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RESEARCH ARTICLE

Yang et al: miR-542-3p and neuronal protection in mice

miR-542-3p attenuates corticosterone-induced hippocampal neuronal damage in depressive mice by modulating PTEN/AKT/GSK3β/β-catenin pathway

Ningbo Yang[1*], Jie Li[2], Hongxia Hu[3] and Xujiang Wang[4]

[1]Department of Psychiatry, First Affiliated Hospital, College of Clinical Medicine of Henan University of Science and Technology University of Science and Technology, Jianxi, China

[2]Department of Emergency Medicine, The First Affiliated Hospital of Henan University of Science and Technology, Luolong, China

[3]Department of Clinical Laboratory Medicine, The First Affiliated Hospital of Henan University of Science and Technology, Jianxi, China

[4]Centre for Psychological Heath Education of Henan University of Science and Technology, Luolong, China

*Correspondence to Ningbo Yang: ynb20241023@hotmail.com.

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ABSTRACT

Depression is a common psychological disease, and nerve injury is the key link of depression. The molecular mechanism involved in this link needs to be explored. miR-542-3p can reduce the degree of hippocampal neuronal damage in rats, but its mechanism in the neural damage of depression is still unclear. HT-22 cell injury was induced by corticosterone (CORT). After overexpression or knockdown of miR-542-3p, CORT-induced HT-22 cell injury was tested by Cell Counting Kit-8 (CCK-8) assay, LDH assay and flow cytometry. Inflammatory and oxidative stress indicator levels were analyzed by kit and flow cytometry. The target genes of miR-542-3p were obtained by database analysis, and the targeting relationship between miR-542-3p and phosphatase and tensin homolog (PTEN) was explored based on dual luciferase assay. After PTEN overexpression or application of AKT pathway agonist MK-2206, the degree of cell damage, inflammation and oxidative stress were detected again. CORT was used to induce depression in mice. Pathological changes of brain tissue structure and neuronal survival were observed by pathological staining. The miR-542-3p, PTEN and AKT/GSK3β/β-catenin pathway protein levels *in vivo* and *in vitro* were detected by qRT-PCR and Western blot. Overexpression/knockdown of miR-542-3p alleviated/aggravated CORT-induced cell injury, inflammation and oxidation levels in HT-22 cells ($P < 0.05$). Meanwhile, overexpressed miR-542-3p can reduce neurological damage of mice. miR-542-3p can target PTEN, and it can trigger the AKT/GSK3β/β-catenin pathway by targeting PTEN expression to reduce CORT-induced nerve injury ($P < 0.05$). miR-542-3p can reduce CORT-induced hippocampal neuronal damage by targeting PTEN and activating the AKT/GSK3β/β-catenin pathway.

Keywords: miR-542-3p; phosphatase and tensin homolog; PTEN; AKT/GSK3β/β-catenin pathway; hippocampal neuronal damage.

INTRODUCTION

Depression is a common mental illness, patients with anxiety, negative weariness, slow thinking, cognitive decline, easy to self-harm or even suicide, the patient's own safety and family life adversely affected[1, 2]. Physiological, psychological, environmental and other factors are the causes of depression. At present, the commonly used anti-depression western medicine has a certain improvement effect, but there are also problems such as drug resistance and a variety of adverse reactions[3]. Depression not only affects the patient's psychological and mental state, but also causes substantial damage to the patient's brain, damaging the patient's brain structure and function. More studies have shown that depression and nerve injury are mutually causal. Depression is also a neurological dysfunction disease, and neuronal damage is a key link. Imaging results show that the density of hippocampus in patients with depression has decreased, and neuronal dendrites have decreased and necrosis[4]. Depression rat model also has hippocampal neuron damage[5]. Therefore, improving neuronal damage and exploring the corresponding mechanism of action is one of the feasible solutions to explore the treatment of depression.

MicroRNA (miRNA) is a non-coding small RNA, it has become a hot spot in nerve cell research[6]. miR-542-3p is expressed in normal brain tissue, and its expression is reduced in human glioma cells, which can inhibit cell migration and invasion[7]. Some scholars have confirmed that miR-542-3p in hippocampal neurons of epileptic rats is down-regulated through basic experimental research and clinical research. The high expression of miR-542-3p can not only reduce the damage degree of hippocampal neurons in epileptic rats, but also reduce the frequency of epileptic seizures and reduce apoptosis[8]. Moreover, up-regulation of miR-542-3p expression inhibits NLRP3-mediated inflammatory activation and reduces neuronal pyroptosis[9]. In view of this, we believe that miR-542-3p can reduce neuronal damage, and speculate that it may also have a protective effect on hippocampal neuronal damage in depression, which provides a certain direction for the exploration and treatment of molecular pathogenesis of depression.

Phosphatase and tensin homolog (PTEN) is a lipid and protein phosphatase that manages cell growth and survival. Most of them appear as tumor suppressors, and the regulation of its position is crucial to its function[10]. The localization of PTEN to the nucleus is conducive to cell growth regulation, which can promote cell survival[11]. Although PTEN has a significant correlation with cancer syndrome, PTEN function also is essential to neurodevelopment. PTEN gene mutation can cause focal abnormalities in white matter, which may lead to

anxiety disorder, developmental delay[12]. PTEN is involved in the development of autism and neurodevelopmental disorders. It can aggravate oxidative injury and hippocampal cell apoptosis[13], and neuronal dysplasia is associated with the occurrence of depression[14, 15]. Therefore, it can be speculated that PTEN can alleviate depression by improving neuronal damage.

PTEN can inhibit PI3K/AKT pathway to promote the growth of the signal cascade[16]. AKT is a serine/threonine kinase connected to cell survival, differentiation and movement. Phosphorylation of AKT can trigger GSK3β activity, which promotes the effective assembly of microtubules in neurite, leading to more branches damaging the synaptic connections of adult-born granule neurons[17]. β-catenin is the primary substrate of GSK3β and has recently been recognized as a crucial factor in behavioral recovery[18]. Studies have shown that selective knockout of β-catenin in the brain encourages depression-like behavior establishment during chronic stress[19], and antidepressants can up-regulate β-catenin in the hippocampus of depressed rats and promote neurogenesis[20]. Inhibition of GSK3β activity stabilizes β-catenin, thereby regulating neurogenesis, which in turn produces antidepressant effects[21]. In summary, we speculate that PTEN can improve the neurological damage of depression through the AKT/GSK3β/β-catenin pathway.

It is unclear whether miR-542-3p can target PTEN and mediate this signaling pathway to regulate nerve injury in depression, which is worth exploring. In view of this, we hypothesize that miR-542-3p and PTEN are connected to the pathogenesis of corticosterone (CORT) -induced neuronal damage in depressed mice, and use CORT-induced hippocampal neuronal damage models to study the expression changes of miR-542-3p and analyze its target genes and regulatory pathways. It is proposed to analyze the impacts of miR-542-3p expression changes on related molecular expression levels and neuronal damage, in order to provide new ideas for molecular target screening for clinical drug treatment of depression.

MATERIALS AND METHODS

Cell culture and processing

Mouse hippocampal neuron cell line HT-22 was purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM medium containing 10% fetal bovine serum (C0235, Beyotime, Shanghai, China) at 37℃ and 5% CO_2.

PTEN overexpression (PTEN) and negative control (vector), miR-542-3p overexpression (mimics) and negative control (mimics NC), miR-542-3p knockdown (inhibitor) and negative control (inhibitor NC) were purchased from RiboBio (Guangzhou, China). Subsequently, the cells were transfected with Lipofectamine 3000 (L3000001, Invitrogen, Austin, TX, USA), and all transfections were performed for 48 h.

HT-22 cells were divided into Control group, Model group, miR-542-3p overexpression/knockdown group, miR-542-3p overexpression+PTEN overexpression (mimics+PTEN) group and miR-542-3p overexpression+AKT inhibitor MK-2206 (mimics+MK-2206) group. The Control and Model groups were cultured with DMEM for 24 h, the other groups were transfected with miR-542-3p and PTEN, and the mimics+MK-2206 group was added with 1 μM MK-2206. Except for the Control group, 200 μM CORT was added to the other groups to induce injury for 24 h.

The effect of CORT on the viability of HT-22 cells

HT-22 cells in logarithmic growth phase were seeded in 96-well plates, 100 μL per well, and incubated for 24 h. Divided into control group and experimental group. HT-22 cells in the control group were cultured in complete medium, and the cells in the experimental group were treated with 50, 100, 200, 250 and 500 μM CORT, respectively. After 24 h of culture, 10 μL CCK-8 working solution (CA1210, Solarbio) was added and mixed gently. The fully mixed cells were placed in a cell incubator and incubated for 2 h. The absorbance of the microplate reader was set to 450 nm, the OD value was detected, the cell survival was evaluated, and the optimal concentration of CORT was screened.

qRT-PCR assay

HT-22 cells were subjected to 50, 100, 200, 250 and 500 μM CORT for 24 h, and total RNA was extracted from HT-22 cells using TransZol Up reagent (ET111-01-V2, TRANS, Beijing, China), and miRNA was extracted by EasyPure miRNA Kit (ER601-01-V2, TRANS). The concentration and quality of RNA were detected using a nucleic acid analyzer (Q5000, quawell, Beijing, China), and the $OD_{260/280\ nm}$ ratio was in the range of 1.8-2.0. AMV reverse transcriptase (2621, TAKARA, Tokyo, Japan) was added for reverse transcription to obtain cDNA. Then TB Green FAST qPCR (CN830S, TAKARA) was used for PCR reaction. At the same time, TransScript Green miRNA Two-Step qRT-PCR SuperMix (AQ202-01, TRANS) was used to synthesize the first-strand cDNA using miRNA as a template, and PCR reaction was performed. Dual distilled water is used as a template-free control (NC) to

monitor any possible contamination. The acquisition cycle threshold (Ct value) represents the number of cycles when the fluorescence signal reaches a specific threshold, which is used for data analysis. The relative level of mRNA and miRNA was computed by $2^{-\Delta\Delta Ct}$ method. *U6* and *GAPDH* can serve as a control.

The primer sequences: *miR-542-3p*: F: 5'-GCGCGATATCGCGACGAGCGACC-3'; R: 5'-TTAAGCGAGCTATCGCGCGCGAGCG-3'; *PTEN*: F: 5'-TGAGTTCCCTCAGCCATTGCCT-3'; R: 5'-GAGGTTTCCTCTGGTCCTGGTA-3'; *U6*: F: 5'-GTTCAGGAAGAGTGACACCA-3'; R: 5'-TTCTCCGCATCTCCATTCTC-3; *GAPDH*: F: 5'-AATGGATTTGGACGCATTGGT-3'; R: 5'-TTTGCACTGGTACGTGTTGAT-3'. The above primers were provided and purified by Sangon Biotech (Shanghai, China).

Bioinformatics analysis

The potential target of miR-542-3p were obtained by miRWalk (http://129.206.7.150/), TargetScan (https://www.targetscan.org/vert_80/), mirRDB (https://mirdb.org/), ENCORI/starBase (https://rnasysu.com/encori/) database. TargetScan predicted the combining region of miR-542-3p and PTEN.

Double luciferase assay

The 3'-UTR fragment of the combining region of miR-542-3p and PTEN was cloned into the plasmid vector for transformation. Plasmids with miR-542-3p and PTEN combining regions were designed on wild-type (WT) and mutant (MUT) sites. Following Lipofectamine 2000 (Invitrogen Inc., Carlsbad, CA, USA) kit, plasmid 200 ng and 30 nM miR-542-3p mimics or mimics NC were extracted and co-transfected at 37°C for 24 h. The fluorescence intensity was tested using a dual luciferase detection system (Promega Corporation, Madison, WI, USA).

Lactate dehydrogenase (LDH) test

LDH cytotoxicity assay kit (C0016, Beyotime, Shanghai, China) was used to test cell damage degree. The HT-22 cell suspension was inoculated in 96-well plates at a density of 1×10^4 cells/well, and the model was established and administered after 24 h. Cell-free culture medium holes (blank control holes) and untreated control holes for measuring the maximum enzyme activity of the sample were also included in the grouping. One hour before the conclusion of the experiment, 20 μL LDH reagent was added to the maximum enzyme activity control hole, and then mixed, and continued to be cultured in the cell culture box

until the end of the experiment. After centrifugation, 120 μL of supernatant was collected and transferred to a new culture plate. The OD value was measured by automatic microplate reader at 490 nm wavelength.

Detection of apoptosis by flow cytometry

The apoptosis was tested by annexin V-FITC/PI apoptosis detection kit (CA1020, Solarbio). The HT-22 cells after different treatments were collected and washed with PBS.About 1×10^6 cells were collected in each group, and 500 μL Binding Buffer was added to gently suspend. Then 5 μL Annexin V-FITC and 10 μL PI were mixed and reacted in dark for 15 min. The samples were transferred to a flow-specific loading tube, and apoptosis was detected by flow cytometry (BD FACSCaliburTM, BD biosciences, San Jose, CA, USA) within 1 h and the apoptosis rate was calculated.

Determination of antioxidant enzyme levels

HT-22 cells after different treatments were collected, after ultrasonic crushing, the supernatant was collected by centrifugation. Superoxide dismutase (SOD) assay kit and malondialdehyde (MDA) assay kit (A001-3-2, A003-1-2, Jiancheng Bioengineering Institute, Nanjing, China) were used to determine the level of antioxidant enzymes. The contents of MDA and SOD in HT-22 cells were operated following instructions. The OD value was read at 450 nm to calculate the enzyme content of each group.

Reactive oxygen species (ROS) level

ROS detection kit (CA1410, Solarbio) was used to detect ROS. HT-22 cells were collected and adjusted to 1×10^6/mL. DCFH-DA was added at a ratio of 1 : 1000 to a final concentration of 1 μmol/L, incubated for 30 min, and stimulated with reactive oxygen species for 30 min. The ROS level was detected by flow cytometry.

Detection of inflammatory factors by ELISA

Interleukin (IL)-6 (SEKM-0007), IL-1β (SEKM-0002), tumor necrosis factor-α (TNF-α, SEKM-0034) ELISA kits were purchased from Solarbio. After the experiment, HT-22 cells were collected, after centrifuged, the supernatant was collected. IL-6, IL-1β and TNF-α levels were tested following instructions.

Animal grouping and processing

The depression-like behavior model of male mice was induced by CORT[22]. All mice were randomly divided into equal groups: Control group, model (CORT) group, miR-542-3p overexpression negative control (agomiR NC) group, miR-542-3p overexpression (agomiR-542-3p) group and miR-542-3p overexpression+AKT inhibitor MK-2206 (agomiR-542-3p+MK-2206) group, n=8 mice per group. Mice in the control group were subcutaneously injected with 0.9% normal saline every day, and mice in the other groups were injected with 20 mg/kg CORT. In addition, mice in the agomiR NC, agomiR-542-3p and agomiR-542-3p+MK-2206 groups were injected with 5 nmol agomiR NC or agomiR-542-3p through the lateral ventricle once a week: After anesthesia with 2% isoflurane, the mice were fixed on the brain stereotaxic instrument, disinfected the skin, made a 0.5 cm incision in the middle of the head, and slowly injected 3 mm vertically at 2.5 mm behind the anterior fontanel and 2 mm on the right side. A microsyringe was used to absorb 2 μL of the corresponding solution and injected into the lateral ventricle of the corresponding group of mice. After the injection, the needle was stopped for 5 min and the syringe was slowly removed. Then the wound was sutured and disinfected. After the mice were awake, they were put back into the cage for feeding. At the same time, the agomiR-542-3p+MK-2206 group was given 120 mg/kg MK-2206 (Beyotime, Shanghai, China) by gavage every day for 21 d. After the behavioral test was completed, the mice were anesthetized and sacrificed by cervical dislocation. The whole brain tissue of the mice was collected. The brain tissue of each group of mice was washed with normal saline, and the hippocampus tissue in the brain tissue was stripped on ice and stored at -80℃.

Observation of depressive behavior

Sucrose preference test (SPT), tail suspension test (TST) and forced swimming test (FST) were used to evaluate the changes of depressive behavior in mice by two evaluators who were unaware of the experimental grouping. SPT was performed according to the experimental method of Zheng Gu[23], and the consumption of sucrose solution and pure water was calculated after 12 h. TST and FST were performed according to the experimental method of Yingying Sun[24], and the total time of mice in water and suspension was noted.

HE staining

The mouse brain tissue was fixed with 4% paraformaldehyde, embedded in paraffin, and then the tissue specimens were cut into 4 μm thick sections. The sections were stained with

hematoxylin (C0107, Beyotime) for 15 min, differentiated in 1% acidic alcohol (containing 70% hydrochloric acid) for 30 s, rinsed with running water, and then stained with 0.5% eosin (G1100, Solarbio) for 3 min. Then the slices were dehydrated by alcohol gradient dehydration and xylene transparent treatment, neutral gum (G8590, Solarbio) sealing, and the hippocampal CA1 region was observed under a microscope.

Nissl staining

Nissl bodies was stained by Nissl staining kit (G1434, Solarbio). The sections of brain tissue were stained with methylene blue for 10 min, and placed in differentiation medium for 1 min, treatment with ammonium molybdate solution for 3 min, and mounting with neutral gum (G8590, Solarbio). The number and morphological changes of Nissl bodies in hippocampal CA1 region were observed under microscope.

TUNEL/NeuN immunofluorescence

Brain tissue sections were dewaxed with xylene, hydrated, and antigen repaired. The sections were blocked with 5% BSA and 0.5% Triton-X100 mixed solution for 2 h. Rabbit anti-neuronal nuclei (NeuN, ab177487, 1 : 1000, Abcam) were added and incubated overnight at 4℃, followed by incubation with goat anti-rat IgG (GB21302, 1 : 500, Servicebio, Wuhan, China) for 2 h. Then, 20 μg/mL proteinase K solution without DNase was added for 30 min, apoptotic cells were labeled with 50 μL Tunel detection solution (C1091, Beyotime) for 60 min in the dark, and then DAPI staining solution was added for 5 min. Seal the film with anti-fluorescence quenching sealing solution, the hippocampal CA1 area was observed and photographed by fluorescence microscope (Olympus, VS200, Shinjuku, Tokyo).

Western blot

After different treatments of HT22 cells and mouse hippocampus tissue were collected, the supernatant was fully lysed. The total protein concentration was measured using BCA quantification kit (PC0020, Solarbio). All samples was detached by electrophoresis, and the protein was then transferred to the PVDF membranes (YA1700, Solarbio). The membranes were incubated with 5% skimmed milk powder (LP0033B, Solarbio) for 2 h, and PTEN (ab267787, 1 : 1000, Abcam), APBB2 (ab137888, 1 : 1000), AKT (ab8805, 1 : 1000), GSK3β (ab93926, 1 : 1000), β-catenin (ab32572, 1∶5000), PSD95 (ab238135, 1 : 2000), SYP (ab32127, 1 : 20000), p-GSK3β Ser9 (ab75814, 1 : 1000), Bax (ab32503, 1 : 2000), p-AKT Thr308 (ab38449, 1 : 1000), Bcl-2 (ab182858, 1 : 2000) and GAPDH (TA-08, 1 : 1000,

ZSGB-BIO, Beijing, China) incubated them at 4℃ for one night. The membranes were washed with TBST buffer (T1082, Solarbio) and incubated with secondary antibody (1 : 20000) for 1 h. After 5 times washed with TBST buffer, ECL (PE0010, Solarbio) reagent was used to react for 2-3 min, and then automatic chemiluminescence imaging system was used for imaging.

Ethical statement

Forty male C57/BL6 mice of SPF grade, 6 weeks old, weighing (20±2) g, were provided by Spyford Biotechnology Co., Ltd. (Beijing, China). Mice were fed under normal conditions (temperature 20 - 24℃, relative humidity 50% - 70%, light and dark 12h/12h). This study was approved by The First Affiliated Hospital of Henan University of Science and Technology Animal Ethics Committee (No.HK-20230417).

Statistical analysis

Three independent biological replicates were used. The Shapiro-Wilk test was used to test the normality of the measurement data. All measurement data were normally distributed and expressed as mean ± standard deviation. Statistical analysis and image drawing were performed using Graphpad 9.0. One-way ANOVA analysis was used to compare multiple groups. If the variance was homogeneous, least significant difference (LSD) test was used for pairwise comparison between groups, and Games-Howell test was used for heterogeneity of variance. Non-normality distribution was tested by non-parametric test. $P < 0.05$ was considered statistically significant, when the results are significant, the Tukey method is used for post-test.

RESULTS

miR-542-3p attenuates corticosterone-induced HT-22 cell injury in mouse hippocampal neurons

Firstly, we used CCK-8 and qRT-PCR assays to test the cell survival rate and *miR-542-3p* level after various levels of CORT treatment for 24 h, respectively. The cell survival rate began to decrease significantly after 200 μM CORT intervention (Fig.1A), and *miR-542-3p* level also decreased significantly (Fig.1B). Combined with previous studies, we selected 200 μM CORT for subsequent experiments. To investigate the impact of *miR-542-3p* on CORT-induced HT-22 injury, we overexpressed/knocked down *miR-542-3p* and verified its transfection efficiency. The *miR-542-3p* level was notably elevated/shrunk (Fig.1C-D),

suggesting that subsequent experiments can be performed. Overexpressed miR-542-3p could obviously improve the decrease of cell viability induced by CORT, and knockdown further aggravated the damage of cell viability (Fig.1E), indicating that miR-542-3p could improve CORT-induced cell damage. LDH is widely used for cytotoxicity detection. The LDH activity was notably increased after CORT treatment. On this basis, the LDH activity was notably declined after overexpressed miR-542-3p, and the LDH level was further significantly increased after knockdown (Fig.1F), showing that miR-542-3p could diminish cell damage degree caused by CORT. Next, we detected the apoptosis rate. The apoptosis rate was significantly increased after CORT treatment. The apoptosis rate was notably declined after overexpressed miR-542-3p, and significantly increased after knockdown (Fig.1G-H). In short, miR-542-3p can regulate nerve injury process induced by CORT, and play a protective role by enhancing cell viability and reducing apoptosis. Inflammation and oxidative stress play a crucial role in neuronal death[25]. SOD and MDA are commonly used indicators to reflect oxidative stress. The SOD content shrunk significantly and MDA content increased significantly after CORT treatment. After overexpression/knockdown of miR-542-3p, the SOD content increased/decreased significantly and the content of MDA decreased/increased significantly (Fig.1I-J). The TNF-α (Fig.1K), IL-1β (Fig.1L), IL-6 (Fig.1M) content and ROS intensity (Fig.1N-O) had the same trend as MDA, which indicated that miR-542-3p could inhibit CORT-induced inflammatory and oxidative stress injury in HT-22 cells, which may be the key to exert cell protective effects.

miR-542-3p targeted regulation of PTEN

Bioinformatics analysis showed that when the miRWalk database targeting score ≥ 0.9, mirRDB database ≥ 90, ENCORI/starBase database ≥ 1.5, the potential targets of *miR-542-3p* were *PTEN* and *APBB2* (Fig.2A), suggesting that *miR-542-3p* could target *PTEN* and *APBB2*. Then we detected PTEN and APBB2 levels, and they were significantly raised after CORT treatment, but the increased effect of APBB2 was not as good as that of PTEN (Fig.2B-C), suggesting that the potential promoting effect of PTEN on nerve injury was more obvious than that of APBB2. After overexpression/knockdown of miR-542-3p, *PTEN* and *APBB2* expression were markedly decreased/increased, but *PTEN* targeting effect was better (Fig.2D-H). The above experiments showed that PTEN targeting effect was better, and APBB2 was not significantly expressed after CORT treatment, so PTEN was selected for subsequent experiments. Next, we used luciferase reporter gene assay to verify whether miR-542-3p and PTEN bind. miR-542-3p overexpression could significantly inhibit the

luciferase activity of WT-PTEN (Fig.2I), denoting that miR-542-3p could bind to PTEN; there is a combining site between them. The TargetScan database showed that context ++ score was-0.20, context ++ score percentile was 88, and weighted context ++ score was -0.19. And the binding sequence is shown in Fig.2J. In summary, miR-542-3p can target down-regulate PTEN.

miR-542-3p inhibits corticosterone-induced HT-22 injury in mouse hippocampal neurons by inhibiting PTEN

Based on the above results, we further explored whether miR-542-3p can target PTEN to affect CORT-induced HT-22 cell damage. We first verified the efficiency of PTEN overexpression, and Western blot results showed that PTEN protein was effectively overexpressed (Fig.3A-B), suggesting that subsequent experiments can be performed. Then the experiment was split into model (mimics NC+vector) group, miR-542-3p overexpression (mimics+vector) group, miR-542-3p overexpression + PTEN overexpression (mimics+PTEN) group. Similar to the above results, miR-542-3p overexpression diminished inflammatory and oxidative stress injury in cell injury and improved cell injury; on this basis, PTEN was overexpressed, and cell viability was significantly decreased (Fig.3C), LDH activity was significantly increased (Fig.3D), SOD content was obviously shrunk (Fig.3E), MDA content was notably raised (Fig.3F), ROS intensity (Fig.3G-H), TNF-α, IL-1β, IL-6 content (Fig.3I) and apoptosis rate (Fig.3J-K) had the same trend as MDA. It indicated that PTEN overexpression significantly attenuated the improvement of miR-542-3p overexpression on cell injury. Combined with bioinformatics results, miR-542-3p could target PTEN to reduce CORT-induced cell damage, and its mechanism is associated with the suppression of oxidative stress and inflammatory response.

Overexpression of miR-542-3p negatively regulates PTEN expression to activate AKT/GSK3β/β-catenin

In order to verify whether miR-542-3p can affect nerve injury by AKT/GSK3β/β-catenin pathway, we divided the experiment into model (mimics NC+vector) group, miR-542-3p overexpression (mimics+vector) group, miR-542-3p overexpression + PTEN overexpression (mimics+PTEN) group and miR-542-3p overexpression + AKT pathway inhibitor MK-2206 (mimics+MK-2206) group. p-AKT Thr308, p-GSK3β Ser9 and β-catenin protein levels were evidently declined after CORT treatment (Fig.4A-B), indicating that the AKT/GSK3β/β-catenin pathway can improve neuronal cell injury. After overexpressed

miR-542-3p, the pathway protein level increased significantly, indicating that miR-542-3p overexpression could activate the AKT/GSK3β/β-catenin pathway. After overexpression of PTEN or application of MK-2206, the level of pathway proteins was decreased (Fig.4C-D), indicating that both PTEN overexpression and AKT pathway inhibitors could weaken the pathway activation caused by miR-542-3p overexpression. In short, miR-542-3p can target down-regulate PTEN to activate the AKT/GSK3β/β-catenin pathway.

To verify the effect of AKT/GSK3β/β-catenin pathway on neuronal cell injury, we found that MK-2206 significantly decreased cell viability (Fig.4E), significantly increased LDH activity (Fig.4F), notably abated SOD content (Fig.4G), markedly enhanced MDA content (Fig.4H), significantly increased ROS intensity (Fig.4I-J), significantly increased inflammatory factor levels (Fig.4K), and significantly increased apoptosis rate (Fig.4L-M). It indicated that AKT pathway inhibitors significantly attenuated the improvement of miR-542-3p overexpression on cell injury. Combined with the experimental results of AKT/GSK3β/β-catenin pathway, suggesting that miR-542-3p could target down-regulate PTEN to trigger AKT/GSK3β/β-catenin pathway and reduce CORT-induced neuronal cell injury. The mechanism is associated with the suppression of oxidative stress and inflammatory response.

miR-542-3p attenuates hippocampal neuronal damage in corticosterone-induced depressive mice

Given the function and related mechanisms of miR-542-3p *in vitro*, we further explored whether it could affect hippocampal neuronal damage in CORT-induced depressive mice. By intracerebroventricular injection of agomiR NC, agomiR-542-3p and MK-2206, the experimental process is shown in Fig.5A, and then related tests were performed. *miR-542-3p* level elevated obviously after injection of agomiR-542-3p, and decreased significantly after application of MK-2206, indicating that *miR-542-3p* level was increased (Fig.5B), suggesting that it was effectively highly expressed *in vivo* and could be used for subsequent experiments. The behavioral observation of mice showed that the sucrose preference rate of mice decreased significantly after CORT treatment, and the time of staying still in water and suspension increased significantly (Fig.5C-E), indicating that the depression model was successfully constructed. After overexpressed miR-542-3p, the depressive behavior of mice was significantly improved, but after application of MK-2206, the depressive behavior was significantly aggravated, denoting that miR-542-3p can improve the depressive behavior. Then, the neuronal damage in the hippocampal was evaluated by HE and Nissl staining. The results showed that after CORT treatment, the nerve cells were swollen and irregular in shape,

with a large number of necrosis, and the morphology of Nissl bodies was disordered. The number was significantly reduced (Fig.5F-G), neuronal apoptosis was significantly increased, and neuronal survival rate was significantly reduced (Fig.5H-K), indicating the phenomenon of nerve tissue damage; after overexpressed miR-542-3p, neuronal necrosis abated, the number of Nissl bodies increased significantly, neuronal apoptosis decreased significantly, and the survival rate increased significantly. However, after application of MK-2206, the damage of nerve tissue was significantly aggravated, denoting that the high expression of miR-542-3p can obviously repair the structural and functional damage of hippocampal neurons. In conclusion, miR-542-3p can alleviate hippocampal neuronal damage in CORT-induced depression mice.

miR-542-3p attenuates hippocampal neuronal damage in corticosterone-induced depressive mice by regulating PTEN to activate AKT/GSK3β/β-catenin pathway

Given that the AKT/GSK3β/β-catenin pathway can reduce CORT-induced neuronal cell damage *in vitro*, we further explored it *in vivo*. The protein level change trend was consistent with the *in vitro* test results (Fig.6A-E). In addition, we also detected apoptosis and neuroplasticity-related proteins, and found that Bax/Bcl-2 protein levels increased significantly after CORT treatment, and SYP and PSD95 protein levels decreased significantly. After overexpressed miR-542-3p, Bax/Bcl-2 levels were significantly reduced, SYP and PSD95 protein levels were significantly increased, but the protein levels were markedly reversed after the application of MK-2206 (Fig.6F-J), indicating that miR-542-3p can reduce CORT-induced neuronal damage by reducing apoptosis and remodeling nerves. In summary, miR-542-3p can down-regulation of PTEN to stimulate the AKT/GSK3β/β-catenin pathway to diminish apoptosis, reshape nerves, and reduce CORT-induced neuronal damage.

DISCUSSION

Depression is a mental disorder. Its clinical symptoms are emotional depression, and reduced language action. Lack of pleasure and low motivation are its main symptoms. With the accelerated pace of life, increased competitive pressure, the prevalence of depression increased rapidly[26]. The depression occurrence is associated with genetic, environmental, psychological and social factors[27]. The pathological mechanism is characterized by central nervous system inflammation, neuronal apoptosis, pyroptosis and autophagy, accompanied by atrophy and dysfunction of cerebral cortex, white matter, amygdala and hippocampus[28, 29]. However, the commonly used drugs in clinical practice have a variety of adverse

reactions[3], so it is very key to find novel therapeutic drugs. Elevated levels of miR-542-3p can diminish neuronal damage on patients with epilepsy, reduce the frequency of seizures and neuronal apoptosis[8], suggesting that miR-542-3p may treat depression by reducing neuronal damage. To clarify the mechanism of miR-542-3p in reducing nerve injury, we used CORT-induced neuronal injury model to survey the action of miR-542-3p and its targets on neuronal injury after modeling.

In this study, after CORT treatment, the cell viability was notably decreased, and cell damage degree and apoptosis rate were notably raised. After overexpression/knockdown of miR-542-3p, the cell viability was notably elevated/declined, and cell injury degree and apoptosis rate were significantly decreased/increased, indicating that miR-542-3p can adjust CORT-induced nerve injury process, and play a protective role by enhancing cell viability and reducing apoptosis. In recent years, neuroinflammation and oxidative stress, as possible causes of mental illness[30], can damage neurons, so we tested this. After CORT treatment, the level of oxidative index SOD was obviously declined, the levels of MDA, ROS and inflammatory indexes were notably enhanced, and the apoptosis was markedly increased. After miR-542-3p overexpression/knockdown, SOD level was markedly increased/declined, MDA, ROS and inflammatory index levels were notably lessened/increased, and apoptosis was significantly decreased/increased. In summary, inflammatory response and oxidative stress occur in CORT-induced nerve injury, and miR-542-3p can reduce nerve injury by inhibiting oxidative and inflammatory injury.

We analyzed the potential targets of miR-542-3p by bioinformatics as *PTEN* and *APBB2*, but found that PTEN targeting effect was better in subsequent experiments, so we chose PTEN for subsequent experiments. Through bioinformatics, there was a combining site between them, indicating that miR-542-3p could bind to PTEN, indicating that miR-542-3p could target PTEN. PTEN aggravated sevoflurane-induced hippocampal cell apoptosis by inhibiting MEK1/ERK pathway[13]. PTEN is important for controlling the regeneration ability of injured neurons. PTEN deletion or inhibition can inhibit the axon growth of a variety of neuronal populations[31]. However, it is not clear whether miR-542-3p can reduce nerve injury by targeting PTEN. Therefore, we overexpressed PTEN on the basis of miR-542-3p overexpression. The cell viability was markedly diminished, the degree of injury and apoptosis were notably boosted, SOD level was notably lessened, MDA, ROS and inflammatory factor levels were obviously elevated, indicating that PTEN overexpression can increase the degree of nerve injury induced by CORT and weaken the improvement effect of

miR-542-3p overexpression. Combined with bioinformatics results, miR-542-3p can target PTEN to reduce nerve injury.

PTEN can mediate oxidative stress, inflammation, autophagy, and neuroprotection by regulating AKT signaling activation. Stimulation of AKT/mTOR signaling by silencing PTEN can reduce autophagy and oxidative stress levels, thereby reducing acute kidney injury in mice[32]. It can also promote the proliferation of brain microvascular endothelial cells and enhance angiogenesis, thereby exerting neuroprotective effects on focal cerebral ischemia rats[33]. In addition, PTEN is also an important inflammatory regulator, which can mediate inflammation and apoptosis by regulating AKT signaling activity. Inhibition of PTEN expression can enhance AKT phosphorylation[34]. Down-regulation of PTEN expression and enhancement of AKT signaling activity can diminish the production of inflammatory cytokines, and protect mice from CIRI by diminishing inflammation and neuronal apoptosis[35]. AKT can promote GSK3β phosphorylation, inhibit the activity of GSK3β[36], stabilize β-catenin, and then translocate into the nucleus to participate in nerve regeneration[37]. Therefore, in this study, we explored the AKT/GSK3β/β-catenin pathway and found that p-AKT Thr308, p-GSK3β Ser9 and β-catenin protein levels were significantly decreased after CORT treatment, indicating that the AKT/GSK3β/β-catenin pathway can improve CORT-induced neuronal cell injury; when miR-542-3p was overexpressed, pathway protein levels increased significantly, indicating that miR-542-3p overexpression could activate AKT/GSK3β/β-catenin pathway; after overexpressed PTEN with miR-542-3p overexpression, pathway protein levels decreased, indicating that PTEN overexpression could weaken the pathway activation caused by miR-542-3p overexpression. MK-2206 is an AKT inhibitor that reduces the phosphorylation level of AKT and its downstream gene GSK3β, while increasing β-catenin level[38]. The addition of MK-2206 on the basis of miR-542-3p overexpression can reduce the protein level. In summary, miR-542-3p can target PTEN to trigger AKT/GSK3β/β-catenin pathway. At the same time, it was found that after the application of MK-2206, the cell viability was markedly declined, the degree of injury and apoptosis were notably raised, SOD level was considerably shrunk, MDA, ROS and inflammatory factors were considerably elevated. It is further explained that miR-542-3p can target PTEN to trigger AKT/GSK3β/β-catenin pathway to reduce CORT-induced neuronal cell injury, and its mechanism is associated with the suppression of oxidative stress and inflammatory response. In the future, we will further clarify whether the AKT/GSK3β/β-catenin pathway is essential for neuronal viability through other experiments

(such as β-catenin knockdown, overexpression, or changes in nuclear β-catenin localization) and more known AKT/GSK3β/β-catenin pathway inhibitors or activators.

Finally, we verified the impact of miR-542-3p in depression *in vivo*. Depression was induced by CORT. The model was evaluated by SPT, TST and FST. After modeling, the sucrose preference rate decreased, and the immobility time in TST and FST elevated, showing obvious anhedonia and behavioral despair, indicating that the mouse depression model was successfully established. Overexpressed miR-542-3p can improve the disappointment behavior, boost the sucrose preference rate, significantly shorten the immobility time, effectively enhance the excitability of mice in self-help, reduce their hopelessness, and have antidepressant effects. Studies have found that hippocampal neuronal damage is closely related to depression pathogenesis[39]. So we detected hippocampal neurons and found that after CORT treatment, nerve cells were swollen and irregular in shape, with a large number of necrosis, a substantial diminish in Nissl body numbers, a considerable enhance in neuronal apoptosis, and a notable decline in survival rate, indicating the occurrence of nerve tissue damage. After overexpression of miR-542-3p, nerve injury was markedly restored, indicating that miR-542-3p could reduce hippocampal neuronal damage in CORT-induced depression mice. At the same time, it was found that AKT/GSK3β/β-catenin pathway protein was consistent with the results of *in vitro* detection, which again indicated that miR-542-3p could activate AKT/GSK3β/β-catenin pathway to reduce CORT-induced neuronal cell damage. In addition, Bcl-2 is considered to be an inhibitor of apoptosis, which can inhibit apoptosis, up-regulate the expression of Bcl-2 to protect nerve cells and inhibit their apoptosis[40]; Bax is a pro-apoptotic gene. Bax binds to Bcl-2 to form a heterodimer, which inhibits Bcl-2 from playing a role[41]. Therefore, Bax and Bcl-2 are considered to be indicators for evaluating apoptosis. The smaller the Bax/Bcl-2 ratio is, the stronger the anti-apoptotic ability is. Synaptic membrane proteins SYP and PSD95 affect synaptic neurotransmitter transmission and participate in synaptic plasticity regulation[42], thereby regulating neural plasticity. In this research, Bax/Bcl-2 protein level increased notably after CORT induction, and the levels of SYP and PSD95 protein decreased significantly, indicating that the anti-apoptotic ability was weak and the neural plasticity was poor. After overexpressed miR-542-3p, Bax/Bcl-2 protein level was significantly reduced, SYP and PSD95 protein levels were significantly increased, but the protein levels were significantly reversed after application of MK-2206, indicating that miR-542-3p can reduce CORT-induced neuronal damage by reducing apoptosis and remodeling nerves. In summary, miR-542-3p could target down-regulate PTEN

to trigger AKT/GSK3β/β-catenin pathway to reduce apoptosis, repair structural and functional damage of hippocampal neurons, reshape nerves, and reduce CORT-induced neuronal damage in depressed mice.

CONCLUSION

This paper reveals the mechanism of miR-542-3p in CORT-induced nerve injury. miR-542-3p can engage the AKT/GSK3β/β-catenin pathway by targeting PTEN expression, thereby reducing ROS and inflammatory factors and increasing antioxidant enzyme activity. MiR-542-3p can reduce CORT-induced HT-22 cell damage and apoptosis by inhibiting oxidative and inflammatory response, and ultimately is significant for neuronal protection. This study supplies a new target for nerve injury in depression treatment, which is helpful to improve the treatment technology with depression. In many previous studies, miR-542-3p was observed to be significantly down-regulated in serum of patients with acute myocardial infarction and in epithelial ovarian cancer tissues, and was also significantly expressed in various cancers, suggesting the application value of miR-542-3p in the clinical environment. However, miR-542-3p level and its target factors in clinical patients with nerve injury and their predictive value for disease severity and prognosis need to be further explored.

Conflicts of interest: Authors declare no conflicts of interest.

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Data availability: The data supporting the findings of this study can be obtained from the corresponding author, upon request.

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FIGURES WITH LEGENDS

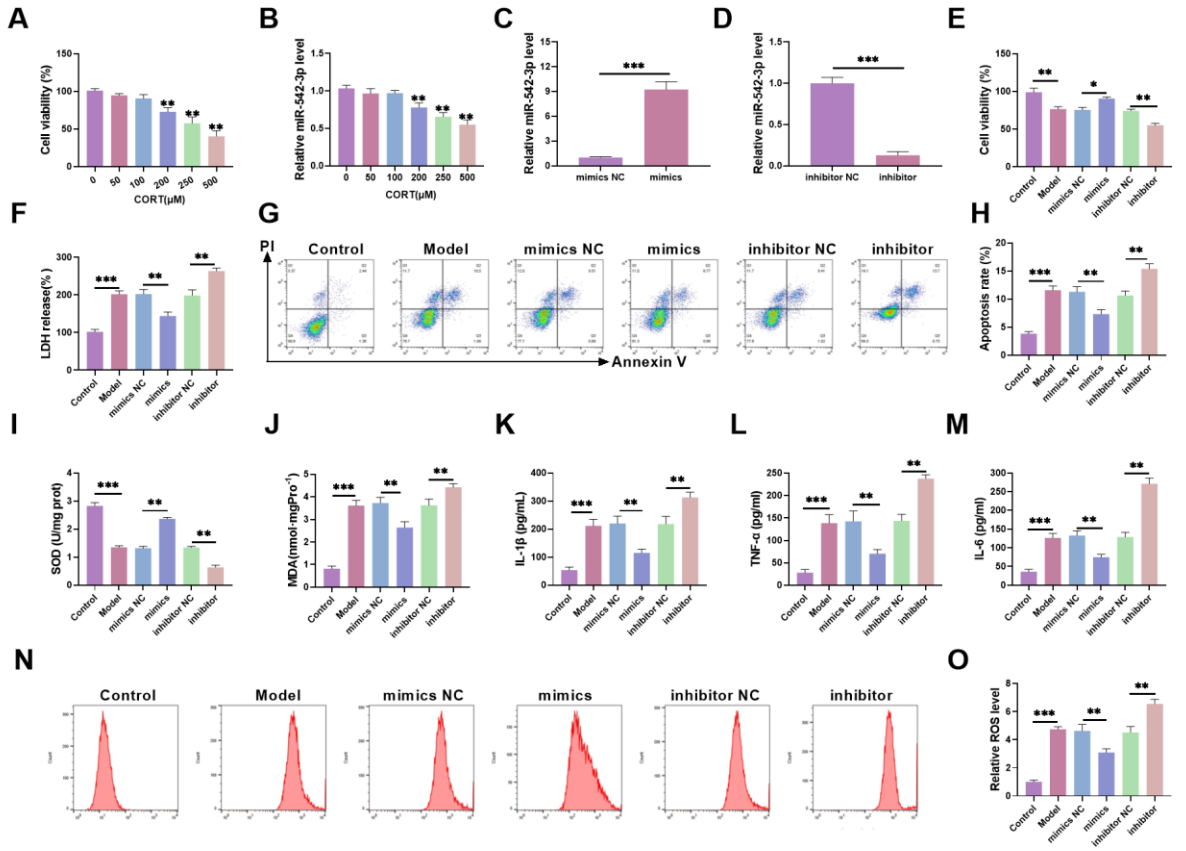

Figure 1. miR-542-3p attenuates corticosterone-induced HT-22 cell injury in mouse hippocampal neurons. (**A**) The cell survival rate was tested by CCK-8 after various levels of CORT treatment for 24 h. It was found that the cell survival rate began to decrease significantly after 200 μM CORT treatment; (**B**) *miR-542-3p* level was tested by qRT-PCR after various levels of CORT treatment for 24 h. The level of *miR-542-3p* began to decrease obviously after 200 μM CORT treatment; (**C-D**) The overexpression/knockdown efficiency of *miR-542-3p* was tested by qRT-PCR, and it was effectively overexpressed/knocked down; (**E**) The proliferation of HT-22 cells was tested by CCK-8 under various treatment conditions. It was found that the cell viability was markedly decreased after CORT intervention, and it was significantly increased/decreased after miR-542-3p overexpression/knockdown; (**F**) The damage degree of HT-22 cells treated with various treatments was detected. It was found that

the release of LDH was markedly raised after CORT treatment, and it was notably declined/increased after overexpression/knockdown of miR-542-3p; (**G-H**) The apoptosis rate was notably elevated after CORT intervention, and it was obviously declined/increased after miR-542-3p overexpression/knockdown; (**I**) The content of SOD was detected by ELISA. The content of SOD was markedly reduced after CORT intervention, and the it was markedly increased/reduced after miR-542-3p overexpression/knockdown; (**J**) The content of MDA was detected by ELISA. MDA content was evidently enhanced after CORT treatment, and it was evidently decreased/enhanced after miR-542-3p overexpression/knockdown; (**K-M**) The TNF-α, IL-1β and IL-6 levels were tested by ELISA. They were considerably elevated after CORT treatment, and they were considerably decreased/raised after miR-542-3p overexpression/knockdown; (**N-O**) The level of ROS was tested by flow cytometry. It was found that ROS level was considerably boosted after CORT treatment, and it was considerably reduced/increased after overexpression/knockdown of miR-542-3p. PTEN: Phosphatase and tensin homolog; CCK-8: Cell counting kit-8; ELISA: Enzyme-linked immunosorbent assay; SOD: Superoxide dismutase; MDA: Malondialdehyde; ROS: Reactive oxygen species; CORT: Corticosterone; qRT-PCR: Quantitative real-time polymerase chain reaction. n=3, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

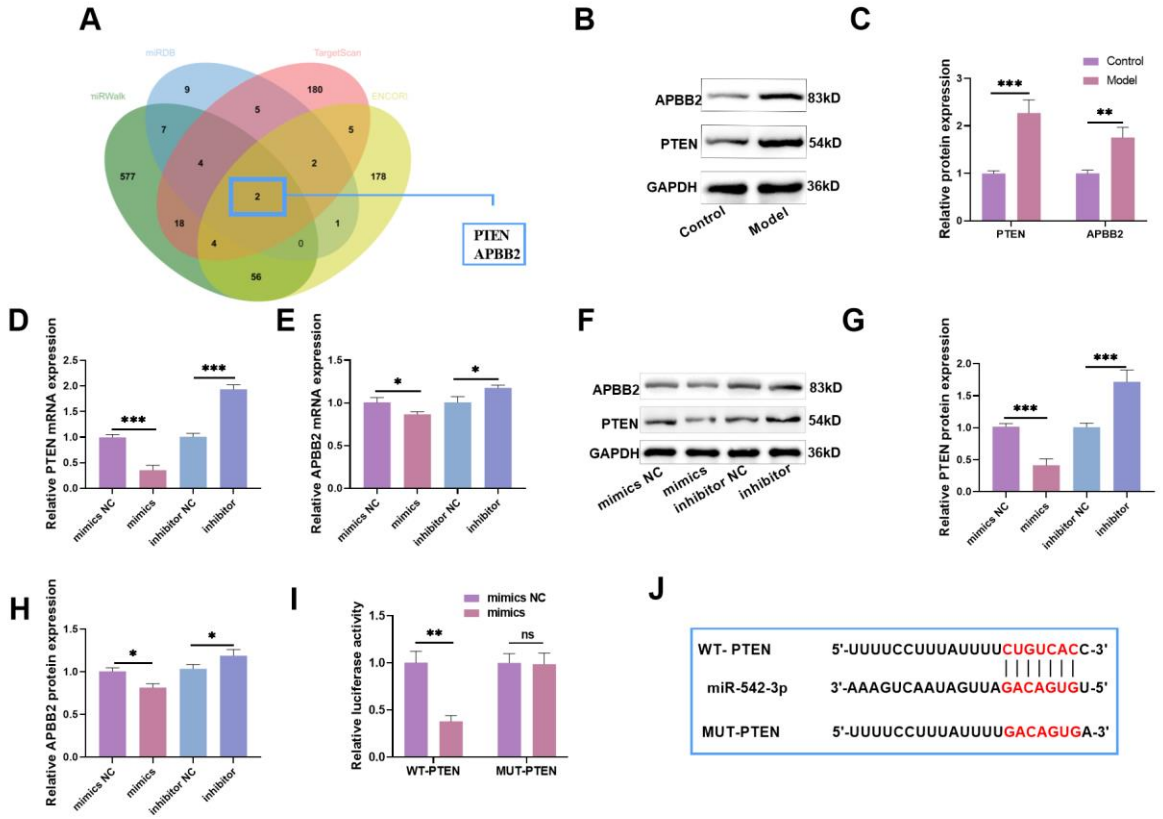

Figure 2. miR-542-3p targeted regulation of PTEN. (**A**) The potential target *PTEN* and *APBB2* of *miR-542-3p* were obtained by the intersection of miRWalk, TargetScan, mirRDB, ENCORI/starBase database; (**B-C**) Western blot showed that PTEN and APBB2 levels were notably elevated after CORT treatment, but the increase effect of APBB2 was not as good as that of PTEN; (**D-H**) *PTEN* and *APBB2* levels were notably declined/increased after miR-542-3p overexpression/knockdown, but the expression of *PTEN* was more notable; (**I**) WT-PTEN and MUT-PTEN luciferase reporter plasmids were co-transfected with mimics NC and mimics into HT-22 cells, respectively. The luciferase activity was tested. Overexpression of miR-542-3p markedly inhibited the luciferase activity of WT-PTEN; (**J**) The binding sequence of miR-542-3p and PTEN. PTEN: Phosphatase and tensin homolog; APBB2: Amyloid beta precursor protein binding family B member 2; CORT: Corticosterone. n=3, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

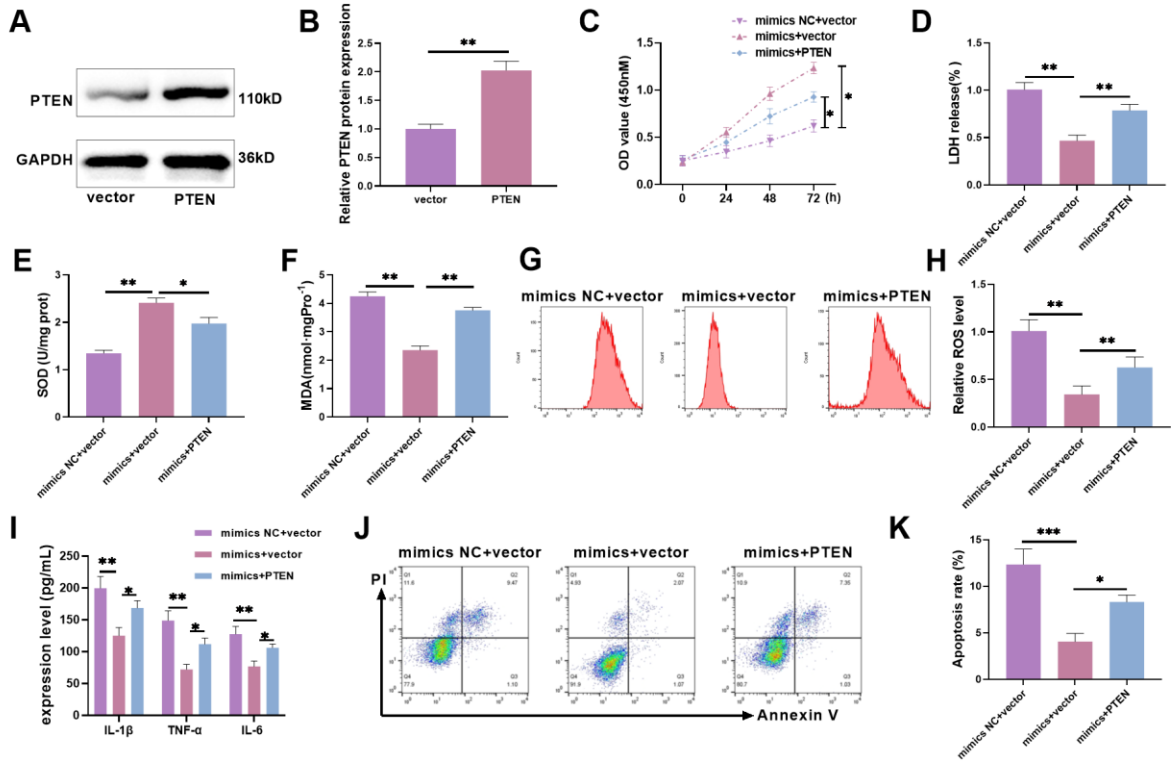

Figure 3. miR-542-3p inhibits corticosterone-induced HT-22 injury in mouse hippocampal neurons by inhibiting PTEN. (**A-B**) The overexpression efficiency of PTEN was tested by Western blot, and PTEN was effectively overexpressed; (**C**) The proliferation of HT-22 cells under various treatment conditions was tested by CCK-8. Compared with miR-542-3p overexpression alone, the cell viability was notably lower after overexpressed PTEN; (**D**) LDH was used to test the damage degree in HT-22 cells treated with different treatments. Compared with miR-542-3p overexpression alone, PTEN overexpression notably elevated cell damage degree; (**E**) The content of SOD was detected by ELISA. Compared with miR-542-3p overexpression alone, it was notably shrunk after PTEN overexpression; (**F**) The MDA content was detected by ELISA; (**G-H**) The ROS level was tested by flow cytometry, ROS level was markedly enhanced after PTEN overexpression; (**I**) The levels of IL-1β, TNF-α and IL-6 were tested by ELISA, they were significantly increased after PTEN overexpression compared with miR-542-3p overexpression alone; (**J-K**) The apoptosis was

tested by flow cytometry. After PTEN overexpression, the apoptosis rate was markedly higher. PTEN: Phosphatase and tensin homolog; CCK-8: Cell counting kit-8; ELISA: Enzyme-linked immunosorbent assay; SOD: Superoxide dismutase; MDA: Malondialdehyde; ROS: Reactive oxygen species; IL: Interleukin; TNF-α: Tumor necrosis factor-α. n=3, * P < 0.05, ** P < 0.01.

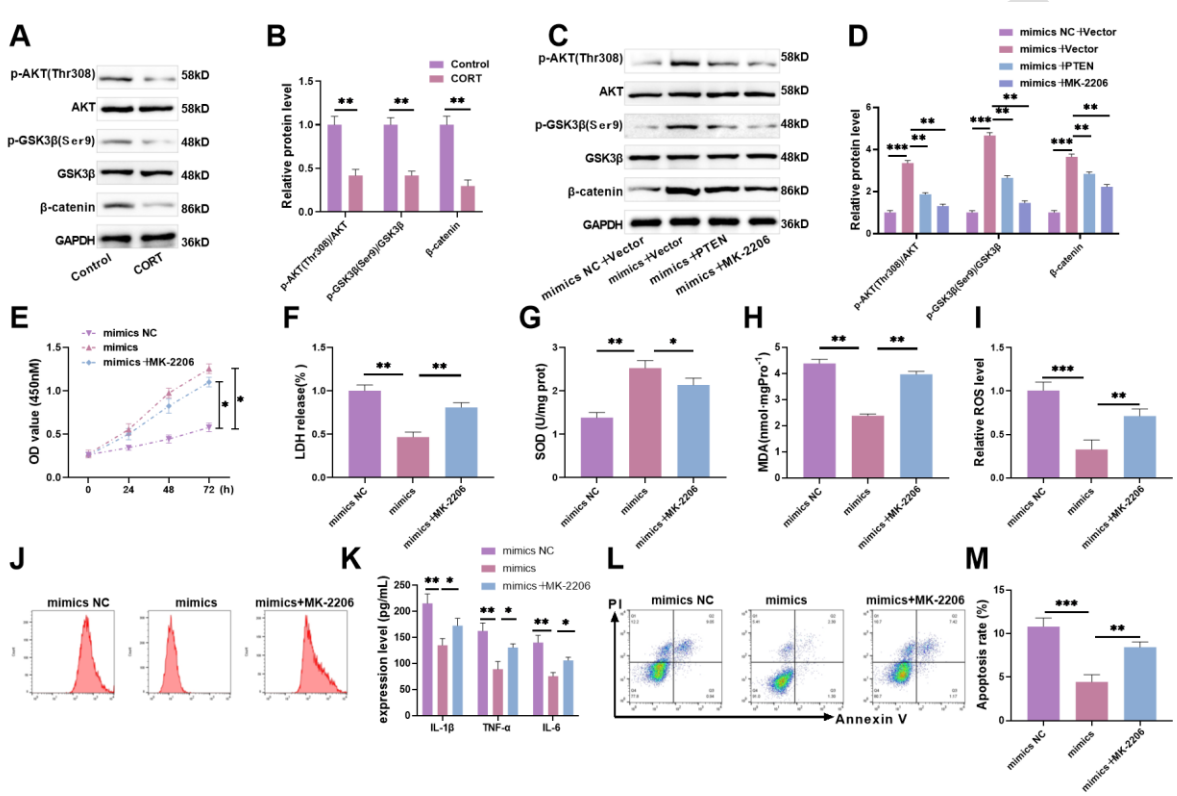

Figure 4. Overexpression of miR-542-3p negatively regulates PTEN expression to activate AKT/GSK3 β / β -catenin. (**A-D**) AKT/GSK3β/β-catenin pathway protein level was tested. The levels of p-AKT Thr308, p-GSK3β Ser9 and β-catenin protein were notably declined after CORT treatment. They were significantly increased after miR-542-3p overexpression. After overexpression of PTEN or application of MK-2206, the levels of pathway proteins were declined; (**E**) The proliferation of HT-22 cells under various treatment conditions was tested by CCK-8. Compared with miR-542-3p overexpression alone, the cell viability was notably lower after the application of MK-2206; (**F**) LDH was used to detect the

degree of damage in HT-22 cells under various treatment conditions. Compared with miR-542-3p overexpression alone, the degree of cell damage was markedly higher after the application of MK-2206; (**G**) The content of SOD was detected by ELISA. It was markedly diminished after the application of MK-2206; (**H**) The MDA content was detected by ELISA. The content of MDA was notably higher after MK-2206 was applied; (**I-J**) The level of ROS was tested by flow cytometry. ROS level was notably raised after the application of MK-2206; (**K**) IL-1β, TNF-α and IL-6 levels were tested by ELISA. They were markedly expanded after the application of MK-2206 compared with miR-542-3p overexpression; (**L-M**) The apoptosis was tested by flow cytometry. After MK-2206 was applied, the apoptosis rate was obviously higher. PTEN: Phosphatase and tensin homolog; GSK3β: Glycogen synthase kinase 3β; CCK-8: Cell counting kit-8; SOD: Superoxide dismutase; MDA: Malondialdehyde; ROS: Reactive oxygen species; IL: Interleukin; TNF-α: Tumor necrosis factor-α; ELISA: Enzyme-linked immunosorbent assay. n=3, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.01$.

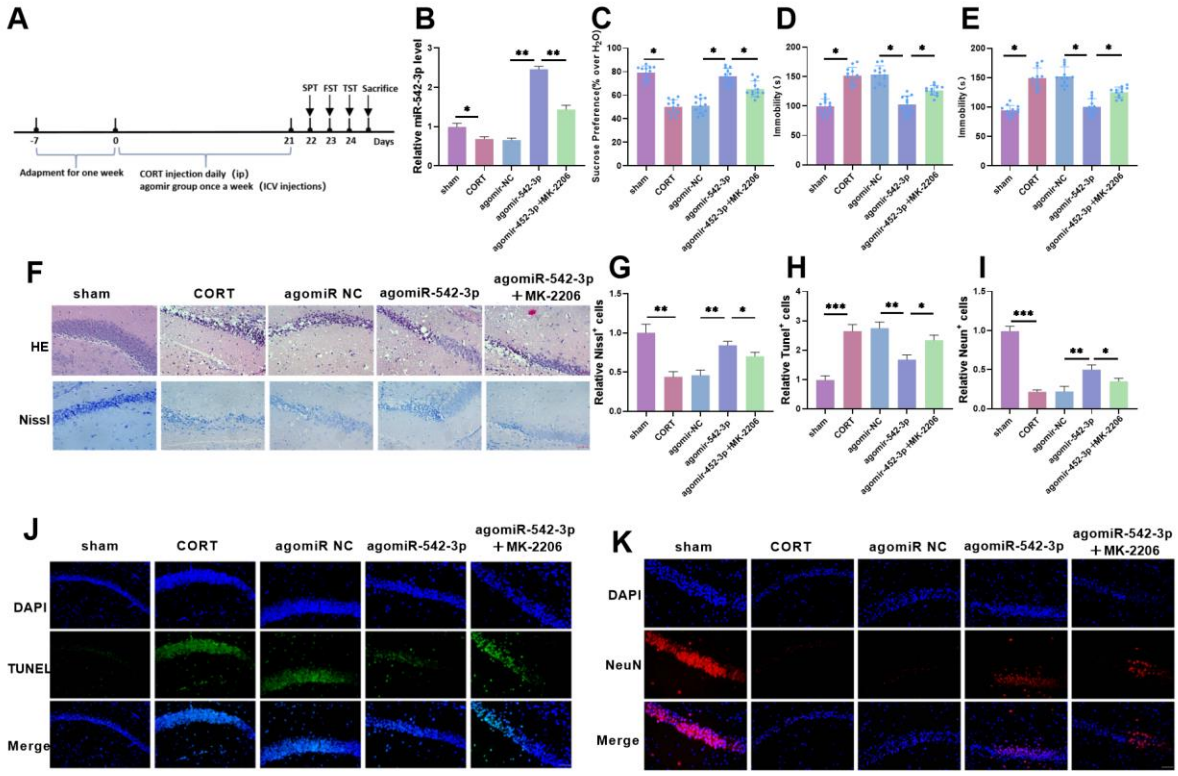

Figure 5. miR-542-3p attenuates hippocampal neuronal damage in corticosterone-induced depressive mice. (**A**) Experimental flow chart; (**B**) *miR-542-3p* level in the hippocampus of mice was tested by qRT-PCR. It was significantly raised after injection of agomiR-542-3p, indicating that it was effectively highly expressed *in vivo;* (**C-E**) The changes of depressive behavior in mice were evaluated by SPT, TST and FST. The sucrose preference rate of mice was significantly decreased after CORT treatment, and the time of staying still in water and suspension was significantly raised. After overexpressed miR-542-3p, the depressive behavior was notably improved, but after application of MK-2206, the depressive behavior was significantly aggravated; (**F-G**) HE staining of hippocampal sections showed that after CORT treatment, nerve cells were swollen, irregular in shape, and a large amount of necrosis occurred. Neuronal necrosis declined after overexpressed miR-542-3p, but increased significantly after application of MK-2206. Nissl staining of hippocampal sections showed that Nissl body numbers declined significantly after

CORT treatment, and increased significantly after overexpressed miR-542-3p, but decreased notably after application of MK-2206 (×40, 50 μm); (**H-K**) The apoptotic cells increased significantly, and NeuN positive cells decreased significantly after CORT treatment. After overexpressed miR-542-3p, apoptotic cells lessened significantly, and NeuN positive cells increased significantly, but the application of MK-2206 was reversed (×40, 50 μm). CORT: Corticosterone; TST: Tail suspension test; HE: Hematoxylin-eosin; SPT: Sucrose preference test; FST: Forced swimming test. n=5, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.01$.

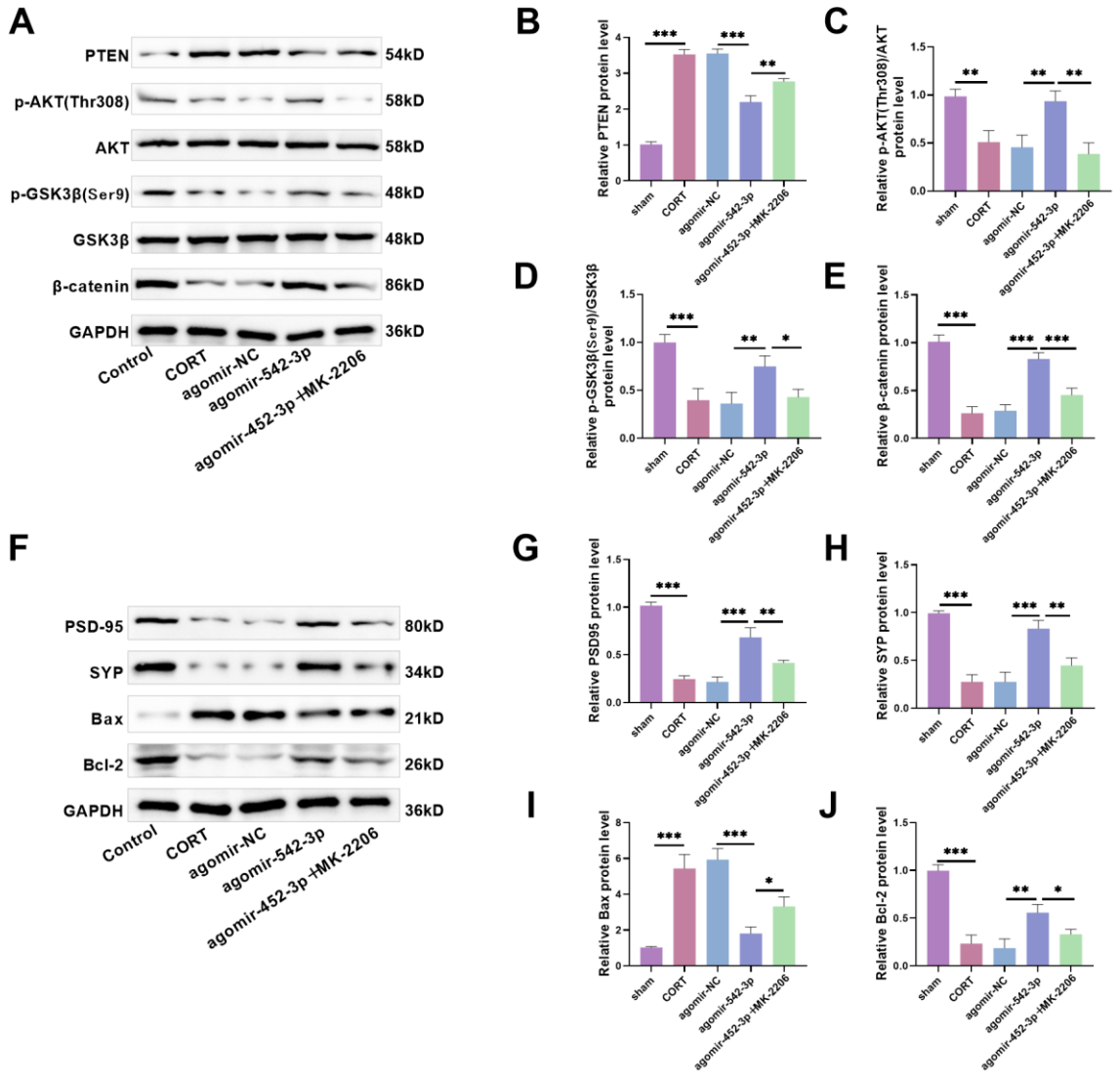

Figure 6. miR-542-3p attenuates hippocampal neuronal damage in corticosterone-induced depressive mice by regulating PTEN to activate AKT/GSK3 β /

β -catenin pathway. (**A-E**) The level of AKT/GSK3β/β-catenin pathway proteins were tested. It was found that the levels of p-AKT Thr308, p-GSK3β Ser9 and β-catenin protein were notably declined after CORT treatment. They was significantly increased after miR-542-3p overexpression. After the application of MK-2206, pathway protein levels were diminished; (**F-J**) The apoptosis and neuroplasticity-related protein levels were tested. Bax/Bcl-2 protein level enhanced significantly after CORT treatment, and the levels of SYP and PSD95 protein decreased significantly. After overexpressed miR-542-3p, Bax/Bcl-2 level was notably shrunk, and SYP and PSD95 protein levels were notably diminished, but the protein level was significantly reversed after application of MK-2206. PTEN: Phosphatase and tensin homolog; Bcl-2: B-cell lymphoma-2; Bax: Bcl2-associated X protein; GSK3β: Glycogen synthase kinase 3β; CORT: Corticosterone; SYP: Synaptophysin; PSD95: Postsynaptic density 95. n=5, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.01$.