European Review for Medical and Pharmacological Sciences 2019; 23: 5941-5948

Loss of microRNA-27a induces cardiac dysfunction through activating FoxO1

X.-D. QIN, L. LIU

Department of Cardiology, Daqing Longnan Hospital, Daqing, China

Abstract. – OBJECTIVE: To elucidate how microRNA-27a and FoxO1 regulate cardiac dysfunction in mice.

MATERIALS AND METHODS: Expression levels of ANP, BNP, β-MHC, α-SMA, Fn1, and Periostin in myocardial tissues of 2-month-old and 8-month-old microRNA-27a-KO mice and age-matched wild-type mice were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot. Dual-luciferase reporter gene assay was conducted in H9C2 cells to verify the binding condition between microRNA-27a and FoxO1. By transfection of microRNA-27a mimics or inhibitor, FoxO1 expression in H9C2 cells was determined at the mRNA and protein levels. HW/BW [ratio of heart weight (mg) and body weight (mg)], HW/TL [ratio of heart weight (mg) and tibial length (mm)], LVPWDT [left ventricular posterior wall diastolic thickness (mm)], LVEDD [left ventricular end-diastolic dimension (mm)], and FS (fractional shortening) in mice treated with or without FoxO1 inhibitor AS1842856 were accessed through echocardiography.

RESULTS: MicroRNA-27a-KO mice had larger LVEDD, HW/BW, and HW/TL, but lower FS and LVPWDT than those of age-matched wild-type mice. Besides, higher levels of ANP, BNP, β-MHC, α-SMA, Fn1, and Periostin were observed in myocardial tissues of microRNA-27a-KO mice compared with those of age-matched wild-type mice. Dual-luciferase reporter gene assay revealed lower luciferase activity in H9C2 cells co-transfected with microRNA-27a mimics and wild-type FoxO1 than that of controls. The expression level of FoxO1 was negatively regulated by microRNA-27a in H9C2 cells at the mRNA and protein levels. After AS1842856 injection, HW/BW, HW/TL, and LVEDD in microRNA-27a-KO mice markedly decreased, whereas FS and LVPWDT elevated. By comparison, AS1842856 injection did not influence cardiac development in wild-type mice.

CONCLUSIONS: MicroRNA-27a knockout could induce cardiac dysfunction in mice through upregulating FoxO1 expression.

Key Words:
MicroRNA-27a, FoxO1, Heart dysfunction, H9C2

Introduction

Cardiovascular and cerebrovascular diseases (CCVD) include hypertension, coronary heart disease, stroke, diabetes mellitus, atrial fibrillation, myocardial infarction, and heart failure[1,2]. It is reported that the global prevalence of CCVD is on the rise. There are 17.3 million people who die from CCVD each year[3]. Due to the high disability rate and mortality rate, cardiovascular diseases consume large medical and social resources, posing a heavy burden on their families and countries. Therefore, actively searching for new biological markers is of great significance for the early prevention and diagnosis of cardiovascular diseases.

MicroRNAs are endogenous, non-coding single-stranded small RNAs[4]. They are involved in organ growth, development, function, and stress response. MicroRNA-27a is closely related to cell apoptosis. Some studies have found that microRNA-27a has profibrotic effects on liver and lung[5,6], and is also associated with cardiac hypertrophy and heart failure[7-9]. A large number of studies[10,11] have shown the important functions of microRNAs in the occurrence and development of cardiovascular diseases.

Transcription factors, such as FoxO1 and FoxO3, have some effects on differentiation, metabolism, and senescence of various types of cells other than cardiomyocytes. Studies[12,13] have shown that congenital FoxO1 and FoxO3 deficient mice usually present embryonic vascular abnormalities or subsequent cardiac hypertrophy. Recent researches pointed out that FoxO1 is the key factor to maintain normal metabolism and survival of cardiomyocytes. FoxO1 is capable of preventing against oxidative stress in cardiomyocytes through the Hippo-YAP signaling pathway.

This work aims to elucidate whether microRNA-27a could inhibit rat cardiomyocyte fibrosis, thus providing new targets for preventing and treating myocardial fibrosis.

Corresponding Author: Lei Liu, BM; e-mail: 13836883166@163.com

Materials and Methods

Experimental Animals

2-month-old and 8-month-old microRNA-27a-knockout mice (microRNA-27a-KO) and age-matched wild-type mice were obtained from Model Animal Research Center of Heilongjiang University. All mice were kept for 2-week habituation. HW/BW [ratio of heart weight (mg) and body weight (mg)], HW/TL [ratio of heart weight (mg) and tibial length (mm)], LVPWDT [left ventricular posterior wall diastolic thickness (mm)], LVEDD [left ventricular end-diastolic dimension (mm)], and FS (fractional shortening) of 2-month-old and 8-month-old mice were recorded. FoxO1 inhibitor AS1842856 was I.V. administered in mice at a dose of 10 mg/kg. This work was approved by the Daqing Longnan Hospital Animal Ethics Committee.

Cell Culture and Transfection

Rat cardiomyocytes H9C2 were obtained from the Cell Culture Center, Institute of Basic Medical Sciences Chinese Academy of Medical Sciences (Beijing, China). H9C2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and maintained at 37°C, 5% CO_2. MicroRNA-27a mimic or microRNA-27a inhibitor was transfected in H9C2 cells until 50-80% of confluence using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Western Blot

Myocardial tissues or H9C2 cells were lysed for extracting total protein. The protein concentration was calculated by bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The extracted proteins were separated on a 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Western blot analysis was performed according to standard procedures. Images were analyzed with the Image J software.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol kit (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA, which was then reversely transcribed into complementary Deoxyribose Nucleic Acid (cDNA). After the cDNA was amplified, qRT-PCR was performed to detect the expressions of related genes with 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 58°C for 30 s, and 74°C for 30 s. Primers used in this study were as follows: FOXO1: F: TCGTCATAATCTGTCCCTACACA; R: CGGCTTCGGCTCTTAGCAAA; ANP: F: TGATAACTGGAGTACATTTCGCC; R: CGGTCATAATGGGTGAGAGTCT; BNP: F: GATGTGCATTCTATGGTGTACC; R: TTTCGGGATTGCTTATCTCAGAC; α-MHC: F: ATTCCACTAGGACCAGACTGCACC; R: GCTGGTGGTACTTATATTGTCCAC; β-MHC: F: TCAAGAGGCGAACACACAAC; R: GGCCTTTTCATTGTTTTCCA; α-SMA: F: ACCCAGCATGACATAACAGTG; R: GGATCCTTCTTCGTTCACAGTTT; Fn1: F: CAGTGCCTCGGAGATGGTG; R: GGTTAGGTTCGCAGAAGTTGG; Periostin: F: GAAGTGCCAAGGGGATCATA; R: TGGCACTTGCTTCACAGAAC; MicroRNA-27a: F: ACACTCCAGCTGGGTTCACAGTGGCTAAG; R: CTCAACTGGTGTCGTGGAGTCCGGCAATTCAGTTGAGGCGGAACT.

Echocardiography

Mice were inhaled anesthetized with 1.5% isoflurane for 3 min and placed on a 37°C thermostat heating plate. The heart rate of mice was maintained at 45-55 beats/min. The chest area was depilated, and the appropriate amount of ultrasonic glue was applied. The ultrasonic probe was placed toward the mouse head. By rotation at 30°-45° counterclockwise, the M echocardiographic image was taken at the papillary muscle level of mitral valve cords. LVEDD, FS, and LVPWDT of mice were recorded. Heart rate (HR) was calculated for the average level based on at least 10 more cycles. Other indexes were recorded as the average level based on 5 HRs by two researchers independently.

Statistical Analysis

Data were analyzed by Statistical Product and Service Solutions (SPSS) 19.0 statistical software (IBM, Armonk, NY, USA). GraphPad Prism (Version X; La Jolla, CA, USA) was introduced for image processing and analyses. The quantitative data were represented as mean ± standard deviation ($\overline{x}\pm s$). The continuous variables were analyzed by the *t*-test between two different groups or one-way ANOVA among different groups, followed by the Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

MicroRNA-27a Knockout Caused Cardiac Remodeling and Dysfunction

We first analyzed the general characteristics of 2-month-old and 8-month-old microRNA-27a-KO mice and age-matched wild-type mice. It is found that 8-month-old wild-type mice present lower body weight, HW/BW, and HW/TL than those 8-month-old microRNA-27a-KO mice (Figure 1A-1C). Through echocardiography determination, microRNA-27a-KO mice had larger LVEDD compared with those of age-matched controls (Figure 1E). However, wild-type mice had higher FS and LVPWDT than microRNA-27a-KO mice (Figure 1D and 1F). These data demonstrated that microRNA-27a is closely related to cardiac development and function.

MicroRNA-27a Knockout Induced Myocardial Fibrosis

To further evaluate the potential mechanism of microRNA-27a in regulating cardiac function, mouse cardiac tissues were harvested for detecting relative gene expressions by qRT-PCR. The data showed higher mRNA levels of ANP, BNP, and β-MHC in cardiac tissues of microRNA-27a-KO mice compared with those of age-matched controls, whereas α-MHC level was lower (Figure 2A). In particular, protein expression of ANP was consistently higher in microRNA-27a-KO mice (Figure 2B). Subsequently, both mRNA and protein levels of α-SMA, Fn1, and Periostin were markedly higher in cardiac tissues of microRNA-27a-KO mice compared with those of age-matched controls (Figure 2C and 2D). The above results revealed that microRNA-27a knockout is involved in the process of myocardial fibrosis.

FoxO1 Was Predicted to be the Target Gene of MicroRNA-27a

Through bioinformatics prediction, FoxO1 was found to be the target gene of microRNA-27a, which contained the potential binding site with microRNA-27a (Figure 3A). Subsequently, the dual-luciferase reporter gene assay was conducted in H9C2 cardiomyocytes to elucidate the binding condition between microRNA-27a and FoxO1. The data elucidated the lower luciferase activity in H9C2 cells co-transfected with microRNA-27a mimics and wild-type FoxO1 than that of controls. However, no significant difference in luciferase activity was found in H9C2 cells co-transfected with microRNA-27a mimics and mutant-type FoxO1 (Figure 3B). Hence, we verified that FoxO1 could directly bind to microRNA-27a. Both qRT-PCR and Western blot results showed that the expression level of FoxO1 is negatively regulated by microRNA-27a (Figure 3C and 3D).

FoxO1 Inhibitor AS1842856 Reversed the Cardiac Dysfunction Induced by MicroRNA-27a Knockout

Finally, we verified the *in vivo* regulatory effect of microRNA-27a on FoxO1 through the administration of FoxO1 inhibitor AS1842856 in mice. The data demonstrated that HW/BW, HW/TL, and LVEDD in microRNA-27a-KO mice markedly decreased than those of age-matched controls after AS1842856 administration (Figure 4A, 4B and 4D). Meanwhile, FS and LVPWDT in microRNA-27a-KO mice injected with AS1842856 significantly elevated (Figure 4C and 4E). By comparison, we did not observe changes in cardiac development in wild-type mice after AS1842856 injection. These *in vivo* results further confirmed that microRNA-27a participates in cardiac development through regulating FoxO1.

Discussion

Some studies have found a variety of microRNAs expressed in the heart, including miR-133a/b, miR-23, miR-29a/b, miR-27a/b, etc.[14]. These microRNAs are associated with many heart diseases such as arrhythmias, myocardial infarction, heart failure, and cardiac hypertrophy. Meanwhile, the significant functions of microRNAs in cardiac development have been well concerned[15-19]. The establishment of cardiomyocyte-specific microRNA transgenic mice provides strong technical support for studying the function and mechanism of microRNAs in heart diseases. MicroRNA-27a, as a well-studied microRNA in heart diseases, exerts an important role in the regulation of cardiomyocytes, vascular cell differentiation, and cardiovascular system. Nishi et al[20] pointed out that microRNA-27a affects the differentiation and proliferation of cardiomyocytes by regulating the expression of β-myosin heavy chain (β-MHC). Thyroid hormone receptor β1 (TRβ1) is a negative thyroid hormone response element that is present on the β-MHC promoter and negatively regulates the β-MHC transcription. MicroRNA-27a upregu-

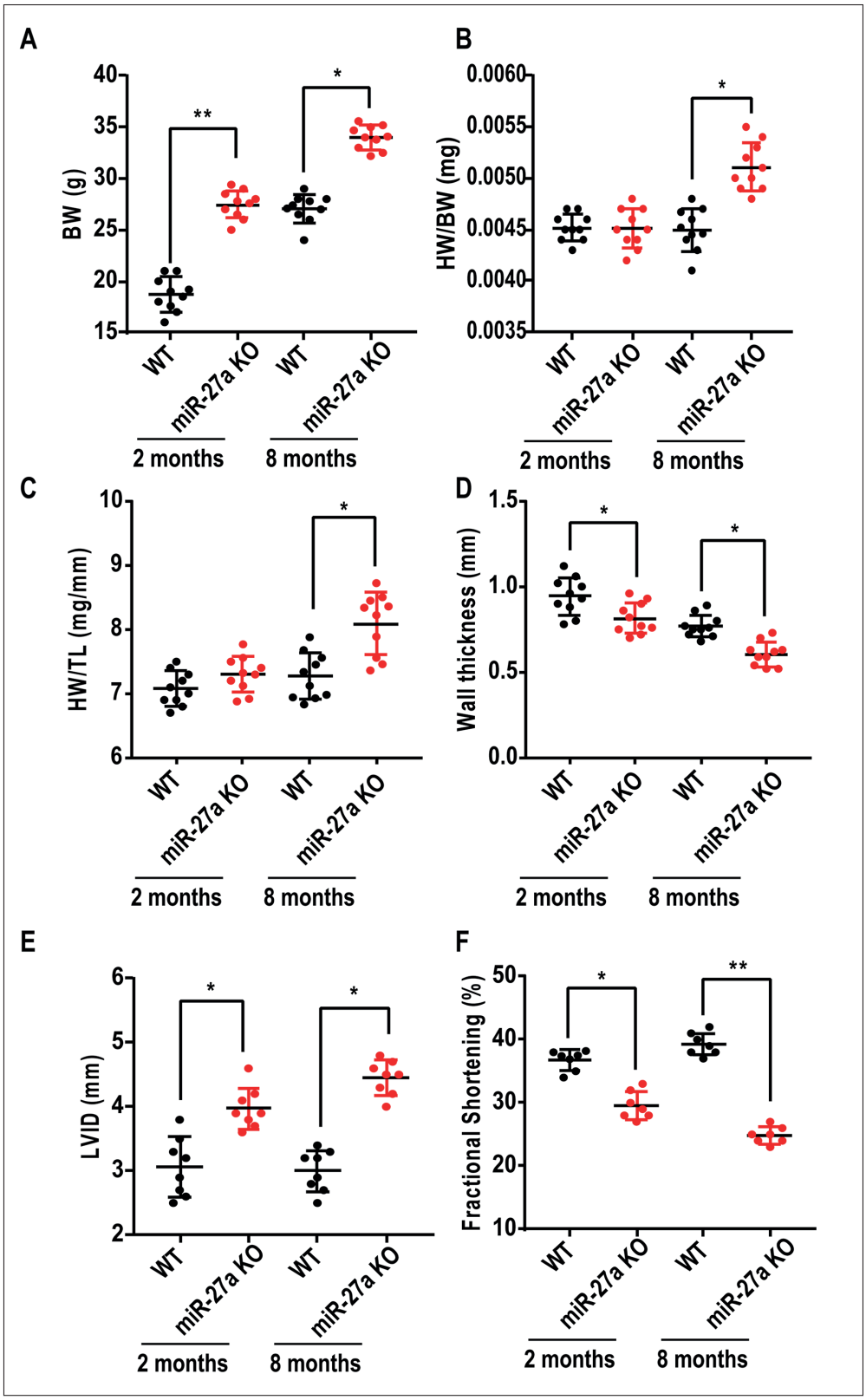

Figure 1. MicroRNA-27a knockout caused cardiac remodeling and dysfunction Body weight (*A*), HW/BW (*B*), HW/TL (*C*), LVEDD (*D*), LVPWDT (*E*), and FS (*F*) in 2-month-old and 8-month-old microRNA-27a-KO mice and age-matched wild-type mice.

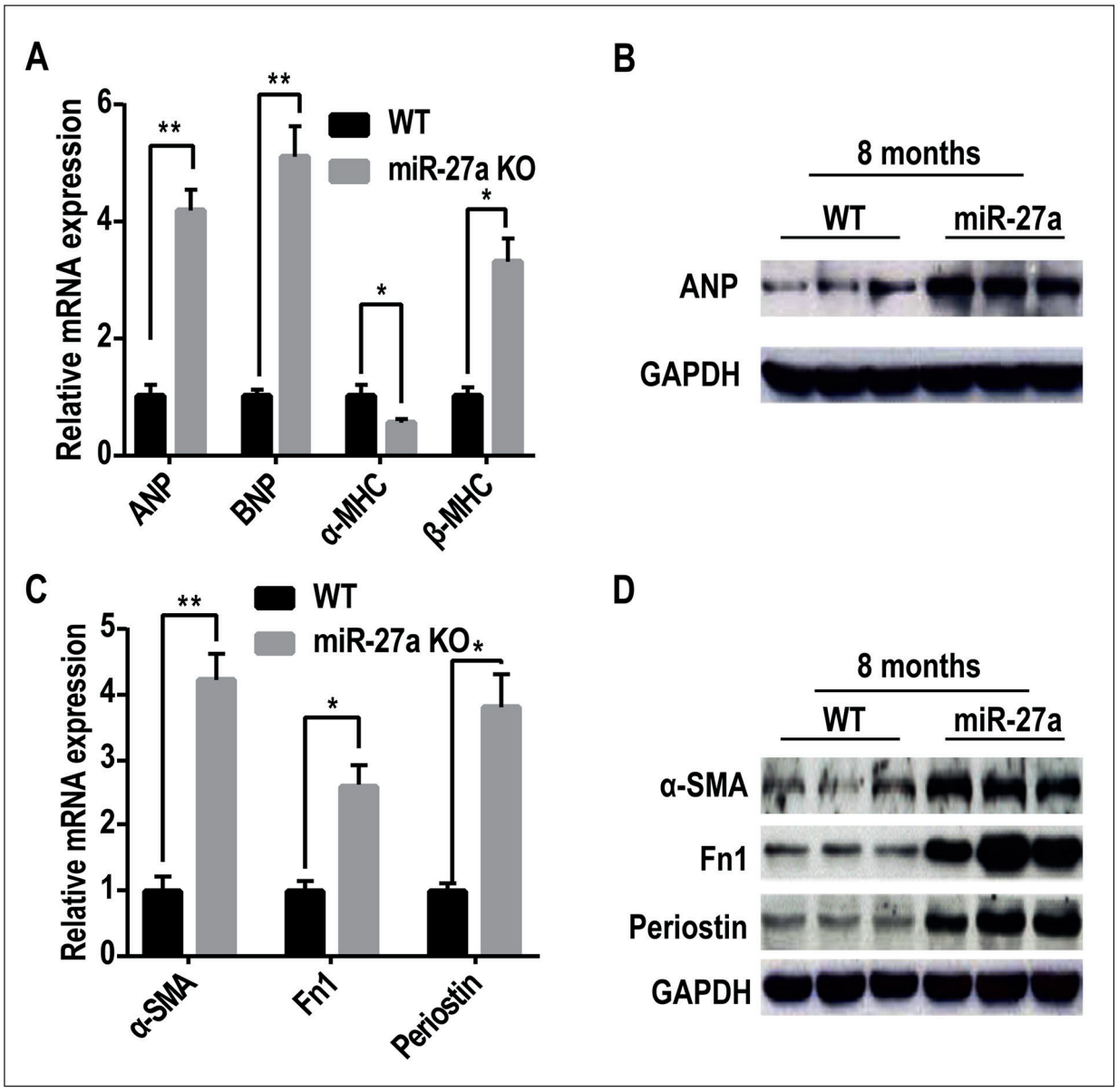

Figure 2. MicroRNA-27a knockout induced myocardial fibrosis. At the 8-week old, cardiac tissues of microRNA-27a-KO mice and age-matched wild-type mice were harvested. ***A,*** The mRNA levels of ANP, BNP, MHC-α, and β-MHC detected by qRT-PCR. **B,** Protein level of ANP detected by Western blot. **C,** The mRNA levels of α-SMA, Fn1, and Periostin detected by qRT-PCR. **D,** Protein levels of α-SMA, Fn1, and Periostin detected by Western blot.

lates the β-MHC expression by downregulating the TRβ1 expression. The overexpression of microRNA-27a during stem cell differentiation exerts a crucial role in cardiac development in mice through regulating β-MHC.

Xue et al[21] established a sepsis model in mice by intraperitoneal injection of lipopolysaccharide. They found that microRNA-27a knockdown protects the myocardium from sepsis injury by upregulating Nrf2 expression. Alvarez et al[22] found that microRNA-27a can reduce the level of low-density lipoprotein cholesterol receptor (LDLR), a significant factor that regulates blood lipid homeostasis thereby reducing the incidence of atherosclerosis. Hence, it is believed that the development of coronary atherosclerotic heart disease can be prevented by regulating the expression level of microR-

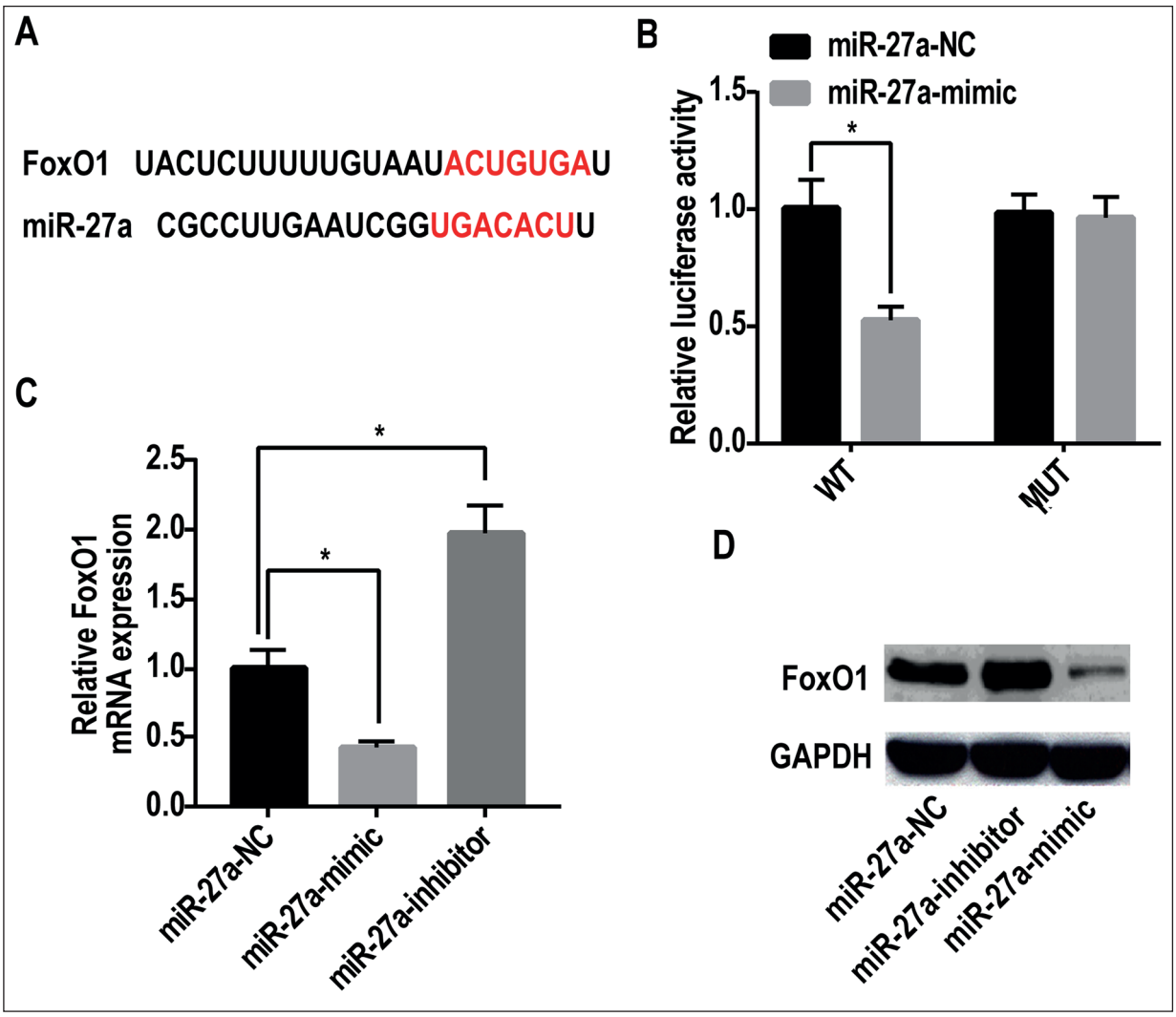

Figure 3. FoxO1 was predicted to be the target gene of microRNA-27a. ***A,*** The binding condition between FoxO1 and microRNA-27a predicted by bioinformatics. ***B,*** Binding condition between FoxO1 and microRNA-27a in H9C2 cells verified by dual-luciferase reporter gene assay. ***C,*** Overexpression of microRNA-27a downregulated mRNA level of FoxO1 in H9C2 cells, and knockdown of microRNA-27a upregulated mRNA level of FoxO1. ***D,*** Overexpression of microRNA-27a downregulated protein level of FoxO1 in H9C2 cells, and knockdown of microRNA-27a upregulated protein level of FoxO1.

NA-27a. Kang et al[23] found that the expression of microRNA-27a in lung tissues of C57BL/6 mice undergoing hypoxia (10%) exposure for 3 weeks greatly increased. Besides, the expression of microRNA-27a target gene PPARγ decreased, resulting in upregulated ET-1 level. These mice presented pulmonary vascular proliferation and pulmonary vascular resistance, which in turn caused pulmonary hypertension and ultimately affected cardiac function. It is suggested that microRNA-27a may exert an important therapeutic role in certain heart diseases, thus serving as a new drug target.

Scholars[24,25] have found that the FoxOs family has important regulatory effects on oxidative free radical damage. FoxO is a major factor in protecting cell damage from antioxidant free radicals. FoxO1 is one of the major members of the transcription factor FoxO subfamily and is called the forkhead box transcription factor. The human FoxO1 gene is located on chromosome 13 and encodes five amino acids. FoxO1 is widely distributed in various tissues and organs of adults, such as the heart, small intestine, colon, and peripheral blood leukocytes. Studies have shown that congenital FoxO1-de-

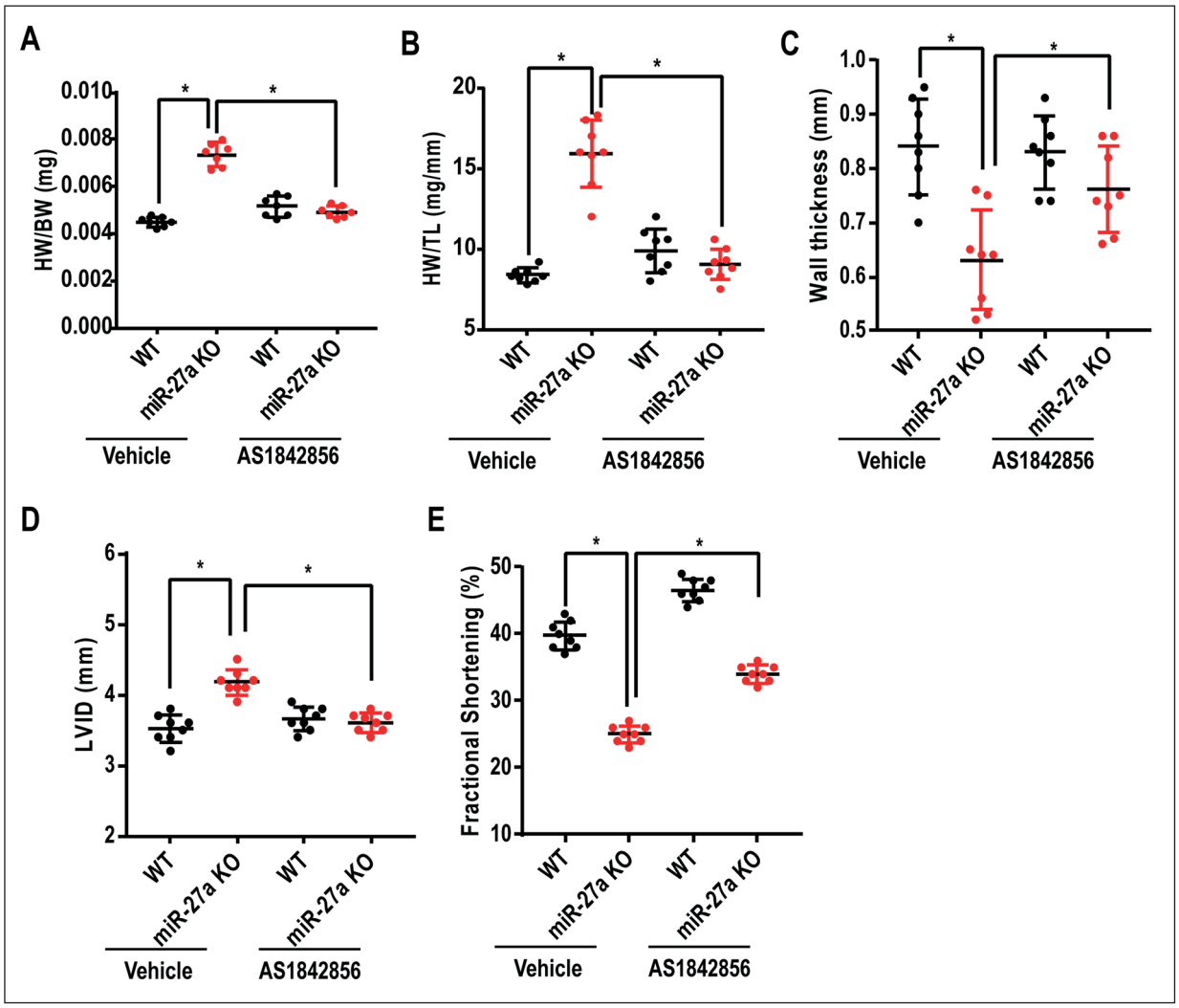

Figure 4. FoxO1 inhibitor AS1842856 reversed the cardiac dysfunction induced by microRNA-27a knockout. HW/BW (***A***), HW/TL (***B***), LVEDD (***C***), LVPWDT (***D***), and FS (***E***) in cardiac tissues of microRNA-27a-KO mice and age-matched wild-type mice after AS1842856 injection. Data were expressed as percentages.

ficient mice usually present fetal vascular abnormalities or cardiac hypertrophy. Recently it has been found that that FoxO1 is the key to maintain the normal metabolism and survival of muscle cells mainly through the Hippo-YAP pathway.

In this work, we compared HW/BW, HW/TL, LVEDD, FS, and LVPWDT between microRNA-27a-KO mice and wild-type mice. MicroRNA-27-KO mice showed higher HW/BW, HW/TL, and LVEDD, but lower FS and LVPWDT than those of age-matched wild-type mice. Moreover, microRNA-27a knockdown markedly upregulated levels of myocardial fibrosis-related genes. By bioinformatics prediction, FoxO1 was screened out to be the target gene of microRNA-27a, which was further verified by qRT-PCR results. Furthermore, treatment of FoxO1 inhibitor (AS1842856) rescued cardiac dysfunction in mice caused by microRNA-27a knockout.

Conclusions

We found that the microRNA-27a knockout could induce cardiac dysfunction in mice through upregulating FoxO1 expression.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Du J, Zhang Z, Ge Y, Zhen J, Leng J, Wang J. VKORC1 and CD-14 genetic polymorphisms associate with susceptibility to cardiovascular and cerebrovascular diseases. Int J Clin Exp Med 2015; 8: 20444-20453.
2) Li Y, Zhu J, Ding JQ. VKORC1 rs2359612 and rs9923231 polymorphisms correlate with high risks of cardiovascular and cerebrovascular diseases. Genet Mol Res 2015; 14: 14731-14744.
3) Li M, Zhang J. Circulating MicroRNAs: potential and emerging biomarkers for diagnosis of cardiovascular and cerebrovascular diseases. Biomed Res Int 2015; 2015: 730535.
4) Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.
5) Garcia-Bates TM, Lehmann GM, Simpson-Haidaris PJ, Bernstein SH, Sime PJ, Phipps RP. Role of peroxisome proliferator-activated receptor gamma and its ligands in the treatment of hematological malignancies. PPAR Res 2008; 2008: 834612.
6) Luconi M, Cantini G, Serio M. Peroxisome proliferator-activated receptor gamma (PPARgamma): Is the genomic activity the only answer? Steroids 2010; 75: 585-594.
7) Siddesha JM, Valente AJ, Sakamuri SS, Yoshida T, Gardner JD, Somanna N, Takahashi C, Noda M, Chandrasekar B. Angiotensin II stimulates cardiac fibroblast migration via the differential regulation of matrixins and RECK. J Mol Cell Cardiol 2013; 65: 9-18.
8) Zhao QD, Viswanadhapalli S, Williams P, Shi Q, Tan C, Yi X, Bhandari B, Abboud HE. NADPH oxidase 4 induces cardiac fibrosis and hypertrophy through activating Akt/mTOR and NFkappaB signaling pathways. Circulation 2015; 131: 643-655.
9) Tsai CF, Yang SF, Chu HJ, Ueng KC. Cross-talk between mineralocorticoid receptor/angiotensin II type 1 receptor and mitogen-activated protein kinase pathways underlies aldosterone-induced atrial fibrotic responses in HL-1 cardiomyocytes. Int J Cardiol 2013; 169: 17-28.
10) Zhang S, Cui R. The targeted regulation of miR-26a on PTEN-PI3K/AKT signaling pathway in myocardial fibrosis after myocardial infarction. Eur Rev Med Pharmacol Sci 2018; 22: 523-531.
11) Jansen F, Yang X, Proebsting S, Hoelscher M, Przybilla D, Baumann K, Schmitz T, Dolf A, Endl E, Franklin BS, Sinning JM, Vasa-Nicotera M, Nickenig G, Werner N. MicroRNA expression in circulating microvesicles predicts cardiovascular events in patients with coronary artery disease. J Am Heart Assoc 2014; 3: e1249.
12) Engler RL, Schmid-Schonbein GW, Pavelec RS. Leukocyte capillary plugging in myocardial ischemia and reperfusion in the dog. Am J Pathol 1983; 111: 98-111.
13) Diaz I, Smani T. New insights into the mechanisms underlying vascular and cardiac effects of urocortin. Curr Vasc Pharmacol 2013; 11: 457-464.
14) Liang Y, Ridzon D, Wong L, Chen C. Characterization of microRNA expression profiles in normal human tissues. BMC Genomics 2007; 8: 166.
15) Sayed D, Hong C, Chen IY, Lypowy J, Abdellatif M. MicroRNAs play an essential role in the development of cardiac hypertrophy. Circ Res 2007; 100: 416-424.
16) Care A, Catalucci D, Felicetti F, Bonci D, Addario A, Gallo P, Bang ML, Segnalini P, Gu Y, Dalton ND, Elia L, Latronico MV, Hoydal M, Autore C, Russo MA, Dorn GN, Ellingsen O, Ruiz-Lozano P, Peterson KL, Croce CM, Peschle C, Condorelli G. MicroRNA-133 controls cardiac hypertrophy. Nat Med 2007; 13: 613-618.
17) van Rooij E, Sutherland LB, Qi X, Richardson JA, Hill J, Olson EN. Control of stress-dependent cardiac growth and gene expression by a microRNA. Science 2007; 316: 575-579.
18) Callis TE, Pandya K, Seok HY, Tang RH, Tatsuguchi M, Huang ZP, Chen JF, Deng Z, Gunn B, Shumate J, Willis MS, Selzman CH, Wang DZ. MicroRNA-208a is a regulator of cardiac hypertrophy and conduction in mice. J Clin Invest 2009; 119: 2772-2786.
19) van Rooij E, Sutherland LB, Liu N, Williams AH, McAnally J, Gerard RD, Richardson JA, Olson EN. A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. Proc Natl Acad Sci U S A 2006; 103: 18255-18260.
20) Nishi H, Ono K, Horie T, Nagao K, Kinoshita M, Kuwabara Y, Watanabe S, Takaya T, Tamaki Y, Takanabe-Mori R, Wada H, Hasegawa K, Iwanaga Y, Kawamura T, Kita T, Kimura T. MicroRNA-27a regulates beta cardiac myosin heavy chain gene expression by targeting thyroid hormone receptor beta1 in neonatal rat ventricular myocytes. Mol Cell Biol 2011; 31: 744-755.
21) Xue WL, Bai X, Zhang L. rhTNFR:Fc increases Nrf2 expression via miR-27a mediation to protect myocardium against sepsis injury. Biochem Biophys Res Commun 2015; 464: 855-861.
22) Alvarez ML, Khosroheidari M, Eddy E, Done SC. MicroRNA-27a decreases the level and efficiency of the LDL receptor and contributes to the dysregulation of cholesterol homeostasis. Atherosclerosis 2015; 242: 595-604.
23) Kang BY, Park KK, Green DE, Bijli KM, Searles CD, Sutliff RL, Hart CM. Hypoxia mediates mutual repression between microRNA-27a and PPARgamma in the pulmonary vasculature. PLoS One 2013; 8: e79503.
24) Paik JH, Kollipara R, Chu G, Ji H, Xiao Y, Ding Z, Miao L, Tothova Z, Horner JW, Carrasco DR, Jiang S, Gilliland DG, Chin L, Wong WH, Castrillon DH, DePinho RA. FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell 2007; 128: 309-323.
25) Puthanveetil P, Wan A, Rodrigues B. FoxO1 is crucial for sustaining cardiomyocyte metabolism and cell survival. Cardiovasc Res 2013; 97: 393-403.