosis and cell viability of primary hippocampal neurons. Cell apoptosis was determined by Hoechst 33342 (blue)/PI (red) nuclear double staining ***(A)***. Normal primary hippocampal neurons cells; ***(B)***. Primary hippocampal neurons with Aβ treatment ***(C)***. Primary hippocampal neurons with Aβ treatment and APP knockdown. ***(D)*** Quantification of cell apoptosis; ***(E)*** Cell viability was determined by MTT assay. $^{*}p < 0.05$ when compared to control group; $^{\#}p < 0.05$ when compared to Aβ treatment group.

pression was found to decrease cell apoptosis and cell viability in the cellular AD model, and this may be attributed to decreased expression of APP in the cellular AD model. These results suggest a possible role for miR-16 in primary neurons that gives insight into the development and pathogenesis of AD.

Previous reports[17] have shown that miRNA plays an important role in multiple, specific neurological processes such as neuroplasticity and stress response. Direct associations can be found between miRNA and AD. According to the amyloid cascade hypothesis in AD, the accumulation of dense plaques and neurofibrillary tangles in the brain leads to progressive dementia. Studies[10] have shown that the differential expression level of miRNAs, such as miR-9, miR-124a, miR-125b, miR-128, miR-146a, miR-219, affect the function and behavior of the aged brain. These data are consistent with the hypothesis that differential expression of mRNA through miRNA regulation may contribute to neural dysfunction in the AD brain. Wang et al[18] showed that miR-107 was involved in accelerated disease progression through targeting of BACE1. In addition, using a cellular model, Hebert et al[19] revealed that miR-29a/b-1 can regulate BACE1/β-secretase to affect the process of APP cleavage into Aβ in patients with sporadic AD. Boissonneault et al[20] demonstrated that miR-298 and miR-328 could bind to sites in the 3'UTR of BACE1 mRNA and regulate BACE1 protein expression in cultured neuronal cells. Recently, several miRNAs, such as miR-20a, miR-17-5p, miR-106a, and miR-106b, were identified as novel endogenous regulators of APP, indicating that miRNAs can influence APP expression in the brain during the development of disease[21,22]. In this study, we found downregulation of miR-16 in the cellular model of AD.

Our bioinformatics analysis indicated that within the 3' UTR of the APP gene, there is a putative binding site for miR-16. By employing multiple molecular assays, we confirmed a substantial correlation between the miRNA and APP levels. Liu et al[23] previously demonstrated that APP is a target of miR-16 and that miR-16 could negatively regulate APP protein accumulation in

AD mice. Our results here are consistent with results from their study. In addition, by using APP siRNA, we found that APP knockdown exhibited a similar effect in the cellular model of AD.

Aβ, a neurotoxic agent, has been verified in both *in vitro* and *in vivo* models of AD[24,25,26]. Furthermore, it was demonstrated that $A\beta_{1-42}$ insult to neuronal cells is one of the major causes of AD. Therefore, we employed this peptide to induce the AD phenotype in primary neurons *in vitro*. In our study, we found nearly 60% rate of cell death and 40% rate of cell apoptosis in primary cultured hippocampal neurons. After transfection with a miR-16 inhibitor, the cultured cells exhibited a slight increase in apoptosis, even in intact cells without exogenous Aβ insult. These results suggested that downregulation of miR-16 may promote the production of endogenous Aβ by upregulating APP expression and inducing similar cytotoxic effects as exogenous Aβ insult.

## Conclusions

Our results demonstrated that the downregulation of miR-16 in primary neurons play an important role in the cellular AD phenotype and might be involved in the pathogenesis of AD. Based on these results, we propose the existence of a molecular event that may explain the pathogenesis of AD. However, further assays should be performed to verify the role of miR-16 *in vivo*.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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