Research Article

Overexpression of microRNA-136-3p Alleviates Myocardial Injury in Coronary Artery Disease via the Rho A/ROCK Signaling Pathway

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Keywords

Coronary artery disease · MicroRNA-136-3p · Cardiac microvascular endothelial cells · EIF5A2 · Rho A/ROCK signaling pathway

Abstract

Objective: Coronary artery disease (CAD) is a cardiovascular disease that poses a fatal threat to human health, and the identification of potential biomarkers may help to delineate its pathophysiological mechanisms. Accumulating evidence has implicated microRNAs (miRNAs) in the pathogenesis and development of cardiovascular diseases. The present study aims to identify the expression of miR-136-3p (miR-136-3p) in CAD and further investigate its functional relevance in myocardial injury both in vitro and in vivo. **Methods:** Initially, CAD models were induced in rats by high-fat diet and intraperitoneal injection of pituitrin. Next, the effect of overexpressed miR-136-3p on cardiac function and pathological damage in myocardial tissue, cardiomyocyte apoptosis, oxidative stress and inflammatory response were assessed in rats. Cardiac microvascular endothelial cells (CMECs) were isolated and cultured by the tube formation method, and the CMEC injury model was induced by homocysteine (HCY). The function of miR-136-3p in vitro was further evaluated. **Results:** miR-136-3p was poorly expressed in the myocardial tissue of CAD rats and CMEC injury models. In vivo assays indicated that overexpressed miR-136-3p could improve cardiac function and alleviate pathological damage in myocardial tissue, accompanied by reduced oxidative stress and inflammatory response. Moreover, in vitro assays suggested that overexpression of miR-136-3p enhanced

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proliferation and migration while inhibiting apoptosis of HCY-stressed CMECs. Notably, we revealed that EIF5A2 was a target gene of miR-136-3p, and miR-136-3p inhibited EIF5A2 expression and activation of the Rho A/ROCK signaling pathway. **Conclusion:** In conclusion, the overexpression of miR-136-3p could potentially impede myocardial injury in vitro and in vivo in CAD through the blockade of the Rho A/ROCK signaling pathway, highlighting a potential miR-136-3p functional relevance in the treatment of CAD.

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Introduction

Cardiovascular disease represents the most frequent cause of death in all developed regions, and the number of cardiovascular disease cases is also increasing in developing regions accompanied by economic growth [1]. As a common cardiovascular disease, coronary artery disease (CAD) ranks as the first cause of mortality across the globe owing to the adverse clinical events [2]. The risk factors contributing to CAD have been well recognized, such as high blood pressure, lipid disorders, cigarette smoking, and decreased physical inactivity [3, 4]. There is a paucity of evidence demonstrating the significant role of oxidative stress in the pathogenesis and development of cardiovascular diseases, including CAD [5]. In addition, the important role of inflammatory reaction has been well established during the progression of CAD [6]. The value of advancements in treating cardiovascular disease are limited when they fail to benefit those with the most urgent need, and the identification of effective biomarker associated with oxidative stress and inflammation could aid in improvement for the diagnosis and therapy of CAD.

MicroRNAs (miRNAs) represent a large family of non-coding RNAs, which posttranscriptionally function as mediators of a variety of biological processes through binding to the 3' untranslated region (UTR) of mRNAs [7]. It is worthy to note that the number of miRNAs has been expanded to be identified as critical mediators of not only physiological processes but also pathophysiological conditions in cardiovascular diseases [10, 11]. Interestingly, increasing studies have provided evidence verifying altered levels of miRNAs in patients suffering from CAD, which could predict the occurrence of cardiovascular events [12, 13]. Specifically, the aberrant expression of microRNA-136 (miR-136) in human vascular smooth muscle cells exerts a role on the development of atherosclerosis [14]. Moreover, the functional relevance of miRNAs in vascular endothelial cell apoptosis has been previously demonstrated, and the alterations of vascular endothelial cells are clearly associated with cardiovascular diseases [15]. He et al. [16] have found that miR-136-5p may act as a significant molecule in the modulation of spinal cord injury lesions in rat models and may be useful for treating the disease. Another study has suggested that expression of miR-136-3p was elevated at 6 h after human chorionic gonadotropin administration in rat ovaries and rat granulosa cells [17]. Besides, through website prediction, eukaryotic translation initiation factor 5A2 (EIF5A2) was verified as a target gene of miR-136-3p. It has been suggested that EIF5A2 could activate the Rho-kinase (ROCK) for enhancing the stress fibers formation [18]. It is reported that upregulation of EIF5A2 induces tumor metastasis in vivo by establishing an experimental mouse model [19]. Another study has revealed that Mg(II)-Cat/si-EIF5A2 had a tumor inhibitory effect in a clinically relevant rat model of in-situ bladder cancer [20]. The Rho/ROCK signaling pathway plays a critical role in the occurrence of atherosclerosis and coronary vasospasm due to its control of endothelial function and the inflammatory reactions [21]. Therefore, we performed this study to detect the expression of miR-136-3p and EIF5A2 in rats and validate the hypothesis that miR-136-3p may be involved in the myocardial injury of CAD through its regulation on EIF5A2 and the Rho/ROCK signaling pathway.

Materials and Methods

Ethics Statement

All animal experiments were approved by the Ethics Committee of Hospital of Traditional Chinese and Western Medicine in Hubei Province and in accordance with the Regulations of the Administration of Affairs Concerning Experimental Animals. All efforts were made to minimize the suffering of the animals included in the study.

Experimental Animals and Grouping

Sixty healthy male Sprague-Dawley rats were acclimated for 7 days and then weighed. Then, 15 rats were randomly selected as the normal group. In the remaining 45 rats, models of CAD were induced by high-fat diet and intraperitoneal injection of pituitrin. The model rats were fed a daily high-fat diet (30 g) for a total of 15 weeks, with free access to water during the experiment. Seventy-two hours before the last feeding, rats in each group were injected with pituitrin (30 µg/kg) once daily for 3 consecutive days. Then, the rats were fasted for 12 h, followed by pathological examination of the left coronary artery and heart of the normal group and the model group to confirm whether the modeling was successful. Forty-five CAD rat models were randomly and evenly classified into three groups (15 rats in each group): CAD group (CAD rat models) and CAD + negative control (NC) group (CAD rat models injected through the tail vein with virus once every 3 weeks for a total of 15 weeks), CAD + miR-136-3p group (CAD rat models injected through the tail vein with virus overexpressing miR-136-3p once every 3 weeks for a total of 15 weeks). The virus overexpressing miR-136-3p was purchased from Shanghai Genechem Co., Ltd. (Shanghai, China). At the 15th week, the heart index was measured. Afterwards, the rats were injected with 10% potassium chloride (2 min) through the femoral vein to stop the heart in the diastolic phase. After the blood was collected, the heart was removed, and the left and right ventricles were separated. After the remaining blood was blotted, the left ventricle weight (LVW) and right ventricle weight (RVW) were measured. The left ventricular weight index (LVWI) and right ventricular weight index (RVWI) represent the ratio of LVW or RVW to the body weight.

Hematoxylin-Eosin Staining

The heart was fastened and fixed in 10% formalin for 12 h. The heart was cut open along the ligature, and the myocardial tissue under the suture was collected. The tissue was dehydrated in 70, 80, 90, and 100% gradient ethanol, and permeabilized in xylene (YB-8499, Shanghai Yubo Bio-Technology Co., Ltd., Shanghai, China). Then, the paraffin-embedded tissue was continuously cut into 4-µm-thick slices. After baking at 60 °C, the slice was dewaxed in xylene for 3 min and hydrated in 100, 95, 80, and 70% gradient ethanol, respectively (5 min each time), followed by soaking in distilled water for 5 min. Hematoxylin (H8070, Solarbio, Beijing, China) was adopted to stain the slice for 4 min, followed by staining in eosin (E8090, Solarbio) for 3 min. After dehydration by gradient ethanol, the slice was permeabilized in xylene and sealed with neutral balsam. The slices were observed and photographed under an optical microscope (DSX100, Olympus, Tokyo, Japan) to examine the staining results.

Transmission Electron Microscopic Observation

The rat myocardial tissue was fixed in 2.5% glutaraldehyde, followed by adding 1% citric acid. Then, tissue was dehydrated with gradient acetone, and embedded in Epon812 epoxy resin, followed by slicing by an ultramicrotome. After lead citrate staining, the pathological conditions of myocardial tissues were observed under a JEM-1400Plus electron microscope.

Transferase-Mediated Deoxyuridine Triphosphate-Biotin Nick End Labeling Staining

Transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) kits (Beyotime Institute of Biotechnology, Nanjing, Jiangsu, China) were used to observe the apoptosis of cardiomyocytes. Paraffin section were dewaxed with xylene, hydrated with gradient alcohol and incubated with 20 µg/mL protease K (p6556, Sigma, St. Louis, MO, USA) solution for 20 min. The sections were incubated with 1× DNase I buffer for 5 min and DNase I buffer containing 10 U/mL DNase I for 10 min. Subsequently, the sections were incubated with 1× equipment buffer to incubate for 30 min and terminal deoxynucleotide transferase (TdT) incubation buffer for 60 min at 37 °C in dark. Next, the sections were incubated with diaminobenzidine substrate solution, incubate for 10 min, counterstained with hematoxylin and sealed. Image were analyzed under a light microscope.

Spectrophotometric Colorimetric Determination

The collected myocardial tissue was homogenized with pre-cooled normal saline to make a 10% homogenate. Then, the homogenate was centrifuged for 10 min to collect the supernatant. The contents of malondialdehyde (MDA) and superoxide dismutase (SOD) in myocardial tissue were examined by spectrophotometry.

Measurement of Serum Factors

Rats were anesthetized with pentobarbital sodium (50 mg/kg, Tyrrell Biotechnology Co., Ltd., Shanghai, China) via intraperitoneal injection for 15 min. The venous blood (15 mL) in the heart of each group was collected, the serum and plasma of which were separated and stored for further use. All the rats in the group were examined for serum levels of cardiac function indices, including creatine kinase MB (CK-MB), cardiac troponin I (cTnI), and inflammatory factors interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α) by using an ELISA kit (Senxiong Biotech. Co., Ltd., Shanghai, China). Nitric oxide (NO) and endothelial nitric oxide synthase (eNOS) antibody (Nanjing JianCheng Bioengineering Institute, Nanjing, China) were applied to evaluate the serum NO and eNOS contents. The contents of NO and eNOS were respectively measured by nitrate reductase assay and a colorimetric method.

Reverse Transcription and Quantitative Polymerase Chain Reaction

Total RNA from myocardial tissue was extracted according to the instructions of a Trizol kit (15596-026, Invitrogen, Gaithersburg, MD, USA). The RNA was reversely transcribed into cDNA according to the instructions of the reverse transcription kit (K1621, Fermentas, Hanover, PA, USA). The obtained cDNA was temporarily stored in a –20 °C refrigerator. The primer sequences of genes of interest and the internal references are displayed in Table 1, which were synthesized by Shanghai GeneChem Co. Ltd., Shanghai, China. The expression of each gene was measured by a real-time PCR kit (Takara, Dalian, China). A real-time PCR instrument (ABI 7500, ABI, Foster City, CA, USA) was employed, with U6 and β-actin as housekeeping genes. The relative expression of each gene of interest was calculated by the $2^{-\Delta\Delta Ct}$ method, and each experiment was repeated 3 times. $\Delta\Delta Ct = (\text{average Ct value of the target group of the experimental group} - \text{average Ct value of the housekeeping gene of the experimental group}) - (\text{average Ct value of the target gene of the control group} - \text{average Ct value of the housekeeping gene of the control group})$. This method was also applicable to the detection of cellular expression levels.

Western Blot Assay

Myocardial tissue samples (50 mg) were cut into pieces and added with protein lysis buffer (R0010, Solarbio, Beijing, China), and homogenized at 3,000 r/min until being fully

Table 1. Primer sequence for RT-qPCR

Gene	Primer sequence
miR-136-3p	Forward: 5'-GGGGACATCATCGTCTCAAAT-3' Reverse: 5'-CAGTGCGTGTCTGGAGT-3'
U6	Forward: 5'-GCTTCGGCAGCACATATA-3' Reverse: 5'-CGCTTCACGAATTTGCGT-3'
IL-1 β	Forward: 5'-GTTTGAGTCTGCACAGTTCCC-3' Reverse: 5'-CAACTATGTCCCGACCATTGC-3'
IL-6	Forward: 5'-TTCTTGGGACTGATGTTTGGAC-3' Reverse: 5'-AATTAAGCCTCCGACTTGTG-3'
TNF- α	Forward: 5'-TGTCTGTGCCTCACTTCTC-3' Reverse: 5'-TTTGGGAACCTCTCTGTGT-3'
EIF5A2	Forward: 5'-CTGCTGACAGAACTGG-3' Reverse: 5'-CACTCATGGC-3'
β -Actin	Forward: 5'-CGGCGCCGCGGACCA-3' Reverse: 5'-TGGGGT-3'

miR-136-3p, microRNA-136-3p; IL-1 β , interleukin 1 β ; IL-6, interleukin-6; TNF- α , tumor necrosis factor- α ; EIF5A2, eukaryotic translation initiation factor 5A2.

lysed. After ice bath for 30 min, the supernatant was collected by centrifugation (5,000 r/min) at 4°C for 15 min. The protein concentration was determined according to the bicinchoninic acid Protein Quantitation Kit (23225, Pierce and Warriner, Rockford, IL, USA), which was adjusted to 1 μ g/ μ L. The processed protein was loaded (1 μ g per well) and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (P1200, Solarbio). The separated protein sample was transferred to polyvinylidene fluoride membrane (HVL04700, Millipore, Bedford, MA, USA) by the semi-dry electrotransfer method, followed by Ponceau S staining (P0012, Solarbio) to observe protein transfer. After being blocked in 5% skimmed milk powder at room temperature for 2 h, the PVDF membrane was incubated with primary antibodies EIF5A2, caspase-3, Bax, Bcl-2, RhoGEP, p-ROCK, β -actin (all 1: 1,000 and from Abcam, Cambridge, UK) and RhoA-GTP (1: 1,000, Cell Signaling Technology, Danvers, MA, USA) in 4°C refrigerator overnight. After TBST rinsing 3 times (10 min each time), horseradish peroxidase-labeled secondary antibody (1:2,000, ab6721, Abcam) was added and incubated for 2 h at room temperature. The protein bands were visualized by diaminobenzidine tetrahydrochloride solution and photographed by a gel imager (Gel Doc XR, Bio-Rad, Hercules, CA, USA). The ratio of the gray value of the protein of interest to that of the internal reference represented the relative expression of the protein. This method was also applicable to the detection of cellular protein expression.

Isolation, Culture, and Characterization of Cardiomyocytes

Rat cardiac microvascular endothelial cells (CMECs) were isolated and cultured by the tissue explant method. The normal Sprague-Dawley rats were intraperitoneally anesthetized by sodium pentobarbital sodium (40 mg/kg). Then, the rats were disinfected in 75% ethanol for 5 min. The chest was opened layer by layer to expose the heart. The pericardium was cut open, and the heart was cut down from the ascending aorta and flushed by the D-Hanks balanced salt buffer to remove blood in the ventricles. The large blood vessels, the left and right atria, the right ventricle, and interventricular septum were removed, but the left ventricle

was retained, with the endocardium and epicardium carefully removed. The remaining ventricular muscle was cut into pieces (about 1 mm³) by iris scissors and supplemented with 1 mL fetal bovine serum (FBS). Afterwards, the tissue blocks were evenly seeded in culture bottles treated with rat tail collagen. The tissues were incubated to allow cells to adhere to the bottle, and high-glucose Dulbecco's modified Eagle medium containing 20% FBS was added 4 h later. After about 48 h, there were cells growing around the tissue block, and plenty of cells grew around the tissue block 72–80 h later. The tissue blocks were removed, and the culture was continued for 36–48 h. After the cells settled at the bottom of the bottle, the cells were digested with 0.125% trypsin containing 0.02% ethylene diamine tetraacetic acid (EDTA) for cell passage [22–24]. Cell morphology was observed under an inverted microscope (IX53, Olympus). High-purity CMECs were obtained through the differential attachment method: cells grew to confluence and were detached by trypsin until the cells returned to seed. The cell suspension was diluted by a complete medium and incubated for 24 h. The non-adherent cells were removed and the medium was replaced. Next, the cells were continuously incubated until they grew to confluence again, then the above steps were repeated twice. The obtained cells were high-purity rat CMECs.

Cell suspension was seeded on slide and allowed to grow to confluence, which was then fixed for 30 min, and incubated with blocking solution containing 0.1% Triton X-100 and 1% FBS for 1 h at room temperature. Subsequently, the cells were incubated with CD31 primary antibody overnight at 4 °C, followed by incubation with fluorescent secondary antibody at 37 °C for 30 min. Then, cells were incubated with DAPI staining solution at 37 °C for 30 min to stain the nucleus, followed by observation under a fluorescence microscope (BX53, Olympus). The subsequent experiments were carried out using cells after passage.

Cell Modeling and Transfection

The rat CMECs were incubated with 100 μmol/L homocysteine (HCY) for 24 h to establish a CAD model. Model cells were divided into blank group (blank control, without transfection of any sequence), mimics NC group (HCY-treated CMECs transfected with the NC sequence of miR-136-3p mimics), and miR-136-3p mimics group (HCY-treated CMECs transfected with miR-136-3p mimics, purchased from Shanghai GenePharma Co., Ltd., Shanghai, China). The cells were seeded in culture flask (10 mL), and the cells were grown to a confluence of 30–50% in complete medium. In the 96-well EP tube, the lipofectamine 2,000 reagent and oligonucleotides were prepared in 100 μL lipofectamine 2,000 + 100 μL serum-free medium (5 min at room temperature); miR-136-3p mimics or mimics NC (50 nmol) + 100 μL serum-free medium (at room temperature) for 10 min to form the complex of liposome). The cells were washed in serum-free medium in the culture flask. The complex was incubated with antibiotics and serum-free medium for transfection at 37 °C in a 5% CO₂ incubator. The medium was renewed after 4 h, then replaced by complete medium and further incubated for the following experiments.

Cell Viability Assay

When the transfected CMECs reached 80% confluence, the cells were routinely trypsinized and pipetted into a single cell suspension. After cell counting, the cells were seeded in a 96-well plate (3 × 10³ to 6 × 10³ cells per well). The volume of each well was 200 μL, and each group was repeated in 6 wells. After incubation at 37 °C in a 5% CO₂ incubator for 24–72 h, the cells were treated with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) solution (5 mg/mL, Sigma, 20 μL per well). Following another incubation at 37 °C in a 5% CO₂ incubator for 4 h, the incubation was terminated, and the culture medium was discarded. Dimethyl sulphoxide (150 μL) was added to dissolve the crystal. After 24, 48 and 72 h, the absorbance of each well was measured on an ELISA microplate reader. The MTT curves were plotted, with the absorbance as the ordinate and the time as the abscissa.

Flow Cytometric Detection of Cell Cycle Progression and Apoptosis

Propidium iodide (PI) single staining assay: 48 h after transfection, CMECs cells of each group were detached by 0.25% trypsin and centrifuged at 4 °C, 1,000 r/min for 5 min, with the supernatant discarded. Then, the cells were resuspended in phosphate buffered saline (PBS), with the cell concentration adjusted to about 1×10^5 cells/mL. Following this, the cells were fixed in 2 mL of pre-cooled 75% ethanol at 4 °C for 30 min and centrifuged to discard the ice ethanol. Then, 100 μ L RNase A was added for a 37 °C water bath for 30 min avoiding the light exposure. The cells were incubated with 400 μ L PI staining solution (P4170, Sigma, USA) for 30 min at 4 °C. A flow cytometer (Gallios, Beckman Coulter, S. Kraemer Boulevard Brea, CA, USA) was adopted to examine the red fluorescence excited at 488 nm for cell cycle detection.

Annexin V fluorescein isothiocyanate (FITC)/PI double staining assay: 48 h after transfection, CMECs of each group were detached by EDTA-free 0.25% trypsin and collected into the flow tube. Cells were centrifuged with the supernatant discarded. According to the instructions of an Annexin-V-FITC Apoptosis Detection Kit (K201-100, Biovison, USA), the Annexin-V-FITC, PI, hydroxyethyl piperazine ethanesulfonic acid (HEPES) buffer solution was formulated into Annexin-V-FITC/PI dye solution at a ratio of 1:2:1. 100 μ L aliquot of the dye solution was adopted to resuspend 1×10^6 cells, which were incubated for 15 min at room temperature. Then, 1 mL of HEPES buffer solution was added, and mixed by shaking. With the excitation wavelength at 488 nm, a flow cytometer was employed to determine FITC fluorescence by a 515 nm band-pass filter, and PI fluorescence by a 620 nm band-pass filter, in order to detect cell apoptosis.

Transwell Migration Assay

After 72 h of transfection, the CMECs were collected and prepared for cell suspension. The cells were seeded in the upper Transwell chamber (Coming, USA), followed by incubation in a 37 °C 5% CO₂ incubator for 24 h. The cells that failed to penetrate the membrane in the upper chamber were gently washed off with a cotton swab. The penetrated cells were fixed in 95% ethanol for 15–20 min and stained with crystal violet dye for 10 min. The cells on the back of the membrane were observed, photographed and counted under a high magnification inverted microscope. The average number of cells in each sample was calculated from 5 randomly selected fields. The number of cells passing through the polycarbonate membrane of each group was used as an index for evaluating the migration ability.

Dual Luciferase Reporter Gene Assay

The possible target genes of miR-136-3p were predicted based on online analysis software, such as TargetScan, miRanda, MiRbase, and Pictar. EIF5A2 3'UTR wild-type sequence (EIF5A2-3'-UTR-WT) and EIF5A2 3'UTR mutant sequence (EIF5A2-3'-UTR-MUT) were designed and synthesized by Shanghai Generay Biotech Co., Ltd. (Shanghai, China). The sequences were connected with luciferase reporter vector pmirGLO and transferred into competent cells after PCR amplification, digestion, and purification. After sequencing, the EIF5A2-WT and EIF5A2-MUT plasmids were obtained (constructed by Generay Biotech), which were then transfected into the HEK293 cell line. The luciferase activity was measured by dual luciferase reporter gene assay reagent (Promega, USA). After 48 h of transfection, the original medium was removed, and cells were washed twice with PBS. Then, 100 μ L of passive lysis buffer was added to each well of the cells and shaken for 15 min at room temperature to collect the cell lysate. The program was set to pre-read for 2 s, and the reading process lasted for 10 s. The prepared LARII, Stop & Glo® Reagent was added to the luminescent plate of the cell lysate (20 μ L per sample) and placed in a Modulus™ luminometer (Turner BioSystems, USA) for detection.

Fig. 1. Expression levels of miR-136-3p in the myocardial tissue of rats in each group. **A** Expression levels of miR-136-3p in the myocardial tissue of normal rats and CAD rats. **B** Expression levels of miR-136-3p in the myocardial tissue of rats in each group with overexpression of miR-136-3p. * $p < 0.05$ vs. the normal group. # $p < 0.05$ vs. the CAD group.

Statistical Analysis

The data were processed by SPSS 20.0 statistical software (IBM Corp, Armonk, NY, USA). The Kolmogorov-Smirnov test was used to determine whether the data were in normal distribution. The results were expressed as mean $\pm$ standard deviation. Comparisons between two groups were performed by the t test, and comparisons among multiple groups by one-way ANOVA followed by pairwise comparison with the Fisher's least significant difference test. The two-tailed test was considered significant if $p < 0.05$.

Results

miR-136-3p Is Poorly Expressed in the Myocardial Tissue of CAD Rats

The results of reverse transcription quantitative polymerase chain reaction (RT-qPCR) identified lower expression of miR-136-3p in the myocardial tissues of modeled rats than that in the normal rats ($p < 0.05$) (Fig. 1A), suggesting that miR-136-3p may be involved in the occurrence of CAD. The expression of miR-136-3p in the myocardial tissues did not differ significantly between the CAD group and the CAD + NC group ($p > 0.05$). Compared with the CAD group and the CAD + NC group, the CAD + miR-136-3p group showed elevated expression of miR-136-3p in the myocardial tissue of the rats ($p < 0.05$) (Fig. 1B), indicating that the virus overexpressing miR-136-3p successfully altered the miR-136-3p expression.

Overexpression of miR-136-3p Improves Cardiac Function and Alleviates Pathological Damage in the Myocardial Tissue of CAD Rats

The results of the cardiac function index measurement (Table 2) identified higher LVWI and RVWI in CAD rats than that in the normal rats ($p < 0.05$). The CAD group and the CAD +

Table 2. The effect of miR-136-3p on the cardiac function index of rats

Group	LVWI, mg/g	RVWI, mg/g
Normal	3.01±0.23	0.65±0.09
CAD	4.79±0.58*	1.31±0.17*
CAD+NC	4.86±0.46*	1.35±0.12*
CAD+miR-136-3p	3.93±0.49*,#	0.96±0.07*,#

CAD, coronary artery disease; NC, negative control; LVWI, left ventricular weight index; RVWI, right ventricular weight index. * $p < 0.05$ vs. the normal group. # $p < 0.05$ vs. the CAD group.

Fig. 2. Overexpression of miR-136-3p improves cardiac function and alleviates pathological damage in the myocardial tissue of CAD rats. **A** HE staining to observe the pathological condition of myocardial tissue in each group ($\times 200$). **B** Transmission electron microscopic observation of the ultrastructure of myocardial tissue in each group ($\times 1,000$). $n = 5$.

Normal group showed no significant difference in LVWI and RVWI ($p > 0.05$). Compared with the CAD group and CAD + NC group, LVWI and RVWI in the CAD + miR-136-3p group were observed with significant degradation ($p < 0.05$), indicating that virus overexpressing miR-136-3p affected the cardiac function of CAD rats.

Hematoxylin-eosin staining was conducted to observe the pathological changes of myocardial tissues (Fig. 2A). The normal group showed neatly arranged myocardial fibers, clear cross striation, as well as continuous and intact myocardium and endocardial cells. Besides, the nucleus was in the middle, and the cytoplasm was evenly stained. No interstitial edema, cell swelling, or inflammatory cell infiltration was observed. In the CAD group and the CAD + NC group, we could observe disordered myocardial fibers and cardiomyocytes, with abnormal structures. The interstitial was moderately swollen, the inflammatory cells infiltrated, and the cytoplasm was unevenly stained. Moreover, the CAD + miR-136-3p group displayed mild pathological damage, with neatly arranged myocardial fibers. Additionally,

Fig. 3. Overexpression of miR-136-3p inhibits cardiomyocyte apoptosis in CAD rats. **A** TUNEL staining for rat cardiomyocyte apoptosis ($\times 200$). **B** Western blot analysis to determine the expression of apoptosis-related proteins in rat myocardial tissue. $n = 5$. Data comparison among multiple groups was performed by one-way ANOVA followed by pairwise comparison with the least significant difference t test. * $p < 0.05$ vs. the normal group. # $p < 0.05$ vs. the CAD group.

the myocardium and endocardial cells were continuous and intact, and the cardiomyocytes were evenly arranged, accompanied by mild interstitial edema, slightly widened interstitial space, and a small amount of inflammatory cell infiltration. The aforementioned results suggested that overexpression of miR-136-3p reduced pathological changes of myocardial tissues caused by pituitrin treatment, inflammatory cell infiltration, and the degree of edema.

The transmission electron microscopic observation (Fig. 2B) identified the ultrastructure of myocardial tissue. In the normal group, the myocardial fibers of the myocardial tissue were arranged neatly with clear bright and dark bands. The shape of the mitochondria was regular, accompanied by intact membrane, dense and regular cristae, intact nuclear membrane, and evenly distributed chromatin. The myocardial fibers in the CAD group and the CAD + NC group were disordered in myocardial fibers, with aggregated and swollen mitochondria, partial membrane rupture, cristae degradation, increased lysosomes, and nucleus pyknosis. The myocardial fibers of the myocardial tissue in the CAD + miR-136-3p group were arranged neatly. No obvious membrane rupture, or degradation of mitochondria were observed. Also, the complete nuclear membrane could be seen. It is reasonable to conclude that overexpression of miR-136-3p can diminish pathological changes caused by pituitrin treatment and maintain normal cardiomyocyte structure.

Overexpression of miR-136-3p Inhibits Cardiomyocyte Apoptosis in CAD Rats

The apoptosis conditions of cardiomyocytes were observed following TUNEL staining (Fig. 3A). There were fewer apoptotic cardiomyocytes in the myocardial tissue of the normal

Fig. 4. Overexpression of miR-136-3p inhibits oxidative stress and inflammatory response in CAD rats. **A–B** Spectrophotometric colorimetric detection of MDA and SOD contents in the myocardial tissue of rats of each group. **C–G** ELISA detection of the levels of CK-MB, cTnI and inflammatory factors in the serum of each group. **H** RT-qPCR detection of the level of inflammatory factors in rat myocardial tissue. **I** Content of serum NO of rats in each group. **J** Content of serum eNOS of rats in each group. $n = 5$. Data comparison among multiple groups was performed by one-way ANOVA followed by pairwise comparison with the least significant difference t test. * $p < 0.05$ vs. the normal group. # $p < 0.05$ vs. the CAD group.

Relative to the normal group, the number of apoptotic cardiomyocytes was significantly increased in modeled rats ($p < 0.05$). Compared with the CAD group, there was no significant difference in the number of apoptotic cardiomyocytes in the CAD + NC group ($p > 0.05$). Compared with the CAD group and the CAD + NC group, the number of apoptotic cardiomyocytes was decreased significantly in the CAD + miR-136-3p group ($p < 0.05$), indicating that virus overexpressing miR-136-3p inhibits cardiomyocyte apoptosis in CAD rats.

The results of Western blot analysis (Fig. 3B) showed that, compared with the normal group, the expression of cleaved-caspase-3 and Bax in the myocardial tissue of the modeled rats was upregulated, and the expression level of Bcl-2 was downregulated ($p < 0.05$). The CAD group was not significantly different from the CAD + NC group in the expression of cleaved-caspase-3, Bax, and Bcl-2 in the myocardial tissue ($p > 0.05$). Relative to the CAD group and the CAD + NC group, the expression of cleaved-caspase-3 and Bax in the myocardial tissue of the CAD + miR-136-3p group was reduced, accompanied by increased expression of Bcl-2 ($p < 0.05$).

Fig. 5. MiR-136-3p inhibits EIF5A2 expression and activation of the RhoA-ROCK signaling pathway in the myocardial tissue of CAD rats. **A** Protein bands of EIF5A2, RhoGEP, RhoA-GTP, and p-ROCK in the myocardial tissue of rats in each group. **B** Expression of EIF5A2, RhoGEP, RhoA-GTP, and p-ROCK in the myocardial tissue of rats in each group. $n = 5$. Data comparison among multiple groups was performed by one-way ANOVA followed by pairwise comparison with the least significant difference t test. * $p < 0.05$ vs. the normal group. # $p < 0.05$ vs. the CAD group.

Overexpression of miR-136-3p Alleviates Oxidative Stress and Inflammatory Response in CAD Rats

The contents of MDA and SOD in myocardial tissue was detected by spectrophotometry (Fig. 4A–B). Increased MDA content and decreased SOD content were found in the myocardial tissue of modeled rats, as compared with the normal rats (both $p < 0.05$). The CAD group showed no significant difference in MDA and SOD contents from the CAD + NC group (all $p > 0.05$). Compared with the CAD and CAD + NC groups, upregulated SOD content and down-regulated MDA content were shown in the myocardial tissue of the CAD + miR-136-3p group (both $p < 0.05$). It indicated that upregulation of miR-136-3p could alleviate oxidative stress injury in CAD rats.

The levels of CK-MB, cTnI, and inflammatory factors IL-1 β , IL-6, and TNF- α in the serum of each group were measured by ELISA (Fig. 4C–G). Compared with the normal group, serum levels of CK-MB, cTnI, IL-1 β , IL-6 and TNF- α were increased in the other groups with modeled rats ($p < 0.05$). There was no significant difference in the serum levels of CK-MB, cTnI, IL-1 β , IL-6, and TNF- α between the CAD and CAD + NC groups (all $p > 0.05$). Moreover, the CAD + miR-136-3p group displayed reduced serum levels of CK-MB, cTnI, IL-1 β , IL-6, and TNF- α , in comparison with the CAD and CAD + NC groups (all $p < 0.05$), indicating that upregulation of miR-136-3p can reduce the inflammatory response in the serum of CAD rats.

At the same time, RT-qPCR was adopted to determine the expression of inflammatory factors IL-1 β , IL-6, and TNF- α in the myocardial tissue of each group (Fig. 4H). Higher expression of IL-1 β , IL-6 and TNF- α was identified in the myocardial tissue of modeled rats than that in the normal rats (all $p < 0.05$). Compared with the CAD group, the expression of IL-1 β , IL-6, and TNF- α in the myocardial tissue of CAD + NC group was not significantly

Fig. 6. Isolation, identification, and modeling of primary rat CMECs. **A** Morphology of primarily cultured CMECs under an inverted optical microscope ($\times 200$). **B** Immunofluorescence staining showing CD31-positive expression in CMECs. **C** miR-136-3p expression after induced stress in CAD rat models and treatment of miR-136-3p mimics. Data comparison among multiple groups was performed by one-way ANOVA followed by pairwise comparison with the least significant difference *t* test. * $p < 0.05$ vs. the blank group. The experiment was repeated three times.

different (all $p > 0.05$). In comparison with the CAD and CAD + NC groups, the expression of IL-1 β , IL-6, and TNF- α in the myocardial tissue of CAD + miR-136-3p group was downregulated (all $p < 0.05$). These findings suggested that overexpressed miR-136-3p attenuated the inflammatory response in myocardial tissue in CAD rats.

We measured the serum NO and eNOS contents in CAD rats, and the results (Fig. 4I, J) indicated that relative to the normal group, the serum NO and eNOS contents were decreased in other model groups (all $p < 0.05$); there were no apparent difference in serum NO and eNOS contents among the CAD group and the CAD + NC group (all $p > 0.05$); in contrast to the CAD group and the CAD + NC group, the serum NO and eNOS contents were enhanced in the CAD + miR-136-3p group (all $p < 0.05$). These data suggested that the upregulation of miR-136-3p could advance the serum NO and eNOS contents of rats.

miR-136-3p Inhibits EIF5A2 Expression and the Activation of the Rho A/ROCK Signaling Pathway *in vivo*

Western blot assay was used to detect the expression of EIF5A2 and the activation status of the Rho A/ROCK signaling pathway (Fig. 5A, B). Compared with the normal group, the expression of EIF5A2 and RhoGEP, RhoA-GTP and p-ROCK was upregulated in the myocardial tissue of modeled rats (all $p < 0.05$). Additionally, the expression of EIF5A2, RhoGEP, RhoA-GTP, and p-ROCK in the myocardial tissue of the CAD + NC group was not statistically different from that in the CAD group (all $p > 0.05$). Relative to the CAD and CAD + NC groups, the expression of EIF5A2, RhoGEP, RhoA-GTP, and p-ROCK in the myocardial tissue of the CAD + miR-136-3p group was remarkably diminished (all $p < 0.05$). This indicates that upregulation of miR-136-3p has the ability to diminish the expression of EIF5A2 and inhibit the Rho A/ROCK signaling pathway in the myocardial tissue of CAD rats.

Fig. 7. Overexpression of miR-136-3p enhances proliferation and migration while inhibiting apoptosis of hypoxia-stressed CMECs. **A** OD values indicating cell proliferation by MTT assay. **B** Flow cytometric cell cycle analysis. **C** Flow cytometric apoptosis analysis. **D** Transwell assay for cell migration analysis. Data comparison among multiple groups was performed by one-way ANOVA followed by pairwise comparison with the least significant difference *t* test. * *p* < 0.05 vs. the blank group. The experiment was repeated three times.

Isolation, Identification, and Modeling of Primary Rat CMECs

When the primarily cultured CMECs settled at the bottom of culture bottle, they were polygonal or fusiform and grew as paving stones (Fig. 6A). After 3 days of culture, the cell confluence reached about 95%. Cells with low confluence were star- or polygon-shaped. Immunofluorescence staining showed that CD31 was mainly distributed in the cytoplasm

Fig. 8. MiR-136-3p inhibits EIF5A2 expression and the activation of the Rho A/ROCK signaling pathway in vitro. **A** Protein bands of EIF5A2, RhoGEP, RhoA-GTP, and p-ROCK in CMECs in each group. **B** Expression of EIF5A2, RhoGEP, RhoA-GTP, and p-ROCK in CMECs in each group. Data comparison among multiple groups was performed by one-way ANOVA followed by pairwise comparison with Fisher's least significant difference test. * $p < 0.05$ vs. the blank group. The experiment was repeated three times.

with red fluorescence (Fig. 6C). These results suggested that relatively pure CMECs can be obtained following primary culture and subsequent experiments.

The expression of miR-136-3p was determined by RT-qPCR (Fig. 6C). Rat CMECs were co-incubated with HCY for CMEC injury models. Relative to unstressed CMECs, the expression of miR-136-3p was downregulated in HCY-stressed CMECs ($p < 0.05$). The blank group and the mimics NC group did not differ significantly in the miR-136-3p expression in HCY-stressed CMECs ($p > 0.05$). The treatment of miR-136-3p mimics elevated the expression of miR-136-3p in HCY-stressed CMECs ($p < 0.05$). These above-mentioned results indicated that the miR-136-3p expression was successfully intervened.

Overexpression of miR-136-3p Enhances Proliferation and Migration while Inhibiting Apoptosis of HCY-Stressed CMECs

MTT assay was employed to assess the inhibitory effect of miR-136-3p on the proliferation of CMECs (Fig. 7A). The OD values of CMECs showed no pronounced difference at 24 h in each group ($p > 0.05$). After 48 and 72 h of CMEC culture, the cell proliferation was not significantly different in the mimics NC group ($p > 0.05$), which was increased in the miR-136-3p mimics group ($p < 0.05$) versus the blank group. Compared with cell proliferation at 24 h, the cell proliferation of each group was facilitated at 48 h and 72 h ($p < 0.05$). Compared with cell proliferation at 48 h, the cell proliferation of each group was also accelerated at 72 h ($p < 0.05$). In conclusion, miR-136-3p resulted in enhanced proliferation of CMECs.

The cell cycle distribution was evaluated by PI single staining assay (Fig. 7B). Compared with the blank group, no pronounced difference was identified in the proportion of cells arrested at the G1, S, and G2 phases in the mimics NC group (all $p > 0.05$). Compared with the

Fig. 9. EIF5A2 is a target gene of miR-136-3p. **A** Bioinformatic prediction of the binding sites of miR-136-3p and EIF5A2. **B** Luciferase activity measured by dual luciferase reporter assay analysis. Data comparison among multiple groups was performed by one-way ANOVA followed by post-hoc comparison with Fisher's least significant difference test. [#]*p* < 0.05 vs. the mimics NC group. The experiment was repeated three times.

blank group, the cells arrested at the G1 phase in the miR-136-3p mimics group were decreased, accompanied by increased cells in the S phase (both $p < 0.05$).

The cell apoptosis conditions were assessed by Annexin V-FITC/PI double staining assay (Fig. 7C). There was no significant difference in the apoptosis rate between the blank group and the mimics NC group ($p > 0.05$). The 13-3p mimics group displayed lower apoptosis rate than the blank group ($p < 0.05$).

Transwell migration assay (Figure 7D) demonstrated that, the cell migration ability did not differ significantly between the miR-136-3p mimics NC group and the blank group ($p > 0.05$). Compared with the blank group and the mimics NC group, the migration ability of the miR-136-3p mimics group was enhanced ($p < 0.05$).

miR-136-3' Inhibits F5A2 Expression and the Activation of the Rho A/ROCK Signaling Pathway in vitro

Western blot analysis was adopted to measure the expression of EIF5A2 and the activation status of the Rho A/ROCK signaling pathway in CMECs (Fig. 8A, B). In comparison with the mimics group, the expression of EIF5A2, RhoGEP, RhoA-GTP, and p-ROCK in the mimics NC group was not significantly different (all $p > 0.05$), while the expression of these factors in the miR-136-3p mimics group was diminished (both $p < 0.05$). This indicates that upregulation of miR-136-3p can repress the expression of EIF5A2 and inactivate the Rho A/ROCK signaling pathway in stressed CMECs in CAD.

EIF5A2 Is a Target Gene of miR-136-3p

The bioinformatics analysis predicted that miR-136-3p could directly target sequences that bind to the 3'-UTR region of EIF5A2 (Fig. 9A, B). The luciferase activity of cells co-transfected with EIF5A2-WT and miR-136-3p mimics was lower than that of cells co-transfected with EIF5A2-WT and mimics NC ($p < 0.05$). There was no significant difference in the luciferase activity of cells co-transfected with EIF5A2-MUT and mimics NC, compared with that of cells co-transfected with EIF5A2-MUT and miR-136-3p mimics ($p > 0.05$). The aforementioned results suggested that miR-136-3p targeted and regulated the 3'-UTR of EIF5A2.

Discussion

Recent studies have suggested that multiple miRNAs are implicated in the pathophysiology of CAD as well as the presentations including myocardial infarction and heart failure [25, 26]. Thus, characterizing the functional relevance of related miRNAs may lead to a better understanding of pathogenesis and provide new insights into diagnosis and prognosis of CAD. Besides, abnormal signaling of vascular endothelial cells has been reported to be a contributor to such common diseases as CAD, atherosclerosis, and hypertension [27, 28]. The activities of CMECs have significant roles to play during the progression of CAD, which have been intensively investigated [29, 30]. Furthermore, a prior study has reported that proinflammatory cytokines are independent risk factors associated with CAD due to the key role of low-grade inflammation in the pathogenesis of CAD [31, 32]. Therefore, this study was performed with the aim of delineating the function of miR-136-3p during the progression of CAD, especially in the oxidative stress and inflammatory response and CMEC behaviors following HCY-induced stress.

It is worthy to report that miR-136-3p was downregulated in the myocardial tissue of CAD rats and CAD-stressed CMECs. Very little research has focused on the expression of miR-136-3p in human or experimental CAD. A previous study on atherosclerosis has indicated that miR-136 was abnormally expressed in vascular smooth muscle cells, and its alteration mediates atherosclerosis through the influence on cell proliferation [14], and ssc-miR-136 has been reported to be broadly decreased in the myocardial tissue of pigs with coronary microembolization [33]. In this current study, we revealed that overexpression of miR-136-3p diminished the cardiomyocyte apoptosis in CAD rat models as evidenced by lowered cleaved-caspase-3 and Bax as well as increased expression of Bcl-2. Previous studies have demonstrated the functional relevance of miRNAs to CAD and its manifestations. For example, a study has revealed that the amplification of miR-136 was able to protect from hypoxia induced apoptosis of vascular endothelial cells [15]. In the current study, it has been identified that the alteration of miR-136 mediates the atherosclerosis through the influence on cell proliferation [14].

The *in vivo* experiment of the present study also revealed that overexpressed miR-136-3p augmented the cardiac function and ameliorate pathological damage in the myocardial tissue of CAD rats. More specifically, the overexpression of miR-136-3p acted to reduce the oxidative stress and inflammatory response in CAD rats, as reflected by upregulated SOD content and downregulated MDA content, reduced serum levels of CK-MB, cTnI, IL-1 β , IL-6 and TNF- α , in addition, it diminished mRNA expression of IL-1 β , IL-6, and TNF- α . Evidence has been presented to show that the levels of biomarkers associated with oxidative stress have the capacity to predict the risk of CAD as well as adverse cardiovascular outcomes [34]. At least as Johnson and Koenig [36] reported, multiple markers related to the systemic inflammation could serve as effective indicators predicting the cardiovascular events, which may be used in the management of CAD. A study by Zhong et al. [37] has documented that overexpression of miR-136-5p could suppress the inflammation in response to oxygen-glucose deprivation and reoxygenation-treated PC-12 cells. Accordingly, lower expression of miR-136-3p may show inverse correlation with serum levels of CK-MB, cTnI, IL-1 β , IL-6, and TNF- α , subsequently with oxidative stress and inflammatory response in CAD rats. It is also significant to note that overexpressed miR-136-3p caused remarkable increase in proliferation and migration, accompanied by diminished apoptosis of HCY-stressed CMECs. These findings were largely consistent with a previously conducted study, where the treatment of miR-136 mimics protected neurocytes against injury that was induced by hypoxia in a spinal cord ischemic injury rat model [38].

Importantly, the targeting relationship of miR-136-3p and EIF5A2 was determined based on bioinformatics prediction and luciferase activity assay. Besides, miR-136-3p was shown

to repress the activation of EIF5A2 and the Rho A/ROCK signaling pathway. Multiple studies have established ROCK as a major downstream effector of the small GTP-binding protein Rho, and it is a significant mediator of angiogenesis and angiogenesis-related disorders [39, 40]. EIF5A2 is a downstream target of Akt [41], and the ROCK signaling shared the activation status with the PI3K/Akt/ROCK pathway [42]. The Rho/ROCK interaction has been implicated in the occurrence of atherosclerosis due to the function exerted in vascular smooth muscle cells, the inflammatory responses, and endothelial function [21]. Thus, the myocardial injury induced by injection of pituitrin or the HCY-induced stress in CMECs could be inhibited when the expression of EIF5A2 and the activation of the Rho A/ROCK signaling pathway were inhibited by the overexpression of elevated miR-136-3p.

The findings of this study revealed a protective effect of miR-136-3p against myocardial injury in CAD rat models and CMECs through the inhibition on EIF5A2 and the Rho A/ROCK signaling pathway. The evidence provided by the study supports the stance that the upregulation of miR-136-3p is likely to present as a new target in the treatment of CAD. However, the research focusing on the role of miR-136-3p in CAD is relatively limited, which may lead to insufficient data and reference materials. Still, future investigations are needed to extend myocardial injury in CAD to other factors.

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Statement of Ethics

All animal experiments were approved by the Ethics Committee of Hospital of Traditional Chinese and Western Medicine, Hubei Province and are in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals. All efforts were made to minimize the suffering of the animals included in the study.

Disclosure of Potential Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Author Contributions

Guarantor of the integrity of the entire study: Yongbo Lin, Jinguo Lu; study design: Yongbo Lin, Hanliang Dan; literature research: Hanliang Dan, Jinguo Lu; experimental studies: Yongbo Lin, Hanliang Dan, Jinguo Lu; data analysis: Yongbo Lin, Hanliang Dan, Jinguo Lu; manuscript editing: Yongbo Lin, Hanliang Dan; manuscript review: Yongbo Lin, Jinguo Lu.

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