Mechanism of fluoxetine downregulation of circMap2k1 to alleviate neurological function after ischemic stroke

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Research Article

Keywords: fluoxetine, circMap2k1, neurological function, cerebral ischemic stroke

Posted Date: August 7th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3209057/v1

Abstract

Background

Ischemic stroke (IS) is known for its high incidence, disability and mortality, and there is an urgent need to investigate the pathophysiological mechanisms and develop novel treatment strategies. We aimed to investigate the mechanisms of the novel circMap2k1/miR-135b-5p/Pidd1 axis in the treatment of IS progression with fluoxetine.

Methods

The middle cerebral artery occlusion (MCAO) model was established, followed by fluoxetine treatment and injecting adeno-associated viruses (AAV)-sh-ctr and AAV-sh-circMap2k1 into bilateral hippocampal tissues of rats. Then cerebral infarction area, weight, Longa score, and neurological injury were evaluated. Dual-luciferase reporter gene assay was employed to confirm the binding between miR-135b-5p and Pidd1. ELISA was performed to measure the concentrations of inflammatory factors TNF-α, IL-6, and IL-1β in the plasma. Finally, we verified the role of circMap2k1 in cellular experiments by overexpression of circMap2k1. Cell viability was assessed using CCK-8 assay, while apoptosis was measured by flow cytometry.

Results

Knockdown of circMap2k1 enhanced the therapeutic effect of fluoxetine on IS injury (cerebral infarction area, weight, and Longa score) in rats. Then knockdown of circMap2k1 enhanced the protective effect of fluoxetine on neurological injury after IS in rats. Dual-luciferase reporter gene assay confirmed the targeting of miR-135b-5p to Pidd1. Additionally, fluoxetine deactivated the adsorption of miR-135b-5p by downregulating circMap2k1, and miR-135b-5p further exerts its inhibitory effect on Pidd1, and finally attenuares the inflammatory response caused by microglial polarization after IS. Cell experiments revealed that overexpression of circMap2k1 repressed cell viability and promoted cell apoptosis.

Conclusions

Fluoxetine downregulated circMap2k1 to ameliorate neurological injury and inflammatory responses induced by microglia polarization after IS.

Introduction

Ischemic stroke (IS) is a disease of the central nervous system caused by the narrowing or occlusion of blood vessels in the brain [1]. It is the primary cause of mortality and a major contributor to acquired physical disabilities worldwide [2]. IS accounts for 70–80% of all stroke cases [3] and is a complex

cascade of events starting with vascular occlusion [4]. The pathogenesis is characterized by thrombosis and neuroinflammation [5]. IS produces a powerful inflammatory cascade response in the brain leading to neuroinflammation [6]. Currently, the diagnosis of IS relies on neuroimaging techniques [7]. Based on its physiological and pathological features, effective IS treatment options are limited [8]. Therefore, there is an urgent need to investigate the pathophysiological mechanisms and develop novel treatment strategies.

Fluoxetine, one of the selective serotonin reuptake inhibitors (SSRIs) [9], is a drug widely used to measure serotonin neurotransmission in central nervous system [10]. Due to pharmacological nature of having multi-target effects, current basic research demonstrates that fluoxetine improves neurological function with increased expression of brain-derived neurotrophic factor [11], modulates the level of nucleus accumbens 5-hydroxytryptamine and its receptors [12], improves motor and functional recovery after stroke [13], and reduces apoptosis in rat hippocampal neuron [14]. In addition, some studies have reported that the effects of fluoxetine on the hippocampus may be related to the improvement of the inflammatory response [15]. However, it remains unclear how fluoxetine targets inflammatory pathway upstream of IS.

Circular RNAs (circRNAs) are a class of highly conserved non-coding RNAs with a closed-loop structure that are more than 200 bases in length [16]. CircRNAs perform biological functions by acting as transcriptional regulators, microRNA (miRNA) sponges, and protein templates [17]. In recent studies, circRNAs have emerged as promising biomarkers for many diseases, including cardiovascular disease, cancer, and IS [18]. Among them, the regulation of circRNAs is involved in the pathophysiological processes behind IS, revealing many potential therapeutic targets and possible clinical biomarkers [19]. Xu X et al. demonstrated that circPHC3 functioned as a sponge for miR-455-5p, thereby activating TRAF3 and facilitating oxygen-glucose deprivation-induced death and apoptosis of human brain microvascular endothelial cells [20]. Dai Q et al. discovered that by targeting miR-133b, knockdown of circHECTOR1 inhibited TRAF3 expression by targeting miR-133b, consequently mitigating cerebral ischemia-induced neuronal injury [21]. Our previous study suggested that fluoxetine modulated circMap2k1/miR-135b-5p/P53-induced protein with a death domain 1 (Pidd1) axis in IS to improve IS [22]. Therefore, we wanted to delve into the mechanism of fluoxetine and circMap2k1/miR-135b-5p/Pidd1 axis in IS.

Based on the above background, we speculate that fluoxetine mediates the circMap2k1/miR-135b-5p/Pidd1 axis to plays a significant role in IS. The objective of this study was to explore the mechanism underlying the therapeutic effect of fluoxetine on the progression of IS by investigating the novel circMap2k1/miR-135b-5p/Pidd1 axis. Our study may enhance the understanding of the molecular mechanisms of fluoxetine mediated IS and provide new targets and strategies for treating IS.

Materials and Methods

Establishment of the middle cerebral artery occlusion (MCAO) model

Sixty-four male SD rats (250 g) were randomly divided into 4 groups: MCAO, MCAO + Fluoxetine, MCAO + Fluoxetine + sh-ctr, MCAO + Fluoxetine + sh-circMap2k1 group, with 16 rats in each group. During the experimental period, animals were housed under natural conditions with a temperature range of 21°C-24°C and humidity maintained at 50%-70%. The MCAO model was established using Zea-Longa method according to our previous study [22]. All rats were preoperatively fasted for 8 h and postoperatively anesthetized by intraperitoneal injection of 2% sodium pentobarbital (2 mL/kg, #57330, Chemical book). Adeno-associated viruses (AAV)-sh-ctr and AAV-sh-circMap2k1 were microinjected into the hippocampal tissue of rats bilaterally [23], and an MCAO model was constructed 14 days later. The MCAO model was maintained for 2 h and perfused for another 14 days, and 10 mg/kg fluoxetine (F131623, aladdin) was injected intraperitoneally 30 min after model formation and once daily thereafter until 14 days. Body weight was recorded. AAV-sh-ctr and AAV-sh-circMap2k1 were provided by GenePharma.

2, 3, 5-triphenyltetrazolium chloride (TTC) staining

To assess the area of cerebral infarction, TTC staining was conducted on the brain sections obtained from each group of rats. Rats were anesthetized with 2% pentobarbital sodium intraperitoneally. After death, intact brain tissues were taken. The brain tissue was rinsed with cold saline and frozen at -20°C for a duration of 10 min. Subsequently, the brain tissue was sliced into sections that were 2 mm thick and immersed in a 2% TTC solution (#17779, Sigma) for 20 min at a temperature of 37°C, shielded from light. After staining was completed, image information was captured using a digital camera. Infarct area was assessed as a percentage of total infarct area.

Neurological function score

Zea-Longa scale was used: 0 - normal, no neurological impairment. 1 - inability to fully extend the left front paw. 2 - turning to the left when walking. 3 - leaning to the left when walking. 4 - inability to walk on its own and showing loss of consciousness. The severity of behavioral impairment in the animal is directly proportional to the score, with higher scores indicating more severe impairment. Before executing each group of SD rats, neurological function was scored and a score of 2.0 was considered successful for the MCAO model [22].

Transmission electron microscopy (TEM)

The neuronal ultrastructure of hippocampal tissue on the infarct side (right side) was examined by TEM. The rat brain was perfused with a fixative consisting of 4% formaldehyde and 2% glutaraldehyde in 0.1 mol/L PBS for 40 min. Coronal sections approximately 1 mm thick were taken from the hippocampal tissue area on the infarct side (right side). Sections were placed in freshly prepared 3% glutaraldehyde at 4°C overnight. After being washed with PBS, sections were placed in 1% osmium tetroxide in 0.1 mol/L PBS and fixed at 4°C for 2 h. The sections were dehydrated and embedded in Epon 812. Ultrathin sections

of hippocampal tissue on the infarct side (right side) were stained with uranyl acetate and lead citrate and observed with TEM [24].

Nissl staining

Nissl staining was utilized to quantify the number of neurons in the hippocampus. Sections were subjected to a baking process at 60°C for 12 h, followed by dewaxing to water. Subsequently, sections were rinsed with distilled water for 5 min. Nissl staining was performed for 0.5-1 min, depending on the shade of staining. The sections were rinsed with distilled water to eliminate floating colors. Differentiation solution (1% glacial acetic acid) was used to differentiate the sections until the background was colorless and the cells and Nissl bodies had a clear outline when viewed under the microscope. Sections were sealed with buffered glycerol and examined microscopically. The neuronal cells were purple-light blue and the Nissl bodies were purple-dark blue.

TUNEL

TUNEL staining was performed to assess apoptosis in the CA1, CA3 and DG regions of the hippocampus. Hippocampal tissue sections were dewaxed to water and subsequently stained following the instructions provided with TUNEL kit (40306ES50, YEASEN). 100 μL Proteinase K working solution was added dropwise to section for 20 min to react at 37°C and sections were rinsed with PBS. After that, sections were incubated with 50 μL of TdT buffer at room temperature for 60 min, followed by rinsing with PBS. After the staining process, the nuclei were counterstained with DAPI working solution for a duration of 10 min. Subsequently, sections were sealed and examined under a microscope, and images were captured.

Immunohistochemistry (IHC)

Rat brain tissue was removed and IHC was performed to detect Iba-1 expression on the infarct side (right side) of the brain. Sections underwent a baking process at 60°C for 12 h, followed by dewaxing to water, and antigen was thermally repaired. PBS was added for 10 min at room temperature to inactivate endogenous enzyme. Three rinses of PBS were performed for three minutes each. Iba-1 (ab178846, Abcam) was incubated overnight at 4°C. Sections were subsequently incubated secondary antibody for 30 min at 37°C, followed by three washes with PBS for a duration of 5 min each. Sections were dehydrated sequentially in alcohol (60–100%), 5 min each step. After removal, sections were immersed in xylene for a period of 10 min. Finally, sections were sealed with neutral gum and examined under a microscope.

Cell treatment

Primary rat brain microvascular endothelial cells (RBMEC, RAT-iCell-n001, iCell) were cultured as described previously [24–26]. 293T (SCSP-502) was purchased from Cell Bank of the Chinese Academy of Sciences and cultured in 90% DMEM and 10% fetal bovine serum. RBMEC and 293T were incubated at

37°C, 5% CO_2, in a saturated humidity incubator. Overexpression of circMap2k1 was performed by transfection of overexpressing circMap2k1 plasmids in RBMEC grouped as EV and circMap2k1-OE groups. Transfection was performed for 48 h according to the instructions of Lipofectamine 3000 reagent (Thermo Fisher Scientific).

Quantitative real-time PCR (qRT-PCR)

The levels of circMap2k1, miR-135b-5p, and Pidd1 were determined using qRT-PCR. Total RNA was isolated using Trizol (15596026, Thermo), and reverse transcription was conducted using PrimeScript 1st strand cDNA Synthesis Kit (#6110A, TaKaRa). qRT-PCR was performed by SYBR Green qPCR SuperMix (#11762100, Invitrogen). GAPDH was employed as the reference gene for circMap2k1 and Pidd1, while U6 served as the reference gene for miR-135b-5p. The relative gene levels were determined using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are: R-circMap2k1-F: AGGCCTGACATATCTACGAGAG, R-circMap2k1-R: TCCTTCAACTCTCCCACCTTC; R-miR-135b-5p-F: TATGGCTTTTCATTCCTA, R-miR-135b-5p-R: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCACAT; R-Pidd1-F: CCCCAGCTTCCTACAACCTG, R-Pidd1-R: GTCATCCCAGGTGCTTGTCA; R-GAPDH-F: GCAAGAGAGAGGCCCTCAG, R-GAPDH-R: GCCCAATACGACCAAATCC; R-U6-F: CTCGCTTCGGCAGCACA, R-U6-R: AACGCTTCACGAATTTGCGT.

Western blot

Western blot was employed to assess the levels of Pidd1 and Iba-1. Total protein was extracted with RIPA (P0013B, Beyotime), and the concentration of proteins was quantified using BCA Protein Assay Kit (BL521A, Biosharp). After SDS-PAGE gel electrophoresis was carried out. Protein was transferred onto the membrane, and membrane was sealed with skim milk. Then membrane was incubated with primary antibody against Pidd1 (ab232773, Abcam), Iba-1 (ab178846, Abcam) and GAPDH (60004-1-Ig, Proteintech) at 4˚C overnight. Next, second antibody Peroxidase-AffiniPure goat Anti-Rabbit IgG (H + L) (111-035-003, Jackson) or Peroxidase-AffiniPure goat Anti-Mouse IgG (H + L) (115-035-003, Jackson) was incubated. Exposure was performed using a supersensitive ECL chemiluminescence substrate (BL520A, Biosharp). GAPDH was utilized as an internal control to determine protein levels.

Enzyme linked immunosorbent assay (ELISA)

The concentrations of inflammatory factors TNF-α, IL-6, and IL-1β were measured in rat plasma. Rat TNF-α (EK0526, BOSTER), IL-6 (EK0393, BOSTER), and IL-1β (EK0412, BOSTER) ELISA kits were applied to determine the concentrations of inflammatory factors TNF-α, IL-6 and IL-1β following the provided instructions.

Cell Counting Kit-8 (CCK-8) assay

RBMEC viability was evaluated by CCK-8 assay at 24, 48 and 72h after transfection. RBMEC were digested, counted, inoculated into 96-well plates at a density of 5×10^3 cells/well (100 μL per well), and incubated at 37°C in 5% CO_2 incubator. CCK8 reagent (T01C, CellCook) was added after 24, 48 and 72 h,

respectively, and the ratio was 1:10, that was, 100 μL culture medium was added to 10 μL detection solution. After incubating at 37℃ with 5% CO_2 for 1.5 h, absorbance at 450 nm was measured using a Bio-Tek microplate reader.

Cell apoptosis

AnnexinV-APC apoptosis detection kit (KGA1022, KeyGen) was utilized to measure RBMEC apoptosis. Simply put, RBMEC were digested and collected without EDTA pancreatic enzymes, and the cells were cleaned twice by PBS. The cells were resuspended with 1×10^6 cells/mL with Binding Buffer. 100 μL suspension was taken into the tube. 5 μL Annexin V and 5 μL 7-AAD (00-6993-50, Invitrogen) was added and mixed well and RBMEC were incubated for 15 min at room temperature away from light. Binding Buffer of 400 μL was mixed and RBMEC were measured by flow cytometry within 1 h.

Bioinformatics prediction and dual-luciferase reporter gene assay

Bioinformatics was conducted to predict the potential binding sites of Pidd1 3 'UTR to miR-135b-5p. Luciferase activity was assayed in 293T cells co-transfected with miRNA-NC or miR-135b-5p mimics and Pidd1 3'UTR wild-type (WT) or mutant (mut) plasmids using the dual-luciferase reporter assay kit (FR201-01, TransGen Biotech). To verify the binding of miR-135b-5p to Pidd1, WT or MUT Pidd1 fragments were generated and inserted into the pmirGLO vector (Promega). Subsequently, we transfected WT (or mut) vectors and miR-135b-5p mimics (or miRNA-NC) into 293 T cells using Lipofectamine 3000 reagent (Thermo Fisher Scientific) following manufacturer's instructions. After 48 h, cells were harvested, and luciferase activity was quantified using dual-luciferase reporter gene assay.

Statistical analysis

GraphPad version8.0 was used for statistical analysis. Measurement data were presented as mean ± standard deviation. Student's t-test was employed for comparisons between two groups, while one-way analysis of variance (ANOVA) was utilized for comparisons among multiple groups, followed by Dunnett post-hoc test. When $P < 0.05$, the difference was deemed statistically significant.

Results

Knockdown of circMap2k1 enhanced the therapeutic effect of fluoxetine on IS injury in rats

To evaluate the effects of knockdown of circMap2k1 on cerebral infarction and body weight in MCAO rats after fluoxetine treatment, we successfully established MCAO model, followed by fluoxetine treatment and injecting AAV-sh-ctr and AAV-sh-circMap2k1 into bilateral hippocampal tissues of rats. According to TTC staining results, MCAO model group exhibited the largest area of cerebral infarction, and the cerebral infarction area decreased after fluoxetine treatment. Fluoxetine treatment and

knockdown of circMap2k1 significantly reduced cerebral infarction area (Fig. 1A and 1B). Compared with the MCAO group, MCAO rats gained weight after fluoxetine treatment. Fluoxetine treatment and knockdown of circMap2k1 significant increased weight in rats (Fig. 1C). In addition, we measured the effect of knockdown of circMap2k1 on neurological function in MCAO rats after fluoxetine treatment. According to Zea-Longa score, we found that MCAO group rats had the highest score and serious behavioral disorders. The score of MCAO + Fluoxetine group was decreased. After fluoxetine treatment and knockdown of circMap2k1, score was lowest and nerve damage was least severe (Fig. 1D). These results suggested that knockdown of circMap2k1 enhanced the therapeutic effect of fluoxetine on IS injury in rats.

Knockdown of circMap2k1 enhanced the protective effect of fluoxetine on neurological injury after IS in rats

Next, we evaluated the protective effect of knockdown of circMap2k1 on neurological injury in MCAO rats after fluoxetine treatment. TEM showed that the nucleus of the MCAO group was solidified, chromatin and nucleolus were severely degraded, electron-dense material appeared, nuclear membrane was distorted, mitochondria were abnormally vacuolated, double membranes were ruptured, cristae were disrupted, and synapses were mostly single synapses and less in number. After fluoxetine treatment, the nucleus was slightly fixed, chromatin and nucleolus were slightly degenerated, mitochondrial membrane structure was intact, cristae were organized, synapses were more numerous, and synaptic complexes were present. After fluoxetine treatment with simultaneous knockdown of circMap2k1, the nucleus was normal, chromatin was well distributed, nucleolus and double-layered nuclear membrane were present, the number of normal mitochondria was the highest, and the number of synapses was also increased, and most of them were synaptic complexes (Fig. 2A). Nissl staining showed degeneration of pyramidal cells and distortion of pyramidal layer in CA1 and CA3 regions of hippocampus in MCAO group, and Nissl bodies number was less. After fluoxetine treatment, the shape of pyramidal cells was normal, the cell degeneration was less, and the number of Nissl bodies was more. After fluoxetine treatment and knockdown of circMap2k1, the cells were basically unchanged and there were a large number of Nissl bodies. In DG region, most of the nuclei of MCAO group were retracted. After fluoxetine treatment, the pyramidal layer was thicker, the cell morphology was more normal, and Nissl bodies number was more. After fluoxetine treatment and knockdown of circMap2k1, nuclear pyknotic cells significantly decreased and the number of Nissl bodies further increased (Fig. 2B). These results suggested that fluoxetine treatment and knockdown of circMap2k1 enhanced the effect of fluoxetine on improving neurological function in IS. In addition, MCAO group displayed a significant presence of apoptotic cells in the hippocampal CA1, CA3, and DG regions. However, the number of apoptotic cells decreased following treatment with fluoxetine. After fluoxetine treatment and knockdown of circMap2k1, apoptosis was significantly reduced (Fig. 2C-2H). These results revealed that knockdown of circMap2k1 enhanced the protective effect of fluoxetine on neurological injury after IS in rats.

Fluoxetine relieved the adsorption of circMap2k1 on miR-135b-5p by downregulating circMap2k1 to weaken the inflammatory response caused by microglial polarization after IS

Our previous study revealed that circMap2k1 could function as a molecular sponge to adsorb miR-135b-5p. Moreover, circMap2k1/miR-135b-5p/Pidd1 axis may be involved in IS [22]. Thus, we further explored the mechanism of role of circMap2k1/miR-135b-5p/Pidd1 axis in IS. Bioinformatics predictions revealed a potential binding site of Pidd1 3'UTR to miR-135b-5p (Fig. 3A). Dual-luciferase results demonstrated that miR-135b-5p could bind to Pidd1 3'UTR and regulate Pidd1 expression (Fig. 3B). qRT-PCR results showed that circMap2k1 and Pidd1 expressions were elevated and miR-135b-5p expression was decreased in the MCAO group. CircMap2k1 and Pidd1 expressions decreased and miR-135b-5p expression increased after fluoxetine treatment. CircMap2k1 and Pidd1 expressions were further decreased and miR-135b-5p expression was increased after fluoxetine treatment with simultaneous knockdown of circMap2k1 (Fig. 3C). Western blot further showed that Pidd1 level was higher in MCAO group, while the expression of the microglial cell marker Iba-1 was also higher, indicating that microglia were activated in the MCAO model. The levels of both Pidd1 and Iba-1 were reduced after fluoxetine treatment. Pidd1 and Iba-1 levels were further reduced after fluoxetine treatment with simultaneous knockdown of circMap2k1 (Fig. 3D and 3E). In addition, IHC showed increased expression of Iba-1 in CA1, CA3 and DG regions of hippocampal tissue on the infarct side (right side) of the MCAO group. Iba-1 expression was decreased after fluoxetine treatment. The lowest Iba-1 expression level was observed after fluoxetine treatment with simultaneous knockdown of circMap2k1 (Figs. 3F and 3G), indicating that knockdown of circMap2k1 enhanced the inhibitory effect of fluoxetine on microglia activation. Additionally, ELISA analysis revealed elevated levels of inflammatory factors TNF-α, IL-6, and IL-1β in MCAO group. After fluoxetine treatment, the levels of these inflammatory factors were observed to decrease. The lowest levels of TNF-α, IL-6, and IL-1β were observed with simultaneous knockdown of circMap2k1 with fluoxetine treatment (Fig. 3H), suggesting that knockdown of circMap2k1 enhanced the inhibitory effect of fluoxetine on inflammation.

circMap2k1 inhibited RBMEC proliferation and promoted its apoptosis

Finally, we verified the role of circMap2k1 in cellular experiments. We found that after transfection of overexpressing circMap2k1 plasmids in RBMEC, circMap2k1 level was increased, miR-135b-5p level was decreased, and Pidd1 level was also increased (Fig. 4A-4C). In addition, cell viability was inhibited and apoptosis was increased after overexpression of circMap2k1 in RBMEC (Fig. 4D and 4E), suggesting that circMap2k1 inhibited cell viability and promoted apoptosis. In conclusion, our results confirmed that elevated circMap2k1 expression suppressed miR-135b-5p expression, promoted Pidd1 expression, and could inhibit cell viability and promote apoptosis.

Discussion

IS is a complex disease and neurological injury after IS has been a hot topic of current research [27]. Inflammatory responses are involved in all stages of stroke, and microglia are the main cells of the post-stroke inflammatory response [28]. Therefore, modulating neuroinflammation by targeting microglia

polarization toward an anti-inflammatory phenotype could potentially serve as a novel therapeutic strategy for IS [3]. In the present study, we explored the specific mechanisms by which fluoxetine mediates circMap2k1/miR-135b-5p/Pidd1 axis in IS through *in vivo* and *in vitro* experiment. We found that fluoxetine downregulated circMap2k1 to ameliorate neurological injury and inflammatory responses induced by microglia polarization after IS. Aadditionally, circMap2k1 inhibited RBMEC proliferation and promoted its apoptosis.

CircRNAs are generated by gene exons through selective mRNA splicing and participate in the pathophysiological process of IS by regulating neuroinflammation [29]. CircRNAs, serving as biomarkers or intervention targets, could provide valuable insights into diagnosing and treating diseases. We previously sequenced the hippocampus of MCAO rat and identified a new homologous circRNA: circMap2k1. CircMap2k1 was up-regulated after MCAO and down-regulated after fluoxetine intervention [22]. In clinical practice, fluoxetine could alleviate the symptoms of post-stroke depression and contribute to the motor recovery of stroke patients [30]. In addition, fluoxetine was associated with better neurological scores [31]. In this study, we found that the area of cerebral infarction increased, the weight growth slowed down, and the neurological injury was serious in MCAO group. Fluoxetine treatment could reduce the neurological injury and slow weight gain caused by MCAO. In addition, the nucleus, mitochondria and synaptic number and structure of nerve cells in MCAO group were seriously damaged, the number of neurons was significantly reduced, and the apoptosis of nerve cells was significantly increased. Fluoxetine treatment could reduce the degree of damage, restore the number of neurons, and inhibit the apoptosis of nerve cells. Knockdown of circMap2k1 could enhance the therapeutic effect of fluoxetine. Our results confirmed that knockdown of circMap2k1 could enhance the therapeutic effect of fluoxetine on IS injury and the protective effect of neurological injury in rats. Therefore, next, we would like to further explore the mechanism of fluoxetine mediating circMap2k1 in IS.

CircRNAs have demonstrated significant roles as miRNA sponges, regulators of gene splicing and transcription, as well as RNA-binding protein sponges and protein/peptide translators [32]. We previously predicted a miRNA enriched in the NF-κB pathway and potentially binding to circMap2k1: miR-135b-5p[22]. In the pathophysiology of IS, inflammation plays a crucial role [33]. IS leads to the release of inflammatory factors, including IL-1β, IL-6, and TNF-α, which can worsen brain injury [34]. The initiators of inflammatory pathways in the central nervous system are microglia activated in response to acute ischemic stress [35]. Activated microglia release inflammatory factors and induce neuronal tissue damage [36, 37]. Inflammatory factors IL-1β, IL-6 and TNF-α activate NF-κB signaling pathway, which in turn regulates the expression of genes associated with inflammatory responses [38]. Among them, the abnormal expression of miR-135b-5p is involved in neurological injury [39]. Pidd1 is closely related to inflammatory response [40]. In addition, the activation of the NF-κB pathway by Pidd1 is essential for cell survival and apoptosis. Yang N et al. found that silencing Pidd1 expression inhibited radiation-induced activation of microglia [41]. In this study, we confirmed that miR-135b-5p could target Pidd1, that was, circMap2k1 level was elevated in the MCAO group, and the inhibition of Pidd1 by miR-135b-5p was removed by molecular sponge adsorption. Zhang J et al. demonstrated that fluoxetine could improve brain activity and white matter fiber injury, reduce Iba-1 expression in prefrontal cortex, hippocampus and

substantia nigra, and reduce inflammatory factors IL-1α, IL-6 and TNF-α levels [42]. We found that in MCAO group, high expression of circMap2k1 caused an increase in the expression of Pidd1, which in turn led to an increase in microglia marker Iba-1 expression, that was, microglia were activated in the model group, and the activation of microglia could induce inflammatory factors release and further promote nerve cells apoptosis. Additionally, the levels of inflammatory factors TNF-α, IL-6 and IL-1β were increased in MCAO model group. After fluoxetine treatment, its elevation was inhibited. Knockdown of circMap2k1 could further inhibit the release of inflammatory factors, indicating that fluoxetine could inhibit the increase of circMap2k1 expression caused by MCAO and inhibit the activation of microglia, thereby reducing inflammation. Experiments at the cellular level further demonstrated that the increased expression of circMap2k1 could inhibit miR-135b-5p expression, promote Pidd1 expression, and inhibit cell viability and promote cell apoptosis.

Conclusion

In this study, we preliminarily evaluated the effect of fluoxetine mediating circMap2k1/miR-135b-5p/Pidd1 axis on IS. We confirmed that fluoxetine downregulated circMap2k1 to ameliorate neurological injury and inflammatory responses induced by microglia polarization after IS. Moreover, circMap2k1 inhibited the proliferation of RBMEC and promoted its apoptosis. Our findings suggest a novel ceRNA mechanism and also provide a new direction for treating IS.

Abbreviations

Ischemic stroke (IS), Middle cerebral artery occlusion (MCAO), adeno-associated viruses (AAV), selective serotonin reuptake inhibitors (SSRIs), Circular RNAs (circRNAs), microRNA (miRNA), protein with a death domain 1 (Pidd1), 2, 3, 5-triphenyltetrazolium chloride (TTC), Transmission electron microscopy (TEM), Immunohistochemistry (IHC), Quantitative real-time PCR (qRT-PCR), Enzyme linked immunosorbent assay (ELISA), Cell Counting Kit-8 (CCK-8) assay, wild-type (WT), mutant (mut), empty vector (EV), One-way analysis of variance (ANOVA).

Declarations

Ethics approval

This animal experiment was carried out in accordance with the Animal Experiment Ethics Committee of Jinan University.

Competing interests

The authors declare no conflicts of interest.

Authors' contributions

Hui Zhang and jian Deng completed all the experiments of figure1 and figure2 and the writing of the manuscript together. Yibo He completed the experiments of figure3 and Figure4. Zhili Cai completed the sorting of the experimental results. Yitao He designed the work. Zhili Cai and yitao He also completed the data review. All authors reviewed the manuscript.

Funding

This work was supported by National Natural Science Foundation of China (No: 82071463).

Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

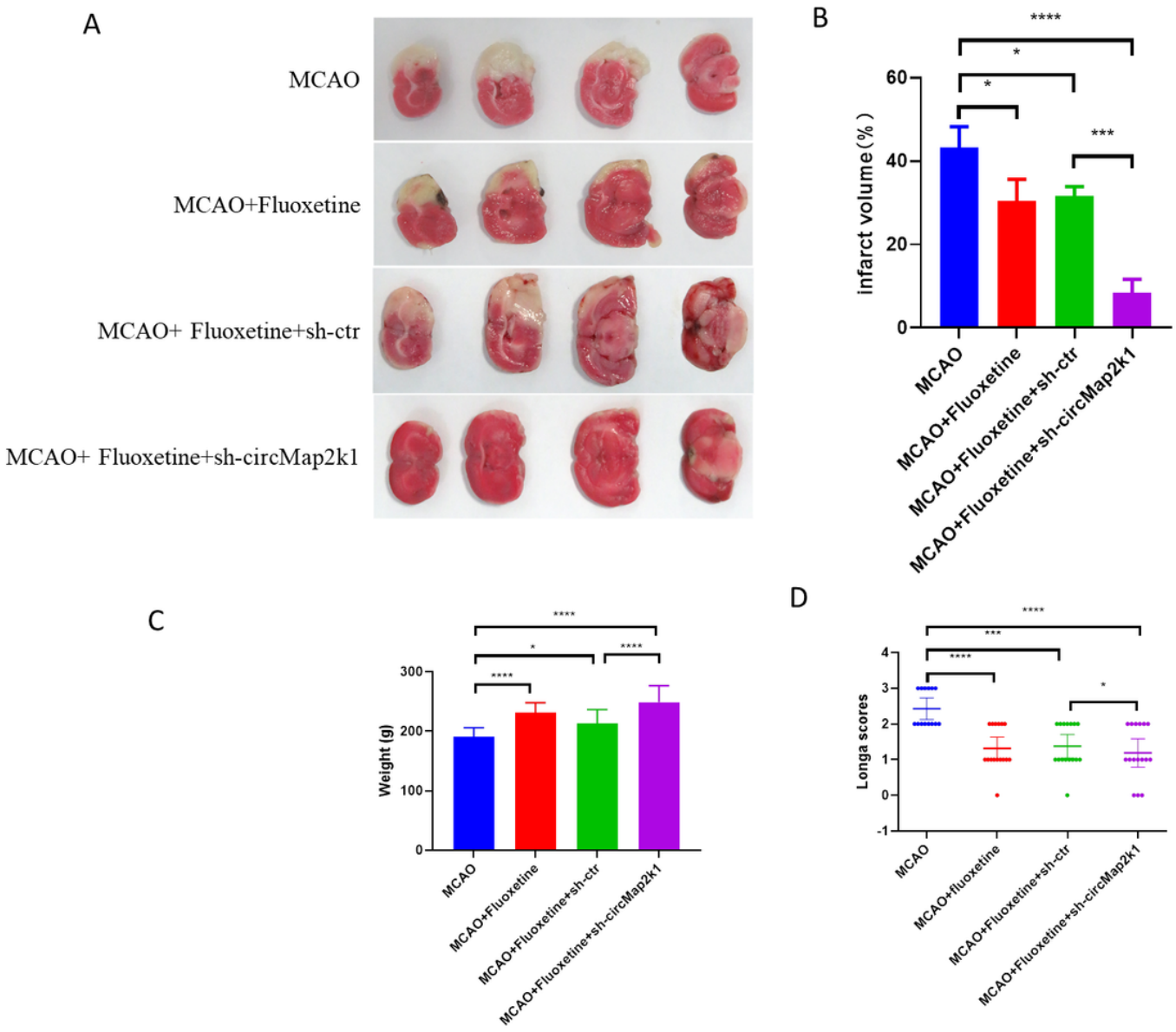

Figure 1

Knockdown of circMap2k1 enhanced the therapeutic effect of fluoxetine on IS injury in rats. A. TTC staining of the infarct area of rats in each group. B. The infarct area was evaluated as a percentage of the total infarct area. C. Body weight of rats in each group. D. Zea-Longa score of rats in each group. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

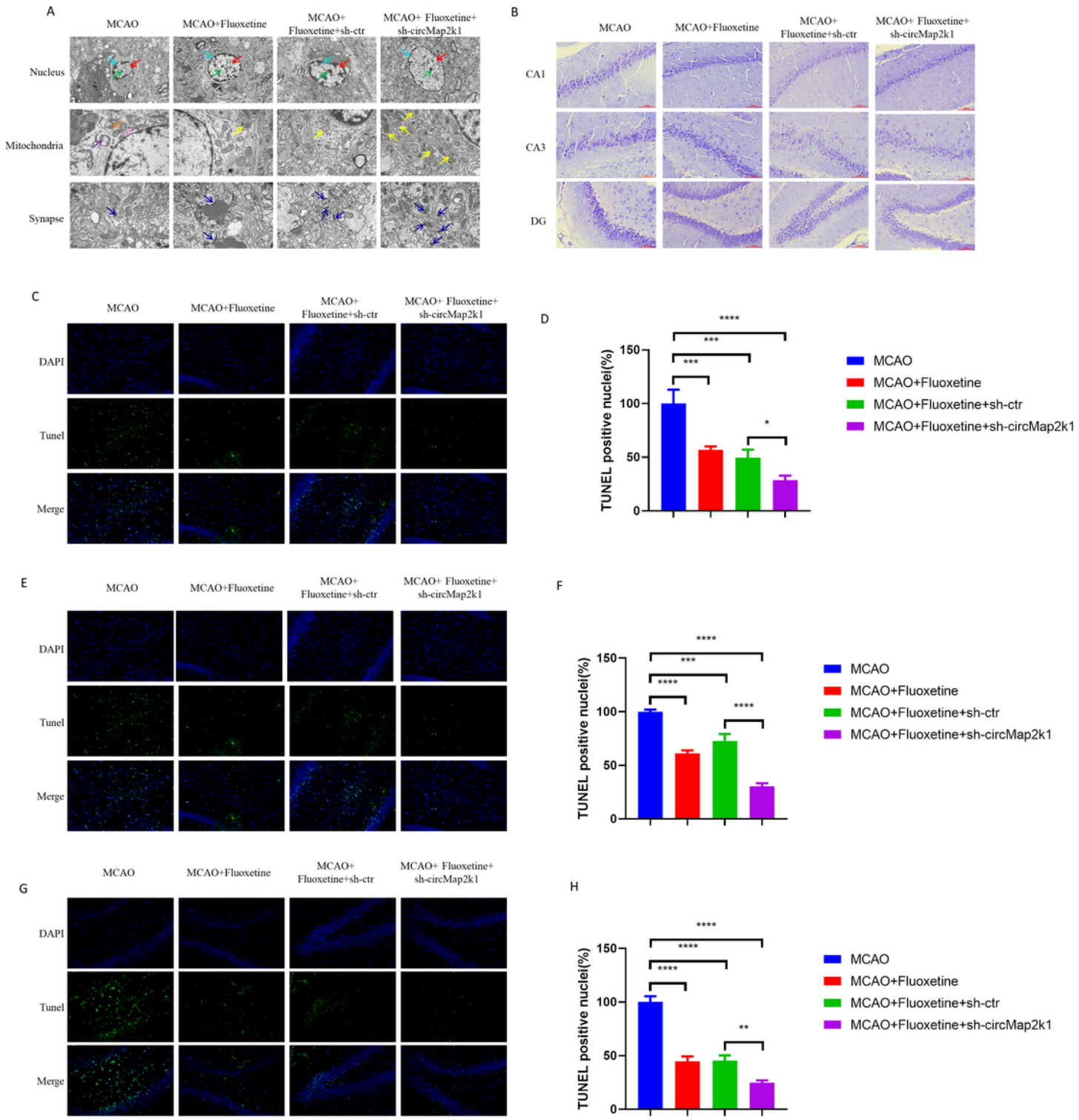

Figure 2

Knockdown of circMap2k1 enhanced the protective effect of fluoxetine on neurological injury after IS in rats. A. TEM of neuronal ultrastructure in hippocampal tissue on the infarct side (right side). Red arrows indicate the nucleus, cyan arrows indicate the nuclear membrane, green arrows indicate the nucleolus, purple arrows indicate vacuolated mitochondria, orange arrows indicate double-membrane ruptured mitochondria, pink arrows indicate cristae disrupted mitochondria, and blue arrows indicate synapses. B.

Nissl staining of the number of neurons in hippocampal region. C. TUNEL staining of neuronal apoptosis in hippocampal CA1 region. D. Quantification of neuronal apoptosis in hippocampal CA1 region. E. TUNEL staining of neuronal apoptosis in the hippocampal CA3 region. F. Quantification of neuronal apoptosis in the hippocampal CA3 region. G. TUNEL staining of neuronal apoptosis in hippocampal DG region. H. Quantification of neuronal apoptosis in the hippocampal DG region. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

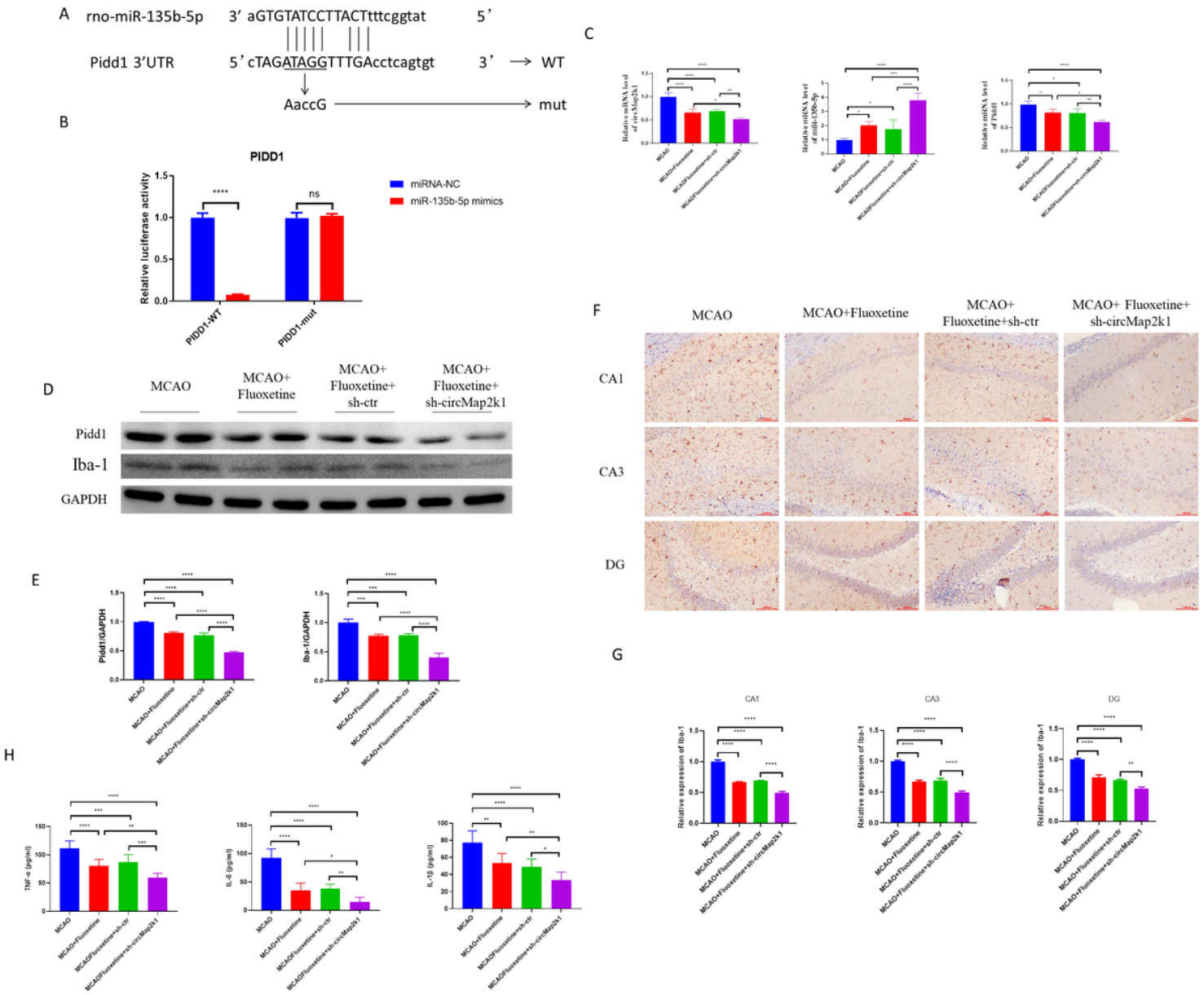

Figure 3

Fluoxetine deactivated the inhibition of miR-135b-5p by downregulating circMap2k1 to weaken the inflammatory response caused by microglial polarization after IS. A. Bioinformatics prediction of potential binding sites of Pidd1 3 'UTR and miR-135b-5p. B. Dual-luciferase report gene assay was utilized to assess luciferase activity after co-transfection of miRNA-NC or miR-135b-5p mimics and Pidd1

3 'UTR wild type (WT) or mutant (mut) plasmid in 293T cells. C. CircMap2k1, miR-135b-5p and Pidd1 levels in hippocampal tissue on the infarct side (right side) were assessed by qRT-PCR. D. Western blot analysis of Pidd1 and Iba-1 levels in hippocampal tissue on the infarct side (right side). E. Quantification of Figure D. F. The expression of Iba-1 in hippocampal tissue on the infarct side (right side) was determined by IHC. G. Quantification of Figure F. H. The levels of TNF-α, IL-6 and IL-1β in plasma was evaluated by ELISA. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

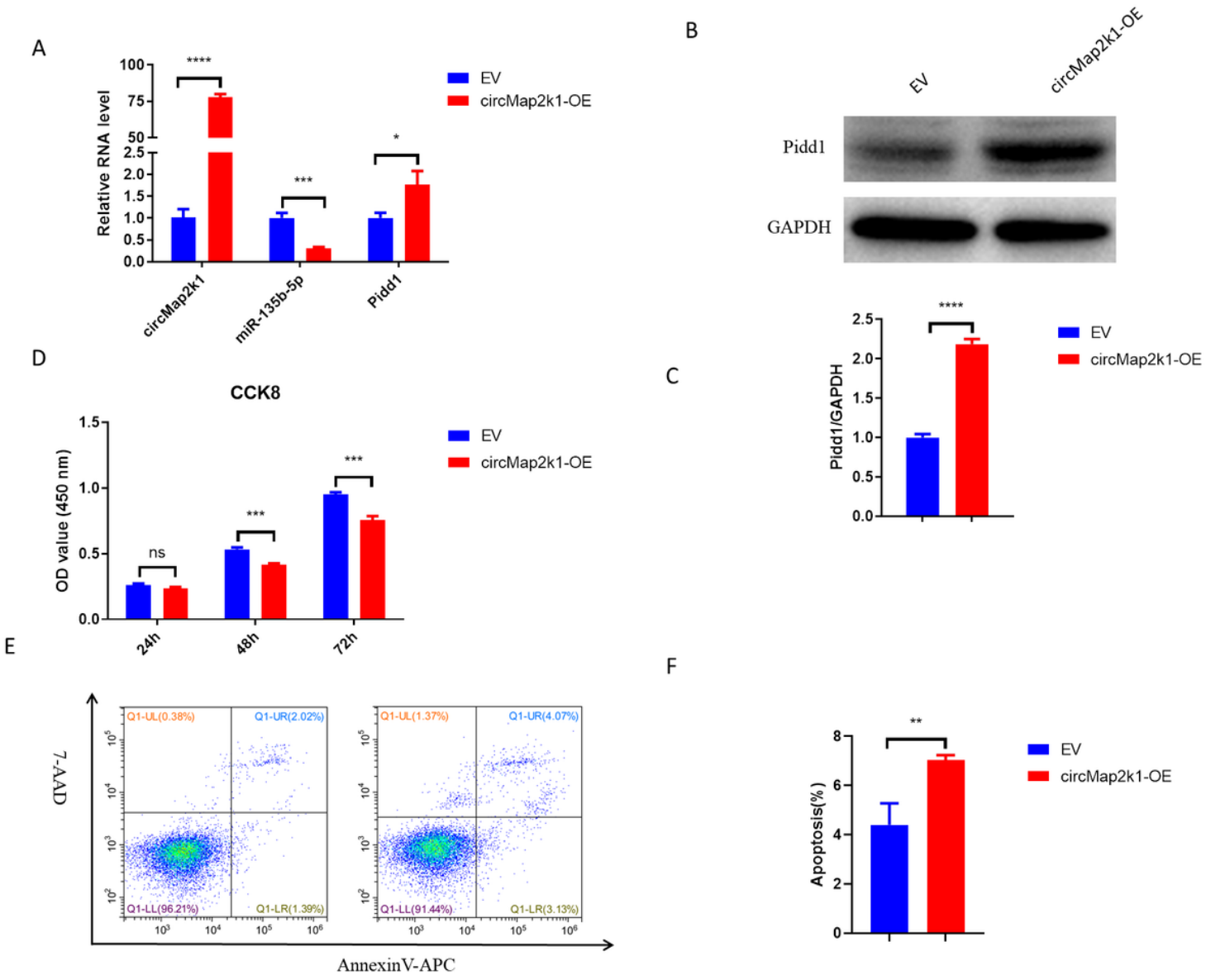

Figure 4

circMap2k1 inhibited RBMEC proliferation and promoted its apoptosis. After transfection of empty plasmids or overexpressing circMap2k1 plasmids in RBMEC for 48 h. A. CircMap2k1, miR-135b-5p and Pidd1 levels were measured by qRT-PCR. B. Western blot analysis of Pidd1 levels. C. Quantification of Figure B. D. Cell viability was determined by CCK-8 at 24, 48 and 72h after transfection. E. Apoptosis was

assessed by flow cytometry. F. Statistics of apoptosis results. ns: no significance, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.