RESEARCH ARTICLE

MiR-124 Regulates Apoptosis and Autophagy Process in MPTP Model of Parkinson's Disease by Targeting to Bim

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Keywords
apoptosis, autophagy, Bax, Bim, miR-124, Parkinson's disease.

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Abstract

Parkinson's disease (PD) is the most prevalent movement disorder characterized by selective loss of midbrain dopaminergic (DA) neurons. MicroRNA-124 (miR-124) is abundantly expressed in the DA neurons and its expression level decreases in the 1-methyl-4-pheny-1, 2, 3, 6-tetrahydropyridine (MPTP) model of PD. However, whether the upregulation of miR-124 could attenuate neurodegeneration remains unknown. Here, we employed miR-124 agomir and miR-124 mimics to upregulate miR-124 expression in MPTP-treated mice and MPP^+-intoxicated SH-SY5Y cells, respectively. We found that loss of DA neurons and striatal dopamine in MPTP-treated mice was significantly reduced by upregulating miR-124. In addition, we identified a target of miR-124, Bim that mediated the neuroprotection of miR-124. Indeed, treatment of miR-124 agomir in MPTP-treated mice inhibited Bim expression, thus suppressing Bax translocation to mitochondria. Moreover, impaired autophagy process in MPTP-treated mice and MPP^+-intoxicated SH-SY5Y cells characterized as autophagosomes (AP) accumulation and lysosomal depletion were alleviated by the upregulation of miR-124. Taken together, these results indicate that upregulation of miR-124 could regulate apoptosis and impaired autophagy process in the MPTP model of PD, thus reducing the loss of DA neurons.

INTRODUCTION

Parkinson's disease (PD) is the most prevalent movement disorder and the second most common chronic and systemic neurodegenerative disorder after Alzheimer's disease (23, 24). PD prevalence increases steadily with age (38), which means the number of PD patients would be larger in an aging population (10). A pathological hallmark of PD is the loss of dopaminergic (DA) neurons within the substantia nigra pars compacta (SNpc) of the basal ganglia (7, 24), which causes abnormal nerve signaling that leads to impaired movement. To date, several lines of evidence from PD research find that pathophysiologic mechanisms underlying DA cell loss are related to mitochondrial dysfunction, oxidative stress and protein aggregation, which each could cause the apoptosis or impaired autophagy of DA neurons (1, 18, 28).Thus, strategies aimed at attenuating the loss of SNpc DA cells might provide an effective cure for PD patients.

MicroRNAs (miRNAs) are highly conserved non-coding RNAs, which modulate transcription of multiple genes via binding to 3′ untranslated regions (3′UTR) (2). Until now, extensive evidence demonstrates that microRNA machinery plays an important role in dopamine neuron biology and diseases of neurodegeneration (16, 21, 31). For example, miR-7 and miR-153 negatively regulate α-synuclein mRNA, a key gene related to inherited PD (11, 19). In addition, miR-133b is deficient in the PD midbrain as well as in mouse models, and miR-34b/34c are decreased in several affected brain regions in PD and incidental Lewy body disease (21, 29).

miRNA-124 (miR-124) is highly expressed in the brain with abundance (more than 100 times) higher than in other organs (30). Convincing evidence has demonstrated the neuro-protective effect of miR-124 in some CNS diseases, such as experimental autoimmune encephalomyelitis (37), focal cerebral ischemia (9) and stroke (44). In addition, a recent study reveals that miR-124 is downregulated in 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)-induced animal model of PD (20). In view of the evidence cited above, we hypothesized that miR-124 might play a vital role in the pathology of DA neurons in PD. In our study, we find that exogenous delivery of miR-124 maintains the number of DA neurons and midbrain dopamine level in MPTP-treated mice. Furthermore, we identified a novel target of miR-124—Bim, a BH3-only protein that mediates the neuro-protective effect of miR-124.

Table 1. Primer sequences.

Gene	Forward sequence	Reverse sequence
Bim (Human)	5′-TCATCGCGGTATTCGGTTC-3′	5′-GAAGGTTGCTTTGCCATTTG-3′
β-actin (Human)	5′-GGCATCCTCACCCTGAAGTA-3′	5′-GGGTGTTGAAGGTCTCAAA-3′
Bim (Mouse)	5′-CCCGGAGATACGGATTGCAC-3′	5′-CAGCCTCGCGGTAATCATTTG-3′
β-actin (Mouse)	5′-CTTTGATGTCACGCACGATTTC-3′	5′-GGGCCGCTCTAGGCACCAA-3′
miR-124	5′-GCGAGGATCTGTGAATGCCAAA-3′	
U6	5′-GCTTCGGCAGCACATATACTAAAAT-3′	

MATERIAL AND METHODS

Animals and treatment

Eight ~10-week-old male C57BL/6 mice were purchased from Guangdong Medical Laboratory Animal Center. The mice received one intraperitoneal injection of MPTP-HCl per day (30 mg/kg free base; Sigma, MO, USA) for five consecutive days. Control mice received saline injections only. Mice were killed at different timepoints after MPTP intoxication: 0 (immediately after the last MPTP injection), 1, 2, 4, 7, and 21 days after the last MPTP administration (41). Six mice were used in each group. Mice were decapitated and, once the brain was removed, the ventral midbrain, which contained the SNpc, was dissected and stored at −80°C for further experiment. Regarding the experiment of exogenous delivery of miR-124 in animal model, the right lateral ventricle of mice was surgically implanted with a stereotactic catheter. The stereotactic intraventricular injection site was chosen as previously reported (2 mm rostral to the bregma, 2 mm lateral to the sagittal suture, and 3 mm below the skull surface) (40). After 1 week of recovery, mice were given one treatment of agomir (RiboBio, Guangzhou, China) miR-124-3p (20 nM of ribonucleotide in a total volume of 5 μL) through the catheter per day for five consecutive days. Agomir-negative control sequences were injected into the right lateral ventricle as negative control. The treatment of agomir was performed 2 days prior to injection of MPTP.

Cell culture and oligonucleotide transfection

SH-SY5Y cells were obtained from Central Laboratory of Nanfang Hospital (Guangzhou, China). Cells were maintained in DMEM/F12 (HyClone, Logan, UT, USA) plus 10% fetal bovine serum (Gibco, Grand Island, NY, USA). Besides the mimics/negative control, inhibitor/negative control of miR-124-3p and SiRNA duplex oligonucleotides targeting human Bim 5′-CCCTACAGACAGAGCCACA-3′ mRNA were synthesized by Ribobo, then were transfected into SH-SY5Y cells by using riboFECT™ CP (RiboBio, Guangzhou, China) according to the manufacturer's protocol.

RT-PCR

Total RNA was extracted from selected mouse brain regions and cells. Complementary DNA was amplified using the Roche LightCycler480 (Roche Diagnostics, Mannheim, Germany). The primer sequences used were as listed in Table 1.

Luciferase reporter assay

A 2522-bp segment from the 3′UTR of the Bim gene containing the two miR-124 binding sites was produced by PCR with the forward primer 5′-GCGGTTCTCTTGTGGAGGGG-3′ and the common reverse primer 5′-AGAAGGGGAAACGGCAGACA-3′, and then cloned into the XhoI/NotI site of psi-CHECK™-2 vector (Promega, Madison, WI, USA). For mutant construct of Bim 3′UTR, deletion mutagenesis and fusion-PCR were performed. Four fragments, including two mutant miR-124 binding sites and two middle segments, were first produced by PCR (Supporting Information Figure S1); the primers are shown in Table 2. The full length of Bim promoter, containing the two mutant miR-124 binding sites, was obtained by mixing the two fragments produced from the first-step PCR and then using them as the template in the second PCR reaction with the outermost primers, and then cloned into the XhoI/NotI site of psi-CHECK™-2 vector too. All of the constructs were confirmed by sequencing. HEK293 cells were co-transfected with 100 ng/mL psi-CHECK™-2 vector and Luciferase vector, including the 3′UTR of Bim (with either wild-type or mutant-type miR-124 binding sites) and miR mimics or control (RiboBio) at a final concentration of 100 nM using riboFECT™ CP as described by the manufacturer. Luciferase assays were performed with a Dual-Luciferase Reporter Assay System (Promega) 48 h after transfection. Renilla luciferase activity was normalized to that of firefly luciferase.

Western blotting

Total protein samples were collected from SH-SY5Y cells or selected mouse midbrain with cell lysis buffer. Additional protein extractions of mitochondria were performed with the Mitochondria Isolation Kit (Beyotime, Jiangsu, China) following manufacturer's description. The lysosomes from SH-SY5Y cells were isolated by a lysosome isolation kit (Sigma, St. Louis, MO, USA) with differential centrifugation, followed by a density gradient centrifugation and Ca^{2+} precipitation, as detailed in the

Table 2. Primers of fusion-PCR.

Name	Sequence
Bim-F1	GCGGTTCTCTTGTGGAGGGG
Bim-R4	AGAAGGGGAAACGGCAGACA
Bim-mut1-F2	GCCAGCTAACTTAAAAAG**CT**TTATTTTTAGAGATTAC
Bim-mut1-R1	GTAATCTCTAAAAATAA**AG**CTTTTTAAGTTAGCTGGC
Bim-mut2-F4	GGAAACTTACTGCAGGTTT**TG**ACAACCTCTCCCTATGTG
Bim-mut2-R3	CACATAGGGAGAGGTTGT**CA**AAACCTGCAGTAAGTTTCC
Bim-F3	GCCCAGGGAGACCCAAGAAAGAG
Bim-R2	CTCTTTCTTGGGTCTCCCTGGGC

Bold represents the depletion of miR-124 biding sites.

manufacturer's instruction (34). Western blotting was carried out as previously described (8). Antibodies used were as following: Bim, Bax, β-actin and Cox-IV (Cell Signaling Technology, Danvers, MA, USA); LC3II (Novus Biologicals, CO, USA); Lamp1 and Lamp2 (Abcam, MA, USA); and Beclin1 (Sigma).

Immunohistochemistry

Immunostaining was performed with a primary antibody to tyrosine hydroxylase (Millipore, MA, USA). The total number of tyrosine hydroxylase (TH)-positive neurons and apoptotic neurons in the SNpc of selected mice were counted as previously described (36).

High performance liquid chromatography

Briefly, tissue samples were obtained by dissection and immediately homogenized on ice in 300 μL of perchloric acid 0.4 N and 0.1% (w/v) sodium metabisulfite, followed by a 10 min centrifugation step at 4000 × g at 4°C. The supernatants were filtered and preserved at −70°C until used for chromatographic analysis (36).

In situ hybridization combined with Immunofluorescence

In situ hybridization (ISH) was carried out on deparaffinized brain tissues using the previously published protocol which includes a digestion in pepsin (1.3 mg/mL) for 30 min. The miCURY LNA™ microRNA detection probe was obtained from EXIQON (Vedbaek, Denmark). The sequence of the probe was (5′ ~ 3′): /5DigN/ATCAAGGTCCGCTGTGAACACG. The probe and tissue miRNA were co-denatured at 60°C for 5 min, followed by hybridization at 37°C overnight and a stringency wash in 0.2X SSC and 2% bovine serum albumin at 4°C for 10 min. The probe target complex was seen due to the action of alkaline phosphatize on the chromogen nitroblue tetrazolium and bromochloroindolyl phosphate (NBT/BCIP). No counterstain was used to facilitate co-labeling of TH proteins. After *in situ* hybridization for miR-124, the slides were analyzed for immunofluorescence using the optimal conditions for TH (1:100, 30 min).

Immunoprecipitations

Co-precipitation of Beclin1 and Bim was done from SH-SY5Y cells. Whole cell lysate was prepared from SH-SY5Y cells from at least 2×10^6 cells. 15 μL of protein G or A beads suspension were washed with TBS-T (0.1%), and mixed with 500 μg cell extract and 5 μg of antibody. The reaction was incubated overnight at 4°C, with rotation. The mix was centrifuged for 30 s at 4°C and beads separated from the mixture using a magnetic stand. Beads were washed three times with TBS-T (0.1%) before adding the sample buffer. The beads were boiled in sample buffer for 5–10 min, cooled, and the IP mix removed from the beads and used for SDS-PAGE and immunoblot, or stored at −20°C until use.

LysoTracker labeling

To label lysosomes, LysoTracker Red (Invitrogen, Carlsbad, CA, USA) was used at a final concentration of 80 nM. Cells intoxicated with 1 mM MPP^+ for 3 h were loaded with LysoTracker Red for 30 min at 37°C. Cells were washed twice in PBS and immediately visualized under fluorescence microscopy.

Electron microscopy

Collected cell pellets were fixed in sodium cacodylate buffer (0.1 M; pH 7.4), 2.5% glutaraldehyde, and 0.25 M sucrose at 4°C, then post-fixed in 1% osmium tetroxide for 1 h at 4°C. The samples were dehydrated, embedded in plastic, and cut into in 70-nm sections for microscopy. Sections were subsequently post-stained with 5% uranyl acetate and viewed with an electron microscope (Hitachi H-7650, Hitachi, Tokyo, Japan).

Statistical analysis

Data are presented as mean ± SEM from three independent experiments. One-way analysis of variance with Dunnett's post-test was used when comparing groups (more than two groups) with only one treatment. Two-way analysis of variance with Bonferroni post-tests were performed among experiments with treatments of MPTP/MPP^+ and agomir/mimics. A *P*-value less than 0.05 was defined as the threshold for significance.

RESULTS

MiR-124 is downregulated in SNpc DA neurons of MPTP-treated mice and MPP^+-intoxicated SH-SY5Y cells

First, we investigated the expression of miR-124 in the midbrain of MPTP-treated mice by RT-PCR. Consistent with a recent study (20), we found that the expression of miR-124 decreased after intraperitoneal injection of MPTP (Figure 1A). As inflammation is involved in PD, and miR-124 is associated with microglia quiescence (37), alteration in microglia might also contribute to the reduction of miR-124 in midbrain. We next determined miR-124 expression in SNpc DA neurons via *in situ* hybridization; the result showed that miR-124 expression was also downregulated in SNpc DA neurons (Figure 1B). The decrease of miR-124 in DA neurons was determined again on MPP^+-intoxicated SH-SY5Y cells *in vitro*. Similarly, MPP iodide induced reduction of miR-124 expression in SH-SY5Y cells compared with control (Figure 1C,D).

Exogenous delivery of miR-124 attenuates neurodegeneration of MPTP-treated mice *in vivo*

Considering the neuroprotection of miR-124 in other CNS diseases, miR-124 agomir or negative control were injected into the right lateral ventricle 2 days before MPTP treatment to up-regulate the miR-124 expression in midbrain (Supporting Information Figure S2). As a result, the density of TH-positive neurons in the miR-124 agomir group was higher than in the negative control group at 21 days after treatment of MPTP (Figure 2A,B). To assess the neurodegeneration, we detected striata levels of dopamine 21 days after MPTP administration, using high-performance liquid

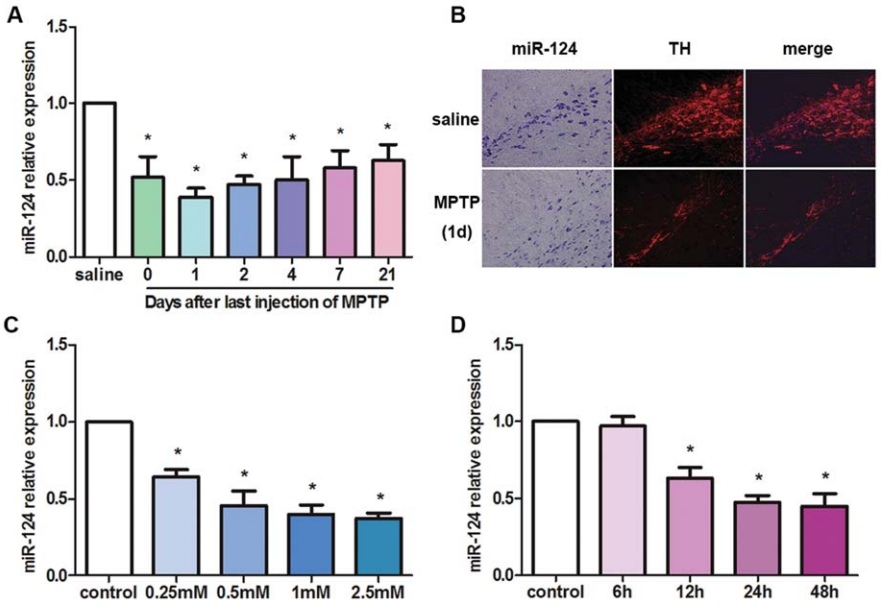

Figure 1. Expression of mir-124 in MPTP-treated mice and MPP^+-intoxicated SH-SY5Y cells. **A.** Total RNA was extracted from midbrain of selected mice and synthesized cDNA, and then analyzed by RT-PCR to determine the miRNA-124 level. miRNA-124 level was normalized to U6. **B.** miR-124 expression in SNpc DA neurons of MPTP-treated mice determined by *in situ* hybridization combined with Immunofluorescence. **C.** miR-124 expression in SH-SY5Y cells with treatment of MPP^+ for 24 h at different concentrations. **D.** miR-124 expression in SH-SY5Y cells exposed to 1 mm MPP^+ for different duration. * < 0.05 compared with control.

chromatography. Comparing with the negative group, loss of striatal dopamine was significantly less pronounced in the miR-124 agomir group (Figure 2C).

MiR-124 targets to Bim

Having found that miR-124 induced neuroprotection in the animal model of PD, we investigated the mechanisms underlying this effect. By using TargetScan software (Whitehead Institute, Cambridge, MA, USA), we analyzed targets predicted for miR-124. Bim, a BH3-only protein involved in apoptosis of DA neurons in MPTP model of PD, was predicted as a putative target with two conserved miR-124 binding sites in its 3′ untranslated region (3′UTR; Figure 3A). When we transfected SH-SY5Y cells with miR-124 mimics, Bim expression was reduced on both the protein (Figure 3B) and the mRNA levels (Figure 3C), as compared with the control. Using the luciferase reporter system, we then detected that miR-124 directly binds the mRNA encoding Bim. Indeed, cells transfected with miR-124 mimics induced a reduction in luciferase activity (Figure 3D).

MiR-124 attenuates mitochondria-dependent apoptotic cell death by targeting to Bim

After indentifying that Bim was the target of miR-124, we further studied whether miR-124 could attenuate MPTP-induced expression of Bim. Compared with the negative control group, upregulation of Bim mRNA level, and protein level induced by MPTP, was reduced by miR-124 agomir (Figure 4A,B). Besides, to determine the specific effects of miR-124 on Bim, we investigated 3 additional BH3-only Bcl2-family members, Puma, Noxa and Bid (Supporting Information Figure S3). And we found there was no change in protein expression of Puma and Noxa in MPTP-treated mice. Actually, Celine Perier *et al*. also found that mRNA levels of Puma and Noxa did not alter after MPTP treatment (36). In addition, although the treatment of MPTP enhanced the cleavage of Bid (45), miR-124 agomir could not counter-regulate that. As the pro-apoptotic effect of Bim is mediated through Bax mitochondrial translocation in the MPTP model of PD (36), we then detected the effect of inhibition of Bim by upregulating

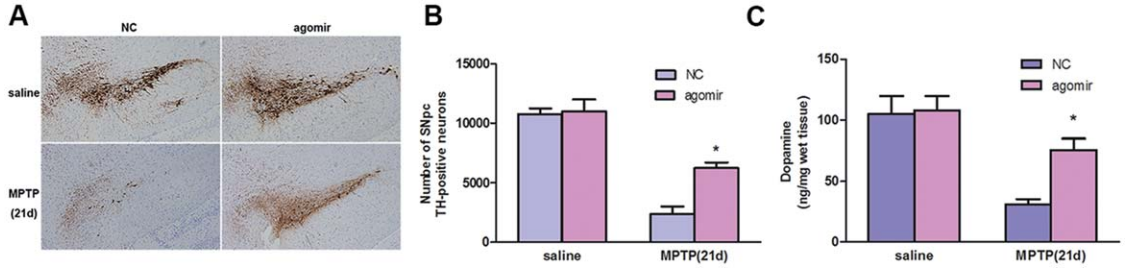

Figure 2. Exogenous delivery of mir-124 rescues DA neurons from MPTP toxicity. (**A** and **B**) Immunostaining and stereological counts of TH-positive neurons in the SNpc were performed in agomir-treated mice and their negative control counterparts at day 21 after the last injection of MPTP. **C.** Striatal dopamine level was determined by HPLC. * < 0.05 compared with negative control in the group of MPTP-treated mice.

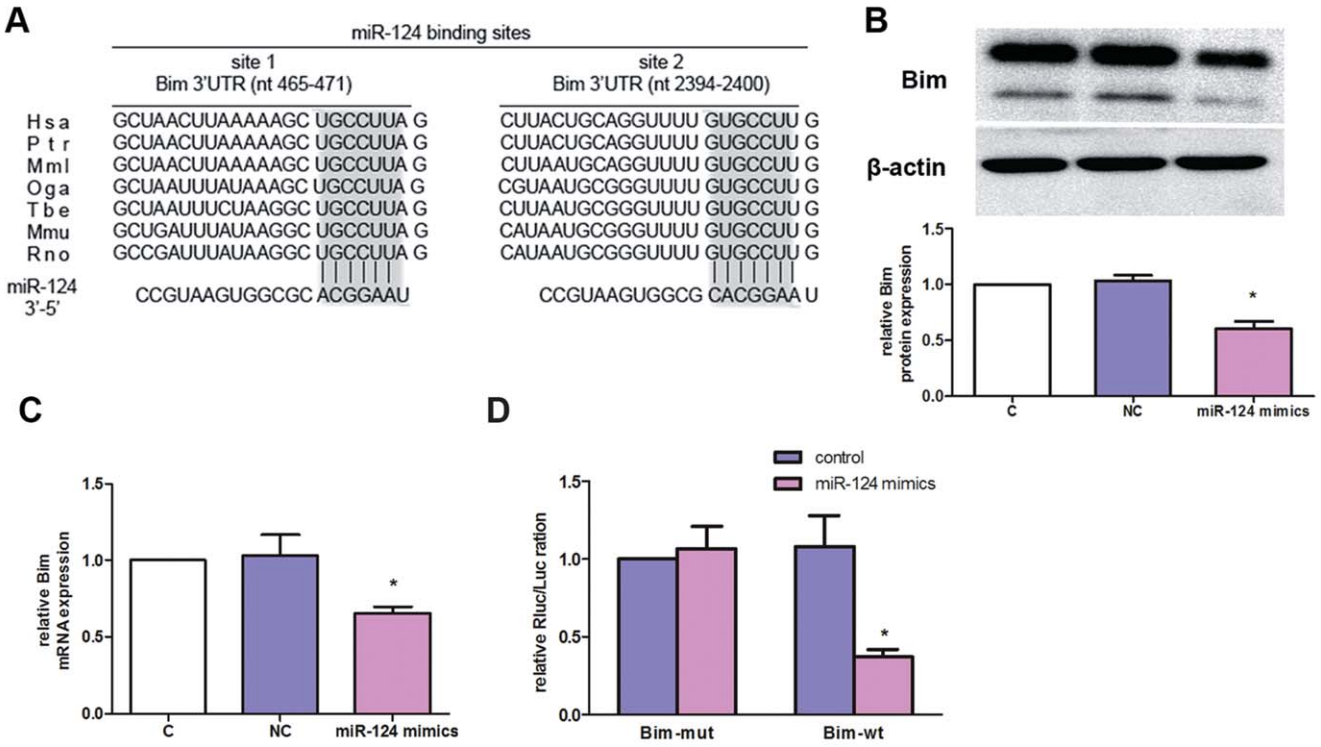

Figure 3. Bim is a target of miR-124. **A.** Alignment of two miR-124 binding sites to Bim 3′UTR is shown for different species predicted by Targetscan. **B.** Western blot analysis of Bim in SH-SY5Y cells transfected with miR-124 mimics (100 nm) or negative control. **C.** mRNA level of Bim determined by PCR in SH-SY5Y cells transfected with miR-124 mimics (100 nm) or negative control. **D.** Luciferase activity in HEK293 cells transfected with reporter constructs containing wild-type (WT) or mutated Bim 3′UTR. The cells were co-transfected with indicated constructs and miR-124 mimics (100 nm) or control, and normalized levels of luciferase activity are shown.* < 0.05 compared with control.

miR-124 on Bax mitochondrial translocation. Interestingly, we observed a similar result as a previous study silencing Bim gene—that Bax mitochondrial translocation caused by MPTP was also reduced by upregulation of miR-124(Figure 4C). Correspondingly, the number of apoptotic cells in SNpc (Figure 4D) was reduced in the miR-124 agomir group.

MiR-124 attenuates autophagosome accumulation and lysosomal depletion

Recently, it has been identified that Bim possesses both anti-autophagy and pro-apoptotic effects (27). We thus examined the effect of exogenous miR-124 on impaired autophagy in MPTP-treated mice, which exhibits as autophagosome accumulation and lysosomal depletion (8). Interestingly, comparing with the control group, miR-124 agomir significantly reduced autophagosome marker LC3II and restored lysosomal marker LAMP1 protein levels (Figure 5A,B). We next wondered whether the effect of miR-124 on autophagy was mediated through downregulation of Bim. Therefore, miR-124 mimics or si-Bim RNA were transfected into SH-SY5Y cells 6 h before MPP^+ treatment. After downregulating Bim (Figure 5C), both miR-124 and si-Bim RNA could attenuate autophagosome accumulation and lysosomal depletion caused by MPP^+ (Figure 5D,E).

Bim mediates Bax translocation to lysosomal membranes

Previous study demonstrated downregulating Bim *in vivo* and *in vitro* would result in enhanced autophagosome formation (27), which seemed paradoxical with our data. Actually, in a steady-state condition, Bim inhibits autophagosome formation depending on Beclin1 (an autophagy regulator) by interacting with Beclin1 directly (27). However, in response to stress, such as starvation—an autophagic stimulus—the interaction between Bim and Bechlin1 is disrupted with Bim ameliorating autophagy inhibition. Therefore, we wondered how it responded to treatment of MPP^+. We found that Bim-Beclin1 interaction was dramatically weakened after 24 h of treatment with MPP^+ (Figure 6A), which indicated that Bim might exhibit autophagy-permissive rather than autophagy-inhibitory effects under stimulation of MPP^+, similar with starvation. Therefore, there might be other mechanisms responsible to affect downregulation of Bim on autophagy. A recent study identified that following MPTP treatment, activated Bax translocates to both lysosomal and mitochondrial membranes causing lysosomal membrane permeabilization (LMP) and mitochondrial outer membrane permeabilization (MOMP), respectively. Bax-induced LMP leads to a decreased number of lysosomes and subsequent

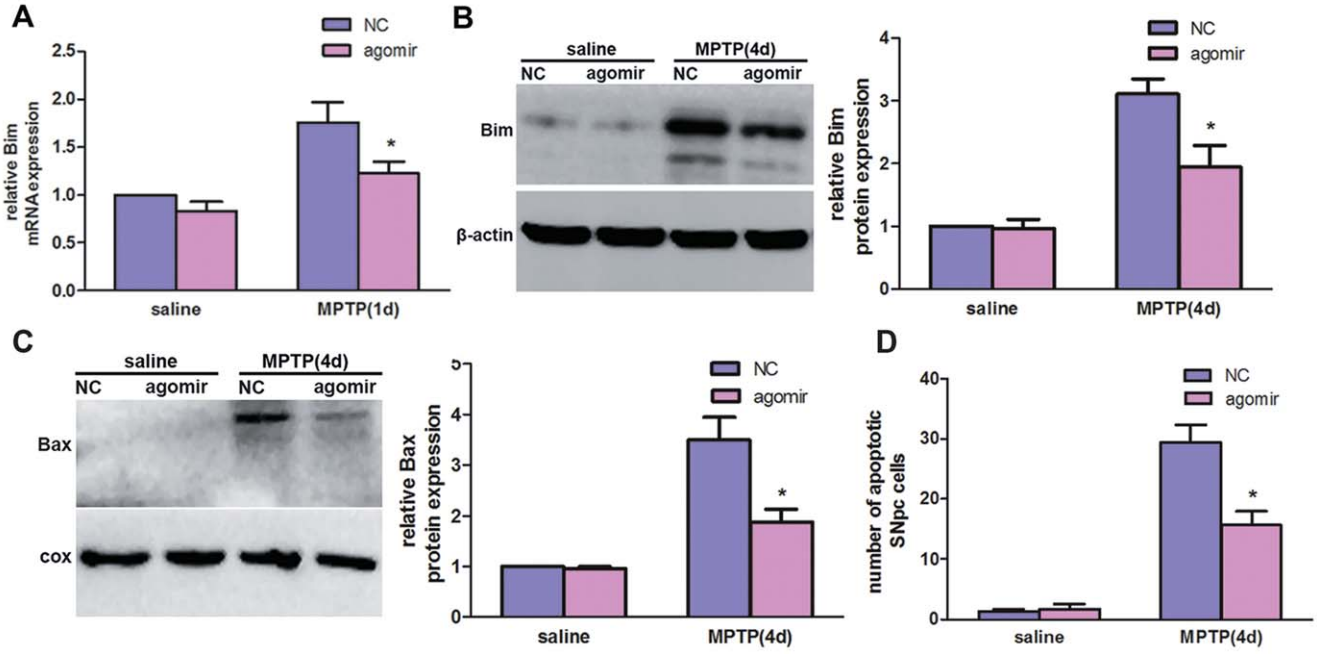

Figure 4. miR-124 inhibits upregulation of Bim and Bax translocation induced by MPTP *in vivo*. **A.** Expression of Bim in ventral midbrain determined by RT-PCR. **B.** Western blot analysis of Bim expression in total protein samples exacted from midbrain. **C.** Western blot analysis of Bax expression in mitochondrial protein isolated from midbrain. **D.** Stereological counts of apoptotic neurons in the SNpc were performed in agomir-treated mice and their negative control littermates at day 4 after the last injection of MPTP. * < 0.05 compared with negative control in the group of MPTP-treated mice.

accumulation of undegraded AP (4). As Bim mediates Bax translocation to mitochondria (36), we then investigated whether Bim participated in Bax translocation to lysosome and finally induced LMP. In our study, we found that downregulating Bim by miR-124 or si-Bim RNA reduced Bax translocation to lysosomal membrane in MPP^+-intoxicated cells (Figure 6B). Accordingly, LMP indicted by lysotracker density was also attenuated by downregulating Bim (Figure 6C).

Inhibiting miR-124 in SH-SY5Y cells causes suppression of basal autophagy

Bim has a key role in inhibiting autophagy under the steady-state condition, so we tested whether up-regulating Bim by inhibiting miR-124 could reduce basal autophagy in SH-SY5Y cells, as loss of basal autophagy in neurons could cause neurodegeneration (15). First, we found that Bim protein level was up-regulated after transfecting miR-124 inhibitor into SH-SY5Y cells (Figure 7A). Then, we assessed the basal autophagy level by measuring LC3II level and electron microscopy. As a result, basal autophagy level decreased in miR-124 inhibitor-treated cells (Figure 7B,C). Unfortunately, although mitoptosis is another mitochondrial death mechanism leading predominantly to activation of autophagy (5, 17), there was no obvious evidence of mitoptosis in our electron microscopy results. In addition, the mitochondrial potential determined by JC-1 staining was also not affected by miR-124 inhibitor transfection in SH-SY5Y cells. (Supporting Information Figure S4)

DISCUSSION

MicroRNAs play a critical role in neuron biology and miR-124 is abundantly expressed in the neurons (31, 43). To date, it has been demonstrated that miR-124 alteration is associated with many CNS diseases, such as injured hypoglossal motor neurons (33), experimental autoimmune encephalomyelitis (37) and cerebral ischemic stroke (44). In addition, we recently found that miR-124 inhibited the growth of high-grade gliomas through posttranscriptional regulation of LAMB1 (6). In the present study, we focused on the role of miR-124 in the DA neurons of MPTP-treated mice and MPP^+-intoxicated SH-SY5Y cells. Consistent with a recent study (20), the expression of miR-124 in selected midbrain tissue of mice was down-regulated. Furthermore, we exclusively investigated the alteration of miR-124 in SNpc DA neurons by employing *in situ* hybridization experiment combined with TH staining, with the result of reduction of miR-124 expression in SNpc DA neurons. In addition, the expression of miR-124 in MPP^+-intoxicated SH-SY5Y cells was also down-regulated. Therefore, the level of miR-124 in DA neurons declines when exposed to PD stimulus MPTP or MPP^+.

Accordingly, Bim, a BH3-only protein that has been identified as a target of miR-124 in our study, is upregulated in MPTP-induced PD model (36). Actually, Bim is a key regulator of neuronal apoptosis, including cerebellar granule neurons (CGNs) deprived of activity (42), sympathetic neurons during removal of nerve growth factor (NGF) (46), and cortical neurons exposed to β-amyloid peptide (48). And transcriptional control is an important

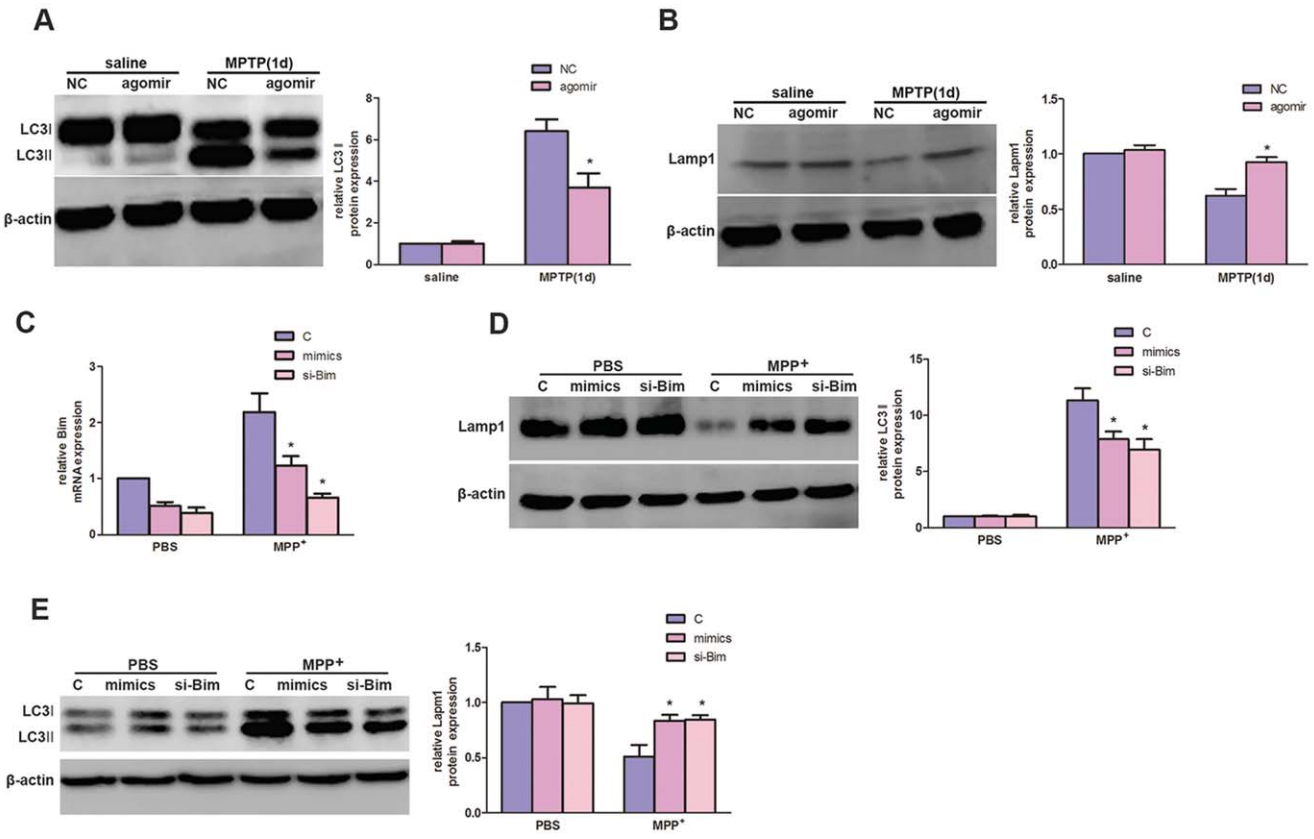

Figure 5. miR-124 attenuates impairment of autophagy in MPTP-treated mice and MPP+-intoxicated cells. Western blot analysis of LC3II (**A**) and Lamp1 (**B**) expression in total protein samples exacted from midbrain at day 1 after the last injection of MPTP. * < 0.05 compared with negative control in the group of MPTP-treated mice. **C.** Bim mRNA level was determined by RT-PCR in PBS or MPP+-treated SH-SY5Y cells transfected with miR-124, si-Bim or control 6 h prior. Western blot analysis of LC3II (**D**) and Lamp1 (**E**) expression in total protein samples exacted from cells treated as in C. * < 0.05 compared with control in the group of MPP+-treated cells.

mechanism regulating Bim expression during neuronal apoptosis, including JNK/c-jun pathway (46), FOXO transcription factors (3) and Egr-1 transcription factor (47). More specifically, the mechanism regulating Bim expression in the MPTP model of PD has been demonstrated to be associated with JNK/c-jun activation, with an induction of Bax translocation to mitochondria (36). Nevertheless, we observed that exogenous delivery of miR-124 could counter-regulate Bim up-regulation induced by MPTP *in vivo*, as miR-124 targeting to Bim demonstrated by our Luciferase reporter assay, and reduce Bax translocation to mitochondria. Indeed, there are several lines of evidence supporting that Bim is regulated by microRNA in other diseases. For example, miR-24 represses Bim and inhibits apoptosis in mouse cardiomyocytes (39), and miR-363 promotes human glioblastoma stem cell survival via direct Inhibition of Bim (12).

Currently, it has been demonstrated that Bim possesses dual effects in inhibiting autophagy and promoting apoptosis (27). Interestingly, we found that downregulation of Bim induced by miR-124 could attenuate autophagosome accumulation and lysosomal depletion in MPTP-treated mice and MPP+-intoxicated SH-SY5Y cells. However, the anti-autophagy effect of Bim is dependent on interaction of Beclin1 under a steady-state condition, which is disrupted under stimulus of MPP+. Therefore, it is possible that there are other mechanisms contributing to the effect of Bim on autophagy in the model of PD. In the scenario of apoptosis, Bim mediates the translocation of Bax to mitochondria, thus releasing cytochrome c and resulting in apoptosis of neurons in MPTP-treated mice (36). In our study, we determined that Bim is likewise correlated to Bax translocation to lysosome, which has just been demonstrated to be associated with lysosomal disruption and impaired autophagy linked to Parkinson's disease (4). In fact, autophagy and apoptosis are two important cellular processes with complex and interconnected protein networks (5, 14, 32). For example, p53 protein possesses dual regulatory function in apoptosis and autophagy, p53 protein induces apoptosis mostly through transcription regulation, while it could activate or suppress autophagy depending on cellular energy status (5). Bcl2-family proteins are another crucial factors governing the crosstalk between autophagy and apoptosis (25). It has also been shown that Bcl-2 not only functions as an anti-apoptotic protein, but also as an anti-autophagy protein via its inhibitory interaction with Beclin1 (35). Indeed, a recent study suggests that prosurvival Bcl-2 family members affect autophagy only indirectly, by inhibiting Bax and Bak (26). Actually, apoptosis and autophagy are different

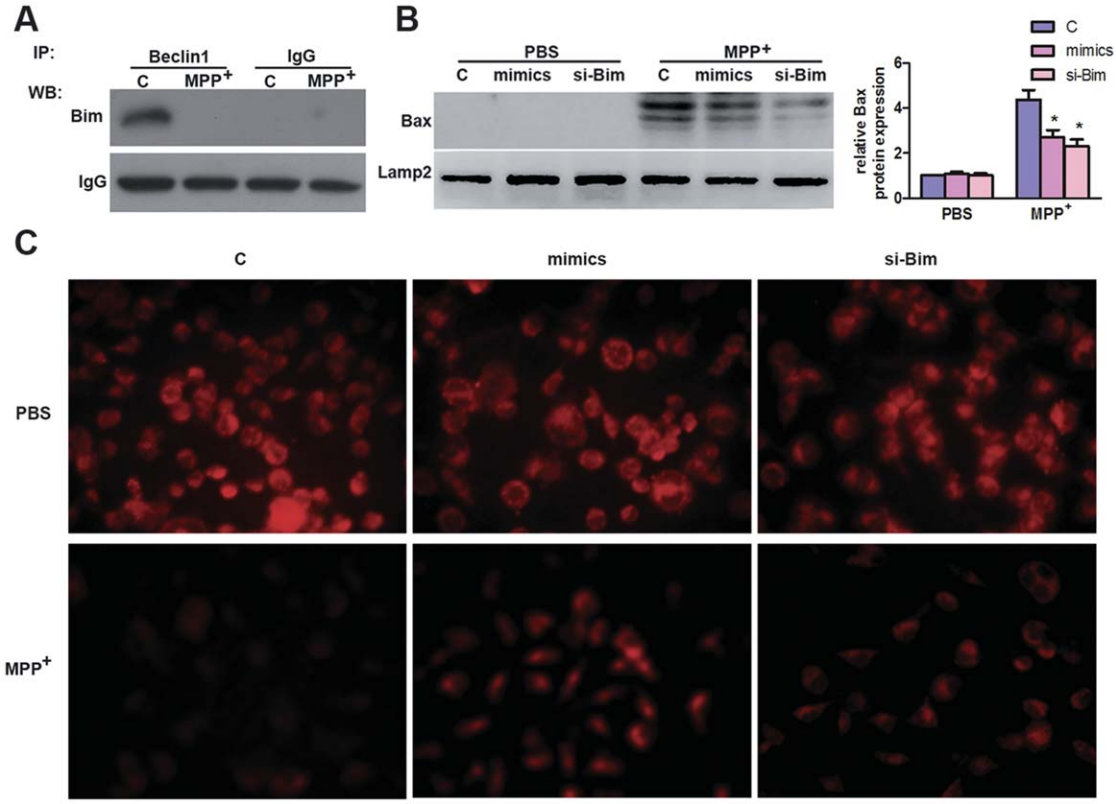

Figure 6. Bim mediates Bax translocation and LMP. **A.** Interaction between Beclin1 and Bim in MPP+-treated SH-SY5Y cells analyzed by immunoprecipitation. **B.** Western blot analysis of Bax in lysosomes isolated from PBS or MPP^+-treated cells transfected with miR-124 mimics or si-Bim 6h prior. * < 0.05 compared with control in the group of MPP^+-treated cells. **C.** LysoTracker Red was used to label lysosome to reflect lysosomal membrane permeabilization (LMP), and visualized by fluorescence microscopy.

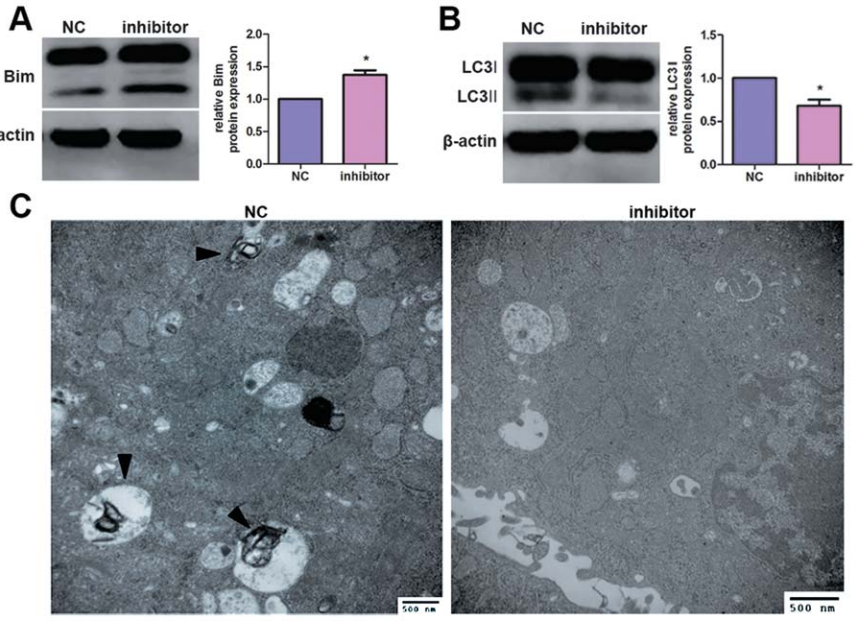

Figure 7. Basal autophagy is affected by inhibiting mir-124 *in vitro*. Western blot analysis of Bim (**A**) and LC3II (**B**) expression in SH-SY5Y cells transfected with miR-124 inhibitor. * < 0.05 compared with negative control. **C.** Basal autophagy level was evaluated by electron microscopy. Arrows indicate autophagosome-like double-membrane vesicles, which contain damaged organelles.

processes of cell death classified according to morphological appearance, enzymological criteria, functional aspects or immunological characteristics (13). In common, mitochondrial outer membrane permeabilization (MOMP) and lysosomal membrane permeabilization (LMP) would respectively cause apoptosis and autophagy. In the MPTP-induced model of PD, apoptosis (named as type I cell death) is attributed to MOMP, which is caused by Bax translocation to mitochondria (36); impaired autophagy (named as type II cell death) is a result of LMP, which is caused by Bax translocation to lysosome (4). Thus, Bax activity is important in the pathogenesis of PD both in the process of apoptosis and autophagy. In our study, translocation of Bax to the mitochondria and the lysosome could be both suppressed by upregulation of miR-124 targeting to Bim, thus attenuating the apoptosis and impaired autophagy of DA neurons in PD.

And last, regarding that Bim possesses apoptosis-inactive/autophagy-inhibitory effects under steady-state condition (27), we found that upregulating Bim in SH-SY5Y cells by inhibiting miR-124 reduced basal autophagy level. Previous studies have demonstrated that suppression of basal autophagy in neural cells causes neurodegenerative disease in mice and loss of autophagy in the central nervous system also causes neurodegeneration in mice (15, 22). So, it would be interesting to perform further experiments to test whether inhibition of miR-124 *in vivo* could result in reduced basal autophagy and neurodegeneration or not, as we have observed downregulation of miR-124 in MPTP model of PD. Besides, it is also worth noting that the mechanisms underlying neuroprotection of miR-124 on PD maybe more complicated, not just only targeting to Bim. For example, miR-124 might suppress microglia activation by inhibiting C/EBP-a (37), and then attenuating inflammation, which is responsible for pathogenesis of PD.

In conclusion, we have found that miR-124 is down-regulated in MPTP-treated mice and MPP^+-intoxicated SH-SY5Y cells, and exogenous delivery of miR-124 could attenuate the loss of SNpc DA neurons in MPTP-treated mice. Furthermore, we demonstrate that miR-124 targets to Bim, a BH3-only protein, which is a vital protein regulating apoptosis and autophagy process of SNpc DA neurons in the pathogenesis of PD (4, 36). The mechanisms underlying the neuro-protection of miR-124 on PD in our study is likely due to the reduction of Bax translocation to mitochondria and lysosome by suppression of Bim, which results in alleviation of apoptosis and impaired autophagy of SNpc DA neurons.

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CONFLICT OF INTEREST

No potential conflicts of interest were disclosed.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Fusion-PCR for mutant construct of Bim 3′UTR. First-step PCR was performed to produce four fragments, including two mutant miR-124 binding sites and two middle segments. And fragments produced from the first-step PCR were mixed to generate the template of second PCR reaction with the outermost primers, and finally the PCR production from previous step was ligated.

Figure S2. The expression level of miR-124 after exogenous delivery of miR-124 agomir. MiR-124 agomir or negative control were injected into right lateral ventricle for 5 days and 2 days prior to MPTP treatment, and total RNA was extracted from midbrain of selected mice and synthesized cDNA, and then analyzed by RT-PCR to determine the miRNA-124 level. MiRNA-124 level was normalized to U6. *<0.05 compared with respective negative control.

Figure S3. Western blot analysis of BH3-only protein (Puma, Noxa and Bid) after treatment of MPTP. Left graph shows the expression of Puma and Noxa in total protein samples is not changed exacted from midbrain at day 4 after the last injection of MPTP. Although MPTP treatment upregulated the expression of Bid, the agomir of miR-124 could not counter-regulate it.

Figure S4. JC-1 staining in the SH-SY5Y cells transfected with miR-124 inhibitor or negative control was analyzed by Fluorescence-activated cell sorting.