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MicroRNA-181c functions as a protective factor in a 1-methyl-4-phenylpyridinium iodide-induced cellular Parkinson's disease model via BCL2L11

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Abstract. – OBJECTIVE: Parkinson is one of the most common neurodegenerative diseases. At present, many studies have pointed out that miRNAs play a very important role in Parkinson's development and process. MiR-181c has been shown to have a significant low expression in blood samples and brain tissues of Parkinson's patients.

MATERIALS AND METHODS: We used 1-Methyl-4-Phenylpyridinium Iodide (MPP(+)) as a tool for constructing the Parkinson's cell model, using mir181c mimics to construct an experimental model of acquisition. The cell viability of PC12 was detected by MTT and CCK8. Reactive oxygen species (ROS) and caspase-3 activity were analyzed. The apoptosis of PC12 was detected by flow cytometry (FCM), and luciferase was used to study the binding of target genes. The protein levels of BCL2L11were measured by Western-blot.

RESULTS: There was a significant low expression of mir181c in MPP(+)-morbid cells. PC12 cell viability was rescued by miR-181c overexpression. Flow cytometry showed that apoptosis in PC12 cells overexpressing miR-181c was significantly decreased. Also, ROS and caspase-3 activity were significantly decreased. Luciferase experiments showed that miR-181c may bind to the 3-'UTR side of BCL2L11 and inhibited its expression. By Western-blot, the BCL2L11 level was markedly decreased by miR-181c.

CONCLUSIONS: miR-181c could promote the cell viability and inhibit the apoptosis of PC12 cells induced by MPP (+) by downregulating BCL2L11, which may play a protective role and provide a new target for PD drug resistance research.

Key Words:
Parkinson's disease, miRNA, *in vitro*, Cell model, Apoptosis.

Introduction

Parkinson's disease (PD), a chronic neurodegenerative disease, is extremely common among elder individuals, which usually manifested as a series of activities including motor or neurobiological disorders, such as resting tremor, exercise retardation, muscle rigidity and postural gait disorders[1]. At present, the treatment of Parkinson's disease still remains a symptomatic therapy and there is no good prevention or other treatment programs[2]. It has been suggested that its pathogenesis is likely to be related to neuronal apoptosis caused by impaired mitochondrial function[3], so the study of neuronal apoptosis may provide a new target for the treatment of PD[4]. 1-Methyl-4-Phenylpyridinium Iodide (MPP(+)) is a commonly used dopamine (DA) neuron poison that can cause the loss of mitochondrial membrane[5], causing reactive oxygen species (ROS) formation, eventually leading to the death of cells, and, therefore, is often used to induce PD *in vitro* model[6]. MiRNA is a sort of non-coding RNAs, which can bind to the 3'-untranslated regions (3'-UTRs) of the target gene, causing a decrease in its protein expression level[7]. Many miRNAs have been found to play an important role in the development of PD. For example, microRNA-124 could enhance brain repair in Parkinson's disease[8], miRNA-205 regulated the expression of Parkinson's disease-related leucine rich repeat kinase 2 (LRRK2) expression[9], and miRNA-7 protected against MPP(+)-induced PD cell apoptosis via KLF4, Bax, and Sirt2[10,11]. According to several studies, miR-181c acted as a common tumor suppressor in many diseases, including

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the gliomas and other neurological diseases[12,13]. At present, miR-181c has been found low expression in the blood of PD patients 14, but the clear role and the specific mechanism in PD are still unclear and need further excavation. Bcl-2-like protein 11 (BCL2L11), a member of BCL family, functions as an apoptotic regulator, and it is involved in many diseases[15]. Several kind of researches identified that BCL2L11 was involved in PD, and promoted the neuronal apoptosis, which accelerated the progress of PD[16]. In our study, we analyzed the expression of miR-181c in MPP(+)-induced dopaminergic (DA) neuroblastoma cell line PC12; the gain of function assay using miR-181c mimics indicated the upregulation of mir181c markedly reduced the MPP(+)-induced apoptosis. Moreover, dual-luciferase reporter assay displayed that miR-181c could directly bind to the BCL2L11 mRNA 3'-UTR region. We next measured the expression of BCL2L11, and we found that upregulation of miR-181c decreased the level of BCL2L11. These findings suggested that miR-181c could act as a protective factor against the MPP(+)-induced apoptosis of neurons via targeting BCL2L11.

Materials and Methods

PC12 Cell Culture and MPP(+) Treatment

PC12 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured in Dulbecco's modified Eagle's medium DMEM containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA). The cultures were incubated at 37°C in 5% CO2 humidified atmosphere. After 48 h, the cells were treated with different concentration of MPP(+) from 0 mM to 2 mM.

Overexpression of miRNA-181c

The miR-181c mimics and negative control (NC) were compounded by Genepharma (Shanghai, China). After cells were cultured to 40% density, PC12 cells were transfected with about 20 nM mimics or NC using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). The cells were replaced with fresh medium for 48 h after incubating for 6 h. Then, the miR-181c level was detected by quantitive Real-time polymerase chain reaction PCR (qRT-PCR).

Transfection of pcDNA

The plasmid pcDNA3.1-BCL2L11 and control were synthesized by Genepharma (Shanghai, China). Cells were seeded in six-well plates for 24 h, and then incubated with pcDNA3.1-BCL2L11 mixed with lipofectamine 2000 in serum-free DMEM. After maintained 48 h in normal atmosphere, the expression of BCL2L11 was confirmed.

Isolation of RNA and qRT-PCR

Total RNAs of PC12 cells were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reserve transfected to cDNAs using Reverse Transcription Kit (Takara, Dalian, China). The level of miR-181c was determined by miRNA Taqman Detection Kit (Haoqin, Shanghai, China) and small nuclear RNA U6 was used as internal control. The expression of BCL2L11 was detected by SYBR Premix kit (TaKaRa, Dalian, China), and GAPDH as internal reference. All the relative expression levels were calculated by the 2-ΔΔCT method. The primers used were: BCL2L11:5'-TAAGTTCT-GAGTGTGACCGAGA-3' (forward), 5'-GCTCT-GTCTGTAGGGAGGTAGG-3' (reverse); GAPDH: 5'-TGTGGGCATCAATGGATTTGG-3'(forward), 5'-ACACCATGTATTCCGGGTCAAT -3'(reverse).

Cell Viability Assays and cck8 Assay

The cell viability was measured using MTT assay. Cells instructed were planted into 96-well plates with 1X104 cells per well in 100 μL medium and cultured with 10 μL reagent for 2 h at standard culture condition. Then, the absorbance of 490 nm was detected using a spectrophotometer. CCK8 (Dojindo Laboratories, Kumamoto, Japan) assay was applied to measure the proliferation of cells. The cells seeded in a density of 1X103 cells with 100 uL medium were cultured for 0, 6, 12, 24 h after transfection. CCK8 reagent was added into the wells and absorbance of 450 nm was measured.

ROS Measurement

The degree of intracellular ROS was detected using the fluorescent probe CM-H2DCFDA. After transfecting with mimics or NC, the PC12 cells were treated with 0 or 2 mM MPP(+), then cultured with 10mM CM-H2DCFDA for 20 min in the dark. Fluorescence level was detected by a fluorescence reader (Saire2, Tecan, Mannedorf, Switzerland) at 485 nm excitation and 535 nm emissions. Normalized by the control, data was carried out by average values.

Caspase-3 Activity Assay

Caspase-3 activity Kit (Beyotime, Jiangsu, China) was used to determine the activity of ca-

spase-3 according to the specific instructions. Cells after transfection were harvested and incubated in lysis buffer for 15 min on ice. The supernatant after 16.000 g for 15 min was added into reaction buffer containing 10 μL Ac-DEVD-pNA (2 mM) and incubated for 1.5 h. The absorbance at 405 nm was detected and measured.

Dual-Luciferase Reporter Assay

The Dual-Luciferase reporter system (Promega, Madison, WI, USA) was used to test the activity of luciferase. The BCL2L11 3'-UTR cDNA fragment containing the wild type or mutant miR-181c binding site was amplified and cloned into pGL3 luciferase vector (Promega, Madison, WI, USA). Then, PC12 cells were co-transfected with the established vector and miR-181c mimics or NC using lipofectamine 2000. Then, the activity of luciferase was determined using luminometer (Promega, Madison, WI, USA) and measured as the fold-change to the basic pGL3 vector relatively.

Cell Apoptosis by flow Cytometry

Cell apoptosis was measured by flow cytometry using an Annexin V fluorescein isothiocyanate (FITC) and propidium iodide (PI) kit (Vazyme, Nanjing, China). Treated PC12 cells were harvested and washed with pre-cooling phosphate buffered saline (PBS) and centrifuged 1000 rpm for 5 min. Then cells were resuspended in 1000 μL of binding buffer adding 10 μL of FITC and PI, respectively. After being maintained in dark for 15 min, cell apoptotic rate was measured by flow cytometry (FACS, BD Biosciences, San Jose, CA, USA), and the percentage of total cells was counted.

Western-blot

To investigate the relative BCL2L11 protein expression level, cells were washed with pre-cooling PBS and then lysed using RIPA (Beyotime, Shanghai, China). Then, the concentration of collected protein was measured by using the BCA kit (Beyotime, Shanghai, China). After being degenerated and chilled on ice, the protein was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and shifted to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). 5% BSA dissolved in TBST buffer was used to block non-specific protein interactions at 25°C for 2 h. The membranes loaded with proteins were incubated at 4°C overnight within the primary antibody (1:1000) against BCL2L11 (Abnova, Taipei, Taiwan). After being washed with tris buffered saline-tween (TBST) for 10 min each time for three times to clean the unbound antibody, the membranes were incubated with secondary antibody conjugated with horseradish peroxide (HRP) at room temperature for one hour. After washing three times in TBST buffer, the bands were visualized using ECL Kit (Millipore, Billerica, MA, USA) following the instructions.

Statistical Analysis

All the data in this study were analyzed with SPSS 16.0 version software (SPSS Inc., Chicago, IL, USA) and presented with GraphPad prism software version 5.0 software (San Diego, CA, USA). The independent samples *t*-test (SPSS Inc., Chicago, IL, USA) was used to perform statistical analysis and quantitative data was shown as mean ± SD. Fisher's exact test was used for comparisons of qualitative variables and $p < 0.05$ was considered having significant difference.

Results

MiR-181c was Downregulated in MPP(+)-treated PC12 Cells

To stimulate the PD-like neuron loss, varying concentrations of MPP(+) were used to seek for the moderate condition. As shown in the Figure 1A, cell viability of PC12 cells was declined with the change of the concentration. We next detected the expression of miR-181c in MPP(+)-treated PC12 cells and understood the level significantly decreased compared to the control group along with the dose of MPP(+) (Figure 1B). According to these results, we chose MPP(+) 2 mM for our main study. These results indicated that miR-181c may affect the function of MPP(+) in PC12 cells.

MiR-181c Protected the Viability and Proliferation of PC12 cell from MPP(+)

To further investigate the affect of miR-181c on cell viability, we overexpressed miR-181c in PC12 cells using miR-181c mimics (Mimics), and the expression level increased about 2.95-fold when compared to the negative control (NC) (Figure 2A). Then, the transfected cells were treated with 0-2 mM MPP(+), and MTT assay was used to measure the viability. Figure 2B displayed that cell viability of PC12 transfected with mimics improved in the groups of treating with 1 mM and 2 mM MPP(+) compared to the NC group. Also, we

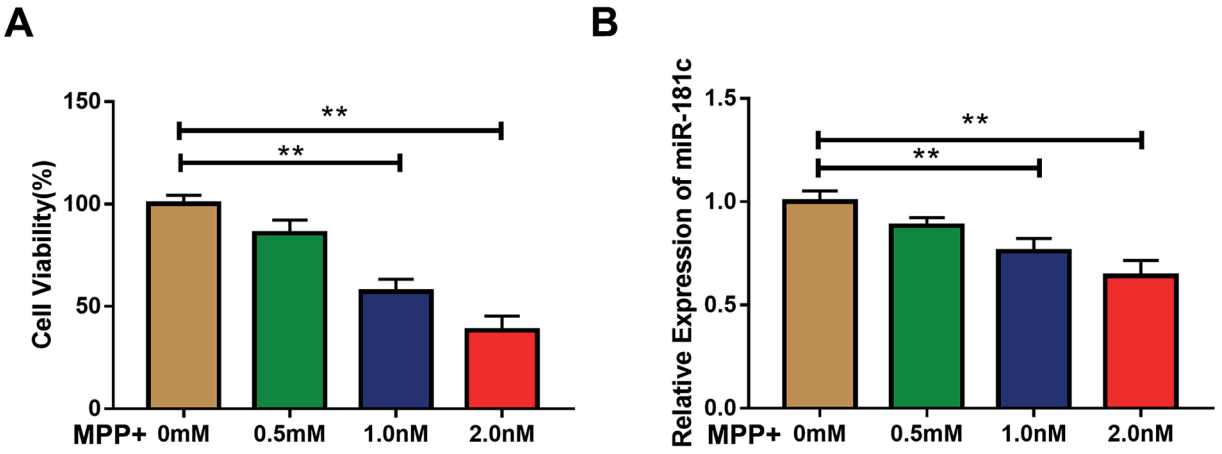

Figure 1. MiR-181c was downregulated in MPP(+)-treated PC12 cells. ***A,*** Analysis of the percentage cell viability in different concentrations of MPP(+). Cell viability of PC12 was significantly decreased along with the evaluated MPP(+) concentration. ***B,*** Analysis of miR-181c expression level in PC12 cells treated with 0-2mM MPP(+). MiR-181c was detected by qRT-PCR and U6 was used as an internal control. Data are presented as the mean ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$.

detected the cell proliferation of established PC12 cells using CCK8 assay at different time points when exposed to 2 mM MPP(+) and found miR-181c protected cell viability as a time-dependent manner (Figure 2C). Furthermore, we determined the ROS activity in the miR-181c overexpression PC12 and found ROS generation was markedly decreased in miR-181c mimics group than the NC group (Figure 2D). All the results showed that miR-181c could protect PC12 cells from MPP(+)-treated toxicity.

MiR-181c Reduced the Apoptosis in MPP(+)-Treated PC12 cells

To determine whether miR-181c could affect the PC12 apoptosis caused by MPP(+), we tested the apoptotic cell rate by flow cytometry in PC12 cells transfected with miR-181c mimics or NC after maintained with 0mM or 2 mM MPP(+) for 24 h. As shown in Figure 3A-B, miR-181c significantly weakened MPP(+)-induced cell apoptosis in PC12 cells. Also, we measured the activity of caspase-3 and showed miR-181c reduced the level of caspase-3 (Figure 3C). The results verified that miR-181c reduced the apoptosis of PC12 cells caused by MPP(+).

BCL2L11 was a Target of miR-181c

To further study the specific way of miR-181c functioned in PC12 cells, we searched for the candidate target gene using bioinformatics analysis and selected BCL2L11 as a potential target (Figure 4A). To confirm the relationship between miR181c and BCL2L11, a dual-luciferase reporter assay was established. The luciferase activity presented a clearly decrease in pGL3-BCL2L11 3′-UTR-transfected cells, but no difference in pGL3-BCL2L11-mut 3′-UTR-transfected cells after overexpressing miR-181c (Figure 4B). Additionally, we verified the BCL2L11 mRNA level using qRT-PCR and protein level using Western-blot. Figure 4C, D and E showed that mRNA and protein of BCL2L11 decreased by overexpressing miR-181c in MPP(+)-challenged PC12 cells. All results indicated BCL2L11 is a direct target gene of miR-181c in a cellular PD model.

MiR-181c Regulated cell Survival and Apoptosis of PC12 via BCL2L11

To validate that BCL2L11 plays a functional role in miR-181c mechanism of action, we overexpressed BCL2L11 using pCDNA-BCL2L11. After transfected with miR-181c mimic, miR-181c NC, or miR-181c mimic and pCDNA-BCL2L11, we treated the cells with MPP(+) and tested the change of apoptotic rate and caspase-3 activity. As shown in Figure 5A, overexpressing BCL2L11 could significantly reduce the protective function of miR-181c in opposition to the apoptosis caused by MPP(+) in PC12 cells. The caspase-3 activity increased too, while overexpressing miR-181c and BCL2L11 increased at the same time, which confirmed the effect of pcDNA-BCL2L11 (Figure 5B). Then, we affirmed the protein level of BCL2L11 by Western-blot and the trends show the same change as we speculated (Figure 5C, D).

All the outcomes revealed that miR-181c could reduce the expression of BCL2L11 and function as a protective factor in MPP(+)-induced PD cellular model.

Discussion

With the extension of life expectancy in the world, Parkinson's disease as a common neurodegenerative disease has gained more and more attention[17]. Because of its lack of early symptoms, the current diagnosis and treatment are still difficult to be detected and need a development stage 18. MiRNAs, as a sort of regulatory factors, have been shown to be involved in the development of multiple diseases in recent years[19]. In neurodegenerative diseases such as Parkinson and Alzheimer's disease, miRNAs have also been shown to be involved in many processes of their development and progression[20,21]. Thome et al[22] revealed miRNA-155 could regulate alpha-synuclein-induced inflammatory responses in PD. Also, miR-126 was reported by Kim et al[23] to regulate growth factor activities to toxic insult in PD. In addition, miR-34b/34c was decreased in several affected brain regions in PD[24]. At the same time, some studies have also pointed out that miRNAs may serve as markers for early diagnosis of PD[25]; for example, blood miRNAs-103a, -30b, and -29a were overexpressed in L-dopa-treated patients with PD[26], and serum miR-221 could serve as a biomarker for Parkinson's disease[27]. Along with the study of exosome, miR-19b, miR-24, and miR-195 could be detected in serum exosome-like

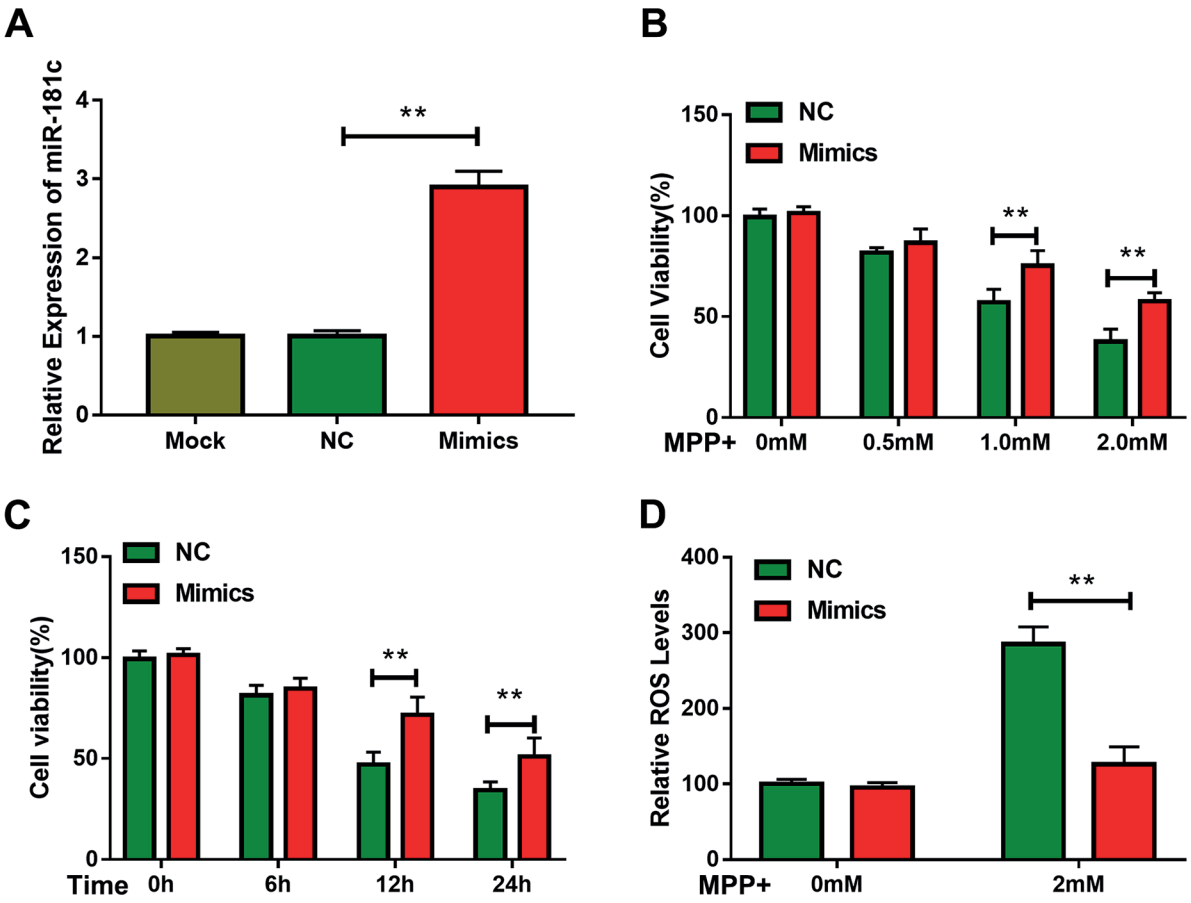

Figure 2. MiR-181c protected the viability and proliferation of PC12 cell from MPP(+). ***A,*** MiRNA level of PC12 cells transfected with miR-181c mimics or NC or not. ***B,*** MTT assay was performed to determine the viability of PC12 cells treating with different concentrations of MPP(+) after transfected with miR-181c mimics (Mimics) or miR-181c NC respectively. ***C,*** CCK8 assay was performed at 0, 6, 12, 24 h time points to determine the viability of PC12 cells co-cultured with 2mM MPP(+) transfected with mimics and NC respectively. ***D,*** ROS levels were detected in variously treated PC12 cells. Data are presented as the mean ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$.

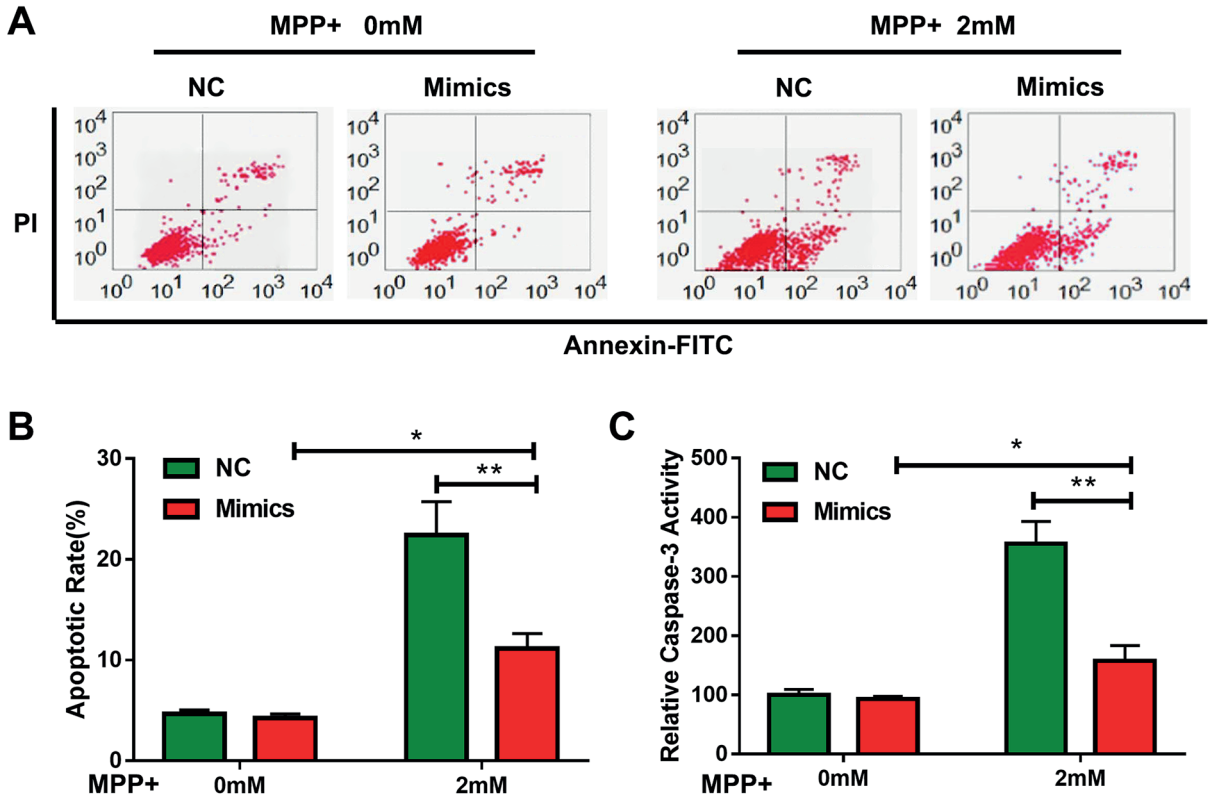

Figure 3. MiR-181c reduced the apoptosis of in MPP(+)-treated PC12 cells. PC12 cells were cultured with or without 2mM MPP(+) after miR-181c mimincs or NC transfection. ***A,*** Flow cytometric analysis was performed to detect the apoptotic rates of established PC12 cells. ***B,*** The apoptotic rate was calculated from the FACS data. ***C,*** The activity of caspase-3 was measured. Data are presented as the mean ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$.

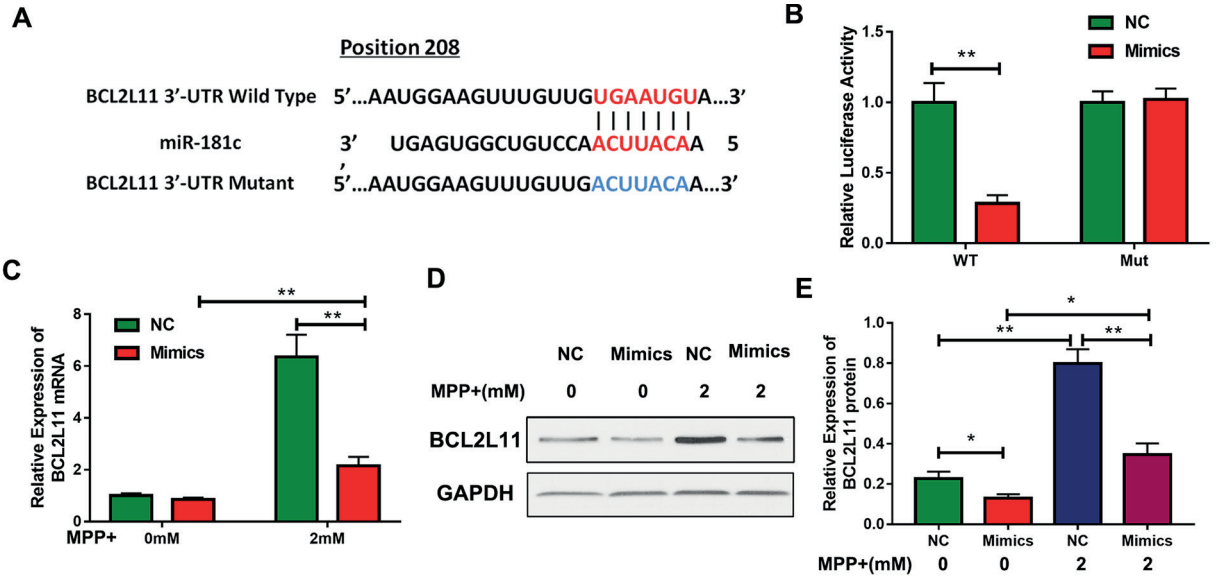

Figure 4. BCL2L11 was a direct target of miR-181c. ***A,*** The predicted binding sites of miR-181c in the 3'-UTR of BCL2L11. ***B,*** Dual-luciferase reporter assay was used to determine the binding site. PC12 cells treated by mimics or NC were transfected with pGL3 construct containing the WT or Mut BCL2L11 3'-UTR region. ***C,*** The expression levels of BCL2L11 mRNA in mimics or NC treated in 0 mM or 2 mM MPP(+)-induced PC12 cells. ***D-E,*** Levels of BCL2L11 protein measured by Western-blot in various PC12 cells. The mRNA levels and the protein levels were normalized to that of GAPDH. Data are presented as the mean ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$.

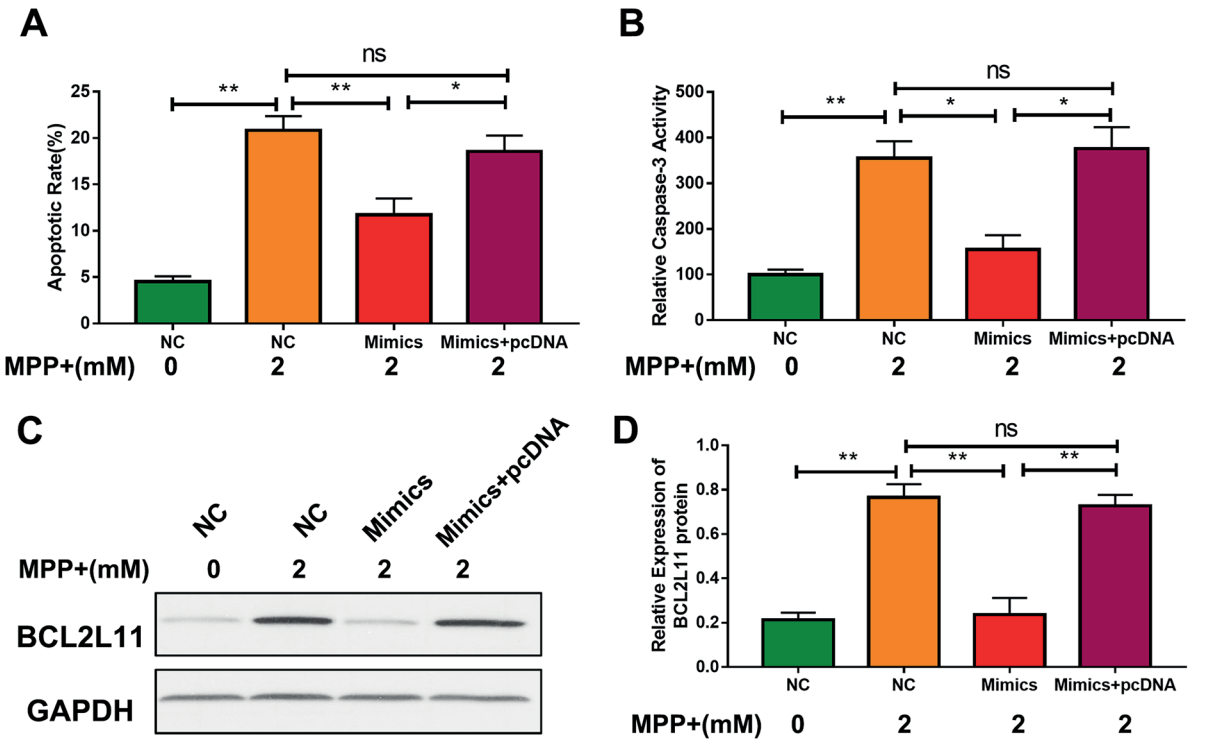

Figure 5. MiR-181c regulated cell survival and apoptosis of PC12 via BCL2L11. The PC12 cells with 0 or 2 mM MPP(+) treatment were transfected with RNA oligonucleotides or with or without pcDNA-BCL2L11. ***A,*** Analysis of the apoptotic rate; ***B,*** Relative activity of caspase-3; ***C,*** Western-blot analyses of BCL2L11 expression level. GAPDH was used as an internal control. ***D,*** Relative protein band densities of BCL2L11. Data are represented as the mean ± SD of three replicates. * $p < 0.05$, ** $p < 0.01$, ns: non-sense.

microvesicles of PD patients due to the findings of Cao et al[28]. MiRNA-181c as a 22-nucleotide miRNA, has been reported to be involved in the progression of many diseases including glioma cancers and Alzheimer's disease. It inhibited glioblastoma cell invasion, migration and mesenchymal transition (EMT) by targeting TGF-β pathway[29]. In sporadic Alzheimer's disease, microRNA-181c regulated serine palmitoyltransferase and, in turn, amyloid β[30]. In PD, miR-181c may serve as a biomarker according to microarray analysis of transcriptome14. Consistence with this, we demonstrated that miRNA-181c was downregulated in MPP(+)-induced PC12 cells. Furthermore, we next detected the effect of miRNA-181c in cell viability and apoptosis by overexpressing miRNA-181c in PC12 cells. As a tumor suppressor in gastric cancer and glioma [9], miRNA-181c functioned as a protective factor against the apoptotic influence caused by MPP(+). MiRNAs could block the translation initiation and reduce the expression of target genes, which is the main mechanism of miRNA activity[31]. We used TargetScan-Human 6.2 to predict the potential targets for miR-181c and predicted that the 3'-UTR of BCL2L11 was a binding site for miR-181c. BCL2L11 (also known as Bim) is an apoptosis facilitator as a member of BCL family, and has been studied to participate in the regulation mechanism through several different pathways to promote the process of PD[8,32]. Dual-luciferase reporter assay confirmed that miRNA-181c could directly binding to the BCL2L11 3'-UTR site. Next, we identified the expression of BCL2L11 mRNA and protein level significantly reduced by miR-181c overexpression with qRT-PCR and Western-blot. In addition, BCL2L11 is involved in the PD regulation network, which co-works with BCL2, Bax, and so on. Further study demonstrated that BCL2L11 overexpression by pcDNA could reduced the protective function of miR-181c against apoptosis in MPP(+)-induced cellular PD model. These results indicated that BCL2L11 was an important molecular of miR-181c, which decreased the negative effect of MPP(+) in PC[12]. Though we have studied the optimistic effect of miR-181c in MPP(+)-indu-

ced cellular PD model, and identify BCL2L11 as a potential target for miR-181c, further studies, especially *in vivo,* are needed for elucidating specific and comprehensive mechanism of miR-181c in PD.

Conclusions

This work determined that miR-181c had a protective effect in MPP(+)-induced PD cellular model by reducing the apoptosis and enhancing the cell viability. Besides, miR-181c could play its protective role via suppressing BCL2L11. Our findings implied that miR-181c may serve as an innovative and prospective diagnosis and treatment target for PD.

Conflict of interest

The authors declare no conflicts of interest.

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