MicroRNA-27a participates in the pathological process of depression in rats by regulating VEGFA

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Abstract. The present study aimed to determine the expression of vascular endothelial growth factor A (VEGFA) and microRNA (miRNA/miR)-27a in hippocampal tissues, and serum from a depression model of rats. In addition, the present study aimed to understand the mechanism of regulation of miR-27a in depression. A total of 40 male rats were selected, and divided into the control and depression model groups. The rats in the model group were subjected to 14 types of stimulations to model depression. By determining the body weight, syrup consumption rate and open field test score, the extent of depression in the rats was evaluated. Quantitative-polymerase chain reaction was used to determine the expression of VEGFA mRNA and miR-27a in hippocampal tissues, and serum. ELISA was used to measure the content of VEGFA protein in serum, while western blotting was employed to determine the expression of VEGFA protein in hippocampal tissues. A dual luciferase assay was carried out to identify the interactions between VEGFA mRNA and miR-27a. The rats in the depression model group showed depression symptoms and the depression model was successfully constructed. Rats with depression had lower VEGFA mRNA and protein expression in the hippocampus, and peripheral blood compared with the control group. Rats in the depression model group had reduced levels of miR-27a in the hippocampus and peripheral blood, which may be associated with the levels of VEGFA. miR-27a was able to bind with the 3'-untranslated region of VEGFA mRNA to regulate its expression. The present study demonstrated that miR-27a expression in hippocampal tissues and blood from rats with depression is upregulated, while the expression of VEGFA mRNA and protein is downregulated. miR-27a may participate in the pathological process of depression in rats by regulating VEGFA.

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Introduction

With the increase of competition pressure in modern society, the number of people suffering from depression is growing day by day (1). As a result, research on depression has attracted widespread concerns (2). Pathophysiological studies show that the volume and nerve density of hippocampus in patients with depression are significantly decreased (3-5). Animal model experiments also confirm that hippocampal neurons in depressed rats are disordered and loose, the nuclei of hippocampal neurons are shrunken, the nuclear membrane is irregular and vague, and mitochondria are swelled and denatured (6,7). The etiology of depression is dominated by monoamine hypothesis, which suggests that the decrease of 5-hydroxy tryptamine, norepinephrine and dopamine functions is its pathological mechanism (8). Some studies have focused on the role of neuronal regeneration in the pathogenesis and treatment for depression. According to the neurotrophic hypothesis of depression, brain derived neurotrophic factor (BDNF), tumor necrosis factor-α (TNF-α) (9), vascular endothelial growth factor (VEGF) (10) or fibroblast growth factor 2 (FGF-2) (11) may participate in the progression of depression.

VEGFA is one of the most effective angiogenic growth factors in human body, and its basic mechanism of action is to promote angiogenesis and to increase blood supply (12). However, more and more studies show that VEGFA can affect neurogenesis in a variety of ways, being a multifunctional growth factor (13-15). It is reported that after VEGFA signal transduction enters the cells, it induces the dimerization of ligand and receptor, and then causes phosphorylation of extracellular structure subunits with its receptor to promote the survival, permeability, migration and proliferation of hippocampal neurons (16).

MicroRNA (miRNA or miR) is single-stranded small-molecule RNA (18-25 nucleotides) that exists in eukaryotic cells and regulates gene expression at post-transcriptional levels (17). miRNA plays important roles in nerve cell development, differentiation, proliferation, and apoptosis. It is reported that the expression of some miRNAs is dysregulated in tissues from patients with depression, and downstream targets of the miRNAs are associated with

depression symptoms (18). miRNA can modulate central nervous activities, including reward feedback, circadian rhythm and cognitive performance, and the abnormalities of these activities are closely related with depression (19-21). miR-27a is a miRNA with abundant functions. Studies show that miR-27a can target epidermal growth factor receptor (EGFR), which is activated in many types of tumors and promotes tumorigenesis (22,23). However, it is never reported whether miR-27a has regulatory effects on VEGFA.

In the present study, we investigate the behaviors of depression rat model, measure the expression of relevant factors in hippocampus and blood of the rats, and try to understand the regulation between miR-27a and VEGFA.

Materials and methods

Animals. A total of 50 male rats (4-6 weeks old; 180-220 g) were obtained from Chongqing Tengxin Biotech Company (Chongqing, China; http://www.cqtx123.com/) with a certificate no. SCXK(Yu)2016-0018. One week before experiments, the rats had free access to food and water to adapt to the environment. The Reduction, Replacement and Refinement Animal Welfare Principle was followed during the experiments. The present study was approved by the Ethics Committee of Zaozhuang Mental Health Center (Zaozhuang, China).

The 50 rats were first raised in cages with circadian rhythm (5 rats/cage) with free access to food and water for three days. After that, body weight, daily intake of food, tropism of syrup, and distance of spontaneous movement within 5 min were recorded as baselines. Eight rats were excluded from the experiments due to abnormal baseline activities. In the end, a total of 40 rats with the closest scores were included in the experiments, and randomly and evenly divided into control group and depression model group (20 rats in each group). The rats in control group were raised normally as described above at a density of 5 rats/cage. Rats in depression model group were raised at a density of 1 rat/cage and used to construct depression model according to methods reported by Willner (24). The rats in model group were subjected to 14 types of stimulations, including day/night reverse (24 and 48 h), repeated tilting of rat cage (direction reverse every 4 for 12 and 24 h), water deprivation (6 and 12 h), food deprivation (12 and 24 h), noise (metal clash for 2 h and rat yawp for 2 h), damp padding (mild and sever for 12 and 24 h), foreign matter, no padding, suspension (6 h), tail clamping (mild), cage sharing, horizontal oscillation, cage exchange, and feeding environment change. Each rat was stimulated with only one method on each day, and raised alone in a cage for 35 days. From the fourth week, the general indicators (weight, drink amount and food amount), percentage of syrup consumption, and open field behavior were observed. Body weight, tropism of syrup, and open field behavior were tested for the two groups. After comparing with baseline results, the rats with and without typical depressive symptoms were noted.

To evaluate body weight, the food intake amount and body weight changes of rats within 24 h (8:00 AM to 8:00 AM on the next day) were recorded on the day before experiment, and days 28 and 35 of the experiment.

To carry out syrup consumption experiment (25), the syrup consumption percentage of rats on the day before experiment, and day 28 and 35 of the experiment were recorded. Total liquid consumption, sugar consumption, pure water consumption, and syrup tropism percentage (syrup consumption/total liquid consumption x100%) were calculated.

For open field test, changes in open field behavior of the two groups of rats were recorded on the day before experiment, and days 28 and 35 of the experiment. The dimension of the open field behavior box was 100x100x40 cm (made by our research group). There were 25 squares with equal sides in the field, and the walls were black. The detailed method was as reported by Lin *et al* (26). Horizontal score was the number of squares being passed by the rats with four paws. Vertical score was the times of vertical activities (front paws leaving the field or climbing the walls). Total score in the open field test was the sum of horizontal score and vertical score.

After fasting for 12 h following activity tests, peripheral blood was collected from all rats and serum was separated. Then, the rats were decapitated and hippocampal tissues were collected. All animal experiments were conducted according to the ethical guidelines of Zaozhuang Mental Health Center.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Tissues (100 mg) were ground using liquid nitrogen and mixed with 1 ml TRIzol (10606ES60; Yeasen, Shanghai, China) for lysis. Serum samples (100 μl) were directly mixed with 1 ml TRIzol (10606ES60; Yeasen, Shanghai, China) for lysis. Then, total RNA was extracted using phenol chloroform method. The concentration and quality of RNA was examined using ultraviolet spectrophotometry (Nanodrop ND2000; Thermo Scientific, Waltham, MA, USA). Then, cDNA was obtained by reverse transcription from 1 μg RNA and stored at -20˚C. Reverse transcription of mRNA was performed using TIANScript II cDNA First Strand Synthesis kit (Tiangen, Beijing, China), and reverse transcription of miRNA was carried out using miRcute miRNA cDNA First Strand Synthesis kit (Tiangen).

SuperReal PreMix (SYBR-Green) RT-qPCR kit (Tiangen) was used to detect mRNA expression of VEGFA. The sequences of VEGFA were 5'-CCAGGAGTACCCCGATGAGATAG-3' (upstream) and 5'-CTGGCTTTGGTGAGGTTTGATC-3' (downstream); and those of β-actin were 5'-ACCCCGTGCTGCTGACGGAG-3' (upstream) and 5'-TCCCGGCCAGCCAGGTCCAT-3' (downstream). PCR reaction system (20 μl) for VEGFA determination was composed of 10 μl RT-qPCR-Mix, 0.5 μl upstream primer, 0.5 μl downstream primer, 2 μl cDNA and 7 μl ddH_2O. PCR conditions for VEGFA determination were: initial denaturation at 95˚C for 2 min; 40 cycles of 95˚C denaturation for 30 sec, annealing at 58˚C for 30 sec and elongation at 72˚C for 30 sec (iQ5; Bio-Rad, Hercules, CA, USA). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of VEGFA mRNA against β-actin. Each sample was tested in triplicate.

Isolation of miR-27a was performed using miRcute miRNA isolation kit (Tiangen). The expression of miR-27a was determined by miRcute miRNA RT-PCR kit (Tiangen), using U6 as internal reference. The sequences of miR-27a were 5'-GCGGCGGTTCACAGTGGCTAAG-3' (upstream) and 5'-ATCCAGTGCAGGGTCCGAGG-3' (downstream); and those of U6 were 5'-CTCGCTTCGGCAGCACATATACT-3' (upstream) and 5'-ACGCTTCACGAATTTGCGTGT

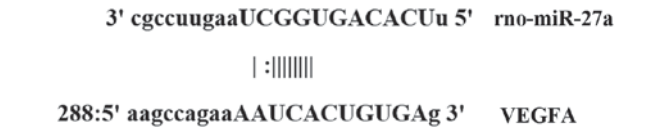

Figure 1. Direct interaction between miRNA-27a and VEGFA. Bioinformatics prediction is a powerful tool for the study of the functions of miRNAs. To understand the regulatory mechanism of VEGFA, we used miRanda, TargetScan, PiTa, RNAhybrid and PICTA to predict miRNA molecules that might regulate VEGFA, and found that miR-27a was able to potentially regulate VEGFA. VEGFA, vascular endothelial growth factor A, miRNA, microRNA.

C-3' (downstream). PCR reaction system (20 μl) for miR-27a determination was composed of 10 μl RT-qPCR-Mix, 0.5 μl upstream primer, 0.5 μl downstream primer, 2 μl cDNA and 7 μl ddH_2O. PCR conditions for miR-27a determination were: initial denaturation at 95˚C for 30 sec; 45 cycles of 95˚C denaturation for 5 sec and annealing at 60˚C for 30 sec (iQ5; Bio-Rad, Hercules, CA, USA). The $2^{-\Delta\Delta Cq}$ method was used to calculate the relative expression of miR-27a against U6. Each sample was tested in triplicate.

Western blot analysis. Precooled Radio-Immunoprecipitation Assay (RIPA) lysis buffer (600 μl; 50 mM Tris-base, 1 mM EDTA, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% TritonX-100, 1% sodium deoxycholate; Beyotime Institute of Biotechnology, Shanghai, China) was used to lyse the samples. After lysis for 50 min on ice, the mixture was centrifuged at 12,000 g/min and 4˚C for 5 min. The supernatant was used to determine protein concentration by bicinchoninic acid (BCA) protein concentration determination kit (RTP7102; Real-Times Biotechnology Co., Ltd., Beijing, China). Protein samples (20 μg) were then mixed with sodium dodecyl sulfate loading buffer before denaturation in boiling water bath for 5 min. Afterwards, the samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The resolved proteins were transferred to polyvinylidene difluoride membranes on ice (100 V, 2 h) and blocked with 5% skimmed milk at room temperature for 1 h. Then, the membranes were incubated with rabbit anti-mouse VEGFA polyclonal primary antibody (1:1,000; Abcam, Cambridge, UK) and rabbit anti-mouse β-actin primary antibody (1:5,000; Abcam) at 4˚C overnight. After extensive washing with phosphate-buffered saline with Tween-20 for 3 times of 15 min, the membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:3,000; Abcam) for 1 h at room temperature before washing with phosphate-buffered saline with Tween-20 for 3 times of 15 min. Then, the membrane was developed with enhanced chemiluminescence detection kit (Abcam) for imaging. Image lab v3.0 software (Bio-Rad, Hercules, CA, USA) was used to acquire and analyze imaging signals. The relative content of VEGFA protein was expressed as VEGFA/β-actin ratio.

Enzyme-linked immunosorbent assay (ELISA). Serum was examined using VEGFA ELISA kit (Abcam). In microplates, standards (50 μl), samples (10 μl sample liquid and 40 μl diluent) and blank were set into predefined wells. In the wells for standards and samples, horseradish peroxidase-labelled conjugates (100 μl) were added before sealing the plates for incubation at 37˚C for 1 h. After washing the plates 5 times, substrates A (50 μl) and B (50 μl) were added into each well. After incubation at 37˚C for 15 min, stop solution (50 μl) was added into each well, and absorbance of each well was measured at 450 nm within 15 min.

Bioinformatics. Bioinformatics prediction is a powerful tool for the study of the functions of miRNAs. To understand the regulatory mechanism of VEGFA, we used miRanda (http://www.microrna.org/microrna/home.do), TargetScan (http://www.targetscan.org), PiTa (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html), RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) and PICTA (http://pictar.mdc-berlin.de/) to predict miRNA molecules that might regulate VEGFA, and found that miR-27a was able to potentially regulate VEGFA (Fig. 1).

Dual luciferase reporter assay. According to bioinformatics results, wild-type (WT) and mutant seed regions of miR-27a in the 3'-UTR of VEGFA gene were chemically synthesized *in vitro*, added with Spe-1 and HindIII restriction sites, and then cloned into pMIR-REPORT luciferase reporter plasmids. Plasmids (0.8 μg) with WT or mutant 3'-UTR DNA sequences were co-transfected with agomiR-27a (100 nM; Sangon Biotech, Shanghai, China) into 293T cells. After cultivation for 24 h, the cells were lysed using dual luciferase reporter assay kit (Promega, Fitchburg, WI, USA) according to the manufacturer's manual, and fluorescence intensity was measured using GloMax 20/20 luminometer (Promega). Using *Renilla* fluorescence activity as internal reference, the fluorescence values of each group of cells were measured.

Statistical analysis. The results were analyzed using SPSS 18.0 statistical software (IBM, Armonk, NY, USA). The data were expressed as means ± standard deviations. Data were tested for normality. Multigroup measurement data were analyzed using one-way ANOVA. In case of homogeneity of variance, Least Significant Difference and Student-Newman-Keuls methods were used; in case of heterogeneity of variance, Tamhane's T2 or Dunnett's T3 method was used. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Rats in depression model group exhibited depression symptoms and the depression model was successfully constructed. To confirm the successful construction of depression rat model, the body weight, syrup consumption rates and open field test scores of the two groups were compared. The data showed that the body weight of the two groups was not significantly different from each other before stimulations, but the body weight of rats in depression model group was significantly lower than that in control group on days 28 and 35 ($P<0.01$) (Table I). In addition, there was no significant difference in syrup consumption rate between the two groups before stimulations, but the syrup consumption rate in depression model group was significantly reduced than that in control group on days 28 and 35 ($P<0.05$) (Table II). Of note, open field test

Table I. Body weight.

Groups	No.	Before (g)	Day 28 (g)	Day 35 (g)
Control	20	205.21±23.02	372.58±35.23	419.59±36.95
Depression model	20	199.69±21.63	315.47±29.16[a]	349.78±31.03[a]

[a]P<0.01 compared with control group.

Table II. Syrup consumption rate.

Groups	N	Before (%)	Day 28 (%)	Day 35 (%)
Control	20	95.6±2.3	93.7±4.2	92.5±6.2
Depression model	20	94.9±2.8	85.5±6.1[a]	79.8±8.0[a]

[a]P<0.05 compared with control group.

Table III. Open field test total score.

Groups	N	Before	Day 28	Day 35
Control	20	130.6±25.5	123.7±20.3	118.6±18.1
Depression model	20	132.1±23.6	65.8±18.9[a]	59.3±12.0[a]

[a]P<0.01 compared with control group.

scores of the two groups were not significantly different from each other before stimulations. However, the total score of open field test in depression model group was significantly decreased than that in control group on days 28 and 35 (P<0.01) (Table III). These results suggest that the rats in depression model group have shown manifest depression symptoms and the depression model is successfully constructed.

Rats with depression have lower VEGFA mRNA expression in the hippocampus and peripheral blood than control group. To determine the expression of VEGFA mRNA, RT-qPCR was carried out. The data showed that the levels of VEGFA mRNA in hippocampal tissues and serum from the rats in depression model group were significantly lower than those in control group, respectively (P<0.01) (Fig. 2). The result indicates that rats with depression have lower VEGFA mRNA expression in hippocampus and peripheral blood than control group.

Depression model rats have decreased VEGFA protein expression in the hippocampus and peripheral blood. To measure the expression of VEGFA protein in hippocampal tissues and serum, western blotting and ELISA were performed, respectively. The data showed that VEGFA protein expression in hippocampus and serum from depression model rats were significantly reduced than that from control group (P<0.05) (Fig. 3). The result suggests that depression model rats have decreased VEGFA protein expression in hippocampus and peripheral blood.

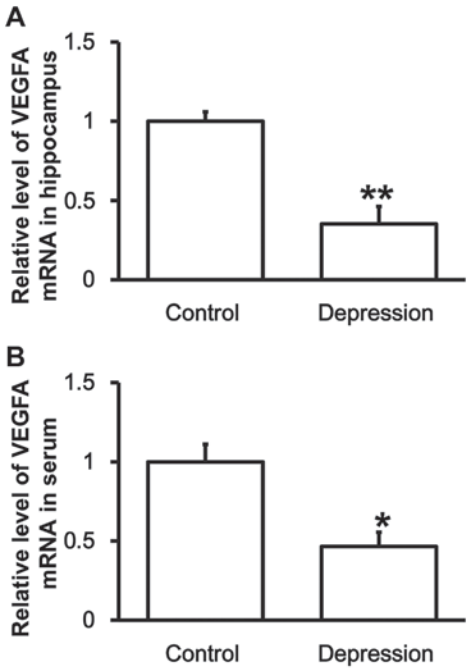

Figure 2. Expression of VEGFA mRNA in (A) hippocampal tissues and (B) serum from rats in control group and depression model group. RT-qPCR was used to measure the expression of mRNA in tissues and serum. *P<0.05 and **P<0.01 compared with control group. VEGFA, vascular endothelial growth factor A, miRNA, microRNA.

Rats with depression have higher levels of miR-27a in the hippocampus and peripheral blood. To test the expression of miR-27a in hippocampal tissues and serum, RT-qPCR was employed. The data showed that the levels of miR-27a in hippocampal tissues and serum from depression model rats were significantly upregulated compared with those from control group (P<0.05) (Fig. 4). The result indicates that rats with depression have higher levels of miR-27a in hippocampus and peripheral blood, which may be correlated with the levels of VEGFA.

miR-27a can bind with the 3'-UTR seeding region of VEGFA mRNA to regulate its expression. To identify the interaction between miR-27a and the 3'-UTR of VEGFA mRNA, dual luciferase reporter assay was performed. The fluorescence value of cells co-transfected with agomiR-27a and pMIR-REPORT-WT luciferase reporter plasmids was significantly lower than that in negative control group (P<0.05). By contrast, the fluorescence value of cells co-transfected with agomiR-27a and pMIR-REPORT-mutant luciferase reporter plasmids was not significantly different from that in negative control group (P>0.05) (Fig. 5). The result indicates that

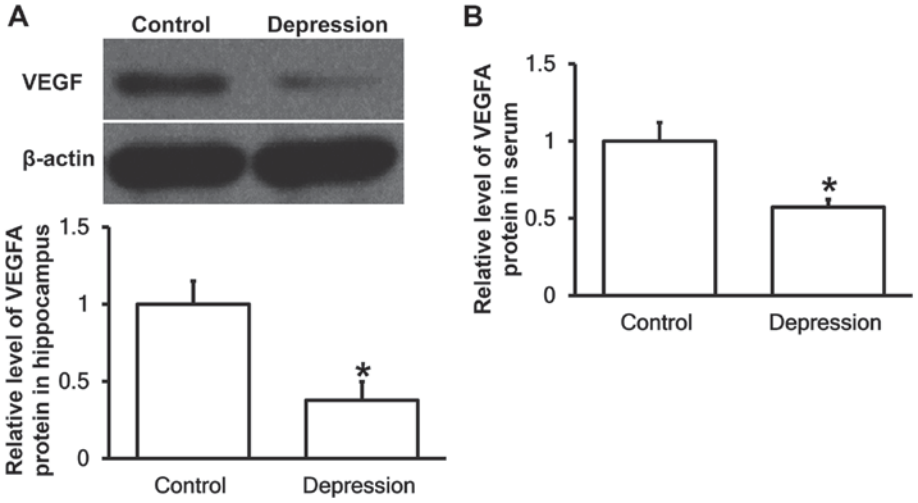

Figure 3. Expression of VEGFA protein in (A) hippocampal tissues and (B) serum from rats in control group and depression model group. VEGFA protein in hippocampal tissues was determined by western blotting, while that in serum was determined by ELISA. *P<0.05 compared with control group. VEGFA, vascular endothelial growth factor A; ELISA, enzyme-linked immunosorbent assay.

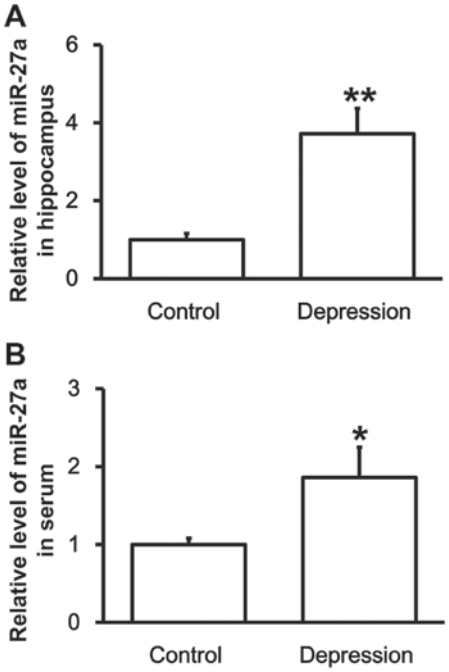

Figure 4. Expression of miR-27a in (A) hippocampal tissues and (B) serum from rats in control group and depression model group. RT-qPCR was used to measure the expression of miR-27a in both types of samples. *P<0.05 and **P<0.01 compared with control group. mir, microRNA.

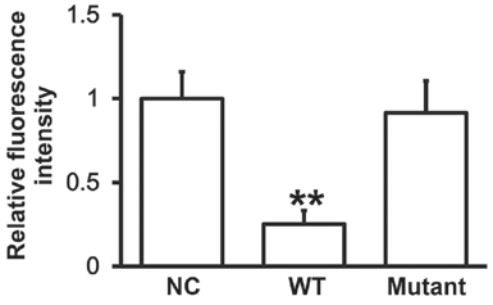

Figure 5. Identification of interaction between miR-27a and VEGFA using dual luciferase reporter assay. Plasmids (0.8 μg) with WT or mutant 3'-UTR DNA sequences were co-transfected with agomiR-27a into 293T cells. After cultivation for 24 h, the cells were lysed using dual luciferase reporter assay kit, and fluorescence intensity was measured using GloMax 20/20 luminometer. Using *Renilla* fluorescence activity as internal reference, the fluorescence values of each group of cells were measured. **P<0.01 compared with NC group. VEGFA, vascular endothelial growth factor A, miRNA, microRNA; WT, wild-type; NC, negative control.

miR-27a can bind with the 3'-UTR seeding region of VEGFA mRNA to regulate its expression.

Discussion

Emotional disorders are usually accompanied by cognitive or behavioral changes or disorders. Cognitive impairment associated with depression mainly includes declines in attention, learning ability and memory. It is reported that learning ability and memory of rats with depression are changed, and this may be caused by damages of limbic system, in which hippocampus is an important component (27). Establishing animal models of central nervous diseases is one of the most commonly used research methods (24). Chronic unpredictable mild stress (CUMS) model proposed by Willner (24), and Willner and Mitchell (28) is one of the most widely used models of depression, and we have used this method to construct depression model in the present study. VEGFA receptor, fetal liver kinase-1 (Flk-1), is the major receptor that exerts the biological roles of VEGFA. Flk-1 is mainly expressed in vascular endothelial cells and neuron precursor cells of hippocampus. It is reported that lack of Flk-1 receptors is important for the survival of neural stem cells (29). VEGFA can affect the complex processes of learning and memory (30), and play a role in regulating neurite growth and maturation during brain development (31). The role of VEGFA in neurogenesis may be mediated by its interaction with downstream effector genes (32). In the present study, our data show that VEGF mRNA and protein expression in hippocampal tissues and serum are downregulated in depression model rats, suggesting that downregulation of VEGFA plays a key role in the depression of rats.

Regulation of mRNA transcription and expression is a complex process of multiple factors. miRNAs cut mRNAs and inhibits their translation to achieve negative feedback regulation (33,34). miRNAs are important regulators in normal development and physiology, and many miRNAs have become biomarkers of various diseases (35,36). Using bioinformatics, we discover that miR-27a is an upstream regulator of VEGFA. miR-27a has various biological functions. It is discovered that miR-27a can regulate the expression of tumor-suppressor gene FOX1 in breast cancer cells (37). In addition, miR-27a inhibits the expression of zinc finger protein ZBTB10, and promotes the accumulation of SP protein in breast cancer cells (38,39), leading to abnormal cell cycles. Single nucleotide polymorphism occurring in miR-27a precursor prevents its transformation to mature form, and families with this have reduced probability of breast cancer (40). By contrast, expression of miR-27 is decreased in several other types of tumors, including acute promyelocytic leukemia (41), colorectal cancer (42,43), malignant melanoma (44), oral squamous cell carcinoma (45) and prostatic carcinoma (46). These reports suggest that miR-27a may also act as a tumor-suppressor in these tumors. miR-27a promotes fibrosis in organs such as liver (47) and lung (48), and is associated with myocardial hypertrophy and heart failure (49,50). Our results in the present study show that VEGFA mRNA and protein expression in depression model rats are downregulated, while miR-27a expression in depression rats is upregulated. Indeed, dual luciferase reporter assay has shown the direct interaction between miR-27a and the 3'-UTR of VEGFA mRNA. Considering the results in open field tests, we discover that miR-27a, VEGFA, and changes in learning ability and memory have regulatory connections with each other.

In conclusion, the present study demonstrates that the mechanism of depression in rats may be the upregulation of miR-27a expression in hippocampal tissues and blood, which results in the downregulation of VEGFA mRNA and protein expression. As a regulator of VEGFA, miR-27a may become a target for the prevention and amelioration of depression.

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