miR-132-5p regulates apoptosis and autophagy in MPTP model of Parkinson's disease by targeting ULK1

Jianli Zhao[a,*], Manyi Yang[a], Qi Li[a,*], Xiaorui Pei[b] and Xiaodong Zhu[a]

Parkinson's disease (PD) is a neurodegenerative disorder that is characterized by a loss of dopaminergic neurons in the substantia nigra of the brain. Numerous investigations have focused on the underlying mechanism involved in the progression of PD in recent decades. miR-132 is abnormal expression in many diseases including PD. However, the functional role and molecular mechanism of miR-132-5p in PD pathogenesis are still not elucidated. In our study, we found miR-132-5p was upregulated in 1-methyl-4-pheny-1,2,3,6-tetrahydropyridine (MPTP) model of PD. MTT assay and flow cytometric analysis revealed that inhibition of miR-132-5p increased cell survival ability and reduced MPTP-induced apoptosis of SH-SY5Y cells. Furthermore, inhibition of miR-132-5p could significantly suppressed mRNA and protein expression levels of LC3 and Beclin 1, indicating inhibition of miR-132-5p might restrain autophagy in PD. Subsequently, ULK1 was identified as a target of miR-132-5p and positively regulated by miR-132-5p at both mRNA and protein levels. Additionally, ectopic expression of ULK1 was able to reverse the effects of miR-132-5p inhibition. Taken together, our results demonstrated that miR-132-5p inhibition might exert a protective role in MPTP-treated PD models by targeting ULK1, indicating that miR-132-5p may be a prospective therapeutic target for PD. *NeuroReport* 31: 959–965 Copyright © 2020 Wolters Kluwer Health, Inc. All rights reserved.

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Introduction

Parkinson's disease (PD) is a neurodegenerative disorder and one of its main character is the progressive loss of dopamine neurons within the substantia nigra pars compacta (SNpc) [1]. Previous evidences of PD research have shown that the pathophysiological mechanism of dopamine cell loss is related to protein aggregation, mitochondrial dysfunction and oxygenation stress, which may lead to neuron apoptosis or impaired autophagy. Thus, strategies to reduce dopamine cell loss might provide effective treatment for PD patients. It is well known that the interaction between genetic and environmental factors is crucial to PD. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a contaminator of mitochondrial functions, is often used to establish PD model [2].

MicroRNAs (miRNAs) are endogenous short noncoding RNAs, which play a vital role in the post-transcriptional regulation of target gene expression by interacting with the 3′-untranslated regions (3′-UTR) of target mRNA [3]. More and more evidences have demonstrated that miRNAs play important roles in diseases of neurodegeneration or dopamine neuron biology. For example, overexpression of miR-7 and miR-153 downregulate the level of α-synuclein [4]. miR-124 decreased the apoptosis and autophagy through inhibiting Bim in MPTP treated mice model and MPTP-intoxicated SH-SY5Y model [5]. In addition, miR-34b/c was downregulated in PD and depletion of miR-34b or miR-34c reduced the viability of SH-SY5Y cells [6].

miR-132, a brain-enriched miRNA, is upregulated in PD. Convincing evidence has demonstrated miR-132 was increased in a transgenic model of PD, along with a downregulating on the nuclear receptor-related 1 protein (Nurr1) [7,8]. It is also reported that miR-132 was upregulated in the striatum of parkinsonian rats [9]. In view of the evidence cited above, we hypothesized that miR-132-5p might play a vital role in the pathology of DA neurons in PD. In our research, we identified miR-132-5p was upregulated in MPTP-treated SH-SY5Y cells and SNpc of MPTP-treated mice. Functional analysis of miR-132-5p demonstrated that inhibition of miR-132-5p reduces apoptosis and suppressed autophagy. Furthermore, we identified a novel target of miR-132-5p – ULK1.

Materials and methods

Animals and treatment

Nine adult (8-week-old) male C57BL/6 mice were purchased from the Beijing Huafukang Biotechnology Company. All animals were treated in strict accordance with the NIH Guide for Care and Use of Laboratory Animals. The studies were approved by the Animal Care Committee of Tianjin Medical University.

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For studying the role of antago-miR-132-5p (RiboBio, Guangzhou, China) in animal model, a catheter (RWD, Shenzhen, China) was implanted into the stereotactic intraventricular injection site (2 mm rostral to the bregma, 2 mm lateral to the sagittal suture, and 3 mm below the skull surface). After 7 days of recovery, mice were treated with antago-miR-132-5p through the catheter per day for five consecutive days. As negative control (NC), antago-miR-NC was injected into the right lateral ventricle. The treatment of antagomir was performed 2 days before injection of MPTP. Then, the mice were intraperitoneally injected with saline or MPTP for five consecutive days (30 mg/kg; Solarbio, Beijing, China). After the last injection of MPTP, the mice were killed. The mice were decapitated and once the brain was removed, the ventral midbrain containing SNpc was dissected and stored at –80°C for further experiments.

Cell lines, transfection, and RNA extraction

The SH-SY5Y cells were cultured at 37°C and CO_2 in RPMI 1640 media (Gibco, Gaitherburg, Maryland, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Transient transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol. miR-132-5p antisense oligonucleotides (ASO-miR-132-5p) was purchased from Gene Pharma (Shanghai, China). A mirVana miRNA Isolation Kit (Ambion, Austin, Texas, USA) was used to obtain miRNAs from SH-SY5Y cells or midbrain according to the manufacturer's recommendations. TRIzol reagent (Invitrogen) was used to extract total RNA.

Plasmid constructions

The gene encoding ULK1 was amplified from cDNA isolated from SH-SY5Y cells with the primer sequences sense 5′-GCG GAAT TCGA GCC GGCCGC GGCGGCAC-3′, antisense 5′-GGCTA TCT AGA ACTGACCGTA GACACG G-3′. The product was ~3150 bp, and it was cloned into pcDNA3 vector sites (*EcoR* I and *Xba* I). The 3′-UTR (containing the binding sites for miR-132-5p) and a mutant 3′-UTR (seven nucleotides were mutated in the binding sites) of ULK1 were annealed and cloned into pcDNA3-EGFP control vector sites (*BamH* I and *Xba* I). The ULK1-UTR sense primer: 5′-GATC CGTGT GTGGTCCCTAT GCCACGGATAAGCTTT-3′; The ULK1-UTR antisense primer: 5′- CTA GAAGCTTA TCCGTG GCATAGGG ACCA CACACG-3′; The ULK1-UTR-mut sense primer: 5′- GATC CGTGTGTGGTCC CTATGATGCTA ATAAG CT T-3′; The ULK1-UTR-mut antisense primer: 5′- CTAGAAG CTTATTAGC ATC ATAG G GA CCACACACG-3′.

Real-time reverse transcription PCR

Gene expression of mRNAs or miRNAs were quantified by SYBR Premix Ex Taq (TaKaRa, Dalian, China) using specific primers. The primers were designed using Primer 5 software and are as follows: ULK1 forward 5′-AAGGGAA GTGCCAGTGAGG-3′, and reverse 5′-TGTCTGC CTGGTCCGTGA-3′; Beclin 1 forward 5′-CTGGACAC GAGTT TCAA GATCCTG-3′, and reverse 5′-GGGC ATGGTAGCA CAGACCTC-3′; LC3 forward 5′-GGAAGAATGACAGATGAC-3′, and reverse 5′-CTTTCAAT CTGTT GGCTG-3′; β-actin forward 5′-CGTG ACATTAA GGAGAAGCTG-3′, and reverse 5′-CTA GAAGCA TTTG CGGTG GAC-3′. The housekeeping gene β-actin (for mRNA) or RNU6B (for miRNA) was measured as an internal control. Relative expression of each gene was determined by $2^{-\Delta\Delta Ct}$ method.

MTT assay

Cells were plated in a 96-well plate at 8000 cells/well after transfection. Cells were cultivated and 15 μl MTT (at a final concentration of 0.5 mg/ml) was added into each well for 4 h. Then, the medium was removed, and the precipitated formazan (Sigma-Aldrich, St. Louis, Missouri, USA) was dissolved in 100 μl DMSO for 20 minutes. Absorbance at 570 nm (A570) was measured by a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, Vermont, USA).

Apoptosis assay

Transfected cells were washed three times in PBS, spun at 1200 rpm for 5 minutes, and resuspended in 500 μl Buffer. Then, the apoptotic of cells was evaluated by Annexin V-FITC/PI apoptosis kit (Sigma-Aldrich). Each sample was incubated with 5 μl of Annexin-V-FITC and 10 μl of PI for 15 minutes at 25°C. Finally, the cells were detected via flow cytometry.

EGFP reporter assay

A fluorescent reporter plasmid and ASO-miR-132-5p, or their control oligonucleotides, together with pDsRed2-N1 (an RFP expression vector plasmid, used as the loading control) were transiently co-transfected into SH-SY5Y cells in 48-well plates. The cells were lysed using radio-immunoprecipitation assay (RIPA) lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.2, 1% Triton X-100, and 0.1% SDS) after transfection for 48 h. Then, the EGFP and RFP intensities were measured via an F4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan).

Western blot analysis

After transfection, cells were washed with ice-cold PBS three times. Then, total protein was extracted using RIPA lysis buffer for 30 minutes at 4°C. Protein electrophoresis was performed in 8% or 20% SDS denaturing PAGE gels and transferred to a PVDF membrane (Millipore, Billerica, Massachusetts, USA). The membrane was incubated with primary antibodies against ULK1 antibody (1:300; Sangon Biotech, Shanghai, China), LC3 and

Beclin 1 (1:500; Sangon Biotech), followed by incubation with an HRP-conjugated secondary antibody (Sangon Biotech). GAPDH (1:1000; Sangon Biotech) was used as a loading control.

Statistical analysis

Data were analyzed using a one-way analysis of variance with Dunnett's post-test or two-way analysis of variance with Bonferroni post-tests. All data were processed using GraphPad Prism 7 software and statistical significance was assessed by comparing mean values (±SD) from three independent experiments. $P < 0.05$ was considered to be significant.

Results

miR-132-5p expression is upregulated in MPTP-treated SH-SY5Y cells and SNpc of MPTP-treated mice

To investigate the expression of miR-132-5p in MPTP-treated models, the expression of miR-132-5p was analyzed using real-time PCR. The results indicated the expression levels of miR-132-5p in SH-SY5Y cells increased in a dose-dependent manner with the increase of MPTP concentrations (Fig. 1a). Similarly, MPTP induced significant increase of miR-132-5p in SNpc of MPTP-treated mice compared with the control (Fig. 1b). These results suggested that miR-132-5p may be upregulated in dopamine neurons during the pathogenesis of PD.

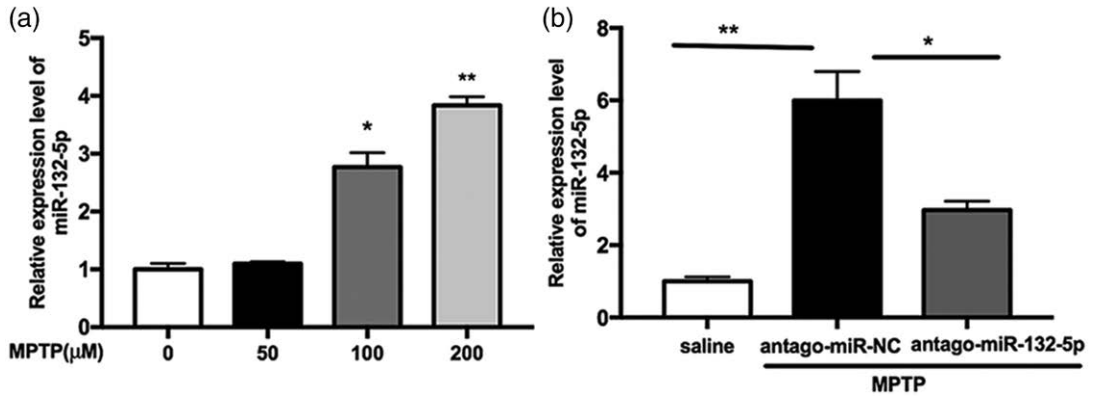
Inhibition of miR-132-5p reduces MPTP-induced apoptosis of SH-SY5Y cells

In order to investigate the role of miR-132-5p in MPTP-induced PD model in vitro, SH-SY5Y cells were transfected with miR-132-5p antisense oligonucleotides (ASO-miR-132-5p) or ASO control (ASO-NC). First, we validated the efficiency of ASO-miR-132-5p by real-time PCR. The results showed that the expression of miR-132-5p in SH-SY5Y cells was significantly decreased following transfection with ASO-miR-132-5p compared with the control group, indicating that ASO-miR-132-5p was effective in MPTP treated SH-SY5Y cells (Fig. 2a). Second, we used MTT assay to assess cell viability. The results showed that inhibition of miR-132-5p reversed MPTP-induced cell apoptosis (Fig. 2b). In addition, flow cytometric analysis also revealed that the apoptosis rate of SH-SY5Y cells treated with MPTP increased significantly, whereas inhibition of miR-132-5p could suppress MPTP-induced cell apoptosis (Fig. 2c). Taken together, these results suggested that miR-132-5p inhibition exerted a protective role in MPTP-induced apoptosis of SH-SY5Y cells.

Inhibition of miR-132-5p suppresses autophagy in MPTP-treated SH-SY5Y cells and SNpc of MPTP-treated mice

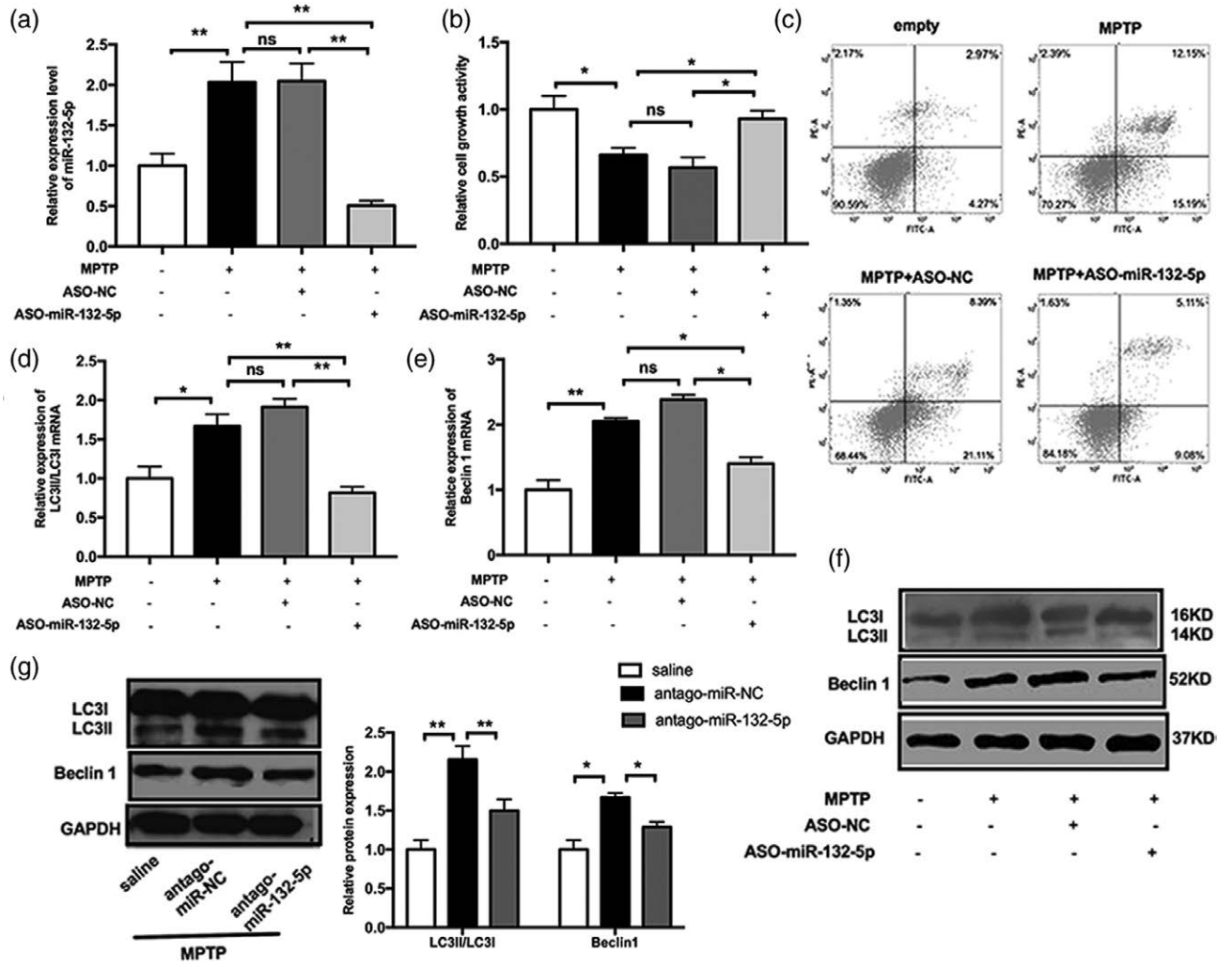
To explore the role of miR-132-5p on autophagy in MPTP-treated SH-SY5Y cells and SNpc of MPTP-treated mice, the expression level of autophagy protein markers (LC3I, LC3II and Beclin 1) were detected by real-time PCR or Western blot. As shown in Fig. 2d and 2e, the mRNA expression levels of LC3II/ LC3I ratio and Beclin 1 were upregulated in MPTP-treated SH-SY5Y cells. Meanwhile, inhibition of miR-132-5p could significantly suppress the MPTP-induced upregulation in mRNA expression levels of these autophagy protein markers. Western blot analysis showed the similar results (Fig. 2f). Consistent with the vitro experiments, inhibition of miR-132-5p can also restrain the transformation of LC3I to LC3II and the protein expression of Beclin 1 in SNpc of MPTP-treated mice compared with

Fig. 1



miR-132-5p expression is upregulated in MPTP-treated SH-SY5Y cells and SNpc of MPTP-treated mice. Real-time PCR was used to determine the expression level of miR-132-5p in MPTP-treated SH-SY5Y cells (a) and SNpc of MPTP-treated mice (b). The experiment was performed three replicates with three wells per replicate in vitro and three replicates with three mice per replicate in vivo. The bars represent the mean ± SD of three independent experiments. miR-132-5p level was normalized to U6 (*$P < 0.05$, **$P < 0.01$). SNpc, substantia nigra pars compacta.

Fig. 2



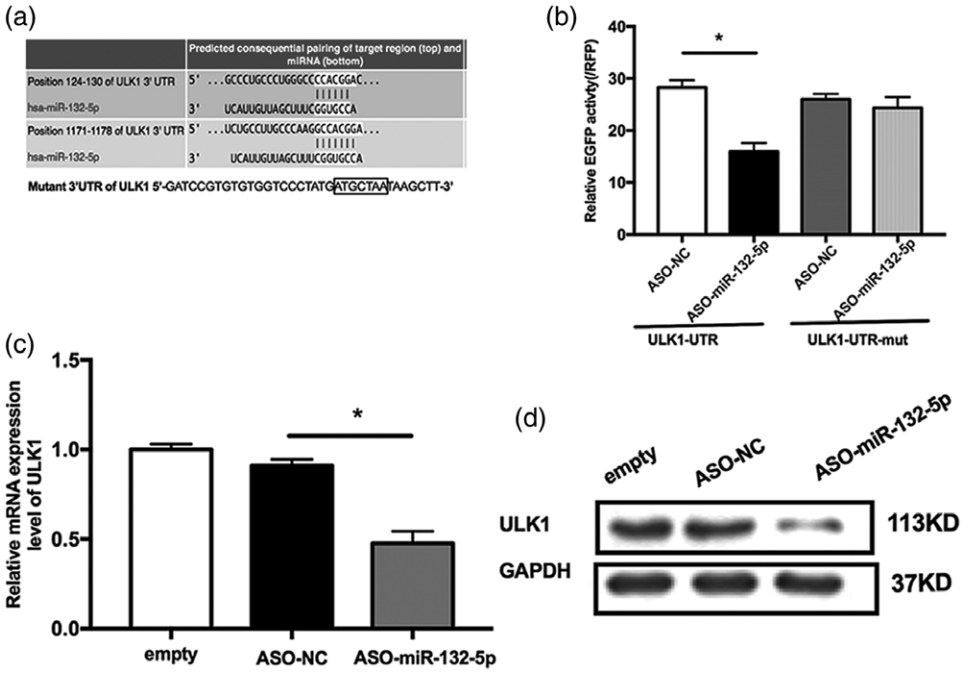
Inhibition of miR-132-5p reduces apoptosis and suppresses autophagy in MPTP-treated SH-SY5Y cells or SNpc of MPTP-treated mice. (a) Real-time PCR was used to assess miR-132-5p expression levels in MPTP-treated SH-SY5Y cells following transfection with ASO-miR-132-5p or ASO-NC. (b and c) The MTT assay and flow cytometry were employed to measure cells viability or cellular apoptosis following treatment with MPTP or transfection with ASO-miR-132-5p or ASO-NC. (d and e) Real-time PCR was performed to detect LC3II/ LC3I and Beclin 1 mRNA level in MPTP-treated SH-SY5Y cells following transfection with ASO-miR-132-5p or ASO-NC. Western blot was used to detect the protein expression levels of LC3II, LC3I and Beclin 1 in MPTP-treated SH-SY5Y cells (f) or SNpc of MPTP-treated mice (g). Statistics analysis of the relative LC3II/LC3I and Beclin 1 protein expression between different group mice (g). All experiments were performed three replicates with three wells per replicate. The bars represent the mean ± SD of three independent experiments (*$P < 0.05$, **$P < 0.01$). SNpc, substantia nigra pars compacta.

the control (Fig. 2g). These data indicated that inhibition of miR-132-5p may suppress autophagy in MPTP-treated SH-SY5Y cells and SNpc of MPTP-treated mice.

ULK1 is a target of miR-132-5p

To investigate the underlying mechanism of miR-132-5p in PD, we used programs (TargetScan, Pic Tar and miRbase Targets) to predict the potential target genes of miR-132-5p. We chose ULK1 as a putative target with two conserved miR-132-5p binding sites in its 3′-UTR, because it is involved in apoptosis and autophagy, which were consistent with the effects of miR-132-5p on the phenotypes of MPTP-treated SH-SY5Y cells (Fig. 3a). First, pcDNA3/EGFP-ULK1-3′UTR or pcDNA3/EGFP-ULK1-3′UTR-mut was co-transfected with ASO-miR-132-5p or ASO-NC into SH-SY5Y cells. As shown in Fig. 3b, the fluorescence intensity of cells co-transfected with ASO-miR-132-5p and pcDNA3/EGFP-ULK1-3′UTR decreased by 50% (Fig. 3b). However, inhibition of miR-132-5p had no significant effect on the fluorescence intensity of cells transfected with pcDNA3/EGFP-ULK1-3′UTR-mut. Next, to verify the regulatory role of miR-132-5p in endogenous ULK1 expression, real-time PCR and western blot were conducted to examine the effects of miR-132-5p on ULK1 mRNA and protein levels. The results showed that when inhibition

Fig. 3



ULK1 is a target of miR-132-5p. (a) Complementary sequences of miR-132-5p to the ULK1 mRNA 3'UTR were obtained using publicly available algorithms. (b) An EGFP reporter assay was performed to confirm direct regulation by miR-132-5p at 3'UTR of ULK1(*$P < 0.05$, **$P < 0.01$). (c and d) Real-time PCR and western blot were performed to detect the ULK1 mRNA and protein levels in SH-SY5Y cells transfected with ASO-miR-132-5p or ASO-NC. The control was normalized to 1. All experiments were performed three replicates with three wells per replicate. The bars represent the mean ± SD of three independent experiments (*$P < 0.05$, **$P < 0.01$).

of miR-132-5p, ULK1 expression was reduced on both mRNA and protein levels, as compared to the control (Fig. 3c and d). Taken together, these results suggested that ULK1 was a direct target gene of miR-132-5p and that inhibition of miR-132-5p could down-regulate the expression of ULK1, indicating that ULK1 was regulated positively by miR-132-5p.

Overexpression of ULK1 restores the influence of ASO-miR-132-5p on MPTP-treated SH-SY5Y cells apoptosis and autophagy

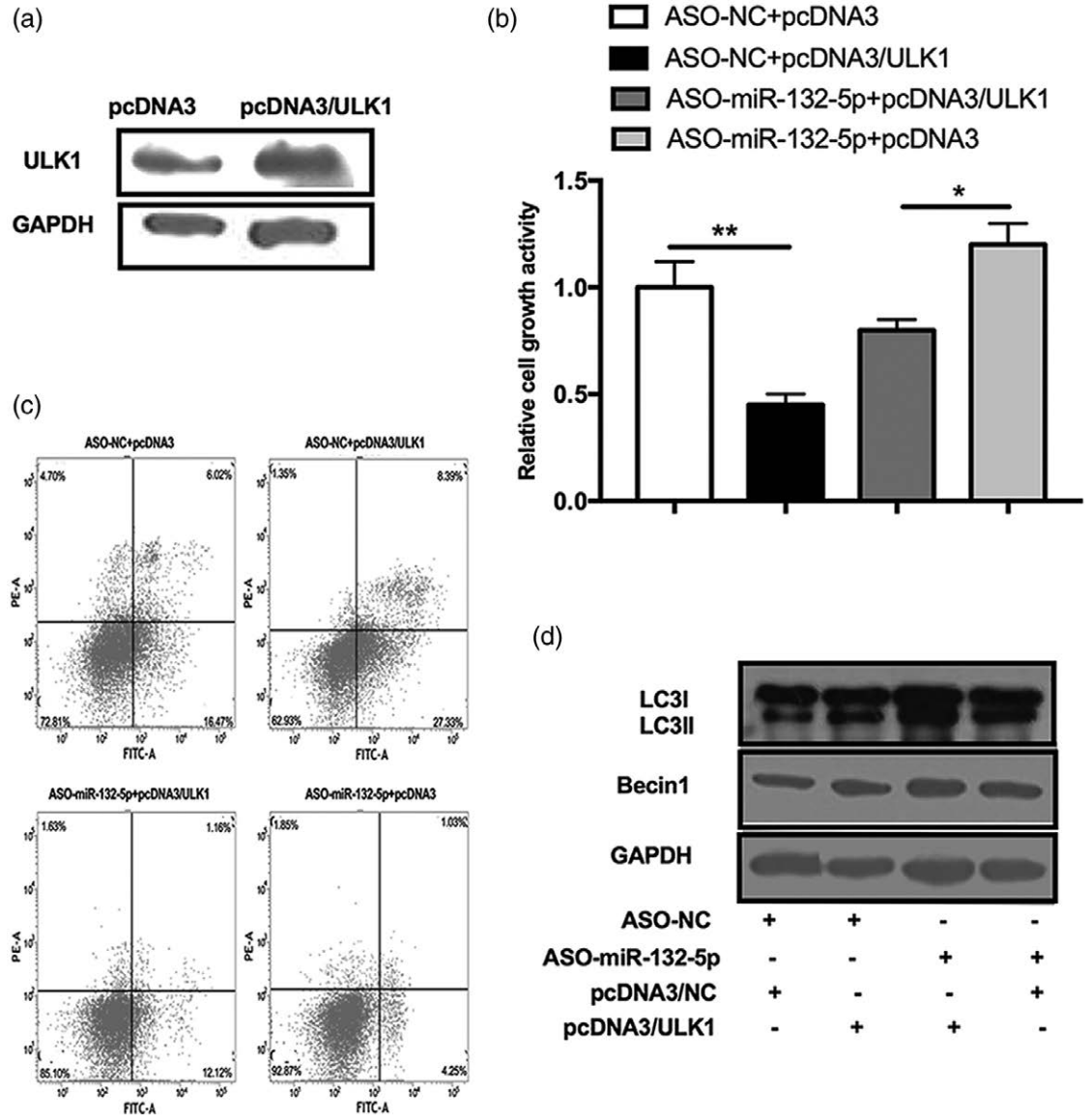
To investigate whether the phenotype of miR-132-5p was mediated by ULK1 rather than by other genes, we performed rescue experiments. First, we constructed an overexpression vector (pcDNA3/ULK1) lacking the 3′-UTR. The results of Western blot confirmed the expression of ULK1 in SH-SY5Y cells (Fig. 4a). Then, MTT and flow cytometry results showed that overexpression of ULK1 decreased the viability of cells and increased the apoptosis rate. Notably, the ectopic expression of ULK1 reversed the effects of ASO-miR-132-5p on cell apoptosis (Fig. 4b and c). Western blot showed overexpression of ULK1 could significantly increase the expression levels of autophagy protein markers. Meanwhile, ectopic expression of ULK1 could reverse the effects of ASO-miR-132-5p on autophagy (Fig. 4d). Thus, these results suggested that ULK1 was involved in the regulation of apoptosis and autophagy by miR-132-5p in MPTP-treated SH-SY5Y cells.

Discussion

It is well known that miRNAs are regulators of many genes at the post-transcriptional level. Abnormal miRNA expression is related to physiological and pathological processes. Identification of aberrant miRNAs and their targets is critical for understanding the progression of PD, which may be aid in the development of new biomarkers for diagnosis and new therapeutic targets.

Numerous reports have demonstrated miR-132 is involved in physiological and pathological processes. In the nervous system, miR-132 is significant for regulating neuronal differentiation, maturation and functioning, and widely participates in axon growth, neural migration ang plasticity [10–12]. Thus, the alteration of miR-132 is associated with many CNS diseases, such as Alzheimer's disease [13,14], cerebral ischemic stroke [15–17] and experimental autoimmune encephalomyelitis [18]. In our reports, we focused on the role of miR-132-5p in MPTP-induced PD model. Consistent with previous studies, the expression of miR-132-5p was elevated both in MPTP-treated SH-SY5Y cells and SNpc of MPTP-treated mice, indicating that

Fig. 4



Overexpression of ULK1 restores the influence of ASO-miR-132-5p on MPTP-treated SH-SY5Y cells apoptosis and autophagy. (a) Western blot was used to detect the efficiency of pcDNA3/ULK1. GAPDH was used as an internal control. (b and c) The MTT assay and flow cytometry were used to measure cells viability or cellular apoptosis following transfection of pcDNA3/ULK1 or co-transfection with ASO-miR-132-5p (*$P <$ 0.05, **$P <$ 0.01). (d) Western blot was used to detect the protein expression levels of LC3II, LC3I and Beclin 1. GAPDH was used as an internal control. All experiments were performed three replicates with three wells per replicate. The bars represent the mean ± SD of three independent experiments (*$P <$ 0.05, **$P <$ 0.01).

miR-132-5p might play a role in PD. Next, we used MTT assay and flow cytometric analysis found that inhibition of miR-132-5p increased cell survival ability and reduced apoptosis in MPTP-treated SH-SY5Y cells. Moreover, we also observed inhibition of miR-132-5p suppressed autophagy by detecting the mRNA and protein expression levels of LC3I, LC3II and Beclin 1.

Identifying the targets regulated by miR-132-5p is important for clarifying our understanding of PD's pathological process. Based on this perspective, we used bioinformatics to predict target genes and selected ULK1 as the candidate target gene. First, we used EGFP reporter assay to confirm the interaction between miR-132-5p and ULK1, revealing that ULK1 was a direct target of miR-132-5p and positively regulated by miR-132-5p. Second, real-time PCR and western blotting assays further confirmed inhibition of miR-132-5p suppressed endogenous ULK1 expression at both the mRNA and protein levels.

UNC51-like kinase (ULK1) is a serine/threonine kinase that initiates the autophagy cascade. Previous studies

have shown autophagy and apoptosis are related in function [19,20]. In neuroblastoma, inhibition of ULK1 significantly reduced cell growth and promoted apoptosis [21]. Meanwhile, overexpression of ULK1 prevented PC-12 cells from hypoxia-induced apoptosis by promoting cell autophagy [22]. However, another research showed that ULK1 expression was increased in the MPP^+ treated MN9D cell and ULK1 knockdown increased neuronal cell viability [23]. In our research, we found overexpression of ULK1 could reverse the inhibition effect of ASO-miR-132-5p on apoptosis and autophagy, indicating miR-132-5p might play a role through regulating ULK1.

Conclusion

We found miR-132-5p might play an important role in PD. Inhibition of miR-132-5p could reduce apoptosis and suppress autophagy through targeting ULK1 in MPTP-intoxicated PD model. On the basis of these, our research provided a valuable clue for developing a prospective therapeutic target for control of PD in the future, although the detail mechanisms of miR-132-5p, ULK1 and PD remained to be fully elucidated.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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