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Original Article

MicroRNA-217/138-5p downregulation inhibits inflammatory response, oxidative stress and the induction of neuronal apoptosis in MPP^+-induced SH-SY5Y cells

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Abstract: Parkinson's disease (PD) is a common neurodegenerative disease. Various microRNAs (miRNAs) have been reported to play important roles in cell growth regulation and inflammatory reaction. However, the detailed roles of miR-217 and miR-138-5p in PD progression remain to be investigated. In the present study, we explored the effects and underlying mechanisms of miR-217 and miR-138-5p on the inflammatory response, oxidative stress and the induction of neuronal apoptosis in an *in vitro* PD cell line model induced by 1-methyl-4-phenylpyridinium (MPP^+). The results of the biological software analysis and luciferase reporter assays demonstrated that sirtuin 1 (SIRT1) was a direct target of miR-217 and miR-138-5p. MiR-217 and miR-138-5p exhibited a negative regulatory effect on the expression of SIRT1 in SH-SY5Y cells. In addition, the expression levels of miR-217 and miR-138-5p were increased, and SIRT1 expression was decreased in SH-SY5Y cells following MPP^+ treatment. Loss-of-function experiments indicated that treatment of the cells with inhibitors against miR-217 and miR-138-5p promoted cell viability and superoxide dismutase (SOD) activity, while the induction of cell apoptosis, lactate dehydrogenase (LDH) activity, and the reactive oxygen species (ROS) release were inhibited in MPP^+-induced SH-SY5Y cells. Moreover, the expression levels of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were reduced in MPP^+-induced SH-SY5Y cells. Treatment of the cells with the miR-217 and the miR-138-5p inhibitors significantly inhibited the ratio of phosphorylated (p)-p65/p65 expression levels in MPP^+-induced SH-SY5Y cells. In summary, the present study demonstrated that the miR-217/miR-138-5p/SIRT1 axis was involved in the progression of PD by regulating the inflammatory response and the induction of oxidative stress and neuronal apoptosis. The data provide new diagnostic and therapeutic strategies for PD patients.

Keywords: microRNA-217, microRNA-138-5p, SIRT1, Parkinson's disease, MPP^+, SH-SY5Y cells

Introduction

Parkinson's disease (PD) is known as the second most common neurodegenerative disease that usually occurs in the elderly [1-3]. This disease is caused due to the loss of neurons in the substantia nigra pars compacta (SNpc) [1-3]. The characteristic manifestations of PD include resting tremor, muscle stiffness, gait and posture disorders and bradykinesia [4-6]. At present, the pathogenesis of this disease is still not fully understood, although aging, oxidative stress, inflammation and apoptosis are examined as major pathogenic factors [7, 8].

MicroRNAs (miRNAs) are a group of small noncoding RNAs that are 20-22 nucleotides long and regulate target gene expression by binding to the 3'-untranslated region (3'-UTR) of mRNAs [9]. A previous study has shown that miRNAs participate in a variety of pathological processes through multiple pathways [10]. MiR-217 and miR-138-5p are two well-known miRNAs that play vital roles in cell growth regulation and the induction of the inflammatory response [11-13]. However, a limited number of reports have been conducted on the roles of miR-217 and miR-138-5p in PD. In addition, previous studies have shown that SIRT1 is downregulated in PD patients and that it ameliorates neuronal apoptosis induced by oxidative stress in PD [14, 15]. Moreover, SIRT1 plays an important role in the inflammatory response and the induction of oxidative stress [16, 17]. These results suggested

that miR-217 and miR-138-5p may play important roles in PD by regulating SIRT1 expression.

In the present study, we investigated whether the miR-217/138-5p axis participated in the regulation of the inflammatory response, the induction of oxidative stress and the induction of neuronal apoptosis by directly targeting SIRT1. The study further aimed to discover whether this miRNA axis could affect the occurrence and development of PD in order to provide new strategies for the diagnosis and treatment of this disease.

Materials and methods

Cell culture

Human neuroblastoma SH-SY5Y cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle medium (DMEM) medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% penicillin/streptomycinin and incubated at 37°C in a humidified atmosphere containing 5% CO_2.

MiRNA target analysis and dual-luciferase reporter assay

The association between SIRT1 and miR-217/miR-138-5p was identified by TargetScan Release 7.1 (www.targetscan.org/vert_71). In order to investigate the interaction of miR-217 and SIRT1, the wild-type (WT) 3'-UTR and mutated-type (MUT) 3'-UTR sequences of SIRT1 were cloned into a miR RB ReportTM dual luciferase reporter gene plasmid vector (Guangzhou RiboBio Co., Ltd., Guangzhou, China). Moreover, the QuikChange Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA) was used according to the manufacturer's instructions that allowed successful point-mutation of the miR-217 binding domain on the 3'UTR of SIRT1. The mimic control or miR-217 mimic sequences were co-transfected with the WT-SIRT1 or MUT-SIRT1 into SH-SY5Y cells for 48 h by Lipofectamine 2000 reagent (Invitrogen, USA). Luciferase activity was analyzed by the dual luciferase reporter assay system (Promega, USA) according to the manufacturer's instructions.

Establishment of PD cell model in vitro

To explore the expression levels of miR-217, miR-138-5p and SIRT1 in the PD cell model, SH-SY5Y cells were treated with 0, 0.25, 0.5, 1 or 2 mM MPP^+ (Sigma, St. Louis, MO, USA) for 24 h, or exposed to 1 mM MPP^+ for 0, 6, 12, 24 or 48 h.

Cell transfection assay

SH-SY5Y cells were seeded at a concentration of 5×10^4 cells/ml in 6-well plates and incubated overnight. The miR-217 and miR-138-5p inhibitors were used to downregulate miR-217 and miR-138-5p expression in SH-SY5Y cells using an inhibitor-control as the negative control. SIRT1-siRNA was used for SIRT1 downregulation. A negative control control-siRNA was also used. The sequences were as follows: control of miR-217 inhibitor (inhibitor control; 5'-GCCUCCGGCUUCGCACCUCU-3'; GenePharma, Shanghai, China), miR-217 inhibitor (inhibitor; 5'-UACUGCAUCAGGAACUGAUUGGA-3'; GenePharma, Shanghai, China), control-siRNA (cat. no. sc-36869; Santa Cruz Biotechnology, Inc.) or SIRT1-siRNA (cat. no. sc-40986; Santa Cruz Biotechnology, Inc.), control of miR-138-5p inhibitor (inhibitor control-2; 5'-CAGUACUUUUGUGUAGUACAA-3'; GenePharma, Shanghai, China), miR-138-5p inhibitor (inhibitor-2; 5'-CGGCCUGATTCACAACACCAGCT-3'; GenePharma, Shanghai, China). The following treatment groups were used: miR-217 inhibitor (inhibitor) + control-siRNA, miR-217 inhibitor (inhibitor) + SIRT1-siRNA, miR-138-5p inhibitor (inhibitor-2) + control-siRNA and ormiR-138-5p inhibitor (inhibitor-2) + SIRT1-siRNA. The sequences were transfected into SH-SY5Y cells using Lipofectamine 2000 reagent (Invitrogen, USA). Following 48 h of cell culture at 37°C, the cells were collected to detect the transfection efficiency using qRT-PCR or cultured further in the presence of 1 mM MPP^+ for 24 h.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total cellular RNA was isolated using the TRIzol reagent (Invitrogen, USA) from SH-SY5Y cells and reverse transcribed into first strand cDNA using the cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. The relative expression levels of miR-217, miR-138-5p and *SIRT1* mRNA were quantified by a

Prism 7000 Real-Time PCR system using Power SYBR Green Master mix (Vazyme, Piscataway, NJ, USA) according to the manufacturer's instructions. The primers were provided by Sangon Biotech (Shanghai, China), and primer sequences were listed as following: SIRT1, forward 5'-AATCCAGTCATTAAAGGTCTACAA-3'; reverse 5'-TAGGACCATTACTGCCAGAGG-3'; U6, forward 5'-GCTTCGGCAGCACATATACTAAAAT-3'; reverse 5'-CGCTTCACGAATTTGCGTGTCAT-3'; GAPDH, forward 5'-CTTTGGTATCGTGGAAGGACTC-3'; reverse 5'-GTAGAGGCAGGGATGATGTTCT-3'; miR-217, forward 5'-TACTGCATCAGGAACTGACTGGA-3'; reverse 5'-GTGCAGGGTCCGAGGT-3'; miR-138-5p, forward 5'-GCGAGCTGGTGTTGTGAATC 3' reverse 5' AGTGCAGGGTCCGAGGTATT 3'. The amplification conditions were as follows: 35 cycles of denaturation at 94°C for 60 sec, annealing at 60°C for 60 sec, and chain extension at 72°C for 1 min, followed by a final extension step at 72°C for 10 min. *GAPDH* and *U6* were used as the internal control genes. The relative expression levels of miR-217, miR-138-5p and *SIRT1* mRNA were calculated by the $2^{-\Delta\Delta Ct}$ method [18].

3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide (MTT) assay

SH-SY5Y cells were seeded into 96-well plates in triplicate and incubated overnight. Subsequently, the culture medium was removed and the cells were treated with 1 mM MPP^+ following cell transfection at 37°C. The cells were incubated with 10 μl MTT solution (Beyotime, Shanghai, China) for 4 h. Subsequently, 100 μl dimethyl sulfoxide (DMSO) was added into each well to solubilize the formazan product after the solution was removed. Detection was achieved by monitoring the absorbance at 490 nm by a micro-plate reader (Bio-Rad, Hercules, CA, USA). The relative cell viability was normalized to that of the control group using optical density values.

Apoptosis analysis

The detection of cell apoptosis was performed by flow cytometry (FCM). SH-SY5Y cells were seeded into 6-well plates overnight, and collected by trypsinization following treatment. The cells were washed once with PBS and subsequently resuspended in 1 × binding buffer. A total of 100 μl cell suspension was transferred to a 5 ml tube and mixed with 5 μl fluorescein isothiocyanate (FITC)-Annexin V and 5 μl propidium iodide (PI) (BD Biosciences, San Diego, CA) respectively, according to the manufacturer's specifications. The induction of cell apoptosis was analyzed by a FACSCalibur flow cytometer (BD Biosciences, USA) within one hour and the data were analyzed using the FlowJo software (version 7.6.1; FlowJo LLC).

Lactate dehydrogenase (LDH) release assay

LDH was used as an indicator of estimated cell death. SH-SY5Y cells were cultured with 1 mM MPP^+ for 24 h and the activity of LDH released into the culture medium was detected with a lactate dehydrogenase assay kit (Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. The absorbance at 490 nm was recorded using a microplate reader (Bio-Rad, Hercules, CA, USA).

ELISA

SH-SY5Y cells were treated with MPP^+ for 24 h, harvested and centrifuged in order to detect TNF-α and IL-1β expression levels using an ELISA kit (BioLegend, Inc., CA, USA) according to the manufacturer's protocol. The absorbance was measured at 450 nm using a micro-plate reader (Bio-Rad, Hercules, CA, USA).

Measurement of intracellular reactive oxygen species (ROS) levels

The intracellular ROS levels were quantified by the oxidation-sensitive fluorescent probe 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Briefly, the treated cells were incubated with 10 μM DCFH-DA (Sigma) at 37°C in the dark for 45 min. The fluorescence intensity was quantified with a fluorescence micro-plate reader (Labsystems Oy, Helsinki, Finland) using an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Detection of superoxide dismutase (SOD) activity

SH-SY5Y cells were collected and dissolved using a cell lysis buffer (Beyotime, Shanghai, China). The SOD activity assay kit (Jiancheng Bioengineering Institute, China) was used to determine the activity of SOD following the manufacturer's instructions.

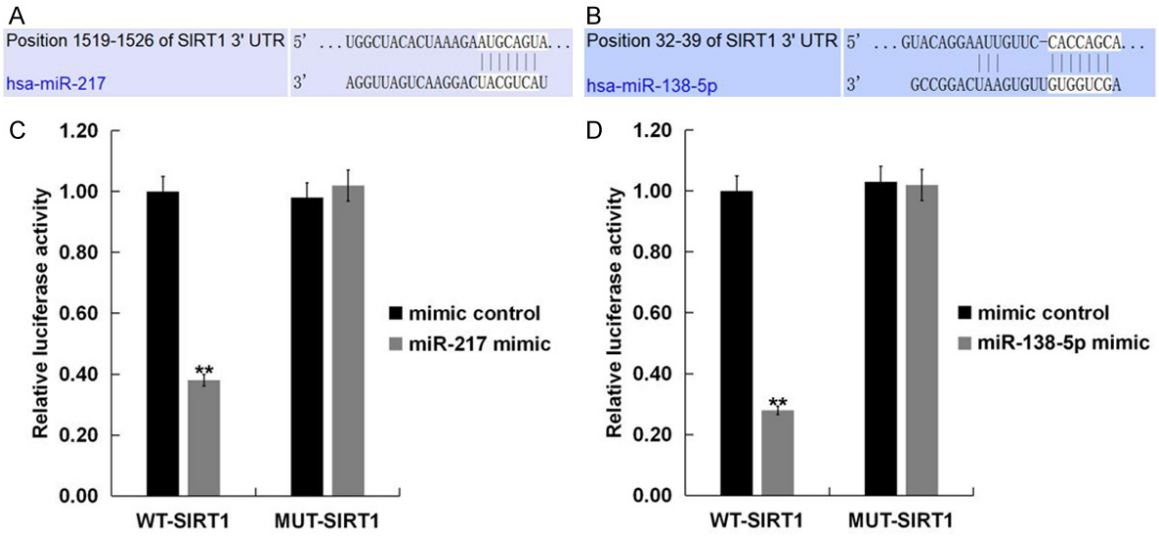

Figure 1. miR-217/miR-138-5p directly targeted SIRT1. A and B. The predicted miR-217/miR-138-5p binding sites in the 3'UTR of SIRT1. C and D. Dual-luciferase reporter assay was used to reveal the relationship between miR-217/miR-138-5p and SIRT1. **P < 0.01 *vs.* mimic control.

Western blot analysis

Following transfection and cell culture for 48 h, SH-SY5Y cells were treated with 1 mM MPP^+ for 24 h, washed twice with cold PBS and immediately lysed with RIPA buffer (Beyotime, Shanghai, China). The lysis products were centrifuged at 12,000 rpm for 15 min at 4°C in order to obtain the total protein levels, which were quantified by a bicinchoninic acid (BCA) protein kit (Pierce, USA). Equal amounts of protein were subjected to polyacrylamide gel electrophoresis (PAGE) using a 12% sodium dodecyl sulphate (SDS) gel and subsequently transferred to PVDF membranes. Following blocking with 5% nonfat milk for 1 h, the membranes were incubated with SIRT1 (1:1,000; cat. no. 9475; Cell Signaling Technology, Inc., Danvers, MA, USA), p-p65 (1:1,000; cat. no. 3033; Cell Signaling Technology, Inc.), p65 (1:1,000; cat. no. 8242; Cell Signaling Technology, Inc.) or GAPDH (1:1,000; cat. no. 5174; Cell Signaling Technology, Inc.) anti-bodies, respectively overnight at 4°C. The following morning, the membranes were washed 3 times in PBST and incubated with the horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.) for 1 h at 37°C. The protein bands were visualized using the ECL luminescent substrate (Pierce) according to the manufacturer's instructions. The experiments were repeated the experiment for 3 times at least.

Statistical analysis

The data were presented as the mean ± standard deviation (SD) of at least three independent experiments. Statistical analysis was performed using the SPSS 18.0 software package (SPSS Inc, USA). The differences between two groups were determined using Student's t-test, whereas one-way analysis of variance with a Bonferroni post hoc test was used to analyze the differences between multiple groups. A *p*-value lower than 0.05 (P < 0.05) was considered for significant differences.

Results

SIRT1 is a target of miR-217/miR-138-5p

To study the molecular mechanisms of miR-217 and miR-138-5p in PD, the association between SIRT1 and miR-217/miR-138-5p was examined. TargetScan results indicated the binding sites between SIRT1 and miR-217/miR-138-5p (**Figure 1A**, **1B**). In addition, a luciferase reporter assay was performed in SH-SY5Y cells in order to confirm the predicted binding sites of miR-217 or mR-138-5p on the 3'-UTR sequence of SIRT1. The data indicated that treatment

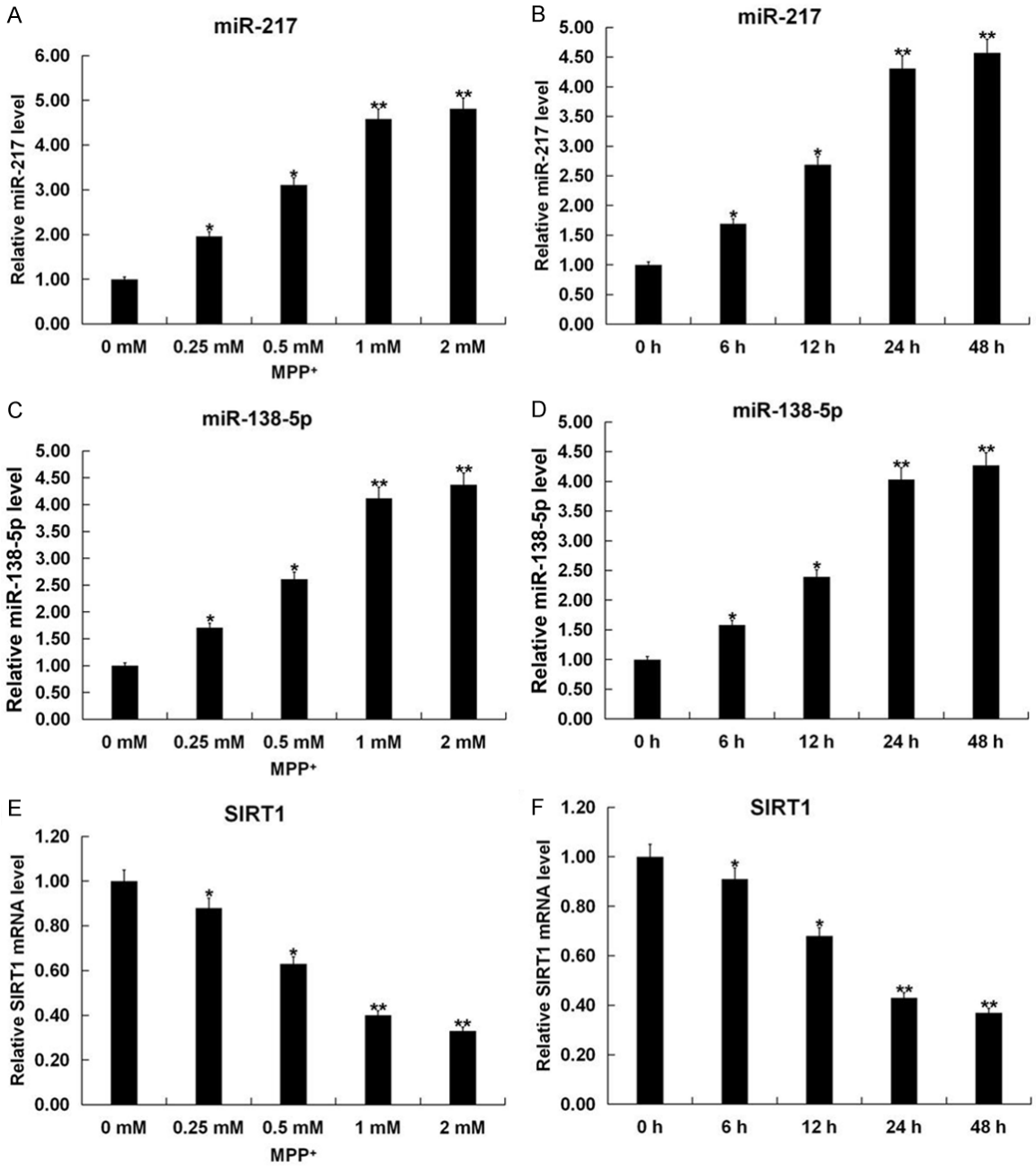

Figure 2. Expression of miR-217/miR-138-5p/SIRT1 in MPP+-induced SH-SY5Y cells. The expression of miR-217 (A), miR-138-5p (C) and SIRT1 (E) mRNA levels were analyzed by qRT-PCR in SH-SY5Y cells treated with different doses of MPP+ for 24 h. The expression of miR-217 (B), miR-138-5p (D) and SIRT1 (F) mRNA levels were also determined by qRT-PCR in SH-SY5Y cells cultured with 1 mM MPP+ for different times. *, **P < 0.05, 0.01 vs. 0 mM/0 h MPP+ treatment group.

with miR-217/miR-138-5p mimic markedly reduced the luciferase activity of SIRT1 wild-type 3'-UTR, whereas it exhibited no effect on SIRT1 3'UTR mut-type compared with that noted in the control group (**Figure 1C**, **1D**). These results indicated that SIRT1 was a direct target of miR-217/miR-138-5p.

MPP+ treatment to SH-SY5Y cells causes an increase in the expression levels of miR-217/miR-138-5p and a decrease in the expression levels of SIRT1

To study the roles of miR-217/miR-138-5p and SIRT1 in PD, miR-217/miR-138-5p and SIRT1

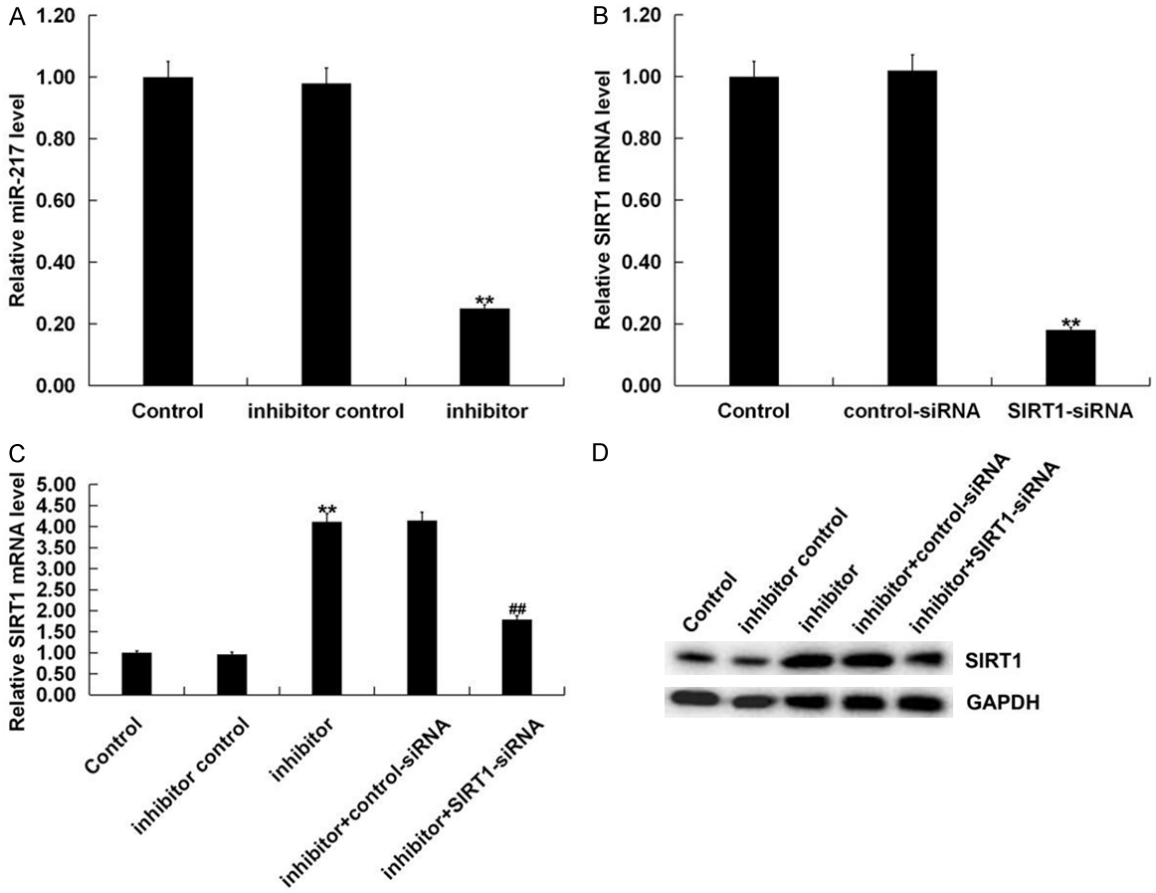

Figure 3. Effects of miR-217 down-regulation on expression of SIRT1 in SH-SY5Y cells. A. The level of miR-217 in SH-SY5Y cells was detected by qRT-PCR. B and C. The mRNA level of SIRT1 in treated SH-SY5Y cells was detected by qRT-PCR assay. D. The protein level of SIRT1 in treated SH-SY5Y cells was detected by western blot assay. **P < 0.01 *vs.* Control; ##P < 0.01 *vs.* inhibitor.

expression levels were detected by qRT-PCR in SH-SY5Y cells, which were treated with different concentrations of MPP^+ (0, 0.25, 0.5, 1, or 2 mM) or exposed to 1 mM MPP^+ at different time points (0, 6, 12, 24 or 48 h). The results indicated that the levels of miR-217 (**Figure 2A**, **2B**) and miR-138-5p (**Figure 2A**, **2B**) were significantly increased in a dose-dependent and time-dependent manner. Conversely, the expression levels of SIRT1 were decreased with increasing concentration and time of MPP^+ treatment in SH-SY5Y cells (**Figure 2E**, **2F**).

Downregulation of miR-217 induces SIRT1 accumulation in SH-SY5Y cells

In order to explore the contribution of miR-217 and SIRT1 to the development of PD, three initial groups, namely inhibitor control, miR-217 inhibitor, control-siRNA or SIRT1-siRNA were prepared by transfection of the corresponding sequences into SH-SY5Y cells. Following transfection, the cells were divided into six groups as follows: inhibitor control group, miR-217 inhibitor group, control-siRNA group, SIRT1-siRNA group, miR-217 inhibitor and control-siRNA co-transfection group and miR-217 inhibitor and SIRT1-siRNA co-transfection group. The transfection efficiency was evaluated by the qRT-PCR assay. The expression levels of miR-217 were dramatically decreased in SH-SY5Y cells containing the miR-217 inhibitor (**Figure 3A**). Moreover, the mRNA levels of SIRT1 were markedly reduced in SH-SY5Y cells in the SIRT1-siRNA group compared with those of the control group (**Figure 3B**). In comparison with the control group, the miR-217 inhibitor significantly increased the expression levels of SIRT1 in SH-SY5Y cells (**Figure 3C** and **3D**). This effect was abolished by SIRT1-siRNA treatment (**Figure 3C** and **3D**).

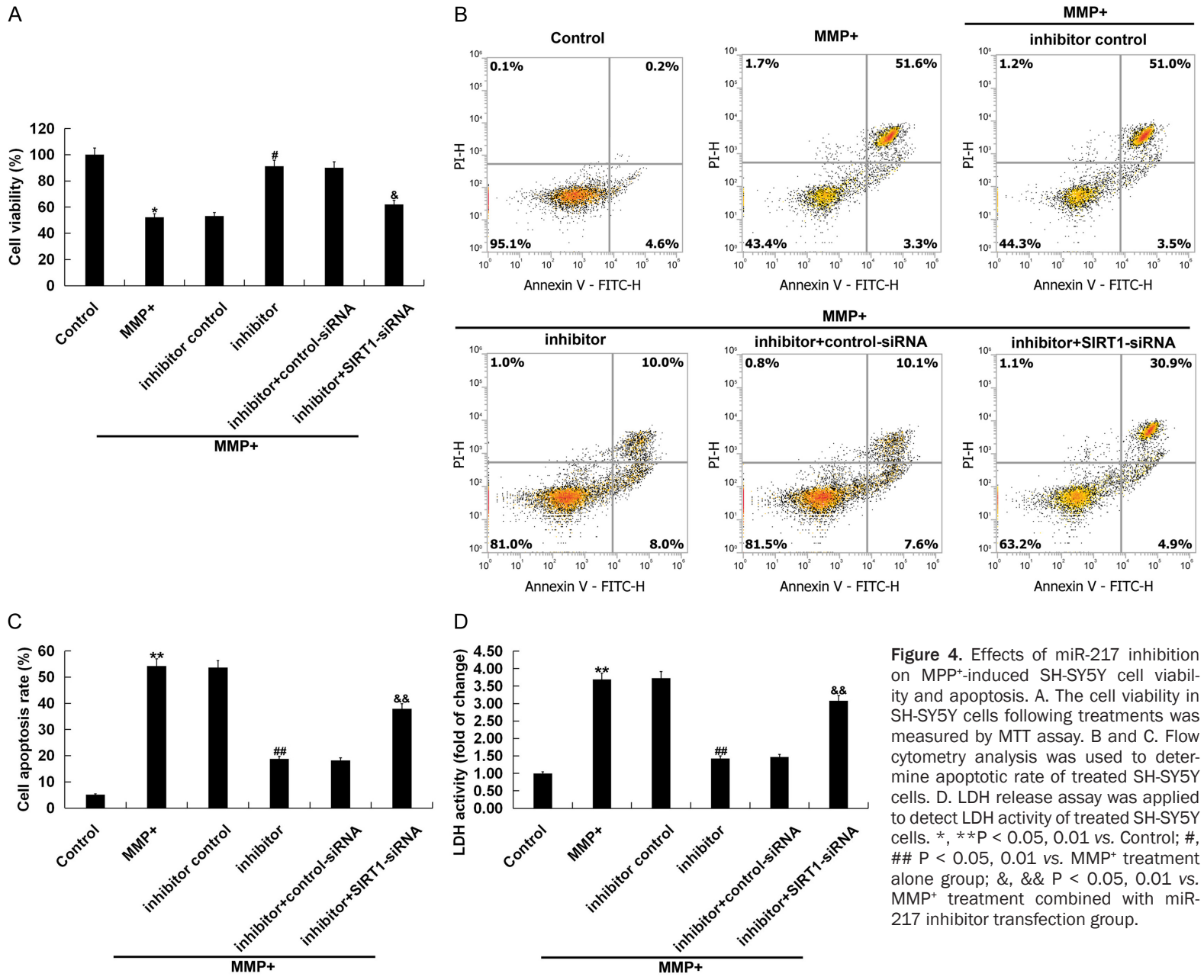

Figure 4. Effects of miR-217 inhibition on MPP^+-induced SH-SY5Y cell viability and apoptosis. A. The cell viability in SH-SY5Y cells following treatments was measured by MTT assay. B and C. Flow cytometry analysis was used to determine apoptotic rate of treated SH-SY5Y cells. D. LDH release assay was applied to detect LDH activity of treated SH-SY5Y cells. *, **P < 0.05, 0.01 *vs.* Control; #, ## P < 0.05, 0.01 *vs.* MMP^+ treatment alone group; &, && P < 0.05, 0.01 *vs.* MMP^+ treatment combined with miR-217 inhibitor transfection group.

MiR-217 inhibition influences the inflammatory response and the induction of oxidative stress and neuronal apoptosis in MPP⁺-induced SHSY5Y cells

MiR-217 was transfected into SH-SY5Y cells that were cultured with 1 mM MPP⁺ for 24 h in order to establish the *in vitro* PD model. MTT assays demonstrated that cell viability was considerably reduced due to MPP⁺ treatment compared with that noted in the control group. In addition, treatment of the cells with the miR-217 inhibitor significantly increased their cell viability compared with that of the MPP⁺-treated group, while this effect was eliminated by co-transfection with SIRT1-siRNA (**Figure 4A**). The cell apoptotic rate was detected by flow cytometry analysis. The results demonstrated that MPP⁺ induced apoptosis compared with the control group. Co-transfection with SIRT1-siRNA significantly reversed the inhibition of cell apoptosis induced by the miR-217 inhibitor in SH-SY5Y cells compared with the control group (**Figure 4B**, **4C**). Furthermore, the integrity and necrosis of the cell membrane was evaluated by the LDH activity assay. As shown in **Figure 4D**, MPP⁺ treatment led to a significant increase in LDH activity, while SIRT1-siRNA markedly reduced the inhibitory effect of the miR-217 inhibitor on the LDH activity of SH-SY5Y cells. In addition, the induction of inflammation was evaluated in an *in vitro* PD cell model by ELISA following the reduction in the levels of miR-217. The results indicated that MPP⁺ treatment significantly increased the expression levels of TNF-α and IL-1β, while miR-217 inhibitor treatment significantly decreased TNF-α and IL-1β expression levels (**Figure 5A** and **5B**). This decrease was reversed by SIRT1-siRNA treatment (**Figure 5A** and **5B**). The release of ROS and the induction of SOD activity were assessed following miR-217 inhibition in the *in vitro* PD model. MPP⁺ caused a dramatic reduction in ROS levels and reduced SOD activity compared with that of the control group. The miR-217 inhibitor decreased ROS levels and increased SOD activity compared with that of the MPP⁺ treatment group. Nevertheless, the effect of the miR-217 inhibitor on SH-SY5Y cells was reversed in the presence of the miR-217 inhibitor and the SIRT1-siRNA co-transfection group (**Figure 5C** and **5D**). Moreover, the protein expression levels of p-p65 and p65 were detected by western blotting in order to explore the influence of miR-217 on the NF-κB pathway in SH-SY5Y cells. The results indicated that MPP⁺ treatment significantly upregulated the ratio of p-p65/p65 compared with that of the control group. Downregulation of miR-217 resulted in a significant reduction of the p-p65/p65 ratio. This effect was eliminated by co-transfection of SH-SY5Y cells with the miR-217 inhibitor and the SIRT1-siRNA sequence (**Figure 5E**).

miR-138-5p inhibition upregulates SIRT1 expression levels in SH-SY5Y cells

To evaluate whether miR-138-5p interfered with SIRT1 expression *in vitro*, SH-SY5Y cells were transfected with inhibitor control (inhibitor control-2), miR-138-5p inhibitor (inhibitor-2), control-siRNA or SIRT1-siRNA for 48 h. As shown in **Figure 6A**, the levels of miR-138-5p in the miR-138-5p inhibitor group were significantly lower than those of the control group. MiR-138-5p downregulation increased SIRT1 expression in SH-SY5Y cells compared with those of the control group, while co-transfection with SIRT1-siRNA reversed this effect (**Figure 6B** and **6C**). All these data indicated that miR-138-5p inhibition improved SIRT1 expression.

Inhibition of miR-138-5p reduces the levels of the inflammatory markers and the induction of oxidative stress and neuronal apoptosis in MPP⁺-treated SHSY5Y cells

SH-SY5Y cells were transfected with inhibitor control (inhibitor control-2), miR-138-5p inhibitor (inhibitor-2), inhibitor-2 + control-siRNA, or inhibitor-2 + SIRT1-siRNA sequences for 48 h and subsequently exposed to 1 mM MPP⁺ for 24 h. Cell viability and apoptotic rate of SH-SY5Y cells were assessed by the MTT assay and by flow cytometry analysis, respectively. MPP⁺ treatment significantly reduced cell viability and induced apoptosis in SH-SY5Y cells compared with the corresponding activities of the control group (**Figure 6D-F**). In contrast to MPP⁺ treatment, the viability of SH-SY5Y cells that were cultured with the miR-138-5p inhibitor was increased, whereas the apoptotic rate was markedly reduced. These effects were attenuated by co-transfection with SIRT1-siRNA. In addition, the results of the LDH release assay demonstrated that LDH activity of SH-SY5Y cells in the MPP⁺ treatment group exhibited a

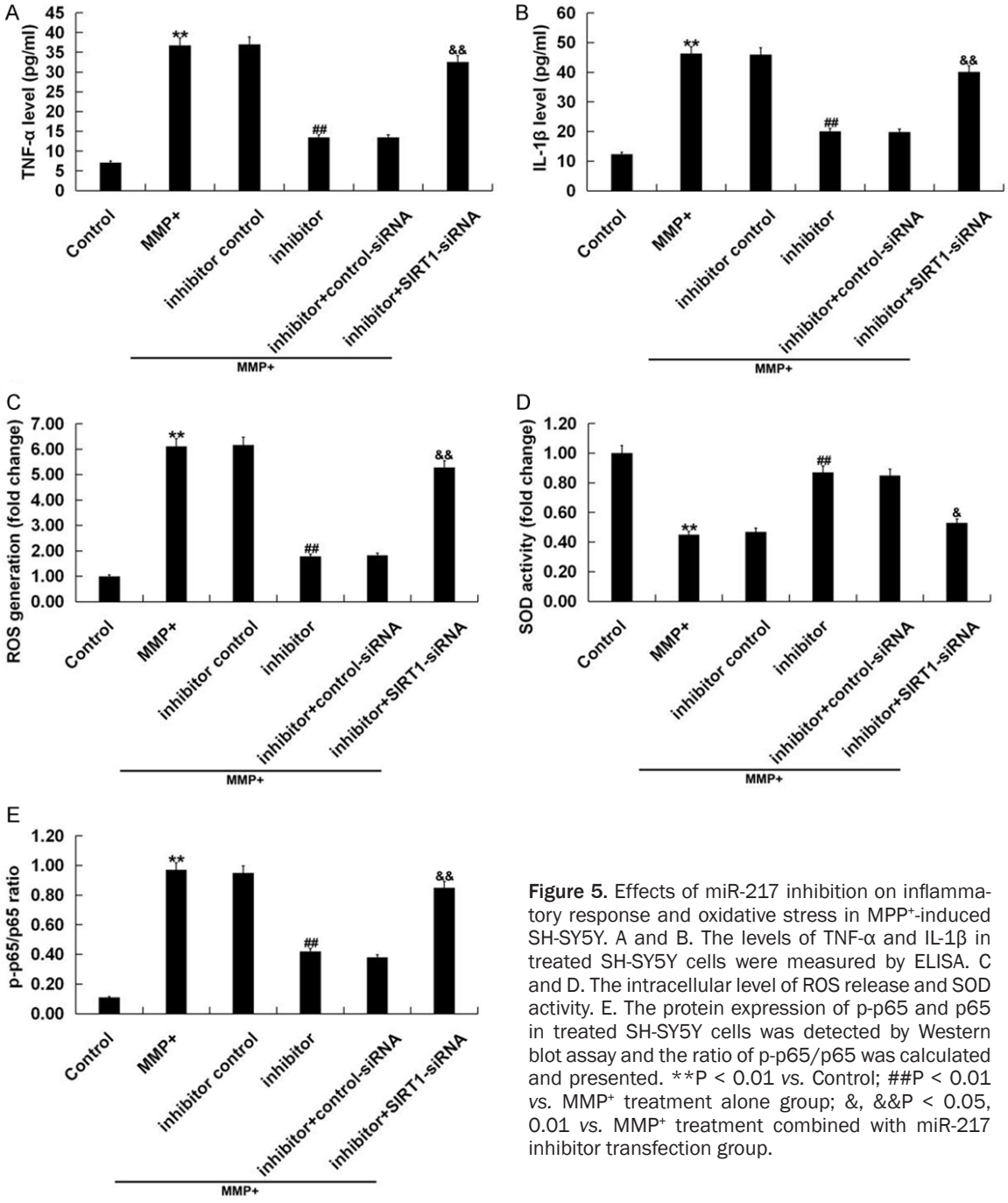

Figure 5. Effects of miR-217 inhibition on inflammatory response and oxidative stress in MPP^+-induced SH-SY5Y. A and B. The levels of TNF-α and IL-1β in treated SH-SY5Y cells were measured by ELISA. C and D. The intracellular level of ROS release and SOD activity. E. The protein expression of p-p65 and p65 in treated SH-SY5Y cells was detected by Western blot assay and the ratio of p-p65/p65 was calculated and presented. **P < 0.01 *vs.* Control; ##P < 0.01 *vs.* MMP^+ treatment alone group; &, &&P < 0.05, 0.01 *vs.* MMP^+ treatment combined with miR-217 inhibitor transfection group.

significant increase compared with that of the control group. In contrast to these findings, downregulation of miR-138-5p reduced LDH activity levels in SH-SY5Y cells and this effect was markedly inhibited by transfection of the cells with the SIRT1-siRNA sequence (**Figure 6G**). In addition, ELISA was used to detect TNF-α and IL-1β expression in the *in vitro* PD cell model. As presented in **Figure 7A** and **7B**, the expression levels of the inflammatory factors TNF α and IL-1β were significantly upregulated in the PD cell model compared with those of the control group. Furthermore, inhibition of miR-138-5p significantly attenuated the expression levels of TNF-α and IL-1β compared with those of the MPP^+ treatment group, while this effect was reversed by application of SIRT1-siRNA to the cells. MPP^+ treatment distinctly

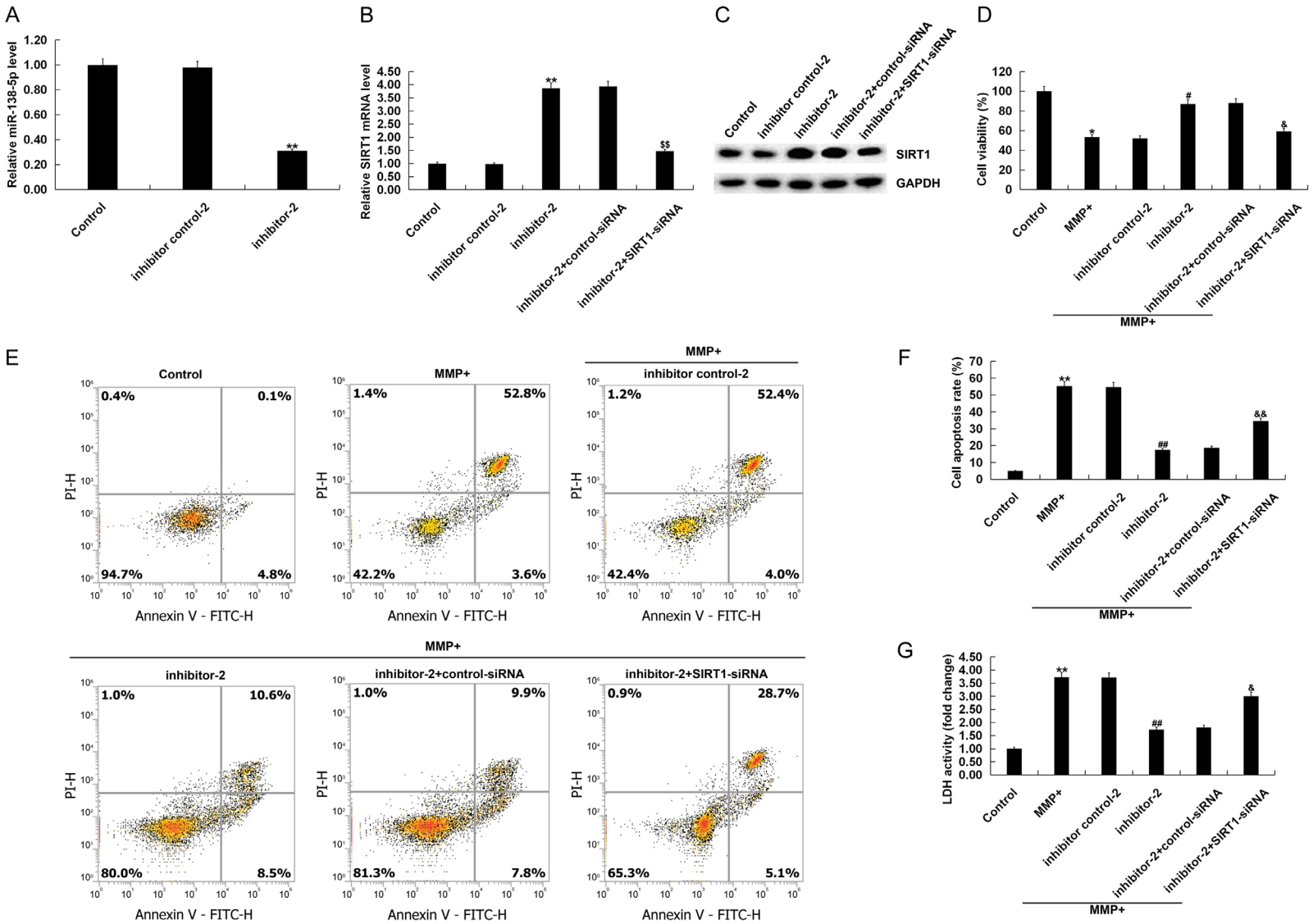

Figure 6. Effects of miR-138-5p inhibition on MPP⁺-induced SH-SY5Y cell viability and apoptosis. A. qRT-PCR assay was used to detect the relative level of miR-138-5p in SH-SY5Y cells. B and C. The mRNA and protein level of SIRT1 in SH-SY5Y cells following transfection was detected by qRT-PCR and western blot assay. D. The cell viability of treated SH-SY5Y cells was determined by MTT assay. E and F. The apoptotic rate of treated SH-SY5Y cells was measured by flow cytometry analysis. G. LDH release assay was used to detect LDH activity of treated SH-SY5Y cells. **P < 0.01 *vs.* Control; ##P < 0.01 *vs.* MMP⁺ treatment alone group; &, &&P < 0.05, 0.01 *vs.* MMP⁺ treatment combined with miR-138-5p inhibitor transfection group.

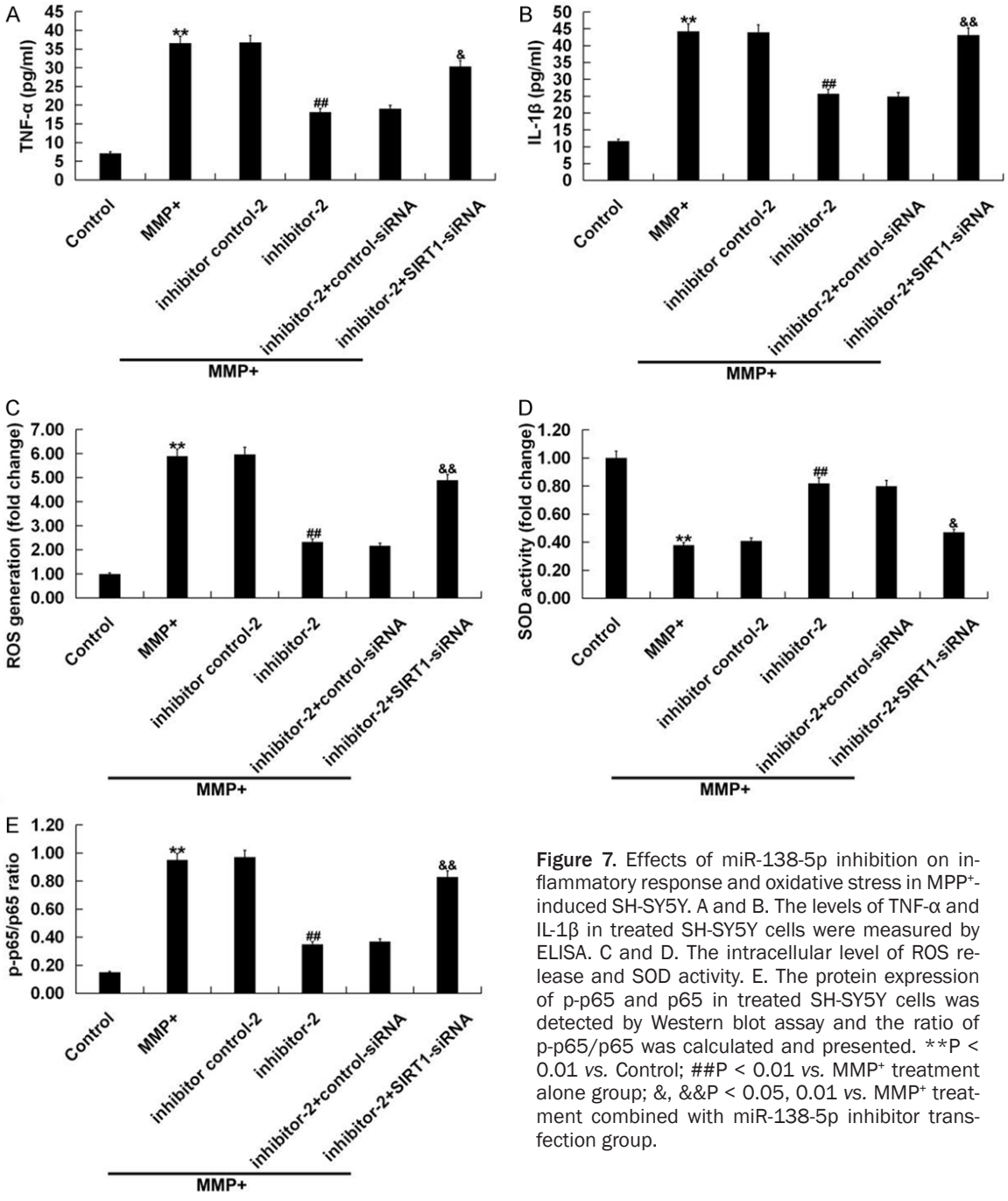

Figure 7. Effects of miR-138-5p inhibition on inflammatory response and oxidative stress in MPP^+-induced SH-SY5Y. A and B. The levels of TNF-α and IL-1β in treated SH-SY5Y cells were measured by ELISA. C and D. The intracellular level of ROS release and SOD activity. E. The protein expression of p-p65 and p65 in treated SH-SY5Y cells was detected by Western blot assay and the ratio of p-p65/p65 was calculated and presented. **P < 0.01 *vs.* Control; ##P < 0.01 *vs.* MMP^+ treatment alone group; &, &&P < 0.05, 0.01 *vs.* MMP^+ treatment combined with miR-138-5p inhibitor transfection group.

increased ROS levels and decreased SOD activity in SH-SY5Y cells compared with those of the control group. The miR-138-5p inhibitor significantly decreased ROS levels and increased SOD activity compared with that of the MPP^+ treatment group (**Figure 7C** and **7D**). These effects were abolished by SIRT1-siRNA treatment (**Figure 7C** and **7D**). Furthermore, we identified that treatment of the cells with MPP^+ caused a significant rise in the ratio of p-p65/p65 levels in SH-SY5Y cells compared with those of the control group, whereas the ratio of the p-p65/p65 levels in the miR-138-5p inhibitor group was considerably lower than that of the MPP^+ treatment group. This effect was reversed in the SIRT1-siRNA group (**Figure 7E**).

Discussion

SIRT1 is a member of the Sirtuin family and has been shown to possess low expression in PD patients [19]. SIRT1 exhibits neuroprotective

effects with regard to the development of PD [19]. Numerous studies have reported that miRNAs participate in the development of various diseases by regulating the expression levels of key factors involved in cell growth and apoptosis [20, 21]. However, the association between the expression levels of the miRNAs and the development of PD has not been fully elucidated. In addition, current treatment strategies for PD have proven ineffective.

In the present study, the data confirmed that miR-217 and miR-138-5p directly targeted SIRT1 via the prediction analysis. The present study indicated that the expression levels of miR-217 and miR-138-5p were significantly increased following an increase in the concentration levels of MPP^+ and in the treatment period, which was in agreement with the results reported in the previous study. In addition, the effects of miR-217 and miR-138-5p were investigated on an *in vitro* PD cell model. The results demonstrated that the downregulation of the expression levels of miR-217 and miR-138-5p improved cell viability significantly and reduced the induction of apoptosis and the levels of LDH activity in MPP^+-treated SH-SY5Y cells. The destruction of the cell membrane structure caused by apoptosis or necrosis results in the release of enzymes from the cytoplasm in the culture medium, including LDH which has relatively stable enzyme activity [22]. LDH release is considered an important indicator of cell membrane integrity and is widely used for cytotoxicity testing. Furthermore, a previous study demonstrated that overexpression of the pro-inflammatory factors could promote apoptosis and further aggravate PD [23]. In the present study, the relative pro-inflammatory factors, including IL-1β and TNFα were detected by ELISA and the protein expression levels of p-p65 were investigated by western blotting. The results indicated that downregulation of miR-217 and miR-138-5p led to the decrease of the expression levels of TNF-α, IL-1β in MPP^+-treated SH-SY5Y cells. Moreover, a decrease was noted in the p-p65/p65 ratio in this cell line model. We further observed that inhibition of miR-217 and miR-138-5p dramatically reduced ROS levels and improved SOD activity in MPP^+-treated SH-SY5Y cells, suggesting that miR-217/miR-138-5p inhibition restrained MPP^+-induced oxidative stress. However, all the aforementioned effects caused by the inhibition of the miR-217/miR-138-5p axis were significantly reversed in MPP^+-treated SH SY5Y cells by SIRT1 silencing. These results indicated that miR-217/miR-138-5p modulated the inflammatory response and the induction of oxidative stress and neuronal apoptosis by targeting SIRT1 in an *in vitro* PD cell model.

In conclusion, we found that the miR-217/miR-138-5p/SIRT1 axis participated in PD progression by regulating the inflammatory response and the induction of oxidative stress as well as the induction of neuronal apoptosis, suggesting that the suppression of miR-217/miR-138-5p may be a potential therapeutic treatment for PD. The present study provides a novel and promising molecular mechanism for PD diagnosis and therapy.

Disclosure of conflict of interest

None.

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References

[1] Keller DL. Neurostimulation for Parkinson's disease with early motor complications. N Engl J Med 2013; 368: 2037-2038.
[2] Pringsheim T, Jette N, Frolkis A and Steeves TD. The prevalence of Parkinson's disease: a systematic review and meta-analysis. Mov Disord 2014; 29: 1583-1590.
[3] Dexter DT and Jenner P. Parkinson disease: from pathology to molecular disease mechanisms. Free Radic Biol Med 2013; 62: 132-144.
[4] Medeiros L, Almeida H, Dias L, Perkusich M and Fischer R. A game-based approach to monitor Parkinson's disease: the bradykinesia symptom classification. Comp Med Sy 2016: 337-342.
[5] Rodriguez-Oroz MC, Jahanshahi M, Krack P, Litvan I, Macias R, Bezard E and Obeso JA. Initial clinical manifestations of Parkinson's disease: features and pathophysiological mechanisms. Lancet Neurol 2009; 8: 1128-1139.
[6] Jankovic J. Motor fluctuations and dyskinesias in Parkinson's disease: clinical manifestations. Mov Disord 2005; 20: S11-S16.
[7] Skaper SD. The brain as a target for inflammatory processes and neuroprotective strategies. Ann N Y Acad Sci 2007; 1122: 23-34.
[8] Lim KL, Dawson VL and Dawson TM. The cast of molecular characters in Parkinson's dis-

ease - felons, conspirators, and suspects. Ann N Y Acad Sci 2010; 991: 80-92.
[9] Santulli G. MicroRNAs and endothelial (Dys) function. J Cell Physiol 2016; 231: 1638-1644.
[10] Li M, Marin-Muller C, Bharadwaj U, Chow KH, Yao QZ and Chen CY. MicroRNAs: control and loss of control in human physiology and disease. World J Surg 2009; 33: 667-684.
[11] Li H, Zhao J, Zhang JW, Huang QY, Huang JZ, Chi LS, Tang HJ, Liu GQ, Zhu DJ and Ma WM. MicroRNA-217, down-regulated in clear cell renal cell carcinoma and associated with lower survival, suppresses cell proliferation and migration. Neoplasma 2013; 60: 511-515.
[12] Yu C, Wang M, Li ZP, Xiao J, Peng F, Guo XJ, Deng YZ, Jiang JX and Sun CY. MicroRNA-138-5p regulates pancreatic cancer cell growth through targeting FOXC1. Cell Oncol 2015; 38: 173-181.
[13] Zhao WG, Yu SN, Lu ZH, Ma YH, Gu YM and Chen J. The miR-217 microRNA functions as a potential tumor suppressor in pancreatic ductal adenocarcinoma by targeting KRAS. Carcinogenesis 2010; 31: 1726-1733.
[14] Singh P, Hanson PS and Morris CM. SIRT1 ameliorates oxidative stress induced neural cell death and is down-regulated in Parkinson's disease. BMC Neurosci 2017; 18: 46.
[15] Tsai KL, Cheng YY, Leu HB, Lee YY, Chen TJ, Liu DH and Kao CL. Investigating the role of Sirt1-modulated oxidative stress in relation to benign paroxysmal positional vertigo and Parkinson's disease. Neurobiol Aging 2015; 36: 2607-2616.
[16] Jia YH, Zheng Z, Wang YC, Zhou Q, Cai WX, Jia WB, Yang LL, Dong ML, Zhu XX, Su LL and Hu DH. SIRT1 is a regulator in high glucose-induced inflammatory response in RAW264.7 cells. PLoS One 2015; 10: e0127605.
[17] Wang WR, Li TT, Jing T, Li YX, Yang XF, He YH, Zhang W, Zhang JY and Lin R. SIRT1 regulates the inflammatory response of vascular adventitial fibroblasts through autophagy and related signaling pathway. Cell Physiol Biochem 2017; 41: 569-582.
[18] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 2001; 25: 402-408.
[19] Shinozaki S, Chang K, Sakai M, Shimizu N, Yamada M, Tanaka T, Nakazawa H, Ichinose F, Yamada Y, Ishigami A, Ito H, Ouchi Y, Starr ME, Saito H, Shimokado K, Stamler JS and Kaneki M. Inflammatory stimuli induce inhibitory S-nitrosylation of the deacetylase SIRT1 to increase acetylation and activation of p53 and p65. Sci Signal 2014; 7: ra106.
[20] Lu X, Cui Z, Liu S and Yin F. MiRNAs participate in the diagnosis, pathogenesis and therapy of Parkinson's disease. Histol Histopathol 2018; 33: 447-453.
[21] Aghabozorg Afjeh SS and Ghaderian SM. The role of microRNAs in cardiovascular disease. Int J Mol Cell Med 2013; 2: 50-57.
[22] van Dijk KD, Persichetti E, Chiasserini D, Eusebi P, Beccari T, Calabresi P, Berendse HW, Parnetti L and van de Berg WD. Changes in endolysosomal enzyme activities in cerebrospinal fluid of patients with Parkinson's disease. Mov Disord 2013; 28: 747-754.
[23] Bank M, Stein A, Sison C, Glazer A, Jassal N, McCarthy D, Shatzer M, Hahn B, Chugh R, Davies P and Bloom O. Elevated circulating levels of the pro-inflammatory cytokine macrophage migration inhibitory factor in individuals with acute spinal cord injury. Arch Phys Med Rehabil 2015; 96: 633-644.