MZF1 alleviates oxidative stress and apoptosis induced by rotenone in SH-SY5Y cells by promoting RBM3 transcription

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ABSTRACT — Objective: To investigate the protective effect of MZF1/RBM3 on rotenone-induced neuronal injury. Methods: Rotenone (1 μM) was used to treat SH-SY5Y cells for 24 hr to simulate the cellular model of Parkinson’s disease (PD), followed by detection of SH-SY5Y cell activities using MTT assay. MZF1 expression in rotenone-treated SH-SY5Y cells was detected by qRT-PCR and Western blot. MZF1 overexpression plasmid or MZF1 overexpression plasmid and RBM3 siRNA was transfected into SH-SY5Y cells, and then the expressions of MZF1 and RBM3 were detected. Oxidative stress (OS) in SH-SY5Y cells was detected using CMH2DCF-DA probes. Cell apoptosis rate was detected by flow cytometry. CHIP assay and dual-luciferase reporter assay were used to detect the binding between MZF1 and RBM3 promoter. Results: The expression of MZF1 was significantly lower in the rotenone-induced SH-SY5Y cells. Overexpression of MZF1 significantly reduced OS and apoptosis in rotenone-induced SH-SY5Y cells. MZF1 was a transcription factor of RBM3, which promoted the transcription of RBM3, and knockdown of RBM3 inhibited the protective effect of MZF1 overexpression on SH-SY5Y cells. Conclusion: MZF1 alleviates OS and apoptosis induced by rotenone in SH-SY5Y cells by promoting RBM3 transcription.

Key words: MZF1, RBM3, Oxidative stress, Apoptosis, Rotenone

INTRODUCTION

Parkinson’s disease (PD) is the second most common and sporadic disorder of neurodegeneration, influencing more than 2% of the population over 65 (Cuenca et al., 2019). In 2015; PD affected approximately 6.2 million people and resulted in about 117,400 deaths globally (GBD 2015 Disease and Injury Incidence and Prevalence Collaborators, 2016; GBD 2015 Mortality and Causes of Death Collaborators, 2016). The pathogenesis of PD is related to the degeneration of midbrain dopamine (DA) neurons (Tao et al., 2021). Pathological accumulation of metal ions (iron species and Mn3+) and abnormal upregulation of monoamine oxidase B (MAOB) induce endogenous DA oxidation, ultimately leading to the selective vulnerability of DA neurons (Zhou et al., 2019). Intracellular iron releases radicals and causes neurotoxic protein oxidation or lipid peroxidation through Fenton reactions, promoting the pathways of oxidative stress (OS) (Han et al., 2021; Minakaki et al., 2020). OS, caused by either abnormal accumulation of reactive oxygen species (ROS) or dysfunction of the antioxidant system, tends to affect the organs with high oxygen consumption and abundant peroxidation-susceptible lipid cells, such as the brain (Kim et al., 2015). The study of Wei et al. strengthened the clinical evidence that PD is accompanied by increased OS, and manipulating concentrations of OS markers should be studied to identify potential therapeutic strategies of the disease (Wei et al., 2018). Therefore, OS is a possible direction for the treatment of PD. A truly effective disease-modifying therapy is crucial, but the lack of targeted delivery to alleviate OS has been a major challenge to overcome anomalies in PD.

MZF1 belongs to the zinc finger protein Kruppel family that can activate or inhibit the expression of target genes to regulate cell differentiation and migration (Brix et al., 2020; Mudduluru et al., 2010). MZF1 is involved in the mechanism of Apomorphine, an anti-PD drug that potently antagonizes D1/D2 DA receptor, preventing the loss of DA neuron (Luo et al., 2009). Upregula-
tion of MZF1 in dorsal root ganglion neurons is involved in the progression of neuropathic pain caused by peripheral nerve injury (Niu et al., 2020). Interestingly, MZF1 could act as a transcription factor of FPN to affect iron efflux and intracellular iron (Chen et al., 2015). MZF1 is therefore hypothesized to be implicated in PD pathogenesis through transcriptionally regulating a target gene. Cold-shock protein RBM3, expressed in human cells and brain tissue, has been proven neuroprotective in neurodegenerative disease models through preventing synapse and neuronal loss (Peretti et al., 2021). RBM3 is considered a signature gene of PD (Zhang et al., 2021), and RBM3 upregulation has been found to ameliorate rotenone-induced cytotoxicity in SH-SY5Y cells (Yang et al., 2019). RBM3 provides neuroprotection in a cellular model of PD, suggesting that RBM3 induction may be a suitable strategy for PD therapy (Yang et al., 2018). An increasing body of evidence has revealed that RBM3 could alleviate OS (Chazarin et al., 2019), and our study found the binding site of MZF1 and RBM3 promoter. However, less research is available as yet concerning the effect of the interaction between MZF1 and RBM on OS, which can provide a new direction for the treatment of PD. Rotenone is a mitochondrial complex I inhibitor that could act as a transcription factor of FPN to affect iron efflux and intracellular iron (Chen et al., 2015). MZF1 overexpression plasmid (pcDNA3.1), RBM3 siRNA (5’-CCAUGAACGGA-GAGUCUCUTT-3′, 2 μg), and its negative control siRNA (5’-UUCCGAAACCGUGACCGUTT-3′) were synthesized by GenePharma Co., Ltd. (Shanghai, China). Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) was used for transfection. All operations were carried out strictly in accordance with the instructions. The following experiments were performed 24 hr after transfection.

**Methyl-tetrazolium (MTT) assay**

SH-SY5Y cells in logarithmic growth phase were digested with trypsin and transplanted onto a 96-well plate with about 5,000 cells per well, and 3 duplicate wells were set up. SH-SY5Y cells were cultured at 37°C, 5% CO2 and with saturated humidity for 24 hr. After the cells were treated by different concentrations of rotenone or for different time periods, the supernatant was discarded, and the 96-well plate was washed with culture medium for 3 times. Then, 100 μL serum-free medium was supplemented to each well, and the cells were cultured for 4 hr. The supernatant was discarded after incubation. A total of 10 μL DMSO dissolved MTT (5 mg/mL) was added into the plate and mixed with the cells before detection of the OD value at 570 nm.

**Cell transfection**

MZF1 overexpression plasmid (2 μg) and empty plasmid (pcDNA3.1), RBM3 siRNA (5’-CCAUGAACGGA-GAGUCUCUTT-3′, 2 μg), and its negative control siRNA (5’-UUCCGAAACCGUGACCGUTT-3′) were synthesized by GenePharma Co., Ltd. (Shanghai, China). Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) was used for transfection. All operations were carried out strictly in accordance with the instructions. The following experiments were performed 24 hr after transfection.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

Total RNA was extracted from cells using TRIZOL (Invitrogen). Reverse transcription kits (TaKaRa, Tokyo, Japan) were used for reverse transcription, and all operations were conducted according to the instructions of the kit. Gene expression was detected using a LightCycler 480 (Roche, Indianapolis, IN, USA) fluorescent quantitative PCR instrument, following the instructions of the fluorescent quantitative PCR kit (SYBR Green Mix, Roche Diagnostics). Thermal cycle parameters were: 10-sec pre-denaturation at 95°C; then 5-sec denaturation at 95°C, 10-sec annealing at 60°C, 10-sec extension at 72°C, a total of 45 cycles; final extension at 72°C for 5 min. Quantitative PCR set 3 replicates per reaction. β-actin was adopted as the reference gene, and the 2-ΔΔCt method was used for data analysis. ΔΔCt for each experimental sample was calculated as ΔΔCt = ΔCt_{experimental}(Ct_{target gene} – Ct_{reference gene}) – ΔCt_{control}(Ct_{target gene} – Ct_{reference gene}). The amplified prim-

Vol. 46 No. 10
er sequences of each gene and its loading controls are shown in Table 1.

### Western blotting

SH-SY5Y cells were lysed using RIPA lysis buffer (Beyotime, Shanghai, China) to extract proteins. After the protein concentration was measured using BCA kits (Beyotime), the corresponding volume of protein was added into and mixed with the loading buffer (Beyotime). The protein was denatured by a boiling water bath for 3 min. Electrophoresis was initially run at 80V for 30 min, and then switched to 120V for 1–2 hr after bromphenol blue entered the separation gel. The protein was blotted onto membranes in an ice bath for 60 min with a current of 300 mA. After membrane transfer, the membrane was rinsed in washing solution for 1–2 min, and then it was placed in the sealing solution at room temperature for 60 min, or for sealing overnight at 4°C. At room temperature, primary antibody against MZF1 (ab64866, 1:1000), RBM3 (ab134946, 1:1000), or β-actin (ab8226, 1 µg/mL, Abcam, Cambridge, MA, USA) was incubated with the membrane on a rotary shaker for 1 hr, and washing solution was used to wash the membrane, 10 min each time. The membrane was then incubated with secondary antibody goat anti-rabbit IgG for 1 hr at room temperature, and washed for 3 times, 10 min each time. Chemiluminescence imaging system (Gel Doc XR, Bio-Rad) was used for detection after the developer solution was dropped onto the membrane.

### Intracellular ROS assay

ROS production in SH-SY5Y cells was evaluated using CMH2DCF-DA probes (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, remove the medium and wash the cells in preheated PBS. Then, PBS containing the probes was used to incubate the cells for 30 min at a concentration of 10 µM before fluorescence intensity of oxidized DCF was detected.

### Flow cytometry (FCM)

SH-SY5Y cell suspension (3 mL, 10^5 cells/mL) was collected in a 10-mL centrifuge tube, and centrifuged at 500 rpm for 5 min, and the culture medium was discarded. Thereafter, the cells were washed with PBS before centrifugation at 500 rpm for 5 min, and the supernatant was discarded. The cells were resuspended with 100 µL of binding buffer, and then homogenized and reacted with Annexin V-FITC (5 µL) and PI (5 µL) at room temperature for 15 min in the dark. FITC fluorescence and PI fluorescence were detected by flow cytometry and the apoptosis rate was analyzed.

### Chromatin immunoprecipitation (CHIP)

CHIP assay was carried out by using a commercial CHIP kit (Millipore, Billerica, MA, USA) to detect the binding of MZF1 and RBM3 promoter. In short, the cross-link between DNA and protein was fixed with formaldehyde for 30 min. The DNA was then isolated and ultrasonically broken into 200–1000 bp fragments. The DNA fragments were incubated with anti-MZF1 or anti-IgG antibody to evaluate the enrichment of MZF1.

### Dual-luciferase reporter assay

The binding sites of MZF1 and RBM3 promoter were predicted by the JASPAR (http://jaspar.genereg.net/) tool. According to the results, the promoter sequences (RBM3 WT and RBM3 MUT) containing the wild and mutated sequences of the binding sites were designed and synthesized. The wild or mutated sequences of the binding site were inserted into luciferase reporter vector (pGL3-basic, Promega, Madison, WI, USA), and then co-transfected with MZF1 overexpression plasmid or its negative control into HEK293T cells, with PRL-TK vector (Promega) as internal reference. After transfection, the activities of Firefly luciferase and Renilla luciferase in each group were detected by a dual-luciferase reporter assay kit (Promega). Renilla luciferase activity was used as internal reference, and the ratio of Firefly luciferase to Renilla luciferase activity was the relative activity of luciferase.

### Statistical analysis

GraphPad Prism7 was used to perform statistical analysis, and all data were expressed as mean ± standard deviation. All experiments were repeated 3 times. T-test was used for comparison between two groups, and one-way analysis of variance was used for multi-group comparison, and Tukey’s multiple comparison test was used for post hoc comparison. \( P < 0.05 \) was considered statistically significant.

### Table 1. Primer sequences.

| Name of primer | Sequences              |
|----------------|------------------------|
| MZF1-F         | CCCCCATGTTGAAAGGCAGATG |
| MZF1-R         | ACCAGGGATACAGGTTGCTA   |
| RBM3-F         | TACCTACCCAGCCCACTTA    |
| RBM3-R         | GATCCACCCCCTAGCAAAC    |
| β-actin-F      | CGCGGCGATATCATCATCCA   |
| β-actin-R      | CGGCTTCTTTTGTCCCCAAT   |

Note: F, forward; R, reverse
RESULTS

Low expression of MZF1 in SH-SY5Y cells induced by rotenone

MTT results showed that cell activity was significantly inhibited after SH-SY5Y cells had been treated with rotenone for 24 hr (Fig. 1A, P < 0.05). The inhibitory effect enhanced with increasing concentration, which was already significant at the concentration of 1 μM. In addition, the results of treating SH-SY5Y cells with 1 μM rotenone for different time periods exhibited that the inhibitory effect had reached a pronounced level at 24 hr, and there was no significant enhancement of the inhibitory effect with longer time (Fig. 2B). According to the results of qRT-PCR and Western blot, the expression levels of MZF1 mRNA and protein were significantly decreased in a concentration-dependent manner in SH-SY5Y cells treated with different concentrations of rotenone for 24 hr (Fig. 1C–D, P < 0.05). Western blot bands with markers were provided in Supplementary material. Therefore, we speculated that MZF1 plays a role in neuronal injury.

Overexpression of MZF1 inhibits OS and apoptosis of SH-SY5Y cells induced by rotenone

MZF1 overexpression plasmid or its negative control was transfected into SH-SY5Y cells. The results of qRT-PCR and Western blot showed that after transfection of MZF1 overexpression plasmid, the expression of MZF1 mRNA and protein in SH-SY5Y cells increased significantly (Fig. 2A–B, P < 0.001). The production of ROS in SH-SY5Y cells was significantly increased after rotenone treatment, while overexpression of MZF1 significantly inhibited the ROS level aroused by rotenone (Fig. 2C, P < 0.05). Flow cytometry results showed that the apoptosis rate of SH-SY5Y cells was significantly increased after...
rotenone treatment, while overexpression of MZF1 significantly inhibited rotenone-induced apoptosis (Fig. 2D, \( P < 0.01 \)). These results indicated that overexpression of MZF1 can inhibit OS and apoptosis of SH-SY5Y cells induced by rotenone.

**Fig. 2.** Overexpression of MZF1 inhibits OS and apoptosis of SH-SY5Y cells induced by rotenone. Note: (A) qRT-PCR and (B) Western blot were used to detect the expression of MZF1 mRNA and protein in SH-SY5Y cells after transfection of MZF1 overexpression plasmid or its negative control; (C) OS in SH-SY5Y cells treated with rotenone or MZF1 overexpression was detected by CMH2DCF-DA fluorescent probe; (D) Apoptosis of SH-SY5Y cells treated with rotenone or MZF1 overexpression was detected by flow cytometry. N = 3, * means \( P < 0.05 \), ** means \( P < 0.01 \), and *** means \( P < 0.001 \). OS, oxidative stress.

MZF1/RBM3 inhibits neurotoxicity

JASPAR (http://jaspar.genereg.net/) predicted the binding sites between MZF1 and RBM3 promoter (Fig. 3A). The qRT-PCR and Western blot showed that the mRNA and protein expression levels of RBM3 were significantly decreased after SH-SY5Y cells were treated with different
concentrations of rotenone for 24 hr (Fig. 3B–C, \( P < 0.05 \)). The binding of MZF1 to the RBM3 promoter was verified by CHIP assay, and the results showed that MZF1 antibody could significantly enrich the RBM3 promoter (Fig. 3D, \( P < 0.001 \)), but the negative control IgG antibody could not pull down the RBM3 promoter sequence. The binding sites of MZF1 and RBM3 promoter were verified by the dual-luciferase reporter assay. After co-transfection with the RBM3 WT and the MZF1 overexpression plasmid into HEK293T cells, the luciferase activity increased significantly. However, the luciferase activity did not change after co-transfection of the RBM3 MUT and the MZF1 overexpression plasmid (Fig. 3E). ChIP assay was performed to examine the change of RBM3 promoter enriched by MZF1 antibody after SH-SY5Y cells were treated with 1 \( \mu \)M rotenone for 24 hr, and the results showed that the RBM3 promoter pulled down by MZF1 antibody was significantly reduced after the treatment (Fig. 3F, \( P < 0.05 \)). MZF1 overexpression vector or its negative control was transfected in SH-

Fig. 3. MZF1 promotes RBM3 transcription. Note: (A) The binding sites of MZF1 and RBM3 promoter predicted by JASPAR; (B) qRT-PCR and (C) Western blot were used to examine the expression of RBM3 mRNA and protein levels in SH-SY5Y cells treated with different concentrations of rotenone for 24 hr; (D) The binding of MZF1 to RBM3 promoter was detected by CHIP assay with IgG antibody as a negative control; (E) The binding of MZF1 to RBM3 promoter was detected by dual luciferase reporter assay; (F) ChIP assay was performed to detect changes in the RBM3 promoter sequence bound to MZF1 after SH-SY5Y cells were treated with 1 \( \mu \)M rotenone; (G) qRT-PCR and (H) Western blot were used to detect the expression of RBM3 mRNA and protein in SH-SY5Y cells transfected with MZF1 overexpression plasmid or its negative control. N = 3, * means \( P < 0.05 \), ** means \( P < 0.01 \), and *** means \( P < 0.001 \).
SY5Y cells, and the results showed that the expression of RBM3 was significantly increased after transfection of MZF1 overexpression vector (Fig. 3G–H, \( P < 0.05 \)). These results indicated that MZF1 is a transcription factor of RBM3 and promotes the transcription of RBM3.

**Knockdown of RBM3 reverses the protective effect of MZF1 on SH-SY5Y cells**

RBM3 siRNA or its negative control siRNA was transfected into SH-SY5Y cells, and qRT-PCR and Western blot results showed that the expression of RBM3 mRNA and protein in SH-SY5Y cells was significantly decreased after transfection of RBM3 siRNA (Fig. 4A–B, \( P < 0.05 \)). While after transfection of RBM3 siRNA, the level of ROS in SH-SY5Y cells was markedly increased and the inhibitory effect of MZF1 overexpression on ROS in SH-SY5Y cells was reversed by co-transfection of RBM3 siRNA or MZF1 overexpression + RBM3 siRNA. N = 3, * means \( P < 0.05 \), and ** means \( P < 0.01 \). OS, oxidative stress.

**Fig. 4.** Knockdown of RBM3 reverses the protective effect of MZF1 on SH-SY5Y cells. Note: (A) qRT-PCR and (B) Western blot were used to detect the expression of RBM3 mRNA and protein in SH-SY5Y cells after transfection with RBM3 siRNA or its negative control siRNA; (C) OS in SH-SY5Y cells after transfection with RBM3 siRNA or MZF1 overexpression + RBM3 siRNA was detected by CMH2DCF-DA fluorescent probe; (D) Flow cytometry was used to detect apoptosis rate of SH-SY5Y cells after transfection with RBM3 siRNA or MZF1 overexpression + RBM3 siRNA. N = 3, * means \( P < 0.05 \), and ** means \( P < 0.01 \). OS, oxidative stress.
itatory effect of MZF1 overexpression on SH-SY5Y cell apoptosis was reversed after co-transfection with RBM3 siRNA (Fig. 4D, $P < 0.01$). The above results suggested that MZF1 exerted a protective effect on SH-SY5Y cells through RBM3.

**DISCUSSION**

PD is the second prevalent age-related neurodegenerative disease of the central nervous system (Yang et al., 2020). The existing therapeutic options include carbidopa-levodopa, MAOB inhibitors, or DA agonists (Armstrong and Okun, 2020). However, there is no appreciable treatment available to date. Current therapies provide symptomatic relief without any influence on the development of the disease (Sharma et al., 2021). OS is ubiquitously involved in PD etiology or progression and associated with DA. Targeting OS is therefore important for the treatment of PD. Here we have shown that MZF1 may alleviate OS and apoptosis induced by rotenone in SH-SY5Y cells by promoting RBM3 transcription.

OS caused by brain iron accumulation is one hypothesized mechanism for the selective susceptibility of specific neuronal populations in PD (Thomas et al., 2021). MZF1 was shown to regulate cell differentiation, proliferation, and programmed cell death (Brix et al., 2020; Mudduluru et al., 2010). A study also proved that Apomorphine (a potent D1/D2 dopamine receptor agonist and an anti-parkinsonian drug) stimulates the FGF-2 promoter via the transcription factor MZF1 (Luo et al., 2009). MZF1 inhibition diminishes FPN expression, the only iron exporter in mammalian cells, which results in intracellular iron retention (Chen et al., 2015). The available investigations indicated that MZF1 plays a role in the therapeutic treatment of PD and it might also relate to the accumulation of intracellular iron. However, the relationship between MZF1 and OS and the possible action mechanism of MZF1 in PD are still unknown. Hence, an extensive study is necessitated for providing a novel therapeutic target for PD. Initially, the results of our experiments showed that the expression of MZF1 mRNA and protein was significantly decreased in SH-SY5Y cells after treatment with rotenone. Despite that the effect of MZF1 on apoptosis has been the subject of intense debate within the scientific community, some studies suggest MZF1 is capable to increase apoptosis and aggravate neuronal injury. For example, peripheral nerve injury increased the expression of Kcnq2 antisense RNA in injured dorsal root ganglion (DRG) by activating MZF1 (Zhao et al., 2013). Nevertheless, targeting DRG-localized MZF1 is also regarded as a promising therapeutic strategy for the treatment of chronic inflammatory pain in the clinic (Niu et al., 2020). These findings suggested that the impact of MZF1 might be different in different cellular context. In our study, MZF1 is beneficial for inhibiting OS and apoptosis. ROS in SH-SY5Y cells was significantly increased after rotenone treatment, while overexpression of MZF1 significantly inhibited the increase of ROS level induced by rotenone as well as the apoptosis rate of SH-SY5Y cells. These results come to a demonstration that rotenone significantly induced OS, and overexpression of MZF1 inhibits OS and apoptosis in a PD cellular model. Our findings add to the preexisting literature since the effect of MZF1 on attenuating OS in PD has not been investigated.

Furthermore, the result of CHIP and dual-luciferase reporter assay concurrently verified the binding of MZF1 and RBM3. Moreover, the expression of RBM3 was negatively regulated by MZF1. These results indicated that MZF1 is a transcription factor of RBM3 and promotes the transcription of RBM3. The inhibitory effect of MZF1 overexpression on ROS and neuronal apoptosis was reversed by co-transfection of RBM3 siRNA, suggesting that MZF1 exerted a protective effect on SH-SY5Y cells through RBM3. RBM3 protects neuroblastoma cells from NO-induced apoptosis by suppressing p38 signaling, which mediates apoptosis through miR-143 induction (Yang et al., 2017). RBM3 mediates hypothermic neuroprotection against rotenone induced neurotoxicity via inhibition on MAPK signaling (Yang et al., 2019).

In conclusion, our study provided evidence that overexpression MZF1 inhibits rotenone-induced OS and apoptosis of SH-SY5Y cells. We also demonstrate that MZF1 promotes the transcription of RBM3, and knockdown of RBM3 reverses the protective effect of MZF1 on SH-SY5Y cells. Collectively, it is believed MZF1 alleviates OS and apoptosis induced by rotenone in SH-SY5Y cells by promoting RBM3 transcription. We propose that MZF1 may be a promising new direction for the disease management of PD. More extensive experiments, such as in vivo experiments, are required to provide more precise data to confirm the effects of MZF1 through RBM3 transcription on alleviating OS and apoptosis in PD.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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