miR-335 promotes stress granule formation to inhibit apoptosis by targeting ROCK2 in acute ischemic stroke

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Abstract. Under harmful environmental conditions, stress granules (SGs), macromolecular aggregates that are associated with cell survival and death, are produced in the eukaryotic cytoplasm. However, whether and how microRNAs (miRNAs/miRs) modulate SG formation induced by acute ischemic stroke has not been investigated. In the present study, a rat model of middle cerebral artery occlusion (MCAO) was utilized and miRNA array profiling and reverse transcription-quantitative polymerase chain reaction were performed. The results revealed that miR-335 was downregulated during acute ischemic stroke, which was concomitant with reduced SG formation, enhanced apoptosis levels and increased Rho associated protein kinase 2 (ROCK2) expression. In the MCAO rat and serum-free cell models, miR-335 treatment upregulated SG formation, alleviated the ischemia-induced infarction, and decreased ROCK2 protein expression and apoptosis levels. By contrast, when compared with miR-335 treatment, the inhibition of miR-335 resulted in reduced SG formation and higher ROCK2 expression and apoptosis levels. Target prediction analysis and luciferase 3'-untranslated region reporter assay identified ROCK2 as the direct target of miR-335. Furthermore, ROCK2 silencing enhanced SG formation and attenuated the level of apoptosis in the serum-free cell model. In addition, ROCK2 silencing markedly inhibited the effect of miR-335 on SG formation and apoptosis levels. Unexpectedly, the phosphorylation of T-cell intracellular antigen-1 was significantly inhibited by miR-335 in the MCAO rat model, which provides a reasonable explanation for the promotional effect of miR-335 on SG formation by specifically targeting ROCK2. In conclusion, these results demonstrate that miR-335 promotes SG formation and inhibits apoptosis by reducing ROCK2 expression in acute ischemic stroke, which provides a possible therapeutic target for brain injury.

Introduction

Stress granules (SG) are a type of complex and dynamic foci that are generated in the cytoplasm when eukaryotic cells undergo different types of stress including endoplasmic reticulum stress, heat shock, arsenite and acute energy starvation (1). The core components of SGs include transcription factors, non-translational mRNAs, nucleating factors and RNA binding proteins (RBPs) (2). When eukaryotes undergo different types of stress, the α-subunit of eukaryotic translation initiation factor 2 (eIF2α) is phosphorylated, which triggers the formation of SGs (3). Following the phosphorylation of eIF2α, multiple RBPs including T-cell intracellular antigen-1 (TIA1), Ras-GAP SH3 domain-binding protein 1 and human antigen R self-aggregate to form the SG (4). SG formation efficiently protects important mRNAs and proteins from degradation and misfolding, which enhances cellular resistance to apoptosis (5).

Acute ischemic stroke (AIS) is the main cause of adult physical disability and mortality worldwide (6), and the process of pathogenesis involves energy failure, inflammation, excitotoxicity, oxidative stress injury and apoptosis (7). An increasing number of reports have demonstrated that apoptosis has a close association with the pathophysiology of AIS (8). Recent studies have indicated that AIS triggers the apoptotic signaling cascade, which leads to a significant level of neuronal death (9,10). Therefore, it's meaningful to investigate the association between SG and apoptosis in order to understand the disease progression of AIS.

Rho associated protein kinase 2 (ROCK2) is a 160 kDa serine/threonine kinase that possesses three different types of functional domains (11). ROCK2 is particularly abundant in brain tissues, which suggests that ROCK2 may perform a key function in cerebral physiological processes (12). According to previous studies, ROCK2 is involved in multiple cellular processes including vascular inflammation and reconstruction, cell adhesion, cell motility and apoptosis (13-15). In addition, ROCK2 has

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been reported to have a connection with a number of diseases, including cardiac hypertrophy, hypertension, atherosclerosis and ischemic stroke (16-18). However, the role of ROCK2 in the process of SG formation remains poorly understood.

MicroRNAs (miRNAs/miRs) are short non-coding RNAs that combine with the 3’ non-translated region of mRNAs and repress the expression of targeted mRNAs (19). Previous reports have demonstrated that miRNAs perform important functions in the pathogenesis of stroke (20-22), including apoptosis, cell cycle progression and cell proliferation (23). miRNAs act as pivotal mediators in the neuronal apoptosis of ischemic stroke by modulating the translation of apoptosis-associated proteins (24-27). Therefore, investigating the apoptotic mechanism of miRNAs in AIS is of great significance. In the present study, rat middle cerebral artery occlusion (MCAO) and serum-free cell models were used to investigate the anti-apoptosis effect of miRNAs and the correlation between SG formation and apoptosis in AIS.

Materials and methods

Animals and materials. A total of 108 adult male Sprague-Dawley (SD; 250-280 g; 7-8 weeks) rats were purchased from the Animal Center of Guangzhou University of Chinese Medicine (Guangzhou, China). The experiments were approved by the Care and Use of Experimental Animals Committee of Guangzhou University of Chinese Medicine and performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals. All animals were allowed to acclimate for 1 week prior to MCAO surgery. Upon receipt and throughout the study, the animals were conditioned to the same environment during the study. Food and water were available ad libitum. Room humidity (50-70%) and temperature (21-25˚C) were maintained consistently. In addition, the study room was maintained on a 12-h light/dark cycle.

MCAO model preparation and intracerebroventricular injection (ICV) of mimic and inhibitor. Under anesthesia (100 mg/kg; ketamine and 10 mg/kg xylazine, intraperitoneal injection), rats were subjected to 2 h MCAO surgery using intraluminal suture as described in a previous report (28). Body temperature was kept at ~36.5˚C with a heating pad during the surgical process until the animal recovered from anesthesia. The animals were allowed to regain full consciousness in a nursery cage, which was heated (~30˚C) and oxygenated prior to returning to their normal cage. After MCAO surgery, rats immediately underwent ICV of miR-335 mimic and inhibitor under anesthesia. The rats were fixed and one small hole was drilled into the skull of the ischemic hemisphere, in order to deliver the miR-335 mimic and inhibitor into the lateral ventricle. The stereotactic coordinates for ICV injection were as follows: -0.9 mm anterior, 1.8 mm lateral and -3.8 mm from the bregma. A total of 48 male SD rats were randomly divided into four groups (n=12 rats/group): Control group (normal saline), Model group (MCAO + normal saline), Mimic group (MCAO + miR-335 mimic, 100 pmol/rat) and Inhibitor group (MCAO + miR-335 inhibitor, 200 pmol/rat). miR-335 mimic (micrON™ miRNA agomir; 5’-ucaagagcaauaagaaagauu-3’) and miR-335 inhibitor (micrOFF™ miRNA antagomir; 5’-aucuuuuucgaauugcuucu-3’) were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China).

2,3,5-Triphenyltetrazolium chloride (TTC) staining and infarct volume calculation. To observe the dynamic alterations in SG formation, levels of apoptosis and infarct volume in the MCAO model, a total of 36 male SD rats were divided into six groups (n=6 rats/group): Control group, 0 h post-reperfusion group, 6 h post-reperfusion group, 12 h post-reperfusion group, 24 h post-reperfusion group and 36 h post-reperfusion group. Rats from each group were sacrificed with CO2 asphyxiation, and the brains were removed and coronally sectioned into six slices (2 mm/slice). The brain slices were stained in a 2% solution of TTC (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 20 min at 37˚C; the unstained area was defined as the infarct area and the infarct area volume was calculated with ImageJ software (Version 14.8; National Institutes of Health, Bethesda, MD, USA). Considering edema of the infarct hemisphere, the percentage of infarct area was calculated as follows: Infarct area (%) = [contralateral hemisphere area-(ipsilateral hemisphere area-infarct area)]/contralateral hemisphere area x100%.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Following 24 h of reperfusion, rats were sacrificed by CO2 asphyxiation. The cortices were washed with cold normal saline and fixed with 4% paraformaldehyde solution at 4˚C overnight. Frozen sections (20 µm thickness) were used in TUNEL and immunohistochemical staining. TUNEL staining was conducted following the manufacturer's protocol (Boster Biological Technology, Ltd., Wuhan, China). The percentage of positive cells in the pathological images captured was calculated with Image Pro Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA). The histopathological observations were documented using a light microscope (Leica DMI400; Leica Microsystems GmbH, Wetzlar, Germany) at x400 magnification and photographed. Each experiment was replicated 3 times and the data were presented as the mean ± standard error of the mean (SEM).

Immunohistochemical staining. Following peroxidase blocking for 30 min and 5% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) blocking for 2 h at room temperature, frozen sections (20 µm) of the brain were incubated with primary antibody (cat. no. ab140595; rabbit anti-TIA1 antibody 1:1,000; Abcam, Cambridge, MA, USA) overnight. Then these sections were incubated with secondary antibody (cat. no. ab6721; horseradish peroxidase-conjugated goat anti-rabbit; 1:5,000; Abcam) for 2 h at room temperature. The expression of TIA1 was detected using a Streptavidin Peroxidase Immunohistochemical staining kit (Tiangen Biotech Co., Ltd., Beijing, China) with DAB staining. The ratio of positive cells was calculated using Image Pro Plus software (version 6.0; Media Cybernetics, Inc.). The histopathological observations were documented by light microscopy (Leica DMI400; Leica Microsystems) at 400x magnification and images were captured. Each experiment was replicated 3 times and the data are presented as the mean ± SEM.

miRNA microarray assay. A total of 24 male SD rats were randomly divided into control and model groups (n=12 rats/group) to perform miRNA microarray assay and reverse transcription-quantitative polymerase chain reaction
(RT-qPCR). The cerebral cortices were removed from the Control and Model group rats for the miRNA microarray assay. Total RNA (600 ng) was extracted from the cortices of the different groups using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The subsequent steps of the experiment were completed by Guangzhou RiboBio Co., Ltd., according to the manufacturer's protocol. Each experiment was replicated 3 times and the data are presented as the mean ± SEM.

**Extraction of total RNA and RT-qPCR.** Total RNA was extracted from the cerebral cortex using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The concentration and purity of RNA were determined using spectrophotometers (NanoDrop ND-2000; Thermo Fisher Scientific, Inc.). The specific stem-loop primer of miR-335 was used in RT-PCR and the U6 gene was used as the reference. The following primers were used: miR-335, RT primer 5'-GTCGATACGAGGTTCCGGAGGA TTTCGCACTGACAGCACTATT-3', forward 5'-CAGC TGAAAGAATA-3', and reverse 5'-ATCCAG TGCAAGGGTCCAGG-3'; U6, forward primer 5'-GTCGATACGAGGTTCCGGAGGA TTTCGCACTGACAGCACTATT-3', and reverse primer 5'-ATCCAG TGCAAGGGTCCAGG-3'. The reverse transcription was performed by the PrimeScript RT reagent kit (Takara, Biotechnology, co., Ltd., dalian, China) according to the manufacturer's protocol, at 37°C for 15 min and 85°C for 30 sec. Light Cycler 480 SYBR Green I (Roche diagnostics, GmbH, Mannheim, Germany) was applied for fluorescent qPCR. The amplification process was as follows: Denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 15 min and 85°C for 30 sec. Light Cycler 480 SYBR Green I (Roche diagnostics, GmbH, Mannheim, Germany) was applied for fluorescent qPCR. The amplification process was as follows: Denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 15 min and 85°C for 30 sec. Light Cycler 480 SYBR Green I (Roche diagnostics, GmbH, Mannheim, Germany) was applied for fluorescent qPCR. The amplification process was as follows: Denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 15 min and 85°C for 30 sec. Light Cycler 480 SYBR Green I (Roche diagnostics, GmbH, Mannheim, Germany) was applied for fluorescent qPCR. The amplification process was as follows: Denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 15 min and 85°C for 30 sec.

**Cell culture and the serum-free cell model.** PC12 cells were purchased from ATCC and were cultured as described previously (29). The serum-free cell model was established by culturing cells in serum-free medium (RPMI-1640 medium; Gibco; Thermo Fisher Scientific, Inc.) for 0, 6, 12, 18, 24 or 30 h. With arsenite stress serving as the positive control treatment, cells were incubated with sodium arsenite (SA; Sigma-Aldrich; Merck KGaA) at the indicated concentrations (0.5 mM) for 50 min as previously described (3). The serum-free cell model was only stimulated with serum-free culture medium and did not include the arsenite stress step. After PC12 cells were stimulated for 0-30 h to ascertain the appropriate duration of stimulation, the expression of TIA1 was detected by immunofluorescent assay as the hallmark of SG production. Each experiment was replicated 3 times and the data are presented as the mean ± SEM.

**Immunofluorescence staining.** PC12 cells were grown on glass coverslips in 24 well-plates at 1x10^5 cells per well. According to the experimental protocol, cells were transfected or co-transfected with miR-335 mimic, miR-335 inhibitor, small interfering (si)-RNA-ROcK2 and pLUC-ROcK2 plasmids, and then stimulated with serum-free medium for 24 h. Following fixation with 4% paraformaldehyde for 30 min at room temperature, PC12 cells were permeabilized with 0.3% Triton X-100 and blocked with 5% BSA. Then cells were incubated with diluted primary antibody (cat. no. 12133-2-AP; rabbit anti-TIA1 antibody; 1:1,000; ProteinTech Group, Inc.) at 4°C overnight. and incubated with the secondary antibody (cat. no. ab150077; Alexa Fluor488-conjugated goat anti-rabbit antibody; 1:5,000; Abcam) for 1 h in the dark. The PC12 cells were rinsed three times with PBS and stained with DAPI solution (BIOSS, Beijing, China) for nuclear labeling. The staining results were observed with a confocal laser scanning microscope (LSM 800; Carl Zeiss AG, Oberkochen, Germany). The formation of SGs was evaluated by calculating the percentage of positive cells with Image Pro Plus software (Version 6.0; Media Cybernetics, Inc.). Each experiment was replicated 3 times and the data are presented as the mean ± SEM.

**Flow cytometry analysis.** PC12 cells were grown in 6 well-plates (5x10^4 cells per well) and divided into different groups including: Control, model, miR-335 mimic, miR-335 inhibitor, siRNA-ROcK2 and NC groups. Each group was transfected with the respective miRNA or siRNA and then incubated with serum-free medium for 24 h except the control group. Control group was incubated with RPMI-1640 medium containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). An Annexin V-FITC Apoptosis Staining/Detection kit (cat. no. ab14085; Abcam) was used to detect the levels of apoptosis. PC12 cells were digested with 0.25% Trypsin and resuspended with 500 μl binding buffer. Annexin V and propidium iodide (5 μl) were then added into the suspension and labeled for 5 min in the dark. The percentage of apoptotic cells was detected using a flow
cytometer (AccuriC6; BD Biosciences, Franklin Lakes, NJ, USA). Accuri CFlow software (Version 1.0; BD Biosciences) was used to analyze the data. Each experiment was replicated 3 times and the data are presented as the mean ± SEM.

Transfection of siRNA-ROCK2 in PC12 cells. Three different siRNA-ROCK2 inserts were purchased from Guangzhou RiboBio Co., Ltd., which specifically bound and degraded the ROCK2 mRNA. Using Lipofectamine® 2000, PC12 cells were transfected with siRNA-ROCK2-1, siRNA-ROCK2-2 and siRNA-ROCK2-3 (100 nM) for 6 h in 6-well plates (5x10^5 cells per well). After 24 h transfection, western blotting and PCR were used to select the most efficient segment. The sequences of siRNA-ROCK2 were as follows: siRNA-ROCK2-1, 5'-cAT GGT GcA TTG TGA TAc A-3'; siRNA-ROCK2-2, 5'-cAG AAG TGc AAG TcT ATT A-3'; and siRNA-ROCK2-3, 5'-cT c AAG TGCAAGTCTATTA-3'; and siRNA-ROCK2-3, 5'-cT-CT CAAG CTGTTGAAATAAGTT-3'.

Western blotting analysis. The cerebral cortices of the ischemic brain and PC12 cells were collected for immunoblotting analysis and lysed with radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, Inc.) for 20 min on ice. The cell debris were removed by centrifuging at 12,000 x g for 10 min at 4°C and the supernatant was harvested for further evaluation (stored at -80°C). The bicinchoninic acid method was used to detect protein concentration. Total protein (30 μg) was separated by 10% SDS-PAGE and transferred to 0.45 µm polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). After blocking with 5% BSA for 2 h at room temperature, the membranes were incubated with primary antibodies (ROCK2, cat. no. ab71598, 1:1,000; TIA1, cat. no. ab40595, 1:1,000; active caspase-3, cat. no. ab2302, 1:1,000; Bcl-2; cat. no. ab692, 1:1,000; GApDH, cat. no. ab8245, 1:5,000; Abcam) at 4°C overnight. After rinsing with Tris-buffered saline -0.01% Tween-20, the membranes were incubated with the secondary antibodies [horseradish peroxidase (HRP)-conjugated goat anti-rabbit, cat. no. ab6721; HRP-conjugated goat anti-mouse, cat. no. ab6789; 1:5,000; Abcam] for 1 h at room temperature. Enhanced chemiluminescence (ECL) western blotting substrate (Pierce; Thermo Fisher Scientific, Inc.) was applied to visualize the bands. Image acquisition was performed using the Tanon-6200 gel imaging system (Tanon Science and Technology Co., Ltd., Shanghai, China). Image processing software (Imaged; Version 14.8; National Institutes of Health) was used to analyze the images. Each experiment was replicated 3 times and the data are presented as the mean ± SEM.

Immunoprecipitation assays. According to the manufacturer's protocol of the Direct Magnetic IP/Co-IP kit (Pierce; Thermo Fisher Scientific, Inc.), the protein extracted from the rat brain tissues was quantified by a bicinchoninic acid assay following addition of phosphatase and protease inhibitors. A total of 500 μg protein (1-2 mg/ml) was incubated with 25 μl TIA1-coupled magnetic beads for 2 h at room temperature on a rotator. After washing and eluting the binding beads, TIA1 protein was collected and a western blotting assay was used to detect the phosphorylation levels. ECL western blotting substrate (Pierce; Thermo Fisher Scientific, Inc.) was used to visualize the bands. Image acquisition was performed with the Tanon-6200 gel imaging system (Tanon Science and Technology Co., Ltd.). Image processing software (ImageJ; National Institutes of Health) was used to analyze the images. Each experiment was replicated 3 times and the data are presented as the mean ± SEM.

Statistical analysis. The experimental data are presented as the mean ± SEM. SPSS 22.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. The figures were all produced using GraphPad Prism (Version 6.0; GraphPad Software, Inc., La Jolla, CA, USA). Student's t-test was used to analyze the differences between two groups. The comparison between multiple groups was analyzed using one-way analysis of variance followed by Tukey's multiple comparisons test as the post hoc test. P<0.05 was considered to indicate a statistically significant difference. Each experiment was replicated 3 times.

Results

SG formation is reduced and apoptosis levels are elevated in MCAO rats following 24 h of reperfusion. To evaluate the optimal point of reperfusion in the MCAO model, rats subjected to MCAO (n=36 rats, 6 rats/group) were sacrificed at 0, 6, 12, 24 or 36 h following reperfusion. The brain tissues were harvested and infarct volume was detected by TTC staining. SG formation and apoptosis levels were detected by immunohistochemical and TUNEL staining in frozen brain sections, respectively. The results revealed that SG formation peaked at 6 h post-reperfusion, then progressively decreased until 24 h and increased at 36 h (Fig. 1A). Apoptosis levels and infarct volume exhibited similar patterns during different time points following reperfusion, which peaked significantly at 24 h post-reperfusion and reduced at 36 h post-reperfusion (P<0.0001; Fig. 1B and C). At 24 h post-reperfusion, SG formation was at the lowest level, whereas apoptosis and infarct volume were at the highest levels. However, at 36 h post-reperfusion, SG formation was increased, whereas apoptosis and infarct volume were decreased; this may be due to the recovery ability of the rats. These data suggested that MCAO injury may reduce SG formation and elevate apoptosis levels at 24 h. In addition, 24 h post-reperfusion was the optimal time point to investigate the association between SG formation and apoptosis.

The expression of miR-335 is significantly decreased in MCAO rats. A total of 24 male SD rats were randomly divided into control and model groups (12 rats/group). The MCAO model was established using the suture method. Following 24 h of reperfusion, all of the rats were sacrificed by CO₂ asphyxiation, the detailed procedure were performed as described previously (30). The relative expression of miR-335 was measured by RT-qPCR (Fig. S1A). Neurological deficit scoring was performed to evaluate the model. The whole brains were removed for TTC staining. Compared with the control group, the infarct volume and behavioral score significantly increased in the model group (P<0.001 and P=0.0026; Fig. 2A and B), which verified the success of MCAO model establishment. Representative TTC-stained coronal sections of each group were presented in Fig. 2C. An miRNA microarray assay was performed to determine the miRNAs with specific expression
in the ischemic cortices of rats. When compared with the control group, there were 20 miRNAs which included 4 upregulated miRNAs and 16 downregulated miRNAs that exhibited differential expression in the model group. miR-335 exhibited the most obvious differential expression in downregulated miRNAs (Fig. 2D). This experimental result was confirmed by the qPCR assay of multiple miRNAs (miR-335, miR-361-3p, miR-290, miR-292-3p, miR-296-5p and miR-3473) in the two groups. Compared with the control group, the miR-335 expression of the model group was significantly decreased (P<0.0001; Fig. 2E). These findings suggested that miR-335 expression was reduced in MCAO rats.

miR-335 treatment increases SG formation and reduces the ischemia-induced injury, ROCK2 protein expression and apoptosis levels in MCAO rats. To investigate the protective effect of miR-335 in vivo, 48 male SD rats were randomly divided into 4 groups (control, model, miR-335 mimic and miR-335 inhibitor groups; 12 rats/group). The MCAO model was established using the suture method in rats. miR-335 mimics or the miR-335 inhibitor were immediately injected into the left lateral ventricle of the rats in the corresponding groups following neurological deficit scoring at 0 h. Following 24 h of reperfusion, all of the rats were sacrificed and brain tissues were harvested for the subsequent analyses. The TTC

Figure 1. An inverse association between SG formation and apoptosis in a MCAO model following 24 h reperfusion. (A) SG formation was inhibited in the MCAO model after 24 h reperfusion. (B) Apoptosis levels were elevated in MCAO rats following 24 h reperfusion. (C) Infarct volume was the greatest following 24 h reperfusion in the MCAO model. TTC staining was used for the measurement of infarct size. Data are presented as the mean ± standard error of the mean. ***P<0.001 and ****P<0.0001 vs. the control group; #P<0.05 6 h vs. 24 h (one-way analysis of variance test). Each experiment was replicated 3 times. The control group underwent sham surgery (the common carotid artery of rats in control group was isolated with no ligation). Scale bars, 25 µm. SG, stress granules; MCAO, middle cerebral artery occlusion.
miR-335 expression is significantly decreased in the MCAO model. (A) The infarct volume of the model group was significantly increased compared with the control group. Brain tissues were sectioned into 6 slices of 2-mm thickness. TTC staining was used for the measurement of infarct size. **P<0.01 vs. the control group (Student’s t-test). (B) The neurological score of the MCAO model group was increased significantly at 24 h. The 0 (green) and 24 h (red) neurological scores of the control group were considered as the zero points. **P<0.01, 0 h vs. 24 h (Student’s t-test). (C) Representative TTC-stained serial sections (left panel) and the most severely damaged section (right panel) of each group. The third slices of the brain were considered to be the most severely damaged sections. (D) The expression of miR-335 was decreased in the microarray assay. miR-335 was marked with a red rectangle. (E) The expression of rno-miR-335 was significantly reduced in the MCAO model. The qPCR assay of the 6 selected miRNAs (miR-335, miR-361-3p, miR-290, miR-292-3p, miR-296-5p and miR-3473) was performed to determine the differential expression in the MCAO model. Data are presented as the mean ± standard error of the mean.

staining (Fig. 3A) and neurological deficit scores were used to evaluate the MCAO model. Compared with the model group, the infarct volume and behavioral score significantly decreased in the mimic group (P=0.0029 and P=0.0104, respectively; Fig. 3B and C). In addition, when compared with the model group, ROCK2 and caspase-3 expression markedly reduced in the miR-335 mimic group (P=0.0107; P=0.0272), whereas TIA1 and Bcl-2 expression increased (P<0.0001; P=0.0020). The immunohistochemical staining of TIA1 was utilized to detect the production of SG. According to the results of immunohistochemistry, the miR-335 mimic significantly promoted TIA1 expression when compared with the model group (P<0.0001; Fig. 3E). The apoptosis levels were detected by TUNEL staining in frozen brain sections. In contrast to the model group, the miR-335 mimic significantly reduced the apoptotic levels of the ischemic brain (P=0.0236; Fig. 3F). These results indicated that miR-335 increased SG formation and reduced the ischemia-induced injury, ROCK2 protein expression and apoptosis levels in MCAO rats. By analyzing the data, the present study revealed that there was a negative correlation between ROCK2 and TIA1 expression in vivo.

miR-335 inhibits the phosphorylation level of TIA1 in a MCAO rat model. To study the potential mechanism of miR-335...
promoting SG assembly, the present study conducted immunoprecipitation to detect the phosphorylation level of TIA1 in the ischemic cortex of MCAO rats. As presented in Fig. 4A, TIA1 phosphorylation was increased in the model and inhibitor groups when compared with control group. In addition, TIA1 phosphorylation was significantly inhibited in the miR-335 mimic group compared with the model (P=0.0011). The mechanism underlying how miR-335 promotes SG formation...
and the regulatory relationship between ROCK2 and TIA1 phosphorylation is presented in Fig. 4B. These results indicated that miR-335 inhibits the phosphorylation level of TIA1 by decreasing the kinase activity of ROCK2 in MCAO rats.

miR-335 directly targets the 3'-UTR of ROCK2 mRNA in PC12 cells. Using bioinformatics software (TargetScan, www.targetscan.org; miRBase, www.mirbase.org), the present study demonstrated that there were predicted binding sites of miR-335 in the 3'-UTR of ROCK2 mRNA (Fig. 5A). To verify this result, a dual luciferase reporter system assay was performed in PC12 cells. Cells transfected with miR-335 NC and pLUc-ROCK2 WT plasmid were treated as the NC. The plasmid profile is presented in Fig. 5B. Compared with the NC group, the relative luciferase activity of cells co-transfected with pLUc-ROCK2 plasmid and miR-335 mimic was significantly decreased (P=0.0022; Fig. 5C). These results confirm that the 3'-UTR of ROCK2 mRNA may be the target of miR-335 in PC12 cells.

miR-335 promotes SG formation, reduces ROCK2 expression and suppresses cell apoptosis in serum-free cells. To investigate the protective effect of miR-335 in vitro, a serum-free cell model was used in the present study. The stimulatory time experiments indicated that 24 h stimulation generated the most obvious SG formation (Fig. 6A). Then the PC12 cells were cultured in 6/24-well plates and divided into 6 groups: Control, model, miR-335 mimic, miR-335 NC, miR-335 inhibitor and miR-335 inhibitor NC groups. The relative expression of miR-335 was measured by RT-qPCR (Fig. S1B). Following transfection with an miR-335 mimic, mimic NC, inhibitor and inhibitor NC respectively, the cells were cultured with serum-free stimulation for 24 h in RPMI-1640 medium. As presented in Fig. 6B, compared with the model group, ROCK2 expression significantly decreased and TIA1 expression increased significantly in the miR-335 mimic group (P<0.0001; P<0.0001). According to the results of immunofluorescence staining (Fig. 6C), the miR-335 mimic significantly promoted SG production (P<0.0001). As presented in Fig. 6D, compared with the model group, apoptosis levels were significantly reduced in the miR-335 mimic group (P<0.0001). These results indicated that miR-335 promoted SG formation, decreased ROCK2 expression and prevented cell apoptosis in the serum-free cell model.

ROCK2 silencing enhances SG formation and reduces the apoptosis level in serum-free cells. To analyze the effect of ROCK2 in vitro, the present study performed RNA interference (RNAi) to silence the expression of ROCK2
in serum-free cells. PC12 cells were transfected with siRNA-ROCK2-1, siRNA-ROCK2-2 and siRNA-ROCK2-3 (100 nM) for 6 and 12 h in 6-well plates (1x10^5 cells per well) to select the most effective segment and transfection time point. The relative expression of ROCK mRNA was measured by RT-qPCR (Fig. SIC). Western blotting results demonstrated that siRNA-ROCK2-1 transfected for 6 h was the most effective method to silence ROCK2 expression (Fig. 7A). Furthermore, compared with the model group, ROCK2 expression significantly decreased and TIA1 expression significantly increased in the siRNA-ROCK2 group (P=0.0013; P=0.0046; Fig. 7B). As presented in Fig. 7C, in contrast to the model group, SG formation was increased in the siRNA-ROCK2 group (P=0.0002). Flow cytometry analysis was used to evaluate apoptosis levels in the cell model. Compared with the model group, the apoptosis level significantly reduced in the siRNA-ROCK2 group (P<0.0001; Fig. 7D). These results indicated that ROCK2 silencing promoted SG formation, elevated TIA1 protein expression and prevented cell apoptosis in serum-free cells. ROCK2 silencing significantly attenuates the effect of miR-335 on SG formation and apoptosis. According to the aforementioned results, the present study revealed that miR-335 promoted SG formation and suppressed cell apoptosis in PC12 cells with serum-free stimulation. Previous studies have demonstrated that the target gene can be regulated by multiple miRNAs (31,32). Therefore, to investigate the association between the protective function of miR-335 and ROCK2, co-transfection of miR-335 and siRNA-ROCK2 was performed in the serum-free cell model. As presented in Fig. 8A, compared with the model group, ROCK2 expression was significantly inhibited in the co-transfection and siRNA-ROCK2 groups (P=0.0024; P=0.0035), and TIA1 expression was significantly increased in these two groups (P=0.0009; P=0.0030). Furthermore, there was no significant difference in ROCK2 and TIA1 expression between the siRNA-ROCK2 and co-transfection groups (P=0.9885; P=0.7090). In contrast to the model group, SG formation was markedly increased in the co-transfection and siRNA-ROCK2 groups (P=0.0019; P=0.0034), however,
no significant difference was identified between these two groups (P=0.9591; Fig. 8B). Flow cytometry analysis was used to detect the level of apoptosis. Compared with the model group, the apoptotic level was significantly downregulated in the co-transfection and siRNA-ROCK2 groups (P=0.0003; P=0.0008), however, no significant difference
was identified when comparing these two groups (P=0.7818; Fig. 8C). These results indicated that miR-335 specifically targeted the ROCK2 3′-UTR to promote SG formation, reduce ROCK2 expression and suppress cell apoptosis.

**Discussion**

Ischemic brain injury is mediated by multiple mechanisms (33) and SG assembly may serve a protective role in the delayed...
progression of ischemic brain injury. However, few studies have focused on the roles of miRNAs in the SG assembly of stroke. The present study used MCAO and serum-free cell models to mimic various pathological features in the early stages of stroke to further investigate the roles of miRNAs in SG assembly in AIS. The major results of the present study were as follows: i) An inverse association between SG formation and apoptotic level was identified in MCAO rats; ii) miR-335 was decreased in the MCAO rats and the mRNA of ROCK2 was the effective target of miR-335; and iii) the SG formation promoted by miR-335 inhibits apoptosis levels by inhibiting ROCK2 expression in AIS. The results also demonstrated that the miR-335-ROCK2 signaling pathway may serve an important role in SG formation and apoptosis levels in stroke and this pathway may be a pivotal target for treatment in AIS.

An important result of the present study was that an inverse association between SG formation and apoptosis was observed in MCAO rats. Previous studies have demonstrated that TIA1 expression could be used as a hallmark for SG presence (34-36). SG formation peaked at 6 h following reperfusion and decreased at 24 h after reperfusion; apoptosis levels were...
the lowest at 6 h post-reperfusion and peaked at 24 h following reperfusion. These observations suggest that decreasing SG formation may promote apoptotic levels and aggravate brain injury in MCAO rats. These results are consistent with a recent study that revealed that SGs have a dynamic structure and exert an anti-apoptosis function in stressed cells (37). The results of the present study suggested that the dynamic regulation of SGs may serve as a protective pathway in stroke.

Another important finding of the present study was that miR-335 promoted SG formation in MCAO rats and PC12 cells with serum-free stimulation. Microarray data and qPCR detection identified that miR-335 was specifically reduced in MCAO rats. These results are consistent with the results of previous study that suggested that dysregulation of miRNAs can be linked to ischemic stroke (38). Furthermore, it has been reported that miRNAs can affect the formation of SGs (39). However, the role of miR-335 on SG formation in response to stroke remains unknown. The present study demonstrated that miR-335 treatment increased SG formation and reduced the apoptotic level in animal and cell experiments, which protects the brain from ischemic injury. This conclusion was consistent with a previous report (30). Under stress conditions, SG formation enhances cell survival and prevents apoptosis (40). If the stress is persistent, SG will be degraded and followed by apoptosis initiation (41). There have been several studies investigating the association between SG and apoptosis (42-44). Therefore, increasing SG formation promoted by miR-335 may be a therapeutic strategy for stroke. The results of the present study suggested that the target gene silencing function of miR-335 may act as a mediator between SG formation and apoptosis in vivo and in vitro. This finding may indicate the molecular mechanism underlying the anti-apoptotic effect of SG formation in AIS.

In the present study, an important mechanism was revealed as miR-335 was observed to regulate SG formation by targeting the mRNA of ROCK2. Bioinformatics analysis predicted that ROCK2 mRNA is targeted by miR-335 and ROCK2 served an important role in ischemic stroke (45,46). ROCKs can lead to the phosphorylation of downstream substrates and induce alterations in cellular function. The present study demonstrated that ROCK2 increased along with the elevated levels of apoptosis and was negatively correlated with SG formation in vivo and in vitro. The observations of the present study suggest that ROCK2 functions as a mediator between apoptosis and SG formation. To verify ROCK2 functions in cells, RNAi experiments were performed with ROCK2 silencing and miR-335 co-transfection in this study. The results of the present study confirm that ROCK2 inhibited the expression of TIA1, which resulted in the reduction of SG formation and increased apoptosis level. In previous studies (47-50), the association between the dynamic generation of SG and the post-translational modification of crucial RBPs has been investigated. These studies demonstrated that SG nucleating factors can be phosphorylated in the serine and threonine residues by specific kinases, which generally reduce the interactions between RBPs and mRNAs, and finally decrease SG assembly. It should be noted that miR-335 inhibited TIA1 phosphorylation in MCAO rats in the present study. This may be explained by the potential mechanism that the serine/threonine residue sites of TIA1 may be phosphorylated by the kinase activity of ROCK2, which in turn impacts the assembly of SG under stress conditions.

There are several important clinical implications of these results. Firstly, miR-335 may be a potential biomarker for the early diagnosis of stroke. miR-335 was decreased in the ischemic cortices of MCAO rats, which is consistent with a previous study which revealed that plasma miR-335 expression decreased in patients with AIS (51). Secondly, there are currently few therapies to protect against the progression of stroke. The present study has revealed that miR-335 inhibited apoptosis by promoting SG formation in AIS. Finally, it is important to identify therapeutic targets for the effective protection against cerebral ischemic impairments. In conclusion, the results of the present study support novel potential therapeutic targets for AIS based on the regulation of the miR-335-ROCK2 signaling pathway in SG formation.

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Availability of data and materials

The data and materials generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

WS performed the experiments and wrote the manuscript. SY performed the experiments and analyzed the data. ZR, XL, ZW and YL performed the cell and animal experiments. JZ and RD provided the reagents/materials. SZ performed the histological examinations of the brain. YwL designed the experiments and analyzed the data. DC conceived and designed the experiments. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Care and Use of Experimental Animals Committee of Guangzhou University of Chinese Medicine and performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Patient consent for publication

Not applicable.

Competing interest

All authors declare that they have no competing interest to disclose.
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