

## Original Article

# LncRNA ROR is involved in cerebral hypoxia/reoxygenation-induced injury in PC12 cells via regulating miR-135a-5p/ROCK1/2

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**Abstract:** Ischemic stroke is a common cerebrovascular disease with high morbidity, disability and mortality. LncRNAs were involved in ischemia/reperfusion injury. The present study aims to investigate whether lncRNA ROR can promote the cerebral hypoxia/reoxygenation (H/R) injury in vitro, a cellular model of cerebral ischemia/reperfusion injury, through inhibiting the expression of miR-135a-5p or upregulating the expression of ROCK1 and ROCK2. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to detect the lncRNA ROR expression in PC12 cells induced by H/R and verify the transfection effect. ROS, LDH, SOD and MDA levels were detected by respective kits. CCK-8 assay and flow cytometry analysis respectively detected the cell viability and cell apoptosis. Western blot analysis was to analyze the expression of apoptosis-related proteins (Bcl-2, Bax and cleaved caspase3). Immunofluorescent staining detected the ROCK1/2 expression. As a result, lncRNA ROR expression was increased in the PC12 cells induced by H/R. lncRNA ROR overexpression could aggravate injury of PC12 cells induced by H/R. And, lncRNA ROR overexpression could decrease viability and promote apoptosis of PC12 cells induced by H/R. In addition, miR-135a-5p was demonstrated to be a target of lncRNA ROR and lncRNA ROR improved H/R injury in PC12 cells by up-regulating the expression of miR-135a-5p via down-regulating ROCK1/2 expression. In conclusion, this study indicated that lncRNA ROR could promote the cerebral H/R injury by inhibiting the expression of miR-135a-5p or upregulating the expression of ROCK1/2. And, miR-135a-5p overexpression could improve the cerebral H/R injury by inhibiting the expression of ROCK1/2.

**Keywords:** lncRNA ROR, cerebral injury, ischemia/reperfusion, miR-135a-5p

## Introduction

Ischemic stroke is an arterial blockage in the brain, which is caused by thrombosis and embolism [1]. Ischemic stroke is a common clinical disease which seriously threatening the public health [2, 3]. On the one hand, reperfusion is important for brain function restoration induced by cerebral ischemia. On the other hand, reperfusion can cause overproduction of free radicals which leading to reperfusion injury, called ischemia-reperfusion injury [4]. Therefore, it is necessary to relieve cerebral ischemia/reperfusion injury for the treatment of ischemic stroke.

Long noncoding RNAs (lncRNAs) are transcripts more than 200 nucleotides in length without or with limited protein coding [5]. Previous studies have shown that lncRNAs were the regulatory factor in the development of H/R injury [6-8].

For instance, Yin et al. found that knockdown of SNHG12 inhibited N2a cells proliferation and promoted N2a cells apoptosis by upregulating the expression of miR-199a in the protection of cerebral ischemia/reperfusion injury [9]. Wei et al. showed that silencing of lncRNA AK038897 inhibited DAPK1 expression and OGD/R-induced N2a cell apoptosis by upregulating the expression of miR-26a-5p [10]. Zhang et al. revealed that the expression of lncRNA ROR was increased in myocardial ischemia and hypoxia injury and lncRNA ROR could relieve myocardial cerebral ischemia/reperfusion injury via p38/MAPK signal pathway [11]. Therefore, we predicted that lncRNA ROR maybe also play its role in the cerebral H/R injury.

Apoptosis has been considered as the basic mechanism of many cerebrovascular diseases. The cerebral ischemia/reperfusion injury is often related to the cell apoptosis [9, 10]. The

process of apoptosis is complex. The activation of caspase cascade plays a key role in the process of apoptosis. Activated caspase acting on the substrate makes the substrate to be decomposed, which leading to apoptosis [12]. ROCK1/2 are the pyrolysis product of activated caspase3 and caspase2 [13], which are related to the apoptosis mediated by caspase [14]. Therefore, whether the down-regulation of ROCK1/2 can inhibit the apoptosis process of hypoxic cerebral cells is worthy to be discussed.

MicroRNAs (miRNAs) are non-coding and single-stranded RNA molecules with about 18-24 nucleotides in length which modulate gene expression at the post-transcriptional level [15, 16]. For instance, downregulation of miRNA-29b may deteriorate cardiac functional recovery through increasing susceptibility of myocardium to cerebral ischemia/reperfusion injury in obese mice [17]. Liang et al. indicated that miR-125b was overexpressed in brain cerebral ischemia/reperfusion injury which could be improved by inhibiting the expression of miR-125b via the CK2  $\alpha$ /NADPH oxidative signaling pathway [18]. Many studies demonstrated that miR-135a was involved in the development of multiple cancers and changes of miR-135a expression could regulate the proliferation and apoptosis of cancer cells [19-22]. Liu et al. demonstrated that miR-135a overexpression could alleviate OGD/R-induced injury in neurons by inhibiting the expression of GSK-3 $\beta$  and promoting the expression of Nrf2 [23]. Wang et al. found that miR-135a could reduce the myocardial ischemia-reperfusion injury in rats binding to protein tyrosine phosphatase 1B [24]. Overall, miRNAs play an important role in reperfusion injury in multiple systems. However, little is known about the role of miR-135a-5p in cerebral H/R injury.

Therefore, this study aims to detect the expression of lncRNA ROR in cerebral H/R cells and analyze the effect of lncRNA ROR on ROCK1 and ROCK2 signaling proteins. In addition, whether lncRNA ROR can promote the cerebral H/R injury by inhibiting the expression of miR-135a-5p or upregulating the expression of ROCK1/2 is investigated in this study.

### Materials and methods

#### *Cell culture*

PC12 cells were purchased from the American Type Culture Collection (ATCC, American) and

cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum. The PC12 cells were cultured at 37°C with 5% CO<sub>2</sub> in a humid incubator.

#### *Induction of H/R model*

After cultured at 37°C with 5% CO<sub>2</sub> for 24 h, PC12 cells were placed in hypoxic conditions (3% O<sub>2</sub>/5% CO<sub>2</sub>/92% N<sub>2</sub>) for 2 h. Then, PC12 cells were cultured in normal condition for 12 h. The PC12 cells cultured in normal condition were identified as the control.

#### *Plasmid construction and transfection*

We amplified and cloned the open reading frame (ORF) of lncRNA ROR, siRNA-lncRNA ROR-1 and siRNA-lncRNA ROR-2 into the expression vector pcDNA3.1 (+) to obtain pcDNA-lncRNA ROR plasmid, siRNA-lncRNA ROR-1 plasmid and siRNA-lncRNA ROR-2 plasmid, which were transfected into PC12 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to verify transfection effect.

#### *Cell transfection*

MiR-135a-5p mimic and mimic control (miR-NC) were brought from RiboBio (Guangzhou, China) and transfected into PC12 cells using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The PC12 cells in the control group were received no any treatment. RT-qPCR was used to verify transfection effect.

#### *RT-qPCR analysis*

Total RNA was extracted from PC12 cells with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and Taqman® MicroRNA Assay Kit (Applied Biosystems) were respectively applied for cDNA synthesis and PCR reactions. The amplification conditions were as follows: 95°C for 10 min, followed by 38 cycles of 95°C for 10 sec and 58°C for 60 sec. U6 and GAPDH were used as endogenous controls, respectively. The primer sequences for qPCR were as follows: U6 forward, 5'-CTCGCTTCGGCAGCACA-3', and reverse, 5'-TGGTGTCTGTGAGTCG-3'; miR-135a-5p forward, 5'-ACAGCCTCCATGGGAATGGAAGCAGGTTGA-3', and reverse, 5'-TGGAGTGTGGCGTTCG-3'; GAPDH forward, 5'-GAAGGTG-

AAGGTCGGAGTC-3', and reverse, 5'-GAAGATG-GTGATGGGATTC-3'; lncRNA ROR forward, 5'-AGTCCTGTTTCATCTCCAGT-3', and reverse, 5'-ACTGGGGTCCATCTCTCCGT-3'. The 2<sup>-ΔΔCt</sup> method was for the quantification of gene expression.

## Detection of ROS level

The reactive oxygen species (ROS) level of PC12 cells was detected with ROS ELISA assay kit (Shanghai Duma Biotechnology Co., Ltd, China) in normoxia group, H/R group and H/R treatment following transfecting pcDNA-lncRNA ROR or siRNA-lncRNA ROR-1/2 according to the manufactures' instructions [25].

## LDH, SOD and MDA levels

The lactate dehydrogenase (LDH), superoxide dismutase (SOD) and malondialdehyde (MDA) levels of PC12 cells were detected for evaluation of injury degree of PC12 cells according to the users' instructions [26]. The commercial kits for determining LDH, SOD and MDA were bought from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

## CCK-8 assay

Cell viability was determined by the Cell Counting Kit-8 (CCK-8) Kit (Beyotime Biotechnology, Shanghai, China). Briefly, PC12 cells induced by H/R were seeded in 96-well plates and cultured for 24 h. After transfection, 10 μl CCK8 solution was added and incubated for 2 h at 37°C. The OD value reflecting viability of PC12 cells was measured at 450 nm basing on a Bio-Rad microplate reader (Bio-Rad, Hercules, CA, United States).

## Flow cytometry analysis

After H/R treatment and transfection, PC12 cells were placed into 5 cm plates and the number of PC12 cells in each plate is about  $1 \times 10^5$ . Afterwards, Annexin V/PI apoptosis detection kit (KeyGEN Biotech, Nanjing, China) was used to stain the plates for the examination of cell apoptosis. Finally, stained PC12 cells were analyzed by a FACSCalibur flow cytometer (BD, New Jersey) within 1 h.

## Western blot analysis

After H/R treatment and transfection, total proteins were extracted from PC12 cells using

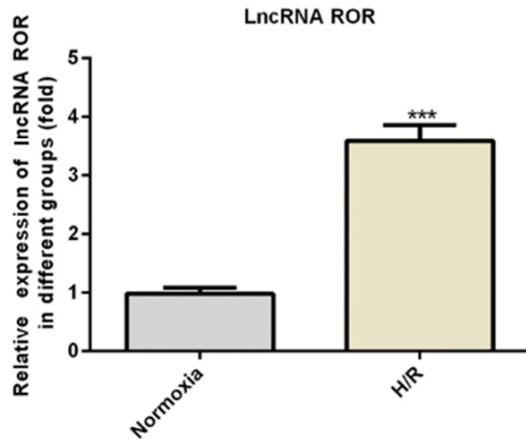
RIPA lysis buffer (Biocolors Biotechnology Co., Shanghai, China). And, BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used to detect the protein concentration. Equal amount of proteins were separated on 12% SDS-PAGE gels. Then, the separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA), followed by 5% nonfat milk in TBST (Tris-buffered saline plus 0.1% Tween 20) incubated for 1 h. The membrane was then incubated with primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, USA) overnight at 4°C. Finally, the membrane was examined by an enhanced chemiluminescence system (Super Signal West Dura Extended Duration Substrate; Thermo Fisher Scientific, Inc.). The primary antibodies against Bcl-2, Bax, cleaved caspase3 and GAPDH were purchased from Cell Signaling Technology.

## Immunofluorescent staining

After H/R treatment and transfection, PC12 cells were fixed with 4% paraformaldehyde (PFA) and then washed with PBS twice. PC12 cells were transparent with 0.5% Triton x-100 and sealed with 10% donkey serum for at least 30 min. The diluted primary antibodies (ROCK1 (rabbit) and ROCK2 (rabbit)) were added and PC12 cells were cultured in a humid incubator at 4°C overnight. Following PBS washing, the relative secondary antibodies were added and PC12 cells were incubated at room temperature for 2 h in the dark. After three times PBS washing, 4', 6-diamidino-2-phenylindole (DAPI) was to stain nucleus of PC12 cells which sealed by mounting medium. Finally, the slides were observed and photographed under fluorescent microscopy (OLYMPUS\_BH2 model).

## Dual-luciferase reporter assay

miRcode (<http://www.mircode.org/>) forecasted that miR-135a-5p was a potential target of lncRNA-ROR. Dual-luciferase reporter assay was used to verify the prediction. PC12 cells were co-transfected with luciferase reporters and a miR-135a-5p mimic or mimic control (NC) vector using Lipofectamine® 2000. TargetScan software (<http://www.targetscan.org>) predicted that ROCK1/2 were the potential targets of miR-135a-5p. Dual-luciferase reporter assay was performed to verify the prediction. PC12



**Figure 1.** lncRNA ROR expression in PC12 cells was detected by RT-qPCR analysis (n = 3). \*\*\*P < 0.001 vs. Normoxia group.

cells were co-transfected with pGL3-ROCK1/2 3'UTR plasmid (containing mutant ROCK1/2 3'UTR or wild-type ROCK1/2 3'UTR) and a miR-135a-5p mimic or mimic control (NC) vector using Lipofectamine® 2000. After incubation for 48 h, luciferase activity was detected by the Dual-Luciferase Reporter Assay System.

#### Statistical analysis

All data were expressed as means  $\pm$  standard deviation. The statistical analysis was basing on SPSS 22.0 software (IBM Corp., Armonk, NY, USA). Differences between multiple groups were analyzed using one-way analysis of variance with Tukey's post hoc test and differences between two groups were analyzed by the T-test. P < 0.05 was considered to be statistically significant difference.

## Results

### *lncRNA ROR expression in PC12 cells*

RT-qPCR analysis was used to analyze the lncRNA ROR expression in H/R treated PC12 cells and normal cells. The result of **Figure 1** indicated that lncRNA ROR expression was up-regulated in H/R treated PC12 cells compared with PC12 cells in Normoxia group.

### *lncRNA ROR overexpression increases the damage of H/R treated PC12 cells*

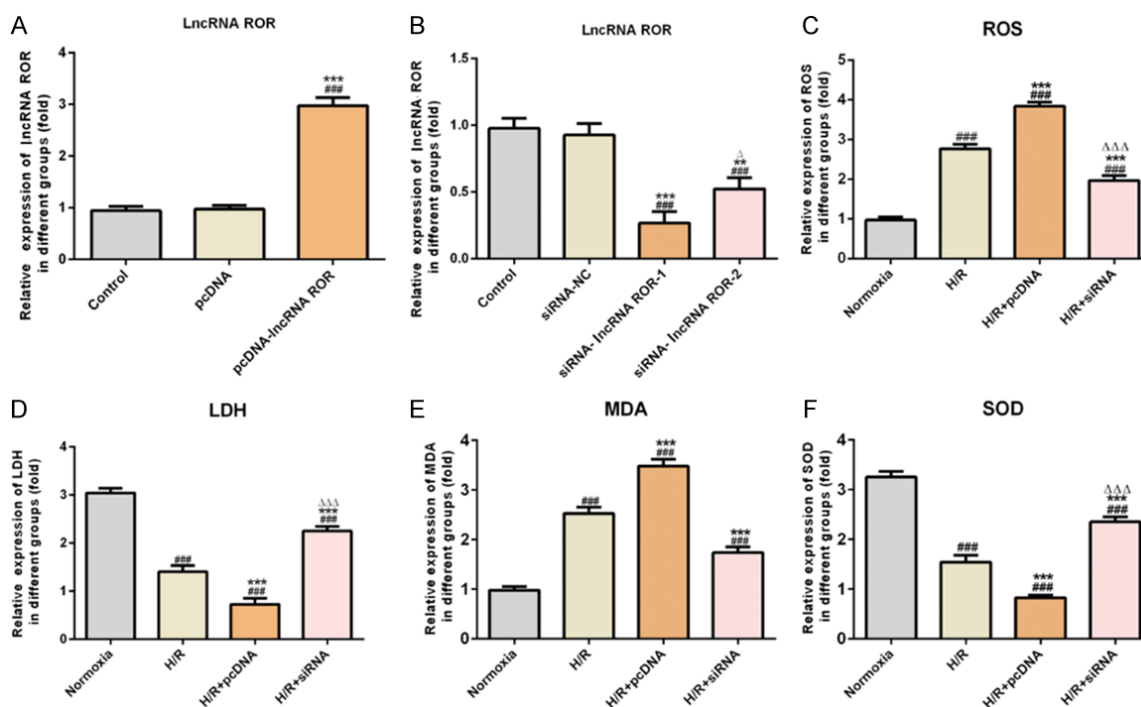
The transfection effects of lncRNA ROR in PC-12 cells were confirmed by RT-qPCR analysis. Compared with the control group and pcDNA

group, the lncRNA ROR expression was obviously increased in PC12 cells transfected with pcDNA-lncRNA ROR (**Figure 2A**) and decreased in PC12 cells transfected with siRNA-lncRNA ROR-1 and siRNA-lncRNA ROR-2 (**Figure 2B**). The lncRNA ROR expression in siRNA-lncRNA ROR-1 group was lower than that in siRNA-lncRNA ROR-2 group. Therefore, siRNA-lncRNA ROR-1 was chosen for the subsequent experiment. As shown in **Figure 2C**, compared with the Normoxia group, ROS level was increased in PC12 cells treated with H/R (H/R group) and ROS level in H/R + pcDNA group was higher than that in H/R group while the ROS level in H/R + siRNA group was decreased but still higher than that in Normoxia group. As shown in **Figure 2D-F**, compared with the Normoxia group, the levels of LDH and SOD were decreased while MDA level was increased in H/R group. The levels of LDH and SOD were obviously decreased and MDA level was increased in H/R + pcDNA group than that in H/R group while the levels of LDH and SOD were obviously increased and MDA level was decreased but could not return to the levels in Normoxia group. Therefore, lncRNA ROR overexpression promotes the oxidative damage of H/R treated PC12 cells and lncRNA ROR inhibition can attenuate the oxidative damage.

### *lncRNA ROR overexpression inhibits viability and promotes apoptosis of H/R treated PC12 cells*

The viability of H/R treated PC12 cells transfected with pcDNA-lncRNA ROR and siRNA-lncRNA ROR at 24, 48 and 72 h was examined by CCK-8 assay. Compared with the Normoxia group, the viability of PC12 cells was decreased after the treatment of H/R and the viability of PC12 cells in H/R + pcDNA group was lower than that in H/R group. And, the viability of PC12 cells in H/R + siRNA group rose up but still lower than that in Normoxia group (**Figure 3A**). After transfection for 72 h, flow cytometry analysis was used to analyze the cell apoptosis. After treatment of H/R, the apoptosis of PC12 cells was increased and the apoptosis of PC12 cells became more numerous in H/R + pcDNA-lncRNA ROR group. The apoptosis of PC12 cells was decreased in H/R + siRNA-lncRNA ROR-1 group while still higher than that in Normoxia group (**Figure 3B**). The western blot analysis for the detection of apoptosis-related protein indicated that compared with the Normoxia group,





**Figure 2.** lncRNA ROR overexpression increases the damage of H/R treated PC12 cells. A. The transfection effect of pcDNA-lncRNA ROR was examined by RT-qPCR analysis (n = 3). \*\*\*P < 0.001 vs. control group. \*\*\*P < 0.001 vs. pcDNA group. B. The transfection effect of siRNA-lncRNA ROR-1 and siRNA-lncRNA ROR-2 was examined by RT-qPCR analysis (n = 3). \*\*\*P < 0.001 vs. control group. \*\*\*P < 0.001 vs. siRNA-NC group. <sup>Δ</sup>P < 0.05 vs. siRNA-lncRNA ROR-1 group. C-F. The levels of ROS, LDH, MDA and SOD were detected by respective kits (n = 3). \*\*\*P < 0.001 vs. Normoxia group. \*\*P < 0.01 and \*\*\*P < 0.001 vs. H/R group. <sup>ΔΔ</sup>P < 0.001 vs. H/R + siRNA group.

the expression of Bax and cleaved caspase3 was increased and Bcl-2 expression was decreased in H/R group. Compared with the H/R group, the expression of Bax and cleaved caspase3 was increased and Bcl-2 expression was decreased in H/R + pcDNA group while the expression of Bax, cleaved caspase3 and Bcl-2 in H/R + siRNA group tended to the levels in Normoxia group but could not reach the levels in Normoxia group. Therefore, lncRNA ROR overexpression decreases viability and increases apoptosis of H/R treated PC12 cells and lncRNA ROR inhibition can reverse these changes.

#### *lncRNA ROR overexpression promotes the damage of H/R treated PC12 cells by activating the expression of ROCK1 and ROCK2*

The expression of ROCK1 and ROCK2 was detected by immunofluorescent staining. As shown in **Figure 4A** and **4B**, compared with the Normoxia group, the expression of ROCK1 and ROCK2 was increased in H/R group and these protein expression was also increased in H/R +

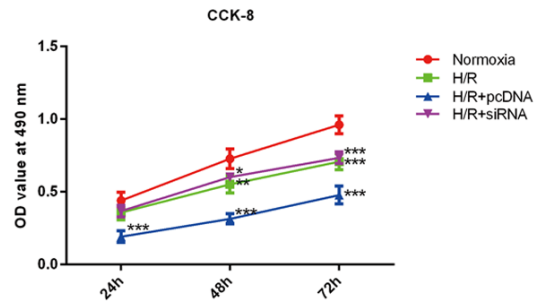
pcDNA group compared with the H/R group. However, the expression of ROCK1 and ROCK2 was decreased in H/R + siRNA group and H/R + pcDNA + GSK group compared with the H/R group.

#### *lncRNA ROR directly targets miRNA-135a-5p*

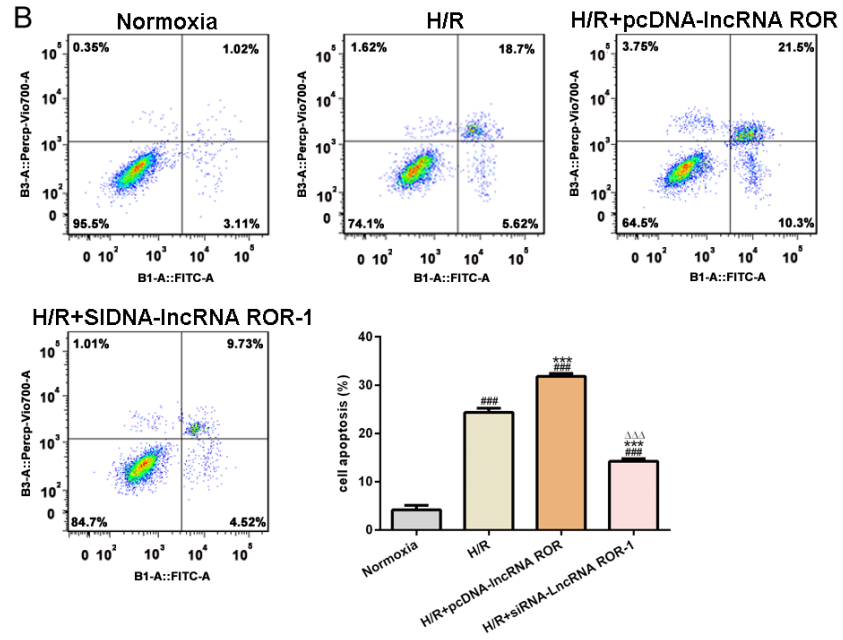
The transfection effects of miR-135a-5p mimic was demonstrated by RT-qPCR analysis and the results showed that miR-135a-5p expression was increased in miR-135a-5p mimic group compared with the control group and miR-NC group (**Figure 5A**). The miRcode tool predicted that lncRNA ROR directly targeted miR-135a-5p and the results of dual-luciferase reporter assay showed that fluorescence intensity was decreased in PC12 cells after transfected with pcDNA-lncRNA ROR and miRNA-135a-5p (**Figure 5B**). As a result, lncRNA ROR directly targets miRNA-135a-5p 3'UTR-WT. And, lncRNA ROR overexpression could reduce the expression of miRNA-135a-5p while lncRNA ROR inhibition could reverse the changes (**Figure 5C**). The lncRNA ROR was downregulat-

# Role of lncRNA ROR/miR-135a-5p in cerebral injury

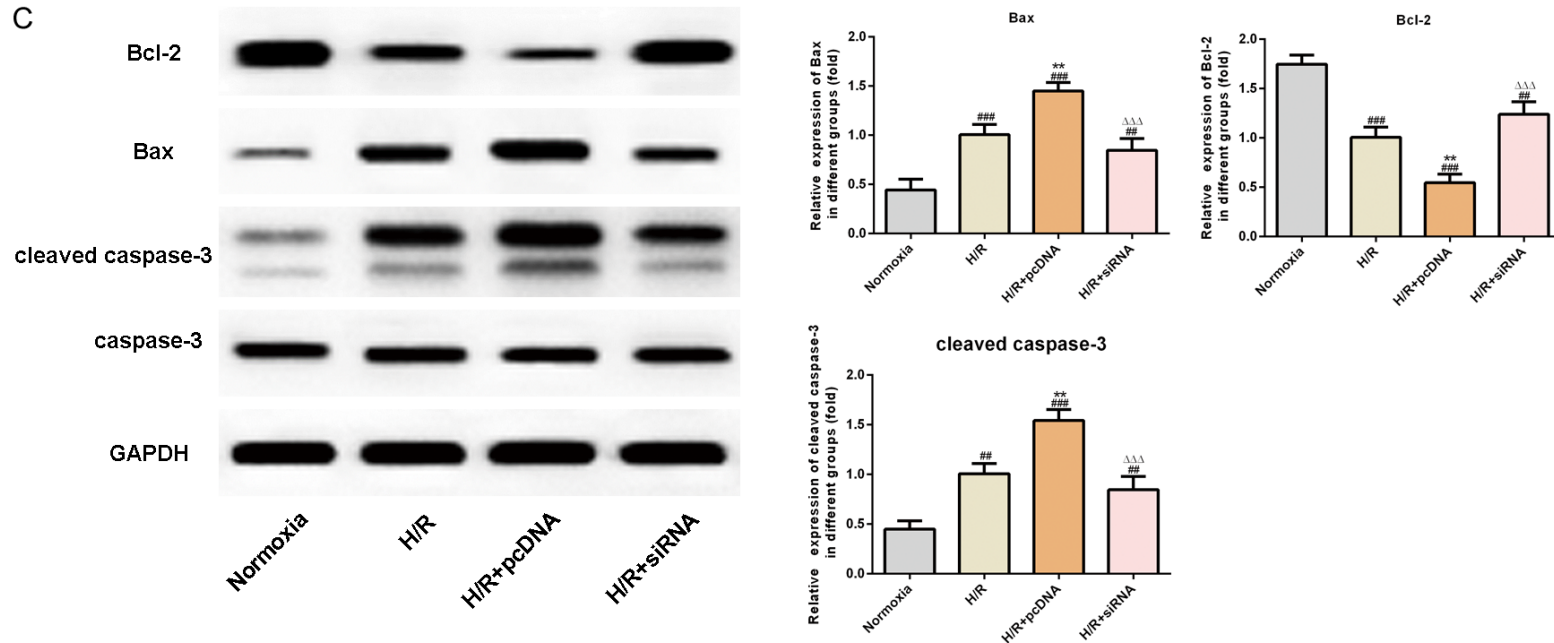
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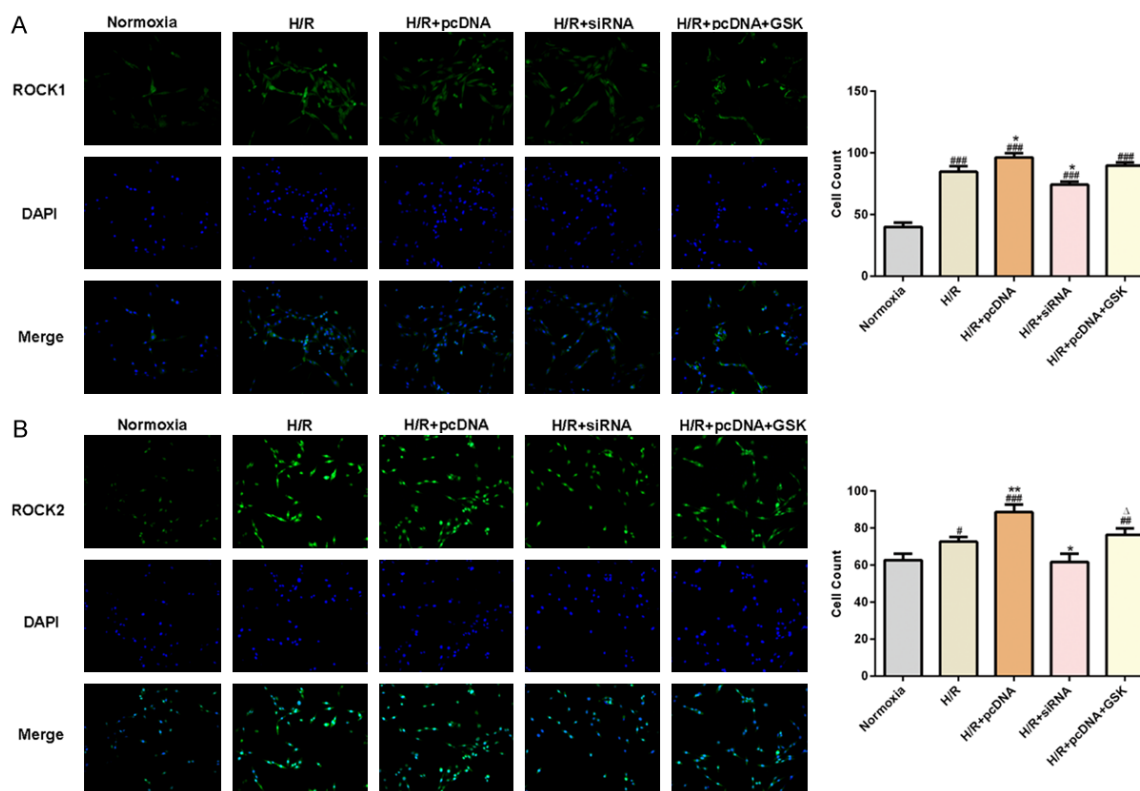
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C



**Figure 3.** lncRNA-ROR overexpression inhibits viability and promotes apoptosis of H/R treated PC12 cells. A. The cell viability of H/R treated PC12 cells after transfection was detected by CCK-8 assay (n = 3). \*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.001 vs. Normoxia group. B. The cell apoptosis of H/R treated PC12 cells after transfection was detected by flow cytometry assay (n = 3). ###P < 0.001 vs. Normoxia group. \*\*\*P < 0.001 vs. H/R group.  $\Delta\Delta\Delta$ P < 0.001 vs. H/R + pcDNA-lncRNA ROR group. C. The apoptosis-related proteins (Bcl-2, Bax and cleaved caspase3) were detected by western blot analysis. ##P < 0.01 and ###P < 0.001 vs. Normoxia group. \*\*P < 0.01 vs. H/R group.  $\Delta\Delta\Delta$ P < 0.001 vs. H/R + pcDNA group.



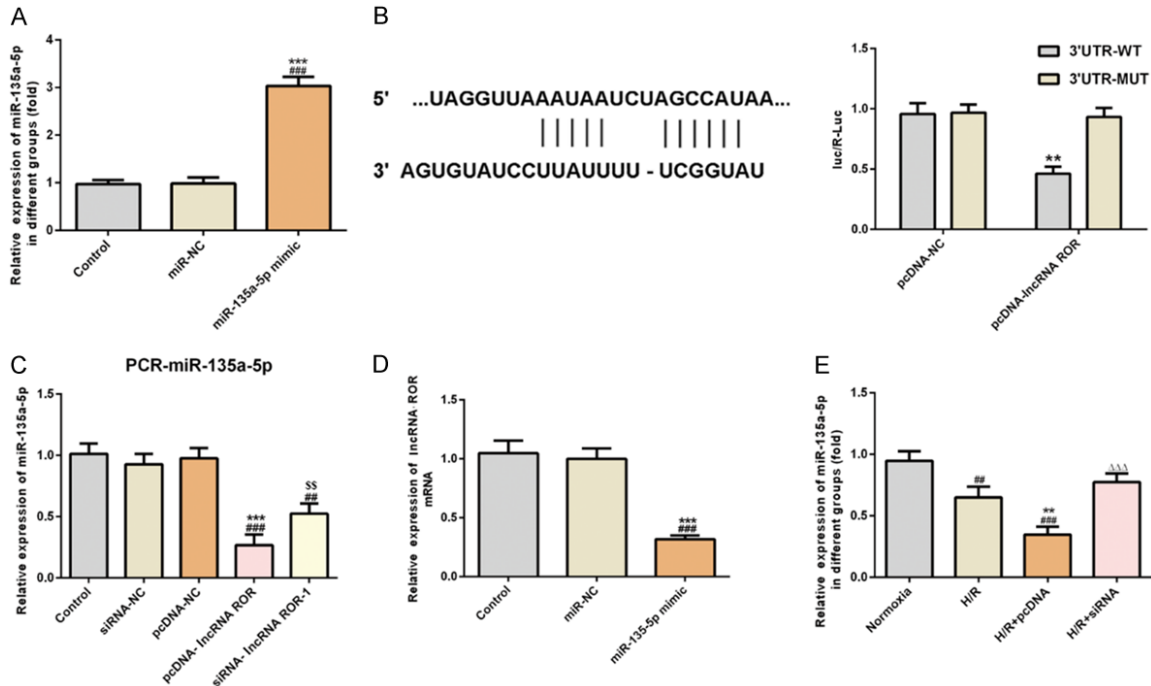
**Figure 4.** lncRNA ROR overexpression promotes the damage of H/R treated PC12 cells by activating the expression of ROCK1 and ROCK2. A. The expression of ROCK1 was detected by immunofluorescent staining (n = 3). B. The expression of ROCK2 was detected by immunofluorescent staining (n = 3).

ed in PC12 cells when miR-135a-5p expression was increased (**Figure 5D**). As shown in **Figure 5E**, compared with the Normoxia group, the miRNA-135a-5p expression was decreased in H/R group and lncRNA ROR overexpression in H/R treated PC12 cells made more decrease of the miRNA-135a-5p expression compared with the H/R group. And, lncRNA ROR inhibition in H/R treated PC12 cells made the miRNA-135a-5p expression rose up compared with the H/R group but still lower than that in Normoxia group.

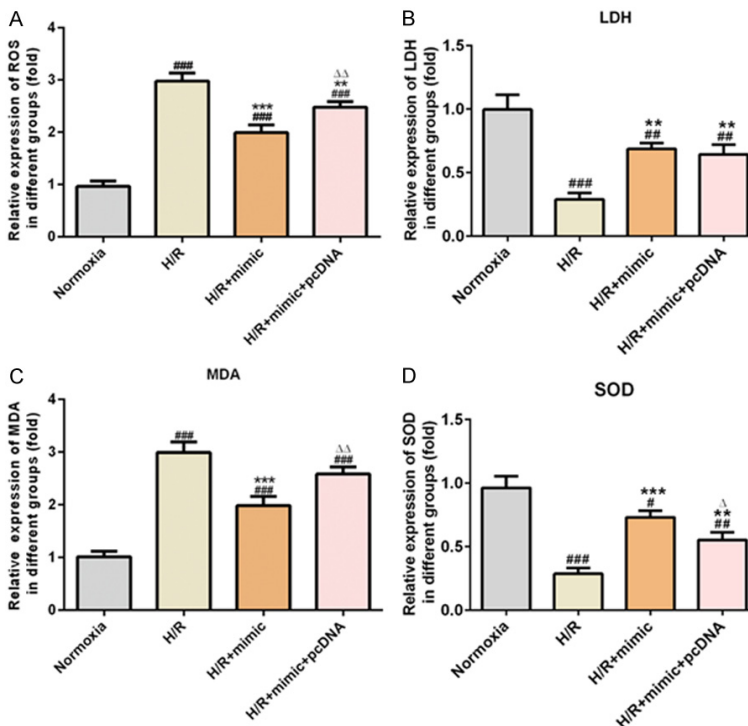
#### miRNA-135a-5p overexpression decreases the damage of H/R treated PC12 cells

The assay kits were used to detect the levels of ROS, LDH, MDA and SOD. As shown in **Figure**

**6A**, compared with the Normoxia group, the ROS level was increased in H/R group and the ROS level was decreased in H/R + mimic group. Transfected with miRNA-135a-5p mimic and pcDNA-lncRNA ROR, PC12 cells treated with H/R showed the increased level of ROS. As shown in **Figure 6B-D**, compared with the Normoxia group, the levels of LDH and SOD were decreased and MDA level was increased in H/R group. Compared with the H/R group, the levels of LDH and SOD were increased and MDA level was decreased in H/R + mimic group. However, the levels of LDH and SOD were decreased and MDA level was increased in H/R + mimic + pcDNA group compared with the H/R + mimic group. Therefore, miRNA-135a-5p overexpression decreases the oxidative dam-



**Figure 5.** LncRNA-ROR directly targets miRNA-135a-5p. A. The transfection effect of miRNA-135a-5p was examined by RT-qPCR analysis (n = 3). ###P < 0.001 vs. control group. \*\*\*P < 0.001 vs. miR-NC group. B. Luciferase activity of a reporter containing lncRNA ROR 3'UTR-WT or 3'UTR-MUT (with a mutation in the miR-135a-5p binding site) (n = 3). \*\*P < 0.01 vs. ROR + miR-NC group. C. The expression of miRNA-135a-5p in PC12 cells after transfected with pcDNA-lncRNA ROR and siRNA-lncRNA ROR was detected by RT-qPCR analysis. ##P < 0.01 and ###P < 0.001 vs. control group. \*\*\*P < 0.001 vs. pcDNA-NC group. \$P < 0.01 vs. siRNA-NC group. D. The expression of lncRNA ROR in PC12 cells after transfected with miR-135a-5p mimic was detected by RT-qPCR analysis. ###P < 0.001 vs. control group. \*\*\*P < 0.001 vs. miR-NC group. E. The change of miRNA-135a-5p expression after transfection of pcDNA-ROR and siRNA-ROR (n = 3). ##P < 0.01 and ###P < 0.001 vs. Normoxia group. \*\*P < 0.01 vs. H/R group. ΔΔΔP < 0.001 vs. H/R + pcDNA group.



**Figure 6.** MiRNA-135a-5p overexpression decreases the damage of H/R treated PC12 cells. The levels of ROS (A), LDH (B), MDA (C) and SOD (D) were detected by respective kits (n = 3). #P < 0.05, ##P < 0.01 and ###P < 0.001 vs. Normoxia group. \*\*P < 0.01 and \*\*\*P < 0.001 vs. H/R group. ΔP < 0.05 and ΔΔP < 0.01 vs. H/R + pcDNA group.

age of H/R treated PC12 cells while lncRNA ROR overexpression will enhance the oxidative damage.

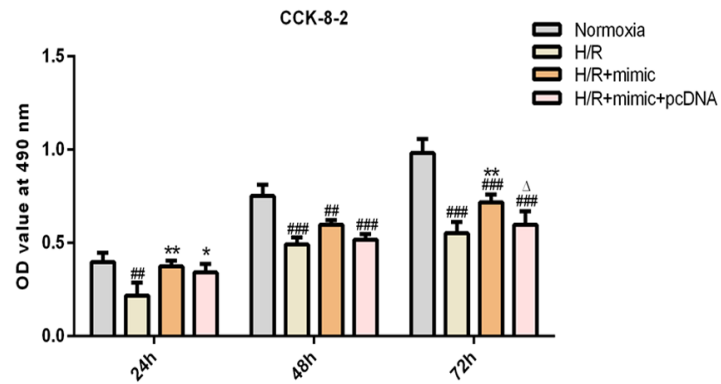
*MiRNA-135a-5p overexpression promotes viability and inhibits apoptosis of H/R treated PC12 cells*

After transfection at 24, 48 and 72 h, the results of CCK-8 assay presented that cell via-

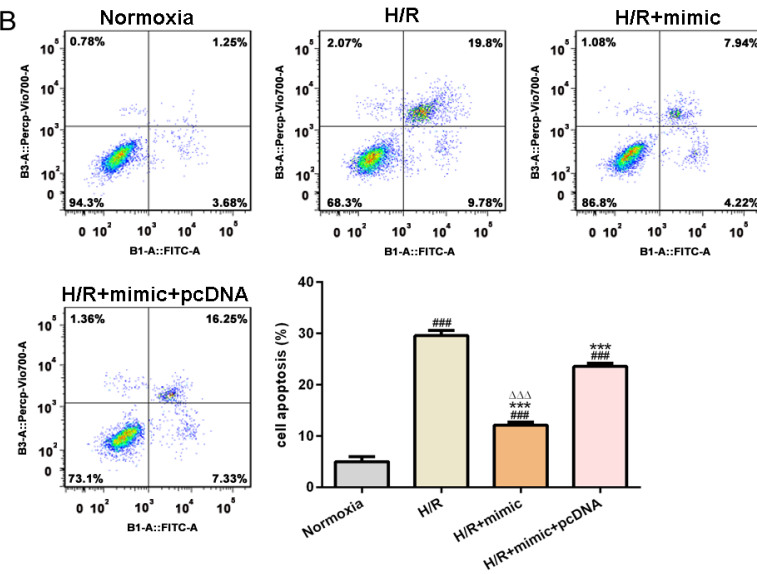


# Role of lncRNA ROR/miR-135a-5p in cerebral injury

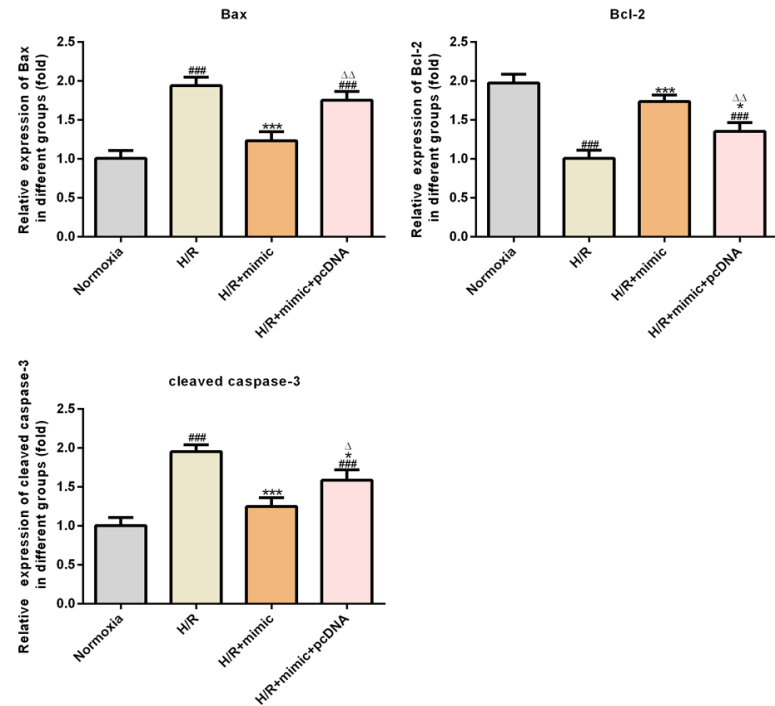
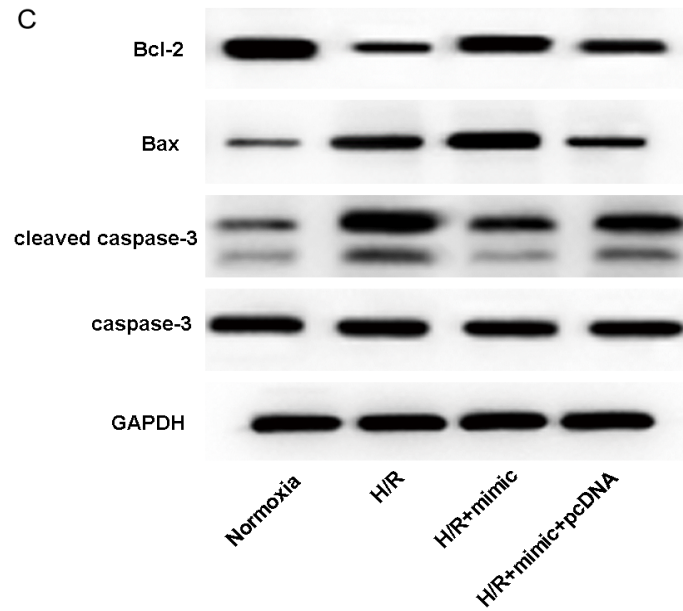
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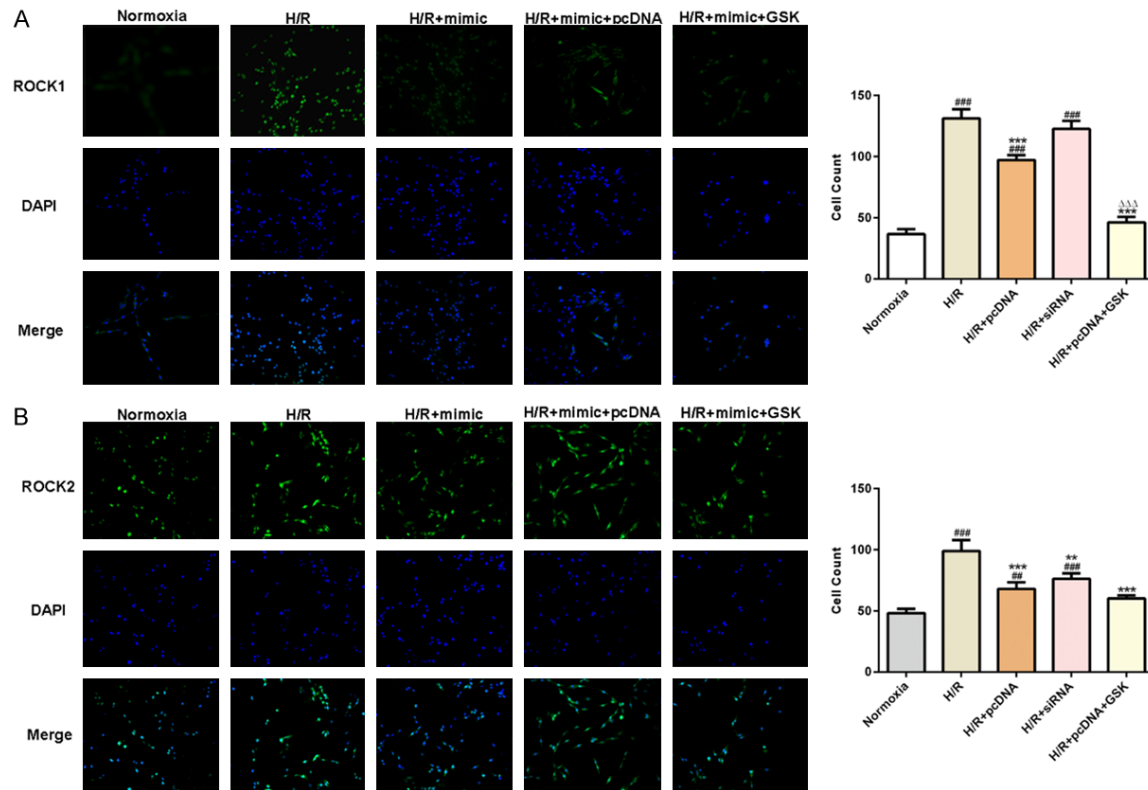
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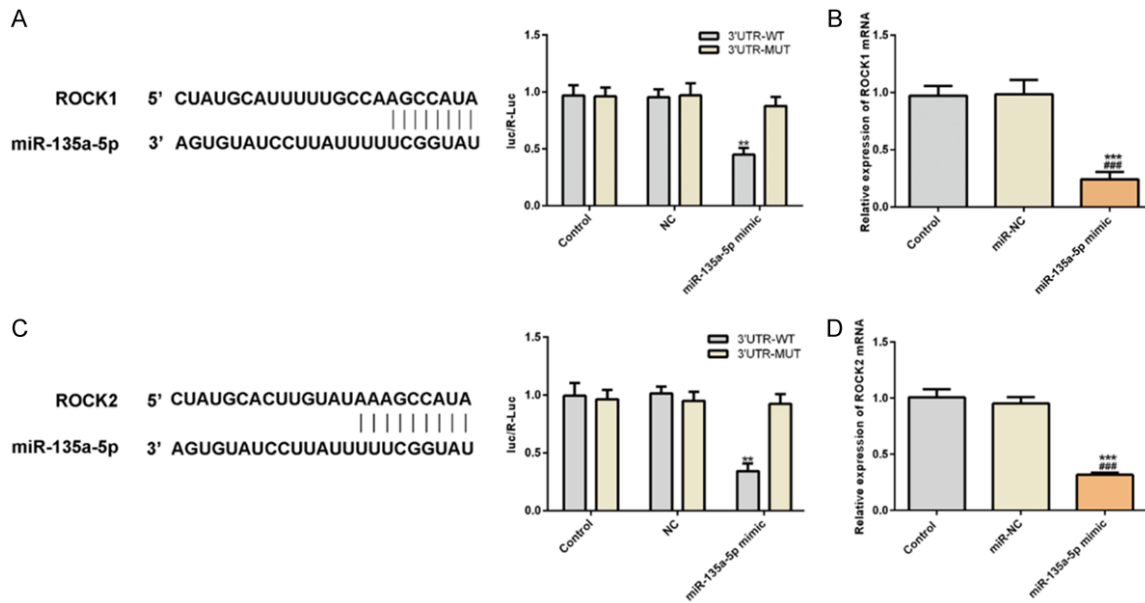
**Figure 7.** MiRNA-135a-5p overexpression promotes viability and inhibits apoptosis of H/R treated PC12 cells. A. The cell viability of H/R treated PC12 cells after transfection was detected by CCK-8 assay (n = 3). ##P < 0.01 and ###P < 0.001 vs. Normoxia group. \*P < 0.05 and \*\*P < 0.01 vs. H/R group.  $\Delta$ P < 0.05 vs. H/R + mimic group. B. The cell apoptosis of H/R treated PC12 cells after transfection was detected by flow cytometry assay (n = 3). ###P < 0.001 vs. Normoxia group. \*\*\*P < 0.001 vs. H/R group.  $\Delta\Delta\Delta$ P < 0.001 vs. H/R + mimic group. C. The apoptosis-related proteins (Bcl-2, Bax and cleaved caspase3) were detected by western blot analysis. ###P < 0.001 vs. Normoxia group. \*P < 0.05 and \*\*\*P < 0.001 vs. H/R group.  $\Delta$ P < 0.05 and  $\Delta\Delta$ P < 0.05 vs. H/R + mimic group.



**Figure 8.** MiRNA-135a-5p overexpression decreases the damage of H/R treated PC12 cells by suppressing the expression of ROCK1 and ROCK2. A. The expression of ROCK1 was detected by immunofluorescent staining (n = 3). B. The expression of ROCK2 was detected by immunofluorescent staining (n = 3).

bility was decreased in H/R group compared with the Normoxia group and miRNA-135a-5p overexpression in H/R treated PC12 cells could increase the cell viability compared with the H/R group while lncRNA ROR overexpression could decrease the cell viability compared with the H/R + mimic group (Figure 7A). As shown in Figure 7B, compared with the Normoxia group, cell apoptosis was increased in H/R group and miRNA-135a-5p overexpression in H/R treated PC12 cells could decrease the cell apoptosis compared with the H/R group while lncRNA ROR overexpression could increase the cell apoptosis compared with the H/R + mimic group. The apoptosis-related protein expression was detected by the western blot analysis and the results showed that com-

pared with the Normoxia group, the expression of Bax and cleaved caspase3 was increased and Bcl-2 expression was decreased in H/R group and miRNA-135a-5p overexpression in H/R treated PC12 cells could decrease the expression of Bax and cleaved caspase3 and increase the Bcl-2 expression compared with the H/R group while lncRNA ROR overexpression could increase the expression of Bax and cleaved caspase3 and decrease the Bcl-2 expression compared with the H/R + mimic group (Figure 7C). Therefore, miRNA-135a-5p overexpression promotes viability and inhibits apoptosis of H/R treated PC12 cells while lncRNA ROR overexpression could reverse the expression of Bax, cleaved caspase3 and Bcl-2.



**Figure 9.** MiRNA-135a-5p directly targets ROCK1 and ROCK2. A. Luciferase activity of a reporter containing ROCK1 3'UTR-WT or 3'UTR-MUT (with a mutation in the miR-135a-5p binding site) (n = 3). <sup>##</sup>P < 0.01 vs. control group. <sup>\*\*</sup>P < 0.01 vs. NC group. B. The expression of ROCK1 in PC12 cells after transfected with miR-135a-5p mimic was detected by RT-qPCR analysis. <sup>###</sup>P < 0.01 vs. control group. <sup>\*\*\*</sup>P < 0.01 vs. miR-NC group. C. Luciferase activity of a reporter containing ROCK2 3'UTR-WT or 3'UTR-MUT (with a mutation in the miR-135a-5p binding site) (n = 3). <sup>##</sup>P < 0.01 vs. control group. <sup>\*\*</sup>P < 0.01 vs. NC group. D. The expression of ROCK2 in PC12 cells after transfected with miR-135a-5p mimic was detected by RT-qPCR analysis. <sup>###</sup>P < 0.01 vs. control group. <sup>\*\*\*</sup>P < 0.01 vs. miR-NC group.

#### MiRNA-135a-5p overexpression decreases the damage of H/R treated PC12 cells by suppressing the expression of ROCK1 and ROCK2

The expression of ROCK1 and ROCK2 was shown in **Figure 8A** and **8B**. Compared with the Normoxia group, the expression of ROCK1 and ROCK2 was increased and miRNA-135a-5p overexpression in H/R treated PC12 cells decreased the expression of ROCK1 and ROCK2 compared with the H/R group while lncRNA ROR overexpression made the expression of ROCK1 and ROCK2 increased and the expression of ROCK1 and ROCK2 was decreased in H/R + mimic + GSK group compared with the H/R + mimic group.

#### MiRNA-135a-5p directly targets ROCK1 and ROCK2

Bioinformatics analysis was performed with the TargetScan tool to predict that MiRNA-135a-5p directly targets ROCK1 and ROCK2 (**Figure 9A** and **9C**). To confirm this prediction, a dual-luciferase reporter assay was used, and the findings indicated that fluorescence intensity was decreased in PC12 cells after transfected with

ROCK1/2 3'UTR-WT and miR-135a-5p mimic. And, miR-135a-5p overexpression could obviously reduce the expression of ROCK1 and ROCK2 (**Figure 9B-D**). As a result, miR-135a-5p directly targets ROCK1 and ROCK2.

#### Discussion

Here, this study investigated whether lncRNA ROR can promote the cerebral hypoxia/reperfusion (H/R) injury by inhibiting the expression of miR-135a-5p or upregulating the expression of ROCK1 and ROCK2. In the present study, we found that lncRNA ROR could promote the cerebral H/R injury by inhibiting the expression of miR-135a-5p or upregulating the expression of ROCK1/2. And, miR-135a-5p overexpression could improve the cerebral H/R injury by inhibiting the expression of ROCK1/2.

Recently, lncRNAs have been reported to be associated with ischaemia-reperfusion injury [27]. For instance, lncRNA HOTAIR expression was increased, which regulating the autophagy in hepatic ischemia/reperfusion injury [28]. lncRNA AK12348 was upregulated in anoxia/reoxygenation (A/R) cardiomyocytes and silenc-

ing of AK12348 could improve the damage induced by A/R [29]. lncRNA AK038897 was upregulated in cerebral ischemia and AK038897 interference could protect against brain injury and neurological deficits in vivo [10]. The oxidative stress injury and cell apoptosis were important in the process of ischemia/reperfusion injury [30-33]. The role of lncRNA ROR in cerebral ischemia/reperfusion injury was examined by upregulating and downregulating the expression of lncRNA ROR. We investigated whether lncRNA ROR could influence oxidative stress injury and cell apoptosis in cerebral ischemia/reperfusion injury. The results manifested that lncRNA ROR expression was increased in response to H/R treatment in PC12 cells and lncRNA ROR overexpression caused the serious oxidative stress injury and a remarkable increase of cell apoptosis, demonstrating lncRNA ROR played an important role in the development of H/R injury.

The present study confirmed that miR-135a-5p was a target gene of lncRNA ROR. Also, many miRNAs played an important role in the development of ischemia/reperfusion injury [34]. For instance, miR-148b-3p expression was significantly up-regulated after H/R treatment and miR-148b-3p interference could alleviate the H/R injury [35]. And, miR-27a-5p was related to liver ischemia-reperfusion injury and miR-27a-5p overexpression may alleviate cell apoptosis in H/R injury by targeting Bach1 in vitro [36]. Therefore, we predicted that miR-135a-5p could also affect the H/R injury because of the lncRNA ROR effect on the H/R injury. Then we found that miR-135a-5p expression was decreased in H/R treated PC12 cells. Consistently, our results showed that miR-135a-5p overexpression alleviated H/R-induced injury by decreasing ROS and MDA, increasing LOH and SOD, improving cell viability and reducing cell apoptosis of PC12 cells. Our results suggested that the decrease of miR-148b-3p expression induced by H/R treatment was harmful to PC12 cells; therefore, the miR-135a-5p overexpression showed a protective effect against H/R injury.

Rho-associated coiled-coil-containing protein kinase is a serine/threonine protein kinase that is more commonly referred to simply as ROCK kinase [37]. ROCK exists as two isoforms, ROCK1 and ROCK2 [38]. ROCK1/2 are the pro-

teins which related to the cell apoptosis. ROCK-1 can regulate the morphological events occurring in the apoptosis, including cell contraction, dynamic membrane blebbing, nuclear disintegration and fragmentation of apoptotic cells into apoptotic bodies [39]. ROCK-2 is cleaved by granzyme B at the IGLD 1131 site during cytotoxic lymphocyte granule-induced apoptosis [40]. In this study, we found that ROCK1/2 expression was proportional to the oxidative stress injury and cell apoptosis, and ROCK1/2 expression was regulated by the change of lncRNA ROR expression and miR-135a-5p expression.

In conclusion, our current work showed a new finding that lncRNA ROR could promote the cerebral H/R injury by inhibiting the expression of miR-135a-5p or up-regulating the expression of ROCK1/2. And, miR-135a-5p overexpression could improve the cerebral H/R injury by inhibiting the expression of ROCK1/2. These results may be important in the treatment of cerebral hypoxia/reoxygenation-induced injury. However, there are still existing some limitations that this study is mainly depending on the cell experiment and further experiments will be made in the animal experiments and clinic to verify the present results.

## Disclosure of conflict of interest

None.

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