MicroRNA-195 triggers neuroinflammation in Parkinson's disease in a Rho-associated kinase 1-dependent manner

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Abstract. Parkinson's disease (PD) is a common progressive neurodegenerative disorder occurring in older individuals. Mechanistically, neuroinflammation is a central pathological change in the progression of PD. Activation of microglia is widely considered to be a major trigger for neuroinflammation. Certain microRNAs (miRs) are key factors in inhibiting or stimulating inflammation during the occurrence and development of PD, among which miR-195 may be a potential crucial biomarker. However, the underlying pathological mechanisms remain unclear. To investigate the pathogenesis of PD, lipopolysaccharide (LPS) was used to establish an *in vitro* model of microglia activation in the present study. It was revealed that miR-195 expression was decreased in LPS-stimulated BV2 cells, suggesting a potential mechanism of action of miR-195 on microglia activation. Furthermore, gain- and loss-of-function experiments were performed by successful transfection of microglia with miR-195 mimics or inhibitors. The results demonstrated that miR-195 overexpression inhibited the release of pro-inflammatory cytokines, including inducible nitric oxide synthase, interleukin-6 (IL-6) and tumor necrosis factor-α, but induced the release of anti-inflammatory cytokines in LPS-treated BV2 cells, including IL-4 and IL-10. In addition, Rho-associated kinase 1 (ROCK1), which is negatively regulated by miR-195, was increased in LPS-stimulated BV2 cells. ROCK1 knockdown with small interfering RNA exhibited the same effect as miR-195 overexpression on regulating microglia status, suggesting that the miR-195/ROCK1 interaction serves a central role in inducing microglia activation. Furthermore, inhibition of ROCK1 impaired cell viability and proliferation but induced cell apoptosis in LPS-treated miR-195-deficient BV2 cells. The present results suggest that miR-195 is a potential therapeutic target for PD.

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Abbreviations: BrdU, 5-bromo-2-deoxyuridine; FCM, flow cytometry; FITC, fluorescein isothiocyanate; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; miR-195, microRNA-195; miR-NC1, microRNA mimics negative control; miR-NC2, microRNA inhibitor negative control; PD, Parkinson's disease; PI, propidium iodide; qPCR, quantitative polymerase chain reaction; ROCK1, Rho-associated kinase 1

Key words: microRNA-195, neuroinflammation, Parkinson's disease, Rho-associated kinase 1, microglia, proliferation, apoptosis

Introduction

Parkinson's disease (PD), a progressive neurodegenerative disorder, affects ~1% of the population aged ≥60 years. Clinically, PD is characterized by bradykinesia, tremor, rigidity and postural instability. Pathologically, these manifestations are primarily caused by loss of dopaminergic neurons in the substantia nigra (1). While significant progress has been made in the elucidation of the etiology of PD, precise targeted therapies interfering with the pathological mechanisms of neurodegeneration are not available at present. Increasing evidence has indicated that chronic inflammation is a key risk factor for aggravating the disease (2). Microglia, the dominant immune cells in the nervous system, maintain a ramified structure in the brain under normal physiological conditions. Once activated by inflammatory cytokines, microglia synthesize and secrete pro-inflammatory cytokines to promote and amplify inflammatory cascades. Gradually, the resulting inflammatory microenvironment facilitates the death of midbrain dopaminergic neurons. Furthermore, the number of microglia is strictly controlled, in part by 'activation-induced cell death' (3). Therefore, inhibiting microglia activation and maintaining a dynamic balance of microglial proliferation and apoptosis has become a strategy for the treatment of PD.

A comprehensive understanding of the molecular mechanisms underlying the occurrence and development of PD is important. MicroRNAs (miRNAs/miRs) have been identified as critical factors that participate in the pathophysiological processes of multiple diseases. The identification of miRNAs over a decade previously has improved the understanding of the post-transcriptional regulation of gene expression. miRNAs are small non-coding RNAs with an average length of 22-25 nucleotides. Compelling evidence has demonstrated that the miRNA machinery in neurodegenerative diseases, particularly in PD, is critical for the survival of neurons (4). Numerous studies have indicated that miRNAs are crucially involved in inhibiting or facilitating inflammation (5), and consequently affect the occurrence and development of PD (6). Of note, miR-195 was identified as a potential key

biomarker for PD (7,8). However, the precise role of miR-195 in PD and the underlying mechanisms remain to be fully elucidated. Preliminary evidence implied that miR-195 inhibits the release of pro-inflammatory factors via diverse signaling pathways (9,10).

Rho-associated kinase 1 (ROCK1) is a major downstream effector of the small GTPase RhoA and serves a pivotal role in cancer, particularly in cell motility, metastasis and angiogenesis. Previously, the pathophysiological effects of ROCK1 regarding other types of cell behaviors and cellular physiological processes have attracted increasing attention (11). It was suggested that ROCK1 may function as a trigger for the inflammatory response (12,13), suggesting the participation of ROCK1 in neuroinflammation associated with PD. Notably, ROCK1 has been demonstrated to be a direct target gene of miR-195 (14). Therefore, in the present study, it was hypothesized that miR-195 inhibits microglia activation, at least in part via inhibition of ROCK1.

Materials and methods

Cell culture. The BV2 cells used in the present study are immortalized microglia and were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). BV2 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China), 100 U/ml penicillin and 100 μg/ml streptomycin (Biosharp, Hefei, China). The BV2 cells were incubated in a cell incubator with a humidified atmosphere of 95% air and 5% CO_2 at 37˚C.

Cell transfection. miR-195 mimics (5'-UAGCAGCACAGAAAUAUUGGC-3'), miRNA mimics negative control (miR-NC1; 5'-UCACAACCUCCUAGAAAGAGUAGA-3'), miR-195 inhibitor (5'-GCCAAUAUUUCUGUCUGCUA-3') and miRNA inhibitor negative control (miR-NC2; 5'-UCACAACCUCCUAGAAAGAGUAGA-3') were purchased from Guangzhou RiboBio Co., Ltd., (Guangzhou, China). ROCK1 small interfering (si)RNA (siROCK1; cat. no. sc-36432) and control siRNA (cat no. sc-37007) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Cell transfection was performed when cell confluence reached 75%. Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used for cell transfection according to the manufacturer's protocols. After 6 h, the transfection mixture was replaced with fresh culture medium and the cells used for subsequent experimentation. The concentration of miR-195 mimics, miR-195 inhibitor and ROCK1 siRNA was 100 nM.

RNA extraction and reverse transcription quantitative polymerase chain reaction (qPCR). Total RNA was extracted from BV2 cells using TRIzol® reagent according to the protocol provided by the manufacturer (Thermo Fisher Scientific, Inc.). RT-qPCR was performed according to a previously described protocol (15). U6 and GAPDH were used as the invariant controls and the mRNA expression of target genes was expressed as fold changes following normalization to GAPDH or U6. The primers used are summarized in Table I.

ELISA. Microglia cells were treated with 1 μg/ml LPS for 24 h and transfection with 100 nM miR-195 mimics for another 6 h. The levels of inducible nitric oxide synthase (iNOS; cat. no., A014-1-1), interleukin (IL)-6 (cat. no. H007), tumor necrosis factor-α (TNF-α; cat. no. H052), IL-4 (cat. no. H005) and IL-10 (cat. no. H009) in whole cell lysate of microglia were determined using corresponding ELISA kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) according to the protocols provided by the manufacturer.

Cell proliferation assay. The cell proliferation rate was determined using a non-radioactive proliferation assay kit (X1327K; Exalpha Biologicals, Inc., Shirley, MA, USA) according the manufacturer's protocol. The ELISA is able to detect the incorporation of 5-bromo-2-deoxyuridine (BrdU) into newly-synthesized DNA in proliferated cells. BrdU solution was added into the culture system following treatment of the cells with 1 μg/ml LPS for 24 h and transfection with 100 nM miR-195 inhibitor and ROCK1 siRNA for 6 h. Subsequently, cells were cultured for an additional 16 h. The cell medium was removed and the BrdU immunoreactivity was detected using prediluted anti-BrdU Detector Antibody and 1X Peroxidase Goat anti-Mouse IgG included in the kit. The absorbance was detected at 450 and 540 nm. The results were expressed as a fold of the vehicle control.

Cell viability assay. Cell viability was detected using a Cell Counting Kit-8 (CCK-8) cell viability assay (Nanjing Jiancheng Bioengineering Institute). Cells were cultured in a 96-well plate ($1x10^4$/well) and then treated with 1 μg/ml LPS for 24 h and transfection with 100 nM miR-195 inhibitor and ROCK1 siRNA for 6 h. Subsequently, 10 μl CCK-8 solution was added to each well, followed by incubation at 37˚C for 1 h. The SpectraMax™ microplate spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA, USA) was used to detect the absorbance at 450 nm.

Cell apoptosis assay. Cell apoptosis was measured by flow cytometry (FCM) using an Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) kit (Vazyme Biotech, Co., Ltd., Nanjing, China). Following treatment with 1 μg/ml LPS for 24 h and transfection with 100 nM miR-195 inhibitor and ROCK1 siRNA for 6 h, BV2 cells were detached from the culture plates with 0.25% trypsin (Beyotime Institute of Biotechnology, Haimen, China) and washed with PBS 3 times. Following removal of the PBS, the cells were suspended in 1 ml binding buffer (Vazyme Biotech, Co., Ltd.) supplemented with 10 μl FITC and PI each. Cells were incubated in the dark for 15 min and then measured by FCM (BD Biosciences, San Jose, CA, USA). Data were analyzed using BD CellQuest Pro software (version 5.1; BD Biosciences, Franklin Lakes, NJ, USA) and expressed as a fold of the vehicle control.

Statistical analysis. All of the experimental data are expressed as the mean ± standard deviation. The results were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). The significance of differences between groups was determined by one-way analysis of variance followed by Dunnett's post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Primers used for analyses of mRNA expression in BV2 cells.

Gene	Orientation	Primers sequences
miR195	Forward	5'-ACGATAGCAGCACAGAAAT-3'
	Reverse	5'-GTGCAGGGTCCGAGGT-3'
iNOS	Forward	5'-CCTTGTTCAGCTACGCCTTC-3'
	Reverse	5'-CTGAGGGCTCTGTTGAGGTC-3'
IL-6	Forward	5'-ATGAACTCCTTCTCCACAAGC-3'
	Reverse	5'-CTACATTTGCCGAAGAGCCCTCAGGCTGGACTG-3'
TNF-α	Forward	5'-ATGAGCACTGAAAGCATGATC-3'
	Reverse	5'-TCACAGGGCAATGATCCCAAAGTAGACCTGCCC-3'
IL-4	Forward	5'-TCTCACCTCCCAACTGCTTC-3'
	Reverse	5'-AGAGGTTCCTGTCGAGCCGTTTCA-3'
IL-10	Forward	5'-AGGGCACCCAGTCTGAGAACA-3'
	Reverse	5'-CGGCCTTGCTCTTGTTTTCAC-3'
U6	Forward	5'-GCTTCGGCAGCACATATACTAAAAT-3'
	Reverse	5'-CGCTTCACGAATTTGCGTGTCAT-3'
GAPDH	Forward	5'-CTATGACCACAGTCCATGC-3'
	Reverse	5-CACATTGGGGGTAGGAACAC-3'

miRNA, microRNA; iNOS, inducible nitric oxide synthase; IL, interleukin; TNF-α, tumor necrosis factor-α.

Results

miR-195 is decreased in lipopolysaccharide (LPS)-stimulated microglia. To preliminarily assess the association between miR-195 expression and microglial activation, the expression of miR-195 in ramified quiescent and activated microglia was initially detected. LPS was used to establish an *in vitro* model of microglia activation. The results indicated that, compared with the control, LPS inhibited miR-195 expression in BV2 cells in a concentration-dependent manner. Notably, no significant difference in miR-195 expression between the 1 and 2 μg/ml LPS-stimulated groups was observed (Fig. 1A). In addition, LPS at the concentration of 1 μg/ml decreased the expression of miR-195 in BV2 cells in a time-dependent manner (Fig. 1B). Taken together, miR-195 expression was decreased in LPS-stimulated BV2 cells in a concentration- and time-dependent manner.

miR-195 suppresses LPS-induced activation of microglia. Microglia activation is a complex process accompanied by extensive release of inflammatory factors. Given that the expression of miR-195 was markedly decreased in LPS-induced activated BV2 cells, the function of miR-195 in microglia activation was then investigated. It was confirmed that, compared with the control, transfection with miR-195 mimics markedly increased the level of miR-195 (Fig. 2A), whereas miR-195 inhibitors decreased the level of miR-195 expression (Fig. 2B), suggesting that the gain- or loss-of-function of miR-195 was successfully achieved in the *in vitro* system. Furthermore, it was revealed that miR-195 mimics inhibited the expression of pro-inflammatory cytokines, including iNOS, IL-6 and TNF-α, but promoted the expression of anti-inflammatory cytokines in LPS-treated BV2 cells, including IL-4 and IL-10 (Fig. 2C-G). However, miR-195 inhibitors enhanced the stimulatory effects of LPS on the expression of these neuroinflammation-associated factors, characterized by an increase in the expression of iNOS, IL-6 and TNF-α, and a decrease in the expression of IL-4 and IL-10 (Fig. 2H-L). Consistent with the qPCR results, the ELISA data indicated similar variations of iNOS, IL-6, TNF-α, IL-4 and IL-10 in microglia following stimulation with LPS and miR-195 mimics or inhibitor (Fig. 2M and N). Taken together, these results demonstrated that miR-195 may have a crucial role in suppressing LPS-induced activation of microglia.

ROCK1 is a potential target of miR-195 in microglia. In view of the pivotal role of ROCK1 in modulating inflammation, the present study investigated whether ROCK1 functions downstream of miR-195 in the inflammatory signaling pathways of in PD. The results indicated that, compared with the control, LPS stimulated the mRNA and protein expression of ROCK1 in a concentration- and time-dependent manner (Fig. 3). Of note, miR-195 mimics markedly decreased the mRNA and protein levels of ROCK1, while miR-195 inhibitors enhanced the expression of ROCK1 mRNA and protein (Fig. 4). These results suggest that ROCK1 expression is positively associated with microglia activation but negatively regulated by miR-195 in microglia.

ROCK1 promotes LPS-induced microglia activation. To systematically investigate the intricate regulatory mechanisms, the potential role of ROCK1 in regulating microglia activation was additionally explored. It was confirmed that, compared with the control, siROCK1 markedly decreased the expression of ROCK1, suggesting that ROCK1 in microglia was efficiently knocked down (Fig. 5A). ROCK1 knockdown inhibited LPS-induced activation of BV2 cells, evidenced by decreased cellular iNOS, IL-6 and TNF-α, and increased IL-4 and IL-10 (Fig. 5B-F). Consistent with the qPCR results, the

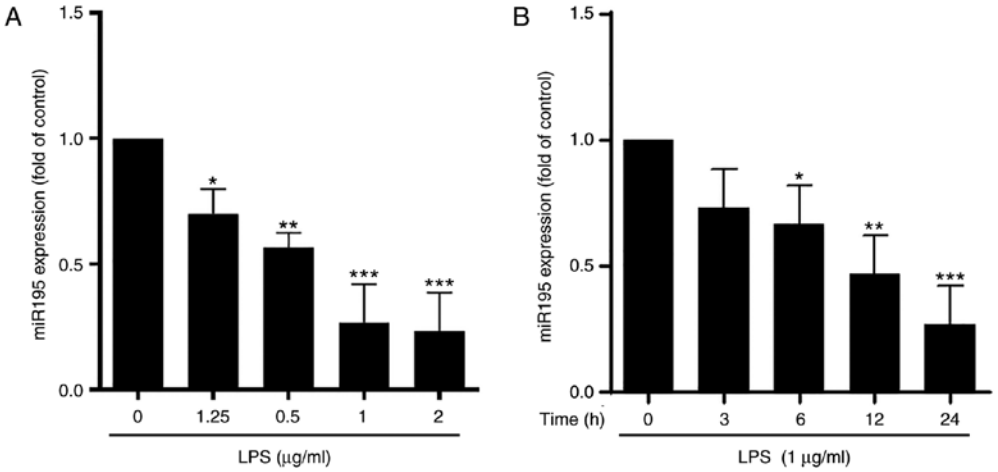

Figure 1. miR-195 is decreased in LPS-stimulated microglia. miR-195 expression in microglia was assessed by reverse transcription quantitative polymerase chain reaction analysis. U6 was used as the invariant control. (A) Concentration-dependent effects. BV2 cells were treated with LPS at the indicated concentrations for 24 h. (B) Time-dependent effects. BV2 cells were treated with LPS at 1 μg/ml for the indicated time intervals. Values are expressed as the mean ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001 vs. 0 μg/ml or 0 h group. miR, microRNA; LPS, lipopolysaccharide.

results of the ELISAs demonstrated similar changes in the levels of iNOS, IL-6, TNF-α, IL-4 and IL-10 in microglia following stimulation with LPS subsequent to transfection with siROCK1 (Fig. 5G). Taken together, these results indicated that inhibition of ROCK1 may prevent microglia activation.

miR-195/ROCK1 pathway regulates the proliferation and viability of BV2 cells treated with LPS. The role of the miR-195/ROCK1 pathway in regulating cell proliferation and viability was then investigated. The results indicated that LPS enhanced the proliferative capacity of microglia, and this effect was additionally increased in miR-195 inhibitor-transfected microglia. Notably, ROCK1 knockdown reversed the effects of miR-195 inhibitor (Fig. 6A). Consistently, the results of the cell viability assay exhibited a similar trend (Fig. 6B). Collectively, these data suggest that the miR-195/ROCK1 pathway has a crucial role in modulating the proliferation and viability of BV2 cells challenged with LPS.

miR-195/ROCK1 pathway regulates apoptosis of LPS-treated BV2 cells. To determine whether cell apoptosis was also affected by the miR-195/ROCK1 pathway, the Annexin V-FITC-PI apoptosis detection kit was used and FCM analysis was performed. The results demonstrated that miR-195 inhibitor suppressed the apoptosis of LPS-treated BV2 cells, which, was reversed by knockdown of ROCK1 (Fig. 7). It was therefore indicated that the miR-195/ROCK1 pathway is involved in regulating LPS-induced microglia apoptosis.

Discussion

PD is the second most common type of neurodegenerative disease in older individuals. However, the pathogenesis of PD remains to be fully elucidated. To the best of our knowledge, the present study was the first to reveal that miR-195 attenuates LPS-induced microglia activation and proliferation, and promotes apoptosis by inhibiting ROCK1.

Microglia are resident immune cells with key roles in regulating inflammation in cerebral diseases (16). When the inflammatory response is initiated, microglia are primarily activated by LPS secreted from adjacent inflammatory cells. Therefore, in the present study, LPS was used to establish an *in vitro* model of microglia activation. An increasing number of studies have indicated that miR-195 is a highly sensitive blood-based diagnostic biomarker for the early detection of PD due to its aberrant expression in the serum of patients with PD (7,8). Furthermore, miR-195 has been identified to participate in multiple diseases of solid organs, particularly in cancer (17) and hepatic fibrosis (18). However, few studies have focused on the role of miR-195 in microglia in the progression of PD. In the present study, the expression of miR-195 was determined in LPS-induced activated microglia. The results clearly indicated that LPS concentration- and time-dependently decreased the expression of miR-195, suggesting a negative association between miR-195 expression and microglia activation. This result was consistent with those of previous studies indicating that miR-195 was downregulated in various types of cancer, including pancreatic (19) and prostate cancer (17). Activated microglia cells serve an important role in adjusting and controlling neuroinflammation. The inflammatory response is triggered by the extensive production and secretion of cytokines and chemokines. Increasing evidence has suggested that iNOS is responsible for the mass production of NO from L-arginine. Furthermore, it has been suggested that the expression of iNOS is significantly increased in inflammatory lesions in multiple inflammation-associated diseases (20). The key pro-inflammatory cytokines also include TNF-α and IL-6, while anti-inflammatory cytokines include IL-4 and IL-10 (21). Subsequently, a gain- or loss-of-function strategy was successfully employed in the present study to determine the role of miR-195 in regulating neuroinflammation during PD. The results indicated a marked decrease in the expression of inflammatory factors and an increase in the expression of anti-inflammatory factors in BV2 cells transfected with miR-195 mimics, which were contrary to those in the miR-195 inhibitor-treated group. These results collectively validated the anti-inflammatory effects of miR-195 in PD. Consistent with this, the anti-inflammatory effects of miR-195 have been

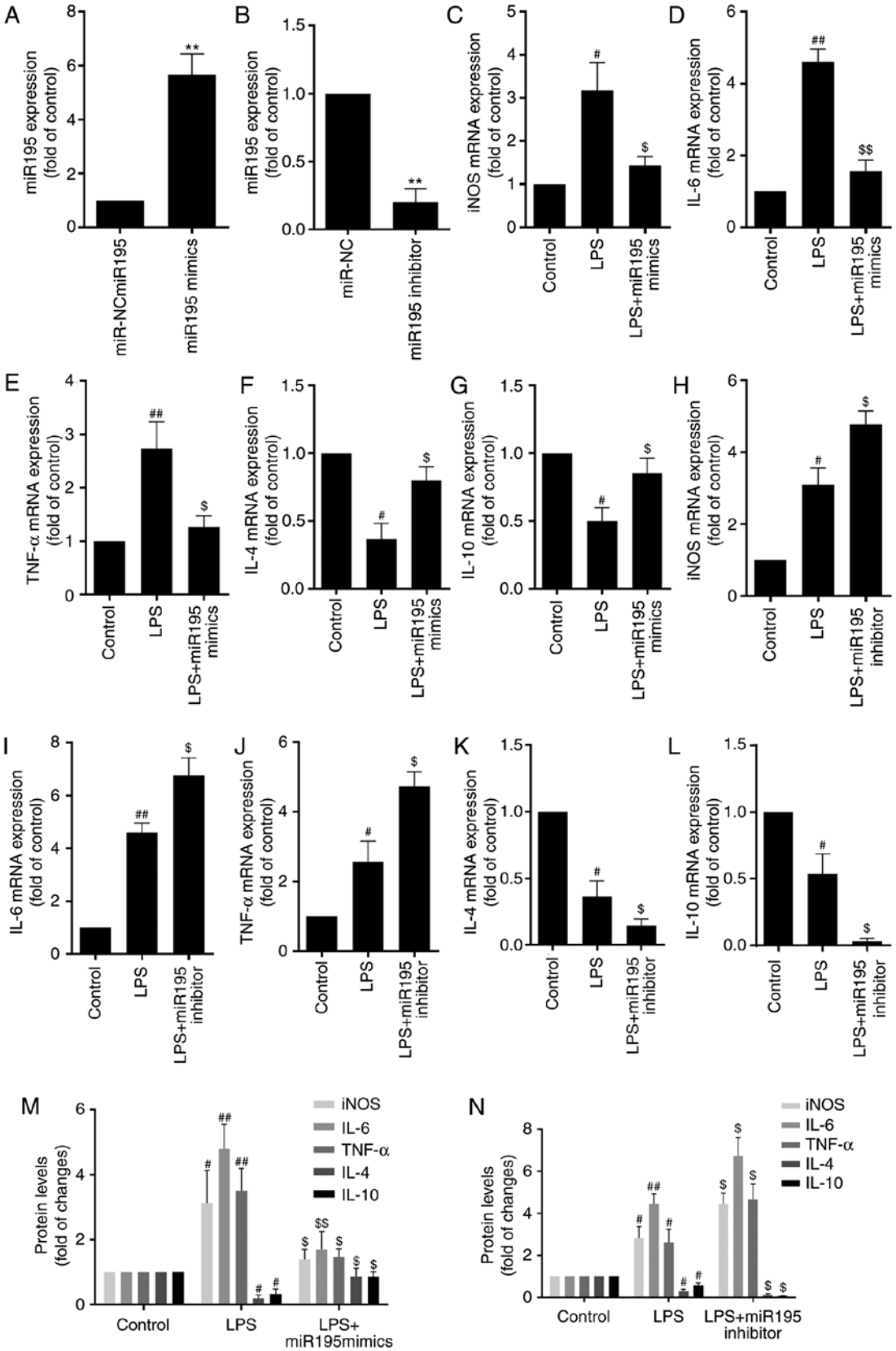

Figure 2. miR-195 suppresses LPS-induced activation of microglia. (A and B) BV2 cells were transfected with (A) miR-195 mimics and miR-195 inhibitor or (B) miR-NC and miR-195 inhibitor. miR-195 expression in microglia was determined by RT-qPCR analysis. (C-L) BV2 cells were transfected with miR195 mimics or miR-195 inhibitors and then treated with LPS (1 μg/ml) for 24 h. The expression of (C) iNOS, (D) IL-6, (E) TNF-α, (F) IL-4 and (G) IL-10 in microglia treated with miR195 mimics was determined by RT-qPCR analysis. Then, the expression of (H) iNOS, (I) IL-6, (J) TNF-α, (K) IL-4 and (L) IL-10 in microglia treated with miR195 mimics was determined by RT-qPCR analysis. GAPDH was used as the invariant control. Analysis of iNOS, IL-6, TNF-α, IL-4 and IL-10 protein expression levels in microglia treated with (M) miR195 mimics and (N) miR195 inhibitors was measured by ELISA. Values are expressed as the mean ± standard deviation. **P<0.01 vs. miR-NC group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. control; $^{\$}$P<0.05 and $^{\$\$}$P<0.01 vs. LPS group. miR, microRNA; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; IL, interleukin; TNF-α, tumor necrosis factor-α; NC, negative control; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

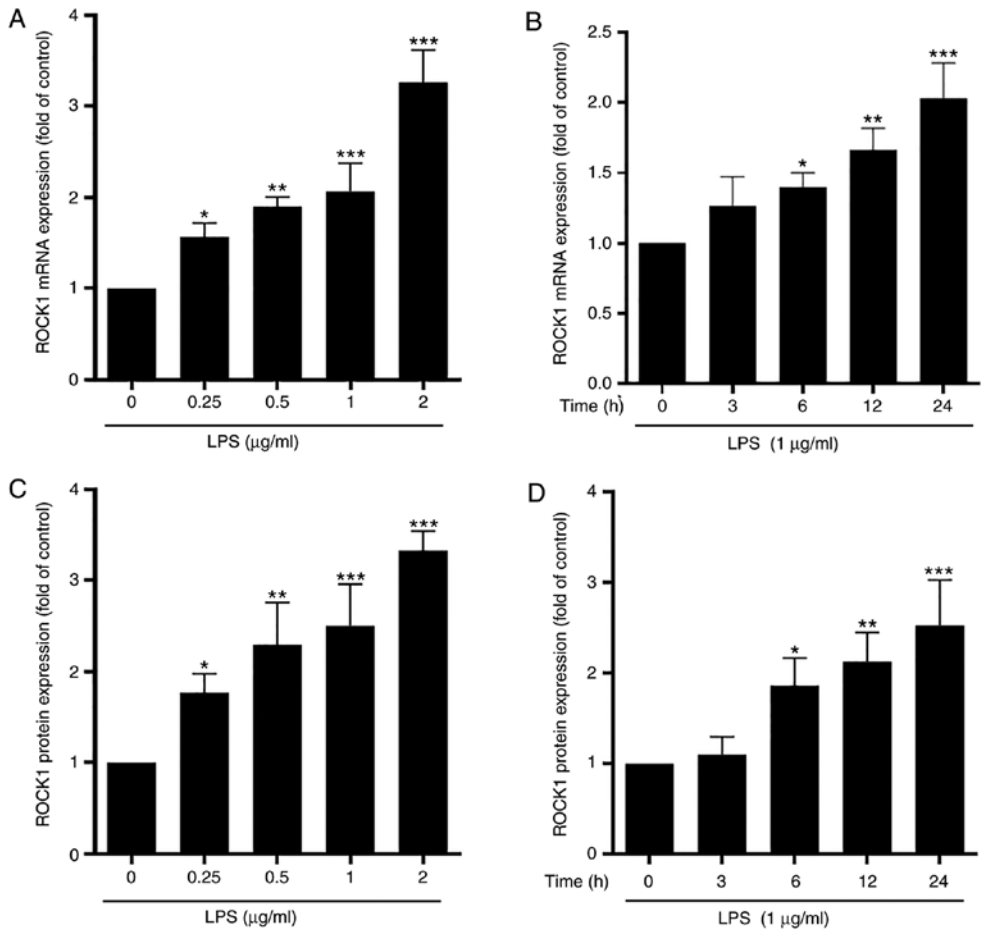

Figure 3. ROCK1 is increased in LPS-stimulated microglia. ROCK1 expression in microglia was assessed by reverse transcription quantitative polymerase chain reaction analysis. GAPDH was used as the invariant control. (A) BV2 cells were treated with LPS at the indicated concentrations for 24 h. (B) BV2 cells were treated with LPS (1 μg/ml) for the indicated time intervals. ROCK1 protein expression in microglia was assessed by ELISA. (C) BV2 cells were treated with LPS at the indicated concentrations for 24 h. (D) BV2 cells were treated with LPS (1 μg/ml) for the indicated time intervals. Values are expressed as the mean ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001 vs. 0 μg/ml or 0 h group. LPS, lipopolysaccharide; ROCK1, Rho-associated kinase 1.

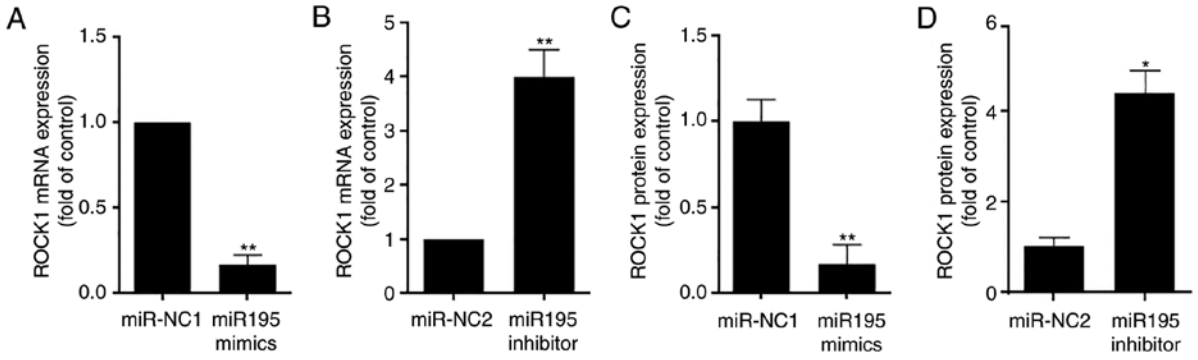

Figure 4. ROCK1 is a potential target of miR-195 in microglia. BV2 cells were transfected with miR-195 mimics, miR-195 inhibitor or the respective miR-NC. (A and B) Reverse transcription quantitative polymerase chain reaction analyses of ROCK1 mRNA expression in microglia following treatment with (A) miR195 mimics and (B) miR195 inhibitors. U6 was used as the invariant control. (C and D) Relative ROCK1 protein expression in microglia following treatment with (C) miR195 mimics and (D) miR195 inhibitors. U6 Values are expressed as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. miR-NC group. NC, negative control; miR, microRNA; ROCK1, Rho-associated kinase 1.

confirmed in various pathological conditions, including sickle cell disease (22), hepatocellular carcinoma (23) and activation of macrophages in atherosclerotic plaques (10).

Extensive studies have demonstrated that inhibition of ROCK1 ameliorates inflammation (12,13). To identify the role of ROCK1 in microglia activation, the expression of ROCK1 was detected in LPS-treated BV2 cells. The results demonstrated that the expression of ROCK1 was positively associated with microglia activation but negatively associated with the expression of miR-195 in microglia. As ROCK1 expression was increased in activated BV2 cells, a loss-of-function experiment was used. As hypothesized, siRNA-mediated ROCK1 knockdown prior

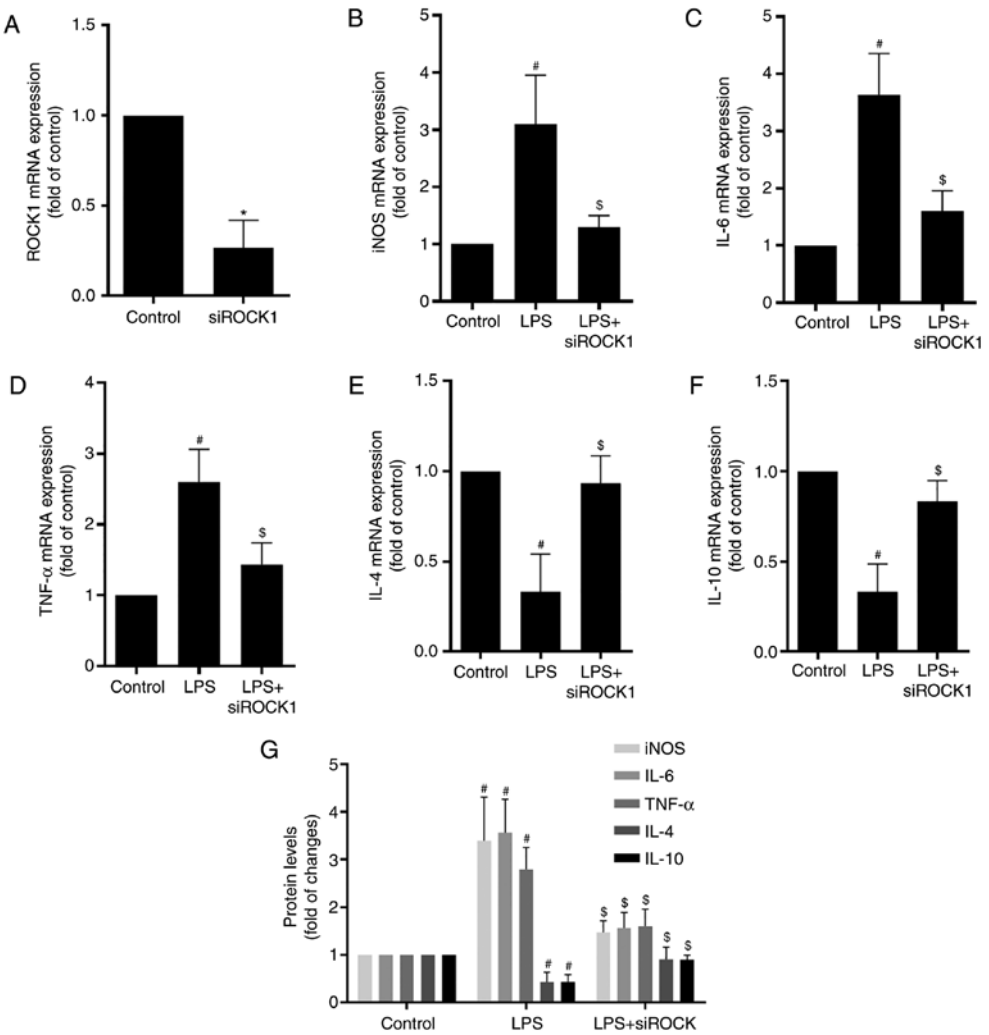

Figure 5. ROCK1 promotes LPS-induced microglia activation. (A) RT-qPCR analysis of ROCK1 expression in microglia. GAPDH was used as the invariant control. BV2 cells were transfected with siNC or siROCK1. (B-F) RT-qPCR analysis of the expression of (B) iNOS, (C) IL-6, (D) TNF-α, (E) IL-4 and (F) IL-10 in microglia. BV2 cells were transfected with siROCK1 and then treated with LPS (1 μg/ml) for 24 h. (G) ELISA of iNOS, IL-6, TNF-α, IL-4 and IL-10 protein levels in microglia. Values are expressed as the mean ± standard deviation. *P<0.05 vs. siNC group; $^{\#}$P<0.05 vs. control group; $^{\$}$P<0.05 vs. LPS group. LPS, lipopolysaccharide; NC, negative control; ROCK1, Rho-associated kinase 1; siROCK1, small interfering RNA targeting ROCK1; miR, microRNA; iNOS, inducible nitric oxide synthase; IL, interleukin; TNF-α, tumor necrosis factor-α; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

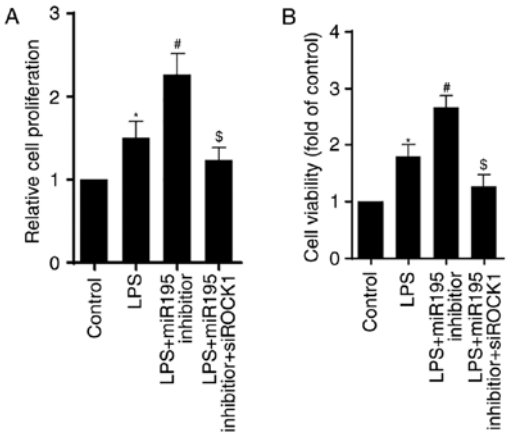

Figure 6. miR-195/ROCK1 pathway regulates cell proliferation and viability of BV2 cells treated with LPS. (A) Cell proliferation determined using a 5-bromo-2-deoxyuridine incorporation assay. (B) Cell viability determined by a Cell Counting Kit-8 cell viability assay. Values are expressed as the mean ± standard deviation. *P<0.05 vs. control group; $^{\#}$P<0.05 vs. LPS group; $^{\$}$P<0.05 vs. LPS + miR-195 inhibitor group. LPS, lipopolysaccharide; miR, microRNA; ROCK1, Rho-associated kinase 1.

to LPS challenge markedly decreased the expression of iNOS, IL-6 and TNF-α, but induced the expression of IL-4 and IL-10 compared with that in control-transfected cells, suggesting that the inhibition of ROCK1 suppressed the inflammation induced by activated microglia. Consistent with this result, a previous study demonstrated that ROCK inhibitor Y-27632 significantly decreased the levels of serum IL-6 and TNF-α, but increased the levels of IL-10 in Murphy Roths Large$^{(+/+)}$/lymphoproliferation mice (24). Therefore, these results suggested that the miR-195/ROCK1 pathway serves an important role in regulating activated microglia-mediated inflammatory responses.

The self-renewal of microglia, which contributes to maintaining a stable number of microglia over a mouse or human lifetime, is achieved based on the homeostasis between proliferation and apoptosis (25). To the best of our knowledge, the present study was the first to determine the role of the miR-195/ROCK1 pathway in the proliferation of LPS-stimulated microglia. It was indicated that miR-195 inhibitors additionally enhanced the proliferation and viability of LPS-treated microglia, while ROCK1 knockdown reversed

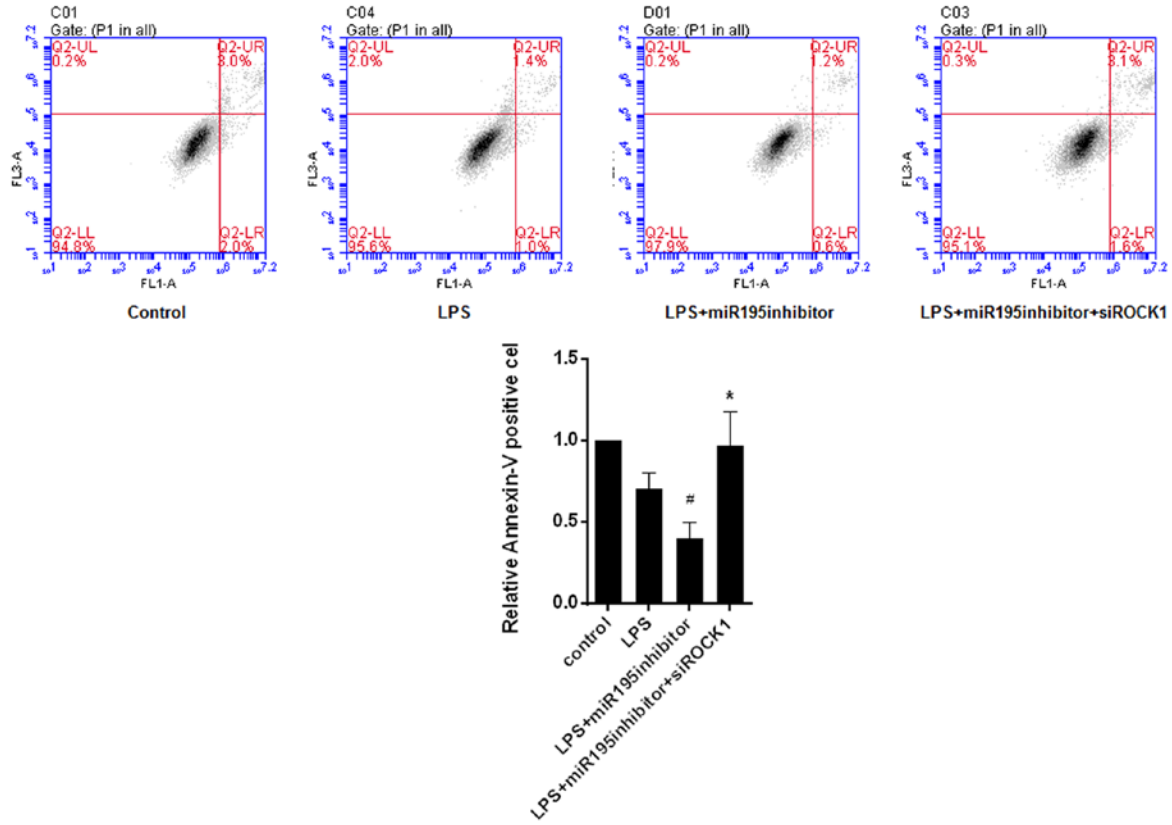

Figure 7. miR-195/ROCK1 pathway regulates apoptosis of LPS-treated BV2 cells. BV2 cells were transfected with miR-195 and/or siROCK1, and then treated with LPS for 24 h. Cell apoptosis was detected using an Annexin V-fluorescein isothiocyanate and propidium iodide kit. Values are expressed as the mean ± standard deviation. #P<0.05 vs. LPS group; *P<0.05 vs. LPS + miR-195 inhibitor group. LPS, lipopolysaccharide; miR, microRNA; siROCK1, small interfering RNA targeting Rho-associated kinase 1.

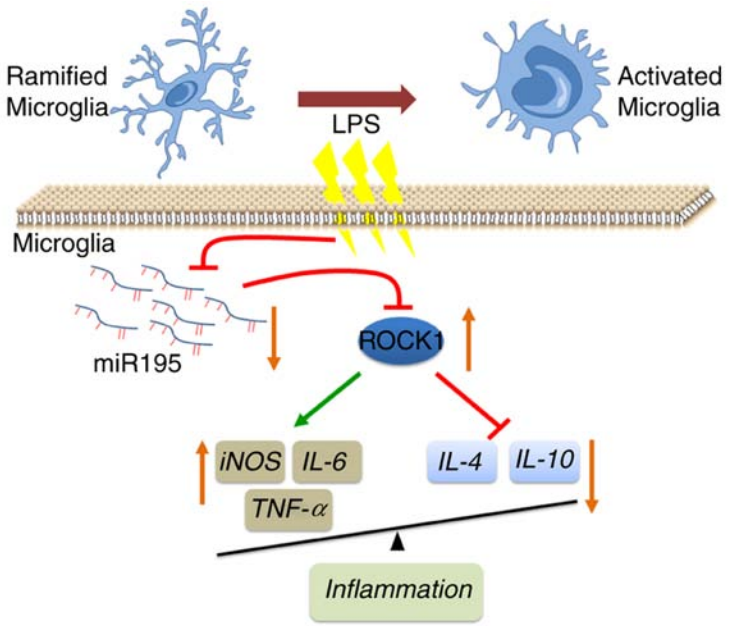

Figure 8. Diagram of the underlying mechanisms of microglia activation in PD. miR-195 deficiency induced high expression of ROCK1, which then promoted microglia activation and triggered neuroinflammation in the present *in vitro* model of PD. PD, Parkinson's disease; miR, microRNA; ROCK1, Rho-associated kinase 1; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; IL, interleukin; TNF-α, tumor necrosis factor-α.

the effects of miR-195 inhibitor. These results suggested that the miR-195/ROCK1 pathway is involved in the regulation of microglia proliferation and viability.

As the extensive production of pro-inflammatory cytokines may aggravate tissue damage and promote the progression of PD, the number of activated microglia is strictly controlled

by apoptosis, which is also called 'activation-induced cell death'. The present results indicated that miR-195 inhibitor decreased the level of apoptosis in LPS-treated BV2 cells, which was reversed by siROCK1. These results suggest that the miR-195/ROCK1 pathway mediates the induction of apoptosis of microglia activated by LPS.

The present study demonstrated that miR-195 expression is decreased in LPS-stimulated BV2 cells. Functionally, the decrease in miR-195 levels increased the expression of inflammatory cytokines, promoted cell proliferation and inhibited apoptosis in LPS-treated BV2 cells. Mechanistically, increased ROCK1 caused by loss of miR-195 promoted the activation of microglia and triggered neuroinflammation in the present *in vitro* model of PD (Fig. 8). However, additional in-depth studies are required in the future. The present results suggest that it may be valuable to explore the potential applications of miR-195 mimics in the clinical treatment of microglia-associated PD.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YR and ML conceived and designed the experiments. YR, HL, and WX performed the experiments. YR and NW analyzed the data. YR wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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