



Long non-coding RNA Mirt2 prevents TNF- α -triggered inflammation via the repression of microRNA-101

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ABSTRACT

Parkinson's disease is normally accompanied by excessive inflammation. Myocardial infarction associated transcript 2 (Mirt2) has an activity to relieve inflammation in numerous cell types. Here, we aimed to investigate whether Mirt2 could elevate the resistance of SH-Sy5y cells to inflammation. Tumor necrosis factor alpha (TNF- α) was used to induce inflammation in SH-Sy5y cells. Mirt2 overexpressed or silenced cells were established. MicroRNA-101 (miR-101) mimic was used to up-regulate miR-101. Viable and apoptotic cells as well as reactive oxidative species (ROS) were detected after staining. Proteins associated with apoptosis, interleukin (IL) and signaling regulators were evaluated by Western blot. IL secretion was assessed by ELISA. Mirt2 and miR-101 were determined by qRT-PCR. We discovered that TNF- α weakened viability of SH-Sy5y cells and resulted in sensitivity to apoptosis with cleavage of PARP and caspase-3. Expression and secretion of IL-6 as well as generation of ROS were facilitated by TNF- α . However, Mirt2 overexpression moderated TNF- α -caused apoptosis associated with inflammation and oxidative stress. Mirt2 suppressed TNF- α -induced accumulation of miR-101, and based on this Mirt2 exhibited anti-inflammatory roles. Additionally, TNF- α -triggered phosphorylation of regulators was blocked by Mirt2 while restored by miR-101 mimic. In short Mirt2 overexpression exhibited anti-inflammatory properties through miR-101 suppression. Through down-regulating miR-101, Mirt2 blocked TNF- α -triggered NF- κ B/p38MAPK pathway.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disease with a predicted prevalence of 1.5 to 8.7 per 100,000 annually in China [1]. Although noteworthy advances have been achieved in understanding etiology and discovering efficient therapeutic options [2], there is no available therapies to entirely halt exacerbation of this disease [3]. It has been known that neuro-inflammation is a major source of oxidative stress which severely contributes to dopaminergic neurotoxicity and neurons loss [4,5]. Based on the pathogenesis and progression of PD, studies have been conducted to attenuate neuro-inflammation as well as excessive oxidative stress [6,7]. Particularly, microRNA (miRNA)-based molecular target therapies have been studied to stimulate the generation of neurons [8]. Blocking neurons loss might provide an opportunity to relieve PD [9].

In the last few years, transcriptome analysis has revealed that multiple long non-coding RNA (lncRNA) genes encompass transcriptional units in proximity to PD-associated protein-coding genes, which

suggests that lncRNA can serve as targets for PD treatment [10]. Recently, lncRNA myocardial infarction associated transcript 2 (Mirt2) has been identified as a negative feedback mediator in response to excessive inflammation, indicating a potency of Mirt2 to be applied as a repressor of over-inflammation [11]. Of importance, a novel molecular mechanism has been confirmed that Mirt2 can sponge miRNA and then indirectly modulate protein expression in non-alcoholic fatty liver disease [12]. However, no studies have addressed the potential involvement of Mirt2 in inflammation of PD, not to mention the mechanism in relevance with miRNAs.

Recent studies have discussed the dysregulation of miRNAs might play key roles in the pathogenesis of PD as well as serve as PD biomarkers, implying their potent application as innovative targets in PD therapy [13]. Mechanically, emerging data confirmed that post-transcriptional modulation by miRNAs is implicated in inflammation reaction of PD [14]. Specifically, microRNA-101 (miR-101) possesses a potential to mitigate the pathological process of another neurodegenerative disorder disease Alzheimer's disease (AD) [15,16]. We

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conjectured that miR-101 might be relevant to the process of PD in a neuro-inflammation-associated mechanism. Besides, lncRNAs-modulated dysregulation of miRNAs have been considered as a pathogenic factor in PD [17,18]. As a consequence, it is imperative to evaluate whether miR-101 could be a downstream responder of Mirt2 in answer to neuro-inflammation.

Herein, our studies verified Mirt2 blocked inflammation-associated apoptosis and identified miR-101 as a medium responder of Mirt2 in answer to inflammation. Our study may help to acknowledge miR-101-associated molecular effects that Mirt2 can exhibit on the PD.

2. Materials and methods

2.1. Cell culture and stimulation

Human neuroblastoma SH-Sy5y cells were purchased from Addexbio (San Diego, CA, USA). According to the information from the supplier, SH-Sy5y cells were derived from metastatic bone marrow. SH-Sy5y cells were cultured in a mixture (1:1) of Dulbecco's modified Eagle medium and Ham's F12 medium (Gibco, Gaithersburg, MD, USA) with the addition of fetal bovine serum (FBS, 15%, Gibco), penicillin/streptomycin (1000 IU/mL), and gentamicin (50 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA). The cells were cultivated in an atmosphere containing 95% air and 5% CO₂ at 37 °C. SH-Sy5y cells were exposed to 20 ng/mL tumor necrosis factor alpha (TNF-α) (Peprotech, Rocky Hill, NJ, USA) for 24 h.

2.2. Cell viability assay

The viability of SH-Sy5y cells was determined by cell counting kit-8 (CCK-8) assay kit (APExBIO, Houston, TX, USA) containing WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt] which produces a water-soluble formazan dye upon reduction in the presence of an electron mediator. The cells were inoculated in a 96-well plate and stimulated with TNF-α. Then, 10 µL of CCK-8 solution was added into the well, and the plate was cultured for 1 h. At last, the absorbance was read at 450 nm using plate reader (Molecular devices, San Jose, CA, USA). The viability was expressed as the percentage of the control.

2.3. Measurement of reactive oxygen species (ROS)

SH-Sy5y cells were grown in 96-well plates and cultured for 24 h. Next, the cells were treated with 20 ng/mL TNF-α for 24 h. The generation of ROS was evaluated using 2,7-dichlorofluorescein diacetate (DCFH-DA), a redox-sensitive dye purchased from Beyotime, Nanjing, China. After cultivation, the cells were washed in phosphate buffered saline (PBS) (Sigma-Aldrich) twice and were stained by 20 µmol/L DCFH-DA for 30 min. Subsequently, the cells were digested using trypsin after washed in PBS. The supernatants were removed after centrifugation, and the precipitate was re-suspended in 500 µL PBS. Then, a flow cytometer (Beckman Coulter, IN, USA) with an excitation wavelength of 488 nm and an emission wavelength of 521 nm was used to measure the fluorescent intensities.

2.4. Apoptosis assay

SH-Sy5y cells were grown and were exposed to TNF-α. The cells were washed in cold PBS twice and collected by centrifugation. Next, 5 × 10⁵ cells were re-suspended in 500 µL 1 × binding buffer, and incubated with 5 µL Annexin V-FITC and 5 µL propidium iodide (Abcam, Cambridge, UK) in the dark for 5 min at room temperature. Finally, Annexin V-FITC binding was detected by the flow cytometry with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The apoptotic cells (Annexin V-FITC+/PI- and Annexin V-FITC+/PI+) were differentiated from necrotic cells (Annexin V-FITC-

PI+) or viable cells (Annexin V-FITC-/PI-). Annexin V-FITC positive cells were counted.

2.5. Western blotting

For protein analysis, Western blotting was used. In short, the cells were collected after TNF-α stimulation for disintegration in lysis buffer (20 Mm Tris, 150 mM NaCl, 1% Triton X-100) (Beyotime) and protease inhibitors (Thermo Scientific, Waltham, MA, USA). The content of protein extract was determined using BCA protein assay kit (Pierce, Appleton, WI, USA). The protein was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis with β-actin as a loading buffer, and transferred onto a nitrocellulose membrane (Millipore, Bedford, MA, USA). The membrane was blocked in 5% bovine serum albumin (BSA) (Thermo Scientific) for 1 h at room temperature and continually probed using primary antibodies (diluted at 1:1000) against poly(ADP-ribose) polymerase (PARP) (Cat. No. 3002-30T; BioVision, Milpitas, CA, USA), caspase-3 (AHP2717; Bio-Rad, Hercules, California, USA), cleaved caspase-3 (AHP2286; Bio-Rad), interleukin (IL)-6 (701028; Thermo Scientific), IL-8 (710256; Thermo Scientific), β-actin (bs-50545R; Bioss, Woburn, MA, USA), p65 subunit of transcription factor NF-kappa B (NF-κB p65) (orb225487; Biorbyt, Cambridge, Cambridgeshire, UK), NF-κB p65 (pSer529) (orb14916; Biorbyt), inhibitor of nuclear factor kappa-B alpha (IκBα) (IP1861; ECM Biosciences, Versailles, Kentucky, USA), IκBα (pTyr305) (IP1041; ECM Biosciences), p38 mitogen-activated protein kinases (p38MAPK) (orb14630; Biorbyt), p38MAPK (pTyr182) (orb14942; Biorbyt) overnight at 4 °C. After washed using PBS supplemented with 0.05% Tween three times, the membrane was detected using horseradish peroxidase-tagged goat anti-rabbit antibody (ab97057; Abcam) (diluted at 1:5000). After washed, the interest proteins were visualized using the enhanced chemiluminescent kit (Bio-Rad). ChemiDoc XRS system (Bio-Rad) was applied to quantify the protein signaling intensity.

2.6. Transfection

The human Mirt2 transcript variant cDNA was amplified by PCR and was ligated into pcDNA[™]3.1/V5-His-TOPO (pcDNA3.1) (Invitrogen, Carlsbad, CA, USA) (pcMirt2). Then plasmids were transduced into SH-Sy5y cells using lipofectamine 3000 transfection reagent (Invitrogen). The HOTAIR stable transduced cells were selected in G418 (Invitrogen). Mirt2 short hairpin RNA (shMirt2) and its negative control (shNC) were synthesized by GenePharma (Shanghai, China). These shRNAs were transduced into SH-Sy5y cells according to the supplier's protocol. miR-101 mimic and its scramble negative miR-101 control (NC mimic), synthesized by GenePharma, were introduced into the cells using lipofectamine. Mirt2 and miR-101 were detected using qRT-PCR assay.

2.7. qRT-PCR assay

Total RNA was isolated from cells using TRIzol reagent (QIAGEN, Hilden, Germany). RNA was reversely transcribed to cDNA using RNA PCR kit (Takara Bio, Otsu, Japan). PCR was performed on 7900 HT sequence detection system (Applied Biosystems, Foster City, CA, USA) with reference to the manufacturer's instruction. The expression of Mirt2 was normalized to GAPDH using 2^{-ΔΔCt} formula, and miR-101 was normalized to U6. The PCR primer was listed as following, Mirt2 forward primer 5'-TCAACACTTCCATAGGT-3' and reverse primer 5'-ATTGTGAGGTCCAGATAG-3'; miR-101 forward primer 5'-GCTGTC AAGGATACGCTA-3' and reverse primer 5'-CAGTACTGTG ATAAC T GAA-3'; U6 forward primer 5'-GTCGGAGTCAACGGATT-3' and reverse primer 5'-AAGCTTCCCGTTCTCAG-3'.

2.8. Enzyme-linked immuno sorbent assay (ELISA)

After co-incubation with TNF- α , the cell supernatants were collected. The concentration of IL-6 and IL-8 was examined using human IL-6 ELISA kit (Abcam) and human IL-8 quantikine ELISA kit (R&D Systems, Abingdon, UK).

2.9. Data analysis

Data analysis was conducted with GraphPad Prism 6.0 software (GraphPad, San Diego, CA, USA). Student's *t*-test was used to compare the difference between two groups. One way analysis of variance followed by Bonferroni's post-test was performed to compare the difference among all groups. Data were expressed as the mean \pm standard deviation. The *P*-values < 0.05 were considered to indicate a statistical significance. The differences were accepted when *P* was < 0.05.

3. Results

3.1. Apoptosis process was triggered by TNF- α in neuroblastoma cells

To examine the function of Mirt2 in over-inflammation, we firstly induced the inflammation overreaction using TNF- α . TNF- α (20 ng/mL, 24 h) caused evident decrease in viable activity (*P* < 0.01) and increase in apoptosis (*P* < 0.001) (Fig. 1A–B). Meanwhile, high levels of PARP and caspase-3 at cleaved forms were detected in TNF- α -treated SH-Sy5y cells (both *P* < 0.001) (Fig. 1C). Next, the exposure to TNF- α contributed to notable secretion of IL-6 and IL-8 (both *P* < 0.001) (Fig. 1D), as well as abundance in SH-Sy5y cells (Fig. 1E). This over-inflammation was accompanied by oxidative stress since TNF- α elevated the cellular generation of ROS (*P* < 0.001) (Fig. 1F). These results showed that TNF- α facilitated apoptosis process associated with over-inflammation and oxidative stress.

3.2. Mirt2 overexpression conferred a protective role against TNF- α -induced apoptosis

Next, Mirt2 was exogenously elevated (*P* < 0.001) or repressed (*P* < 0.01) in SH-Sy5y cells by transduction (Fig. 2A). The transduced cells were then treated by TNF- α (20 ng/mL, 24 h). We found that number of viable cells increased apparently (*P* < 0.05) in Mirt2 up-regulated groups. Inversely, Mirt2 silence resulted in a decrease in viability of SH-Sy5y cells (*P* < 0.05) (Fig. 2B). In addition, Mirt2-overexpressed SH-Sy5y cells exhibited an obvious resistance to TNF- α -caused apoptosis (*P* < 0.05) while Mirt2-silenced cells were sensitive to it (*P* < 0.05) (Fig. 2C). To further investigate the role of Mirt2 in apoptosis, we performed Western blot assay to detect PARP and caspase-3. The results showed that Mirt2 overexpression suppressed the production of cleaved forms induced by TNF- α while Mirt2 silence aggravated the cleavage process of PARP and caspase-3 (both *P* < 0.01) (Fig. 2D). Besides, we found TNF- α induced secretion and abundance of IL-6 and IL-8 were controlled by Mirt2 up-regulation while impeded by Mirt2 knockdown (*P* < 0.05 or *P* < 0.01) (Fig. 2E–F). Consistently, the generation of ROS was blocked by Mirt2 overexpression while exacerbated by its silence (*P* < 0.05 or *P* < 0.01) (Fig. 2G). Thus, the apoptosis of neuroblastoma cells induced by TNF- α could be retarded by artificially up-regulating Mirt2.

3.3. miR-101 was implicated in the anti-apoptotic property of Mirt2 in TNF- α -treated neuroblastoma cells

Next, we assessed miR-101 in transduced cells after TNF- α treatment. We found TNF- α significantly promoted the accumulation of miR-101 in SH-Sy5y cells (*P* < 0.01). However, the up-regulation of Mirt2 contributed to the suppression of miR-101 after TNF- α treatment (*P* < 0.05). By contrast, Mirt2 silence caused the abundance of miR-

101 in TNF- α -administrated cells (*P* < 0.05) (Fig. 3A). Consequently, Mirt2-mediated resistance to apoptosis might be regulated by the down-regulation of miR-101. With this in mind, we up-regulated miR-101 in SH-Sy5y cells (*P* < 0.001) (Fig. 3B). As predicted, simultaneous over-expression of Mirt2 and miR-101 abrogated the ability of Mirt2 to maintain viability (*P* < 0.05) (Fig. 3C). Additionally, miR-101 over-expression motivated SH-Sy5y cells to be sensitive to TNF- α -triggered apoptosis (*P* < 0.05) (Fig. 3D). Meanwhile, we found this process was accompanied by the cleavage of PARP and caspase-3 (both *P* < 0.01) (Fig. 3E). Moreover, miR-101 mimic stimulated neuroblastoma cells to generate and secrete IL-6 and IL-8 to negated the protective role of Mirt2 against TNF- α (both *P* < 0.05) (Fig. 3F–G). As for the production of ROS, miR-101 overexpression restored the high level of ROS induced by TNF- α (*P* < 0.01) (Fig. 3H). Together, miR-101 functioned as a medium responder to TNF- α -induced apoptosis, and through repressing miR-101 Mirt2 exerted a protective effect.

3.4. Mirt2 lead to bluntness of NF- κ B/p38MAPK pathway through repressing miR-101 in response to TNF- α

Since Mirt2 conferred an inhibitory role in inflammation, we analyzed the alteration of associated cascades in SH-Sy5y cells. Firstly, TNF- α visibly induced the phosphorylation of NF- κ B p65, I κ B α and p38MAPK (*P* < 0.001). However, the phosphorylated ratio of NF- κ B p65, I κ B α and p38MAPK was reduced in Mirt2-overexpressed SH-Sy5y cells after TNF- α administration (*P* < 0.05 or *P* < 0.01). Coinstantaneous transduction of Mirt2 and miR-101 precluded Mirt2-caused blockade of phosphorylation in TNF- α -treated SH-Sy5y cells (Fig. 4). These findings showed that Mirt2-mediated suppression of miR-101 functioned in the blockage of NF- κ B/p38MAPK which was activated by TNF- α .

4. Discussion

Mirt2 exhibits an effective anti-inflammatory capacity and balances macrophage polarization in response to excessive inflammation [11]. Currently, we investigated the anti-inflammatory role of Mirt2 in response to TNF- α administration in neuroblastoma cells. Our data confirmed that Mirt2 prevented SH-Sy5y cells from TNF- α -triggered apoptosis associated with inflammation and oxidative stress. The underlying mechanism might be associated with Mirt2-mediated repression of miR-101.

The neuropathology of PD has been confirmed to be largely associated with severe inflammation which is permanently present in PD brains [19]. Neuro-inflammation results in generation and release of reactive species and induces excessive oxidative stress which is a fundamental process in neurons destruction [20]. Hence, relieving over-inflammation will be useful in better management of PD. Given human origin, easiness of culture and catecholaminergic features, SH-Sy5y cells has been widely applied as a cell model for PD study [21]. Correspondingly, SH-Sy5y cells were stimulated by TNF- α to exhibit over-inflammation and oxidation in our study. Besides, we detected the activation of PARP and caspase-3 induced by TNF- α . PARP catalyzes the synthesis of PARP implicated in parthanatos which also happens in neuro-degeneration diseases [22]. Strikingly, excluding executioner property of apoptosis, caspase-3 activation has been uncovered to mediate apoptosis of microglia whose activation triggers pro-inflammatory reaction in PD [23]. Consequently, excessive inflammation triggered the apoptosis process in neuroblastoma cells.

Controlling neuro-inflammation is considered to be of therapeutic benefit for management and treatment of PD [24]. Since lncRNAs regulate the transcription of genes associated with inflammation, some lncRNAs might be able to function as inflammation repressors [25,26]. Mirt2 was initially detected in heart after myocardial infarction [27]. The anti-inflammatory capacity of Mirt2 was then demonstrated in several cell types, for instance, tracheal epithelial cells, hepatocytes,

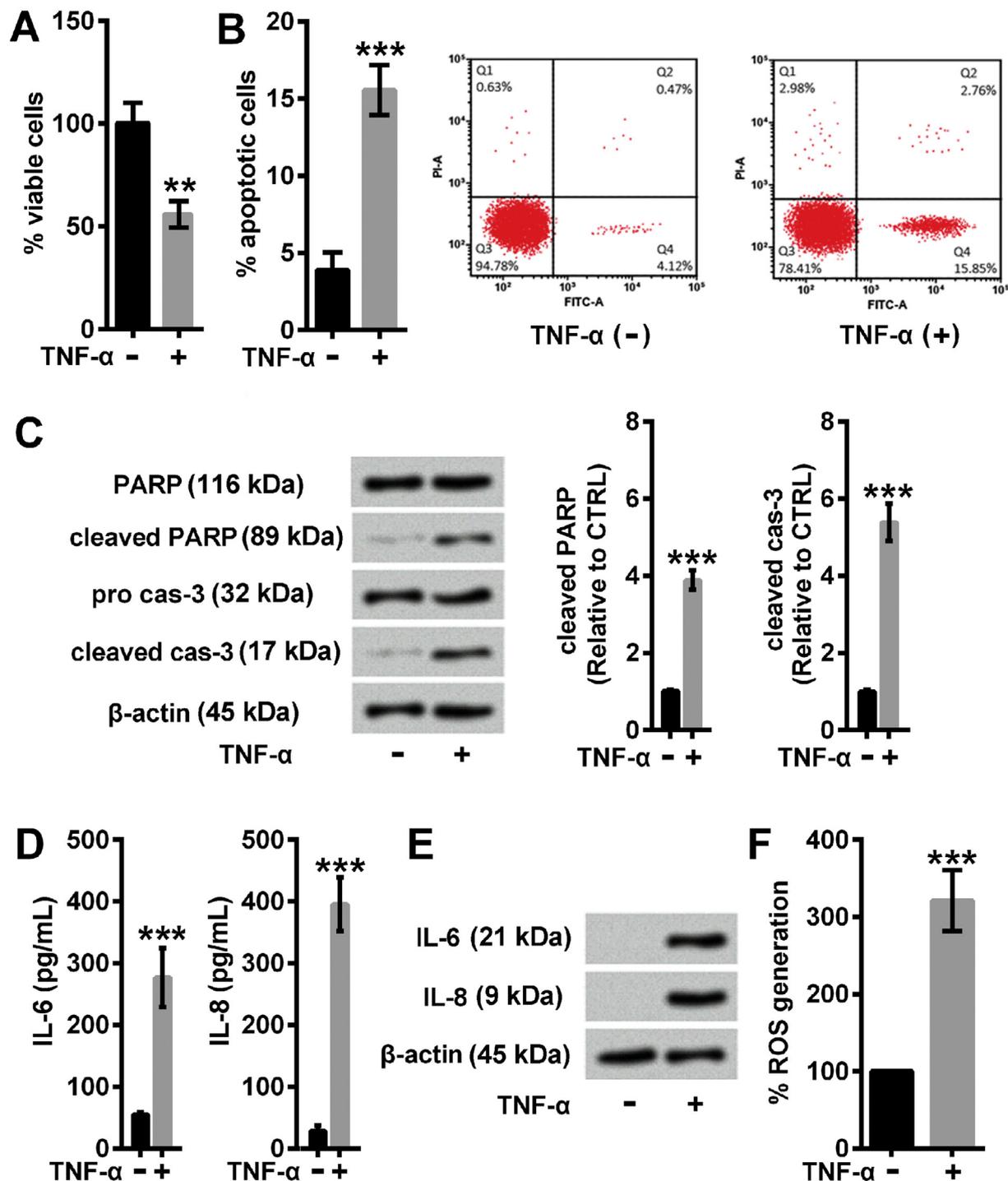


Fig. 1. Tumor necrosis factor alpha (TNF- α) triggered inflammatory apoptosis in SH-Sy5y cells. SH-Sy5y cells were treated with TNF- α (20 ng/mL) for 24 h. **A.** The tetrazolium salt was reduced into formazan orange dye in viable cells and detected by microplate reader at 450 nm. **B.** Phosphatidylserine from apoptotic cells was affiliated by fluorescent-conjugated Annexin V and was observed by flow cytometer. **C.** Lyses from cells were probed with antibodies to the pro or cleaved forms of PARP and caspase (cas)-3. Band intensity was quantified using densitometric analysis and was normalized to β -actin. **D.** The content of IL-6 and IL-8 in cell supernatant was examined by ELISA. **E.** Lyses from cells were probed with antibodies to IL-6 and IL-8 with β -actin as a loading control. **F.** 2,7-dichlorofluorescein diacetate (DCFH-DA) was oxidized by reactive oxygen species (ROS) into bright orange fluorescence which was detected by flow cytometer. Data represented the group mean \pm standard deviation. $n = 3$. ** ($P < 0.01$) and *** ($P < 0.001$) indicated the significant differences.

adipocytes, cardiomyocytes and cardiac fibroblasts by Du et al. [11]. To relieve or block neuro-inflammation of PD, we firstly transduced Mirt2 into SH-Sy5y cells. Surprisingly, Mirt2 also suggested notable anti-inflammatory roles in neuroblastoma SH-Sy5y cells. Not only did Mirt2 repress synthesis of cytokines and ROS, but also apoptosis process. Therefore, Mirt2 up-regulation might be a promising tool for PD

treatment.

miR-101 has been detected in multiple human tissues, and it is highly expressed in brain [28]. Besides, miR-101 was reported as a high up-regulated gene in Huntington's disease, a neurodegenerative disorder [29]. In another neurodegenerative disease AD, studies showed that miR-101 post-transcriptionally regulates the expression amyloid- β

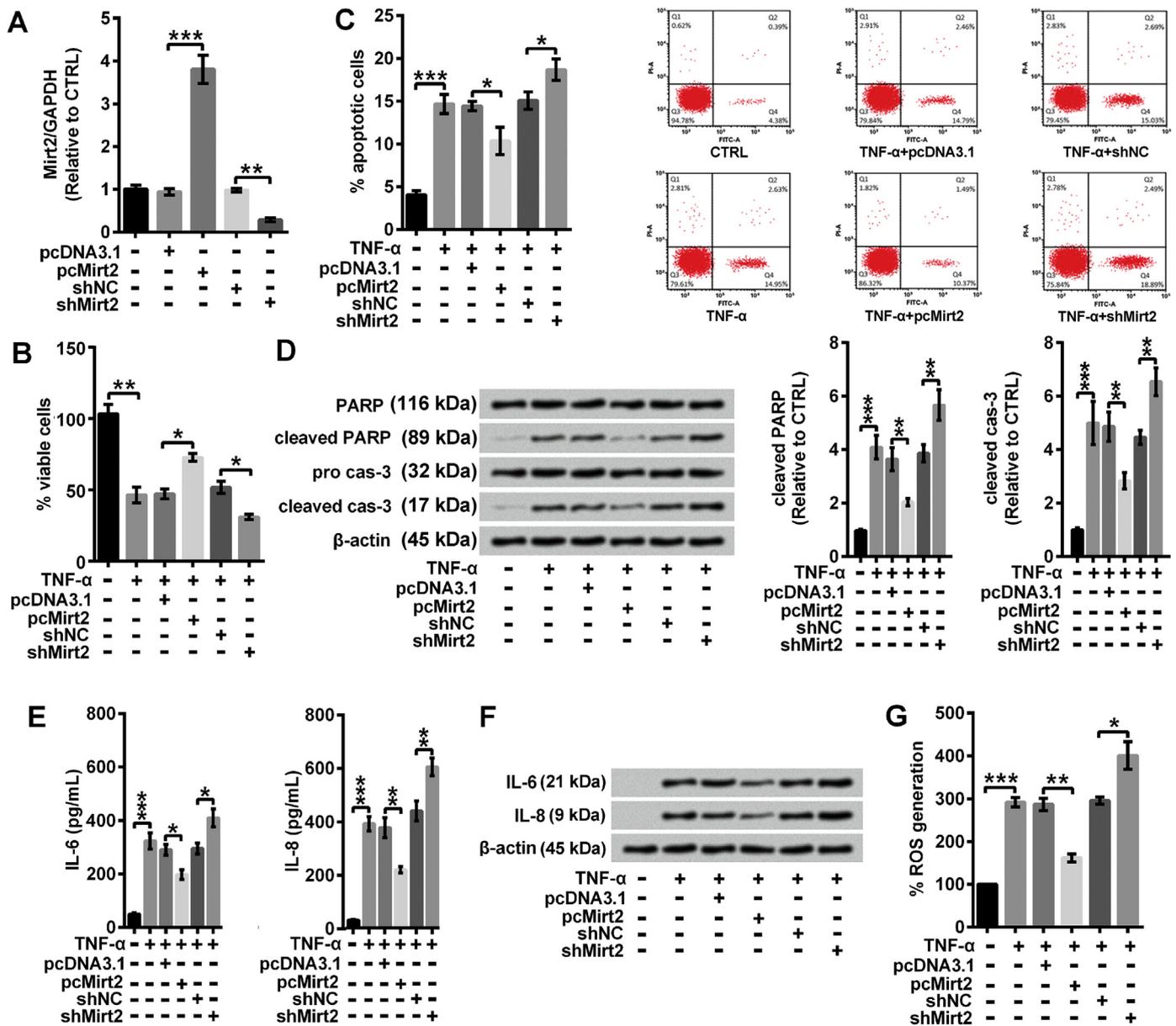


Fig. 2. Myocardial infarction associated transcript 2 (Mirt2) overexpression relieved tumor necrosis factor alpha (TNF-α) induced apoptosis. Mirt2 was artificially overexpressed or silenced in SH-Sy5y cells. **A.** Mirt2 was detected by qRT-PCR and was normalized to GAPDH. The transfected cells were treated by TNF-α. **B.** The tetrazolium salt was reduced into formazan orange dye in viable cells which can be determined by microplate reader at 450 nm. **C.** Fluorescent-conjugated Annexin V was affiliated with phosphatidylserine from apoptotic cells and then was observed by flow cytometer. **D.** The pro or cleaved forms of PARP and caspase (cas)-3 were probed by primary antibodies. Band intensity was quantified using densitometric analysis with β-actin as an internal control. **E.** IL-6 and IL-8 in cell supernatant was quantified by ELISA. **F.** IL-6 and IL-8 was probed by antibodies with β-actin as a loading reference. **G.** Flow cytometer was used to detect bright orange fluorescence which was produced by 2,7-dichlorofluorescein diacetate (DCFH-DA) upon oxidation by reactive oxygen species (ROS). Data represented the group mean ± standard deviation. n = 3. * (P < 0.05), ** (P < 0.01), and *** (P < 0.001) indicated the significant differences.

precursor protein, a component of insoluble amyloid plaques detected in AD brains via a specific site targeting [15,16]. Here, TNF-α treatment resulted in the abundance of miR-101, suggesting that miR-101 accumulation might be attributable to over-inflammation in SH-Sy5y cells. Additionally, simultaneous overexpression of Mirt2 and miR-101 halted the protective impact of Mirt2 against TNF-α-triggered inflammation. Consequently, Mirt2 overexpression-mediated miR-101 down-regulation was a possible causative factor of the anti-inflammatory capacity.

Our study also dissected the alteration in inflammation-associated signaling transduction cascades. In response to various stimuli, such as inflammation and oxidative stress, NF-κB [30] and p38MAPK [31] normally participate in PD, suggesting that cascades can be used as a therapeutic target. Studies found MAPK and NF-κB signaling pathways

play vital role in mediating the expression of pro-inflammatory cytokine production in through toll-like receptor under LPS-stimulated condition [32,33]. In this context, Mirt2 overexpression impeded phosphorylation process of p65, IκBα and p38MAPK initiated by TNF-α. Similarly, in response to LPS-caused inflammation, Mirt2 restricts MAPK and NF-κB through blocking oligomerization and ubiquitination of TNF receptor-associated factor 6 [11]. However, another anti-inflammatory mechanism associated with miR-101 was proposed in our study. We found miR-101 mimic negated Mirt2-elicited blockage of MAPK and NF-κB which are activated in answer to excessive inflammation. Previous studies have presented the possible mechanisms that miR-101 is implicated in the modulation of NF-κB-mediated anti-apoptotic genes [34] and regulates MAPK phosphatase 1 [35].

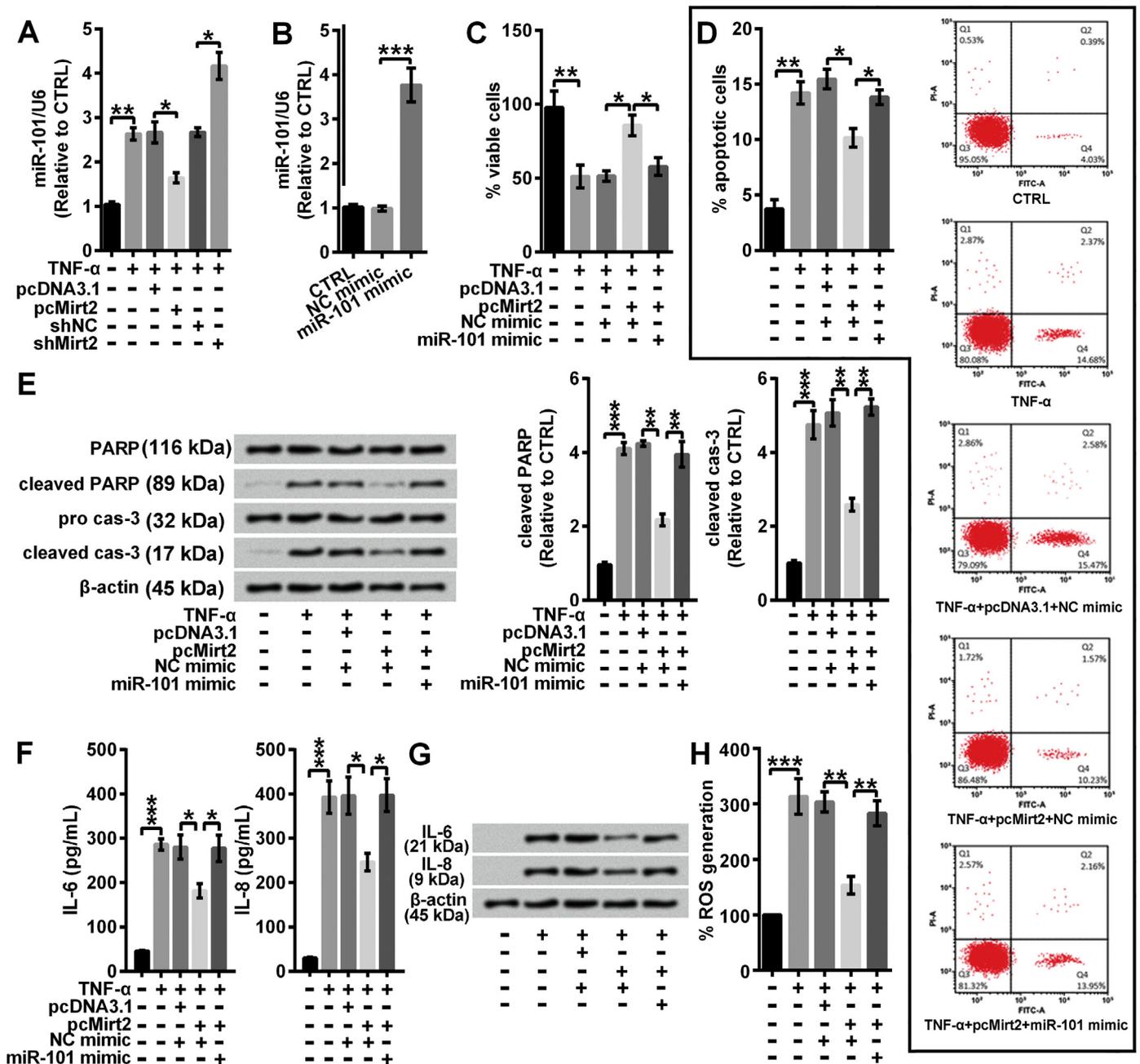


Fig. 3. Tumor necrosis factor alpha (TNF-α)-triggered apoptosis was retarded by Mirt2 overexpression-induced suppression of miR-101. **A.** Mirt2 was artificially overexpressed or silenced in SH-Sy5y cells. After treatment by TNF-α, miR-101 was detected by qRT-PCR. **B.** SH-Sy5y cells were forced to overexpress miR-101, which was confirmed by qRT-PCR. After simultaneously transduced by pcMirt2 and miR-101 mimic, SH-Sy5y cells were administrated by TNF-α. **C.** Microplate reader was exploited to detect formazan orange dye which was produced by tetrazolium salt upon reduction by viable cells. **D.** Phosphatidylserine from apoptotic cells associated with fluorescent-conjugated Annexin V was detected by flow cytometer. **E.** The pro or cleaved forms of PARP and caspase (cas)-3 were detected by primary antibodies and signaling intensity was analyzed by densitometry which was normalized to β-actin. **F.** ELISA proceeded to evaluate the content of IL-6 and IL-8 in cell supernatant. **G.** IL-6 and IL-8 in cell lysates were probed by primary antibodies and β-actin functioned as a loading control. **H.** 2,7-dichlorofluorescein diacetate (DCFH-DA) was oxidized by reactive oxygen species (ROS) to exhibit bright orange fluorescence which can be detected by flow cytometer. Data represented the group mean ± standard deviation. n = 3. * (P < 0.05), ** (P < 0.01), and *** (P < 0.001) indicated the significant differences.

5. Conclusion

In summary, Mirt2 overexpression conferred an elevated resistant activity to neuroblastoma SH-Sy5y cells in response to TNF-α-elicited inflammation and oxidative stress. Mirt2-repressive miR-101 might be a functional link during this process. Inhibition of miR-101 by Mirt2 resulted in the blockage of MAPK and NF-κB cascades which might be a significant implication in PD treatment.

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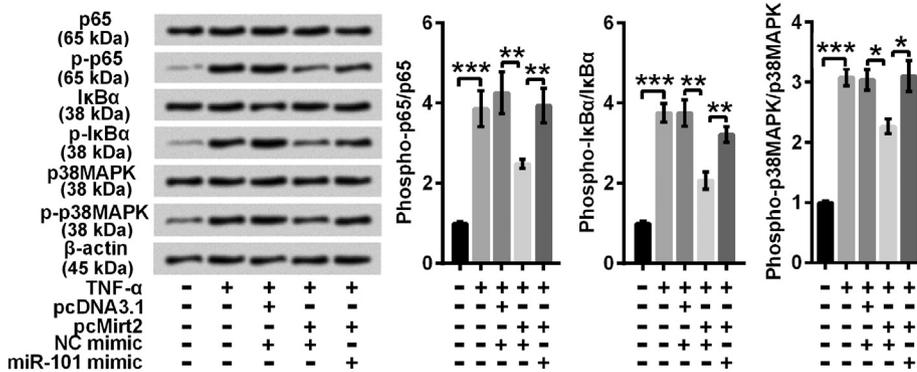


Fig. 4. Activation of NF- κ B/p38MAPK cascade by tumor necrosis factor alpha (TNF- α) was blunted by Mirt2 while negated in miR-101 mimic-transduced SH-Sy5y cells. After simultaneously transduced by pcMirt2 and miR-101 mimic, SH-Sy5y cells were administered by TNF- α . Phosphorylated forms of NF- κ B p65 (pSer529), I κ B α (pTyr305), p38MAPK (pTyr182) were probed by the indicated antibodies and were expressed as the phosphorylated ratio after normalization to β -actin. Data represented the group mean \pm standard deviation. $n = 3$. * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$) indicated the significant differences.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

References

- Y.M. Zou, J. Liu, Z.Y. Tian, D. Lu, Y.Y. Zhou, Systematic review of the prevalence and incidence of Parkinson's disease in the People's Republic of China, *Neuropsychiatr. Dis. Treat.* 11 (2015) 1467–1472.
- N.L. Del Rey, A. Quiroga-Varela, E. Garbayo, I. Carballo-Carbajal, R. Fernandez-Santiago, M.H.G. Monje, I. Trigo-Damas, M.J. Blanco-Prieto, J. Blesa, *Advances in Parkinson's disease: 200 years later*, *Front. Neuroanat.* 12 (2018) 113.
- D.L.M. Radder, I.H. Sturkenboom, M. van Nimwegen, S.H. Keus, B.R. Bloem, N.M. de Vries, Physical therapy and occupational therapy in Parkinson's disease, *The International journal of neuroscience* 127 (10) (2017) 930–943.
- P. Agostinho, R. A. Cunha, C. Oliveira, Neuroinflammation, oxidative stress and the pathogenesis of Alzheimer's disease, *Curr. Pharm. Des.* 16 (25) (2010) 2766–2778.
- E.J. Shin, H.Q. Tran, P.T. Nguyen, J.H. Jeong, S.Y. Nah, C.G. Jang, T. Nabeshima, H.C. Kim, Role of mitochondria in methamphetamine-induced dopaminergic neurotoxicity: involvement in oxidative stress, neuroinflammation, and pro-apoptosis-review, *Neurochem. Res.* 43 (1) (2018) 66–78.
- R. Calvello, A. Cianciulli, G. Nicolardi, F. De Nuccio, L. Giannotti, R. Salvatore, C. Porro, T. Trotta, M.A. Panaro, D.D. Lofruento, Vitamin D treatment attenuates neuroinflammation and dopaminergic neurodegeneration in an animal model of Parkinson's disease, shifting M1 to M2 microglia responses, *J. Neuroimmune Pharmacol.* 12 (2) (2017) 327–339.
- D. Leonoudakis, A. Rane, S. Angeli, G.J. Lithgow, J.K. Andersen, S.J. Chinta, Anti-inflammatory and neuroprotective role of natural product securinine in activated glial cells: implications for Parkinson's disease, *Mediat. Inflamm.* 2017 (2017) 8302636.
- C. Saraiva, L. Ferreira, L. Bernardino, Traceable microRNA-124 loaded nanoparticles as a new promising therapeutic tool for Parkinson's disease, *Neurogenesis (Austin, Tex.)* 3 (1) (2016) e1256855.
- A. Iannielli, S. Bido, L. Folladori, A. Segnali, C. Cancellieri, A. Maresca, L. Massimino, A. Rubio, G. Morabito, L. Caporali, F. Tagliavini, O. Musumeci, G. Gregato, E. Bezzard, V. Carelli, V. Tiranti, V. Broccoli, Pharmacological inhibition of necroptosis protects from dopaminergic neuronal cell death in Parkinson's disease models, *Cell Rep.* 22 (8) (2018) 2066–2079.
- M. Elkouris, G. Kouroupi, A. Vourvoukelis, N. Papagiannakis, V. Kaltezioti, R. Matsas, L. Stefanis, M. Xilouri, P.K. Politis, Long non-coding RNAs associated with neurodegeneration-linked genes are reduced in Parkinson's disease patients, *Front. Cell. Neurosci.* 13 (2019) 58.
- M. Du, L. Yuan, X. Tan, D. Huang, X. Wang, Z. Zheng, X. Mao, X. Li, L. Yang, K. Huang, F. Zhang, Y. Wang, X. Luo, D. Huang, K. Huang, The LPS-inducible lncRNA Mirt2 is a negative regulator of inflammation, *Nat. Commun.* 8 (1) (2017) 2049.
- B. Zhang, H. Li, D. Li, H. Sun, M. Li, H. Hu, Long noncoding RNA Mirt2 upregulates USP10 expression to suppress hepatic steatosis by sponging miR-34a-5p, *Gene* 700 (2019) 139–148.
- L. Leggio, S. Vivarelli, F. L'Episcopo, C. Tirola, S. Caniglia, N. Testa, B. Marchetti, N. Iraci, MicroRNAs in Parkinson's disease: from pathogenesis to novel diagnostic and therapeutic approaches, *Int. J. Mol. Sci.* 18 (12) (2017).
- Y. Zhou, M. Lu, R.H. Du, C. Qiao, C.Y. Jiang, K.Z. Zhang, J.H. Ding, G. Hu, MicroRNA-7 targets Nod-like receptor protein 3 inflammasome to modulate neuroinflammation in the pathogenesis of Parkinson's disease, *Mol. Neurodegener.* 11 (2016) 28.
- J.M. Long, D.K. Lahiri, MicroRNA-101 downregulates Alzheimer's amyloid- β precursor protein levels in human cell cultures and is differentially expressed, *Biochem. Biophys. Res. Commun.* 404 (4) (2011) 889–895.
- C. Barbato, S. Pezzola, C. Caggiano, M. Antonelli, P. Frisone, M.T. Ciotti, F. Ruberti, A lentiviral sponge for miR-101 regulates RanBP9 expression and amyloid precursor protein metabolism in hippocampal neurons, *Front. Cell. Neurosci.* 8 (2014) 37.
- Y. Chen, Y.-j. Lian, Y.-q. Ma, C.-j. Wu, Y.-k. Zheng, N.-c. Xie, LncRNA SNHG1 promotes α -synuclein aggregation and toxicity by targeting miR-15b-5p to activate

- SLAH1 in human neuroblastoma SH-SY5Y cells, *Neurotoxicology* 68 (2018) 212–221.
- W. Liu, Q. Zhang, J. Zhang, W. Pan, J. Zhao, Y. Xu, Long non-coding RNA MALAT1 contributes to cell apoptosis by sponging miR-124 in Parkinson disease, *Cell & Bioscience* 7 (1) (2017) 19.
- A. Gustot, J.I. Gallea, R. Sarroukh, M.S. Celej, J.M. Ruyschaert, V. Raussens, Amyloid fibrils are the molecular trigger of inflammation in Parkinson's disease, *The Biochemical journal* 471 (3) (2015) 323–333.
- A.H. Bhat, K.B. Dar, S. Anees, M.A. Zargar, A. Masood, M.A. Sofi, S.A. Ganie, Oxidative stress, mitochondrial dysfunction and neurodegenerative diseases; a mechanistic insight, *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* 74 (2015) 101–110.
- H. Xicoy, B. Wieringa, G.J. Martens, The SH-SY5Y cell line in Parkinson's disease research: a systematic review, *Mol. Neurodegener.* 12 (1) (2017) 10.
- Y. Lee, H.C. Kang, B.D. Lee, Y.I. Lee, Y.P. Kim, J.H. Shin, Poly (ADP-ribose) in the pathogenesis of Parkinson's disease, *BMB Rep.* 47 (8) (2014) 424–432.
- E. Kavanagh, J. Rodhe, M.A. Burguillos, J.L. Venero, B. Joseph, Regulation of caspase-3 processing by cIAP2 controls the switch between pro-inflammatory activation and cell death in microglia, *Cell Death Dis.* 5 (2014) e1565.
- A. Roy, A. Ghosh, A. Jana, X. Liu, S. Brahmachari, H.E. Gendelman, K. Pahan, Sodium phenylbutyrate controls neuroinflammatory and antioxidant activities and protects dopaminergic neurons in mouse models of Parkinson's disease, *PLoS One* 7 (6) (2012) e38113.
- M.K. Atianand, W. Hu, A.T. Satpathy, Y. Shen, E.P. Ricci, J.R. Alvarez-Dominguez, A. Bhatta, S.A. Schattgen, J.D. McGowan, J. Blin, J.E. Braun, P. Gandhi, M.J. Moore, H.Y. Chang, H.F. Lodish, D.R. Caffrey, K.A. Fitzgerald, A long noncoding RNA lincRNA-EP5 acts as a transcriptional brake to restrain inflammation, *Cell* 165 (7) (2016) 1672–1685.
- F. Li, J. Sun, S. Huang, G. Su, G. Pi, LncRNA GAS5 overexpression reverses LPS-induced inflammatory injury and apoptosis through up-regulating KLF2 expression in ATDC5 chondrocytes, *cellular physiology and biochemistry: international journal of experimental cellular physiology, Biochem. Pharmacol.* 45 (3) (2018) 1241–1251.
- J. Zangrando, L. Zhang, M. Vausort, F. Maskali, P.Y. Marie, D.R. Wagner, Y. Devaux, Identification of candidate long non-coding RNAs in response to myocardial infarction, *BMC Genomics* 15 (2014) 460.
- F. Llorens, M. Banez-Coronel, L. Pantano, J.A. del Rio, I. Ferrer, X. Estivill, E. Marti, A highly expressed miR-101 isomiR is a functional silencing small RNA, *BMC Genomics* 14 (2013) 104.
- E. Marti, L. Pantano, M. Banez-Coronel, F. Llorens, E. Minones-Moyano, S. Porta, L. Sumoy, I. Ferrer, X. Estivill, A myriad of miRNA variants in control and Huntington's disease brain regions detected by massively parallel sequencing, *Nucleic Acids Res.* 38 (20) (2010) 7219–7235.
- P.M. Flood, L. Qian, L.J. Peterson, F. Zhang, J.S. Shi, H.M. Gao, J.S. Hong, Transcriptional factor NF-kappaB as a target for therapy in Parkinson's disease, *Parkinson's disease* 2011 (2011) 216298.
- S.K. Jha, N.K. Jha, R. Kar, R.K. Ambasta, P. Kumar, p38 MAPK and PI3K/AKT signalling cascades in Parkinson's disease, *International journal of molecular and cellular medicine* 4 (2) (2015) 67–86.
- A.D. Bachtetter, B. Xing, L. de Almeida, E.R. Dimayuga, D.M. Watterson, L.J. Van Eldik, Microglial p38alpha MAPK is a key regulator of proinflammatory cytokine up-regulation induced by toll-like receptor (TLR) ligands or beta-amyloid (A β), *J. Neuroinflammation* 8 (2011) 79.
- I. Gujjarro-Munoz, M. Compte, A. Alvarez-Cienfuegos, L. Alvarez-Vallina, L. Sanz, Lipopolysaccharide activates Toll-like receptor 4 (TLR4)-mediated NF-kappaB signaling pathway and proinflammatory response in human pericytes, *J. Biol. Chem.* 289 (4) (2014) 2457–2468.
- E. Farhadi, F. Zaker, M. Safa, M.R. Rezvani, miR-101 sensitizes K562 cell line to imatinib through Jak2 downregulation and inhibition of NF-kappaB target genes, *Tumour biology: the journal of the International Society for Oncodevelopmental Biology and Medicine* 37 (10) (2016) 14117–14128.
- Y. Gao, F. Liu, L. Fang, R. Cai, C. Zong, Y. Qi, Genkwanin inhibits proinflammatory mediators mainly through the regulation of miR-101/MKP-1/MAPK pathway in LPS-activated macrophages, *PLoS One* 9 (5) (2014) e96741.