Cardiac Hypertrophy Is Positively Regulated by MicroRNA miR-23a*

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MicroRNAs (miRNAs) are a class of small noncoding RNAs that mediate post-transcriptional gene silencing. Myocardial hypertrophy is frequently associated with the development of heart failure. A variety of miRNAs are involved in the regulation of cardiac hypertrophy, however, the molecular targets of miRNAs in the cardiac hypertrophic cascades remain to be fully identified. We produced miR-23a transgenic mice, and these mice exhibit exaggerated cardiac hypertrophy in response to the stimulation with phenylephrine or pressure overload by transverse aortic banding. The endogenous miR-23a is up-regulated upon treatment with phenylephrine, endothelin-1, or transverse aortic banding. Knockdown of miR-23a attenuates hypertrophic responses. To identify the downstream targets of miR-23a, we found that transcription factor Foxo3a is suppressed by miR-23a. Luciferase assay indicates that miR-23a directly inhibits the translation activity of Foxo3a 3’ UTR. Introduction or knockdown of miR-23a leads to the alterations of Foxo3a protein levels. Enforced expression of the constitutively active form of Foxo3a counteracts the provocative effect of miR-23a on hypertrophy. Furthermore, we observed that miR-23a is able to alter the expression levels of manganese superoxide dismutase and the consequent reactive oxygen species, and this effect is mediated by Foxo3a. In addition, our results show that miR-23a and Foxo3a bi-transgenic mice exhibit a reduced hypertrophic response compared with the miR-23a transgenic mice alone. Our present study reveals that miR-23a can mediate the hypertrophic signal through regulating Foxo3a. They form an axis in hypertrophic machinery and can be targets for the development of hypertrophic treatment.

Cardiac hypertrophy is a common response to a variety of physiological as well as pathophysiological stimuli, and will eventually lead to heart failure. Maladaptive hypertrophy is considered to be a therapeutic target for heart failure. Nevertheless, the underlying molecular mechanisms of cardiac hypertrophy are still poorly understood. To prevent heart failure, it is necessary to identify and characterize molecules that may regulate hypertrophy.

MicroRNAs (miRNAs)3 are 21–23-nucleotide single-stranded noncoding RNA molecules that are found to be gene expression regulators and can affect mRNA stability or translation efficiency (1–3). Growing evidence has revealed that miRNAs are involved in the control of the hypertrophic program (4–9). The expression profiles of many miRNAs are altered in response to thoracic aortic-banded hearts and in the calcineurin-overexpressed transgenic mice (4, 10, 11). Other hypertrophic stimuli such as angiotensin II, PE, endothelin-1 (ET-1), and isoproterenol also lead to an aberrant expression of miRNAs (12–15).

Functional studies have revealed that some miRNAs are able to antagonize hypertrophy. miR-133 is down-regulated in the mouse model of cardiac hypertrophy as well as in left ventricular tissue of patients with cardiac hypertrophy. miR-133 overexpression can significantly inhibit hypertrophy not only in vitro but also in vivo. Knockdown of miR-133 can easily induce a marked hypertrophic response (16). miR-26 has been recently shown to play an essential role in regulating myocyte survival and hypertrophy through targeting Gata4. miR-26 is significantly down-regulated after pressure overload on the heart. Consequently, the expression of Gata4 is increased during hypertrophy. Modulation of miR-26 expression levels reveals that miR-26 can inhibit ET-1-mediated up-regulation of Gata4. In contrast, knockdown of miR-26 induces an increase in cell size (17).

On the contrary, a variety of miRNAs can provoke hypertrophy. miR-195 is characterized to be involved in inducing hypertrophic growth in the adult heart. In particular, it is up-regulated in both human and mouse hypertrophic hearts (18). miR-208 is a conserved cardiac-specific miRNA, which resides in intron 27 of the α-myosin heavy chain (α-MHC) gene and is

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specifically expressed in both human and mouse hearts (19). Transgenic overexpression of miR-208a specifically in the heart induces hypertrophic growth in mice, with enlarged chambers and thickened ventricular walls (5).

A miRNA may have multiple targets, but it also can use a specific mediator to convey its signal in regulating hypertrophic program. For example, miR-133 exerts its effect through targeting Cdc42, Rho-A, and Nelf-A/WHSC2 that take part in hypertrophic remodeling (16, 20, 21). miR-208a targets thyroid hormone receptor-associated protein 1 (THRAP1), which is a co-factor of the thyroid hormone nuclear receptor (19). Given the important roles of miRNAs in cardiac hypertrophy, it is important to identify the downstream mediators of miRNAs so that therapeutic approaches can be developed by targeting their mediators.

The forkhead family of transcription factors consists of more than 80 members identified in species ranging from *Saccharomyces cerevisiae* to humans, and divided into 19 subgroups (from A to S). They are characterized by the presence of a conserved 100-amino acid DNA binding domain, and participate in regulating diverse cellular functions such as apoptosis, differentiation, metabolism, proliferation, and survival (22). Foxo3a can exert a diversity of effects including cell cycle arrest, oxidative scavenging, and apoptosis. It is expressed in the heart and skeletal muscle (23–25). Recent studies have demonstrated that Foxo3a is involved in the regulation of cardiac hypertrophy (24, 26–28). However, its relationship with miRNAs in the hypertrophic program remains unknown.

Our present work was aimed at finding the downstream targets of miR-23a. The results showed that miR-23a transgenic mice exhibited exaggerated hypertrophic responses, and miR-23a can negatively regulate Foxo3a. miR-23a and Foxo3a bi-transgenic mice demonstrated less hypertrophy than miR-23a transgenic mice. Thus, miR-23a and Foxo3a have a cross-talk in cardiac hypertrophy.

**EXPERIMENTAL PROCEDURES**

**Adenoviral Constructions and Infection**—The constitutively active form of human Foxo3a was kindly provided by Dr. Boudewijn M. T. Burgering. The adenoviruses harboring the constitutively active form of Foxo3a (caFoxo3a) were constructed using the Adeno-X™ expression system (Clontech). The adenoviruses containing β-galactosidase (β-gal) were as described elsewhere (29). To construct adenoviruses encoding miR-23a, a mouse genomic sequence harboring the premiR-23a was amplified using the following primer sets: 5’-GCAAGCTGATTTAGATCTTG-3’, 5’-AACTAGGC- ACTGTGAAACAGC-3’, and then cloned into the adenoviral system. The mouse Foxo3a RNAi target sequence is 5’-CAAGTACACCAGAGGCCGA-3’. A nonrelated, scrambled RNAi without any other match in the mouse genomic sequence was used as a control (5’-TCAGACAGAGACAGACC-3’). The adenoviruses harboring Foxo3a RNAi or its scrambled form were constructed using the pSilencer™ adeno-1.0-CMV System (Ambion) according to the manufacturer’s instructions. The effects of these constructs on Foxo3a expression were tested. All constructs were amplified in HEK293 cells. Adenoviral infection of cardiomyocytes was performed as we described previously (30).

**Cardiomyocyte Culture and Treatment**—Cardiomyocytes were isolated from 1–2-day-old mice as described (31). The cells were plated in 10 μg/ml of laminin-coated culture dishes. Cells were treated with PE at 50 μM, or ET-1 at 100 nM, except as otherwise indicated elsewhere.

**Generation of Cardiac-specific miR-23a Transgenic Mice, caFoxo3a Transgenic Mice, and miR-23a/caFoxo3a Bi-transgenic Mice**—For creating miR-23a transgenic mice, a 312-bp DNA fragment containing murine miR-23a was cloned to the vector, pα-MHC-clone 26 (kindly provided by Dr. Zhongzhou Yang), under control of the α-myosin heavy chain promoter. The primers used to generate miR-23a transgenic mice include, forward primer, 5’-GCAGGAAGAATGGTGCTATTCC-3’; and reverse primer, 5’-GTGATGAGCTCTTCGTTGTT-3’. Microinjection was performed using the following standard protocols. The primers for genotyping miR-23a transgenic mice include the forward primer in the α-MHC promoter, 5’-AGGGAAGTTGTGGTGAAGAA-3’, and the reverse primer in the miR-23a DNA, 5’-AGCACAGGTCAGTTGGAAT-3’.

For creating caFoxo3a transgenic mice, human caFoxo3a was as we described (32), and cloned to the vector pαMHC-clone 26 under control of the α-myosin heavy chain promoter. The primers include the forward primer, 5’-ATGGCAGAGGCGACCGGCTTCC-3’; and reverse primer, 5’-TCAGCCTGGCCACCCAGCTCTGAG-3’. The primers for genotyping Foxo3a transgenic mice include, the forward primer in the α-MHC promoter, 5’-TGCCCAAGCTGGCGCACCCAGCTCTGAG-3’, and the reverse primer in the Foxo3a cDNA, 5’-CCGCCCTTTTCAGGGGCACGT-3’. We crossed miR-23a transgenic mice with caFoxo3a transgenic mice to produce bi-transgenic mice harboring both miR-23a and foxo3a transgenes (miR-23a/Foxo3a bi-transgenic mice).

**Determinations of Cell Surface Areas, Sarcomere Organization, and Protein/DNA Ratio**—The cell surface area of F-actin-stained cells or unstained cells was measured as described (31). Briefly, the cardiomyocytes were fixed in 3.7% formaldehyde in PBS. Cells were dehydrated with acetone for 3 min and treated with 0.1% Triton X-100 for 20 min. They were then stained with a 50 μg/ml of fluorescent phalloidin-TRITC conjugate (Sigma) for 45 min at room temperature, and visualized by a laser confocal microscopy (Zeiss LSM 510 META). 100–200 cardiomyocytes in 30–50 fields were examined in each group. To measure the protein/DNA ratio, total protein and DNA contents were analyzed as described (32).

**Immunoblot**—Immunoblots were performed as described (30). In brief, samples were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. Equal protein loading was controlled by Ponceau red staining of membranes. Blots were probed using antibodies. The anti-Foxo3a antibody was from Cell Signaling. The anti-manganese superoxide dismutase (Mn-SOD) was from Upstate. The horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-goat IgG were purchased from Santa Cruz Biotechnology.
Preparations of the Luciferase Construct of Foxo3a 3' UTR and Luciferase Assay—Foxo3a 3' UTR was amplified by PCR. The forward primer was 5'-GACCTCAAACGTGCGAAGA-3'; the reverse primer was 5'-CCACGTGAAGCCTAAATGCAC-3'. To produce the mutated 3' UTR, the mutations were generated using a QuickChange II XL Site-directed Mutagenesis Kit (Stratagene). The constructs were sequence verified. Wild-type and mutated 3' UTRs were subcloned into the pGL3 vector (Promega) immediately downstream of the stop codon of the luciferase gene.

Luciferase activity assay was performed as described (33). In brief, cells were infected with adenoviral miR-23a or β-gal, and then transfected with plasmid constructs of 150 ng/well of pGL3-Foxo3a-3'UTR or pGL3-Foxo3a-3'UTR-mut using Lipofectamine 2000 (Invitrogen). At 48 h after transfection, cells were lysed and luciferase activity was measured.

Detection of Intracellular ROS Levels—Intracellular ROS levels were analyzed by employing ROS-sensitive dyes, hydroethidine. After cells were incubated with 5 μg/ml of hydroethidine for 30 min at 37 °C, cells were washed with PBS and fluorescent images were acquired from a laser confocal microscope (Zeiss LSM 510 META) by employing its region of interest mean function, and the intensity on regions of interest was measured. Intensity values of the treated cells were determined in comparison with the control cells.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)—Stem-loop qRT-PCR for mature miR-23a was performed as described (34) on an Applied Biosystems ABI Prism 7000 sequence detection system. Total RNA was extracted using TRIzol reagent (Invitrogen). After DNase I (Takara, Japan) treatment, RNA was reverse transcribed with reverse transcriptase (ReverTra Ace, ToyoBo). The results of qRT-PCR were normalized to that of U6. The sequences of U6 primers were forward, 5'-GCTTCGCGACGACATACATAC-TAA-3'; reverse, 5'-AACGCTTCAGAAATTTCGCT-3'. qRT-PCR for ANP and β-MHC was performed as described (35). RNA was reverse transcribed using oligo(dT). Moloney murine leukemia virus reverse transcriptase (ReverTra Ace) was from ToyoBo. The samples were run in triplicate using the Applied Biosystems ABI 7000 sequence detection system according to the manufacturer’s instructions. The results were standardized to control values of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequences of ANP primers were forward, 5’-CTCCGATAGATCTGCCCTTCTT-GAA-3'; and reverse, 5’-GGTACCGGAACTGTGGCAGC-CTA-3'; β-MHC forward primer, 5’-CAGACATAGAGACCTACCTT-3'; and reverse, 5’-CAGCATGCTAAGACCTAG-3'; GAPDH forward primer, 5’-TGTGTCGCGCGGTAATGGA-3'; and reverse, 5’-TGTGTCGCGCGGTAATGGA-3'. The specificity of the PCR amplification was confirmed by agarose gel electrophoresis.

Transfection of Antagomir—miR-23a antagomir and the antagonist negative control (antagomir-NC) were purchased from GenePharma Co. Ltd. The antagonist sequence is 5’-GGAAAUCCUGGGAUAUGAAU-3’. All the bases were 2’-OMe modified, and the 3’-end was conjugated to cholesterol. Chemically modified oligonucleotides 5’-CAGUAC-UUUUGUGUGUACAA-3’ were used as a negative control (antagomir-NC). Cells were transfected with the antagonist or antagomir-NC at 50 nm. The transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Apoptosis Assay—Apoptosis was determined by the terminal deoxynucleotidyl transferase-mediated TUNEL using a kit from Roche Applied Science.

Animal Experiments and Antagomir Delivery—Adult male C57BL/6 mice (8 weeks old) were purchased from the Institute of Laboratory Animal Science of the Chinese Academy of Medical Sciences (Beijing, China). Food and water were freely available throughout the experiments. Experiments were conducted according to a protocol approved by the Institute Animal Care Committee. For pressure-overload, transverse aortic constriction was carried out as described (36). Sham mice were subjected to a comparable operation without tightening of the suture encircling the aorta. Three weeks after the surgery, the hearts were harvested for histological analysis as described below.

Mice were infused with PE (75 mg/kg dissolved in 0.9% NaCl) for 2 weeks, saline-infused mice served as controls. For in vivo transfer of miR-23a antagomir, mice were infused with PE and miR-23a antagomir (30 mg/kg) at the same time. Antagomir-NC served as a negative control, and was subjected to the same procedures as antagonist. The infusions were executed with implanted osmotic minipumps (Alzet model 2001, Alza Corp.). For the experiment with antagomir in the TAC model, antagonist treatment started 24 h after TAC and on three consecutive days of the treated mice received intravenous injections of miR-23a antagomirs or antagonist control at doses of 80 mg/kg body weight in a small volume (0.2 ml) per injection.

Histological Analysis—Histological analysis of the hearts was carried out as described (33). Briefly, the hearts were excised, fixed in 10% formalin, embedded in paraffin, and sectioned into 7-μm slices, and stained with hematoxyline-eosin. To measure the cross-sectional area of the cardiomyocytes, the sections were stained with FITC-conjugated or TRITC-conjugated wheat germ agglutinin (Sigma) according to the method previously described (33).

Echocardiographic Assessment of Cardiac Dimensions and Function—Transthoracic echocardiography was performed on lightly anesthetized mice using a Vevo 770 high-resolution system (Visualsonics, Toronto, Canada) equipped with a 40-MHz RMV 704 scanhead. Two-dimensional guided M-mode tracings were recorded in both parasternal long and short axis views at the level of papillary muscles. Diastolic left ventricular posterior wall thickness, ventricular parameters including diastolic interventricular septal thickness and systolic left ventricular internal diameters were measured and fractional shortening was calculated with the established standard equation. All measurements were made from more than three beats and averaged.

Statistical Analysis—The results are expressed as mean ± S.E. The statistical significance was calculated by one-way analysis of variance, followed by Tukey post hoc test for multiple comparisons. Two groups were evaluated by Student’s t test. p < 0.05 was considered statistically significant.
RESULTS

miR-23a Transgenic Mice Are Susceptible to Undergoing Hypertrophy—To understand the pathological significance of miR-23a in the heart, we produced cardiac-specific miR-23a transgenic mice, and these mice developed normally to adulthood without substantial alterations in morphology (Fig. 1A) and cardiac function (Fig. 1B). However, in response to the stimulation with PE, these mice exhibited more severe hypertrophic responses as revealed by the hypertrophic phenotype (Fig. 1C), cardiomyocyte size (Fig. 1D), and heart/body weight ratios (Fig. 1E). miR-23a transgenic mice also showed an elevated level of hypertrophic specific markers including ANP and β-MHC upon treatment with PE (Fig. 1F). The echocardiographic assessment demonstrated an aggravated cardiac function in miR-23a transgenic mice (Fig. 1G–I). We further employed the hypertrophic model of pressure overload and observed a larger cardiomyocyte size (Fig. 1J) and an increase in heart/body weight ratios (Fig. 1K) in miR-23a transgenic mice. These data suggest that miR-23a can potentiate hypertrophy in the animal model.
Knockdown of miR-23a attenuated sarcomere organization (Fig. 2). miR-23a plays a functional role. To this end, we employed the antagomir of miR-23a and it reduced miR-23a levels (Fig. 2). miR-23a plays a functional role. To this end, we employed the antagomir of miR-23a and it reduced miR-23a levels (Fig. 2). miR-23a plays a functional role. To this end, we employed the antagomir of miR-23a and it reduced miR-23a levels (Fig. 2).

FIGURE 2. miR-23a conveys the hypertrophic signal. A and B, up-regulation of miR-23a upon treatment with PE (A) or ET-1 (B). The level of miR-23a was analyzed by qRT-PCR. C, miR-23a antagonist reduces the levels of miR-23a. Cardiomyocytes were transfected with miR-23a antagonist (anta-23a) or antagonist negative control (anta-NC). 24 h after transfection, cells were treated with 50 μM PE. The expression of miR-23a was analyzed by qRT-PCR. *, p < 0.05 compared with the group of PE alone. D-F, knockdown of miR-23a reduces the levels of miR-23a. Cardiomyocytes were transfected with antagomir or antagomir-NC. 24 h after transfection cells were treated with PE. Representative photos show sarcomere organization (D), bar = 20 μm. E, cell surface area measurement. F, the protein/DNA ratio analysis. *, p < 0.05 compared with the group of PE alone. G, miR-23a antagonist suppresses the expression of miR-23a. Cardiomyocytes were transfected with antagonist or antagonist-NC. 24 h after transfection, cells were treated with PE. The expression of miR-23a was analyzed. *, p < 0.05 compared with the group of ET-1 alone. H, pretreatment with miR-23a antagonist inhibits hypertrophy induced by ET-1. Cardiomyocytes were transfected with antagonist or antagonist-NC. 24 h after transfection cells were treated with ET-1. I, cell surface area measurement (left panel), analysis of the transcripts for β-myosin heavy chain (β-MHC) by qRT-PCR (right panel). *, p < 0.05 compared with the group of ET-1 alone. J, representative photos show sarcomere organization, bar = 20 μm.

PE and ET-1 Require miR-23a to Convey the Hypertrophic Signal in Cellular Models—We analyzed the levels of miR-23a in hypertrophy induced by PE (Fig. 2A) and ET-1 (Fig. 2B), and observed that their levels were elevated. We asked whether miR-23a plays a functional role. To this end, we employed the antagonist of miR-23a and it reduced miR-23a levels (Fig. 2C). Knockdown of miR-23a attenuated sarcomere organization (Fig. 2D), reduced cell surface area (Fig. 2E), and protein/DNA ratio (Fig. 2F) upon PE treatment. miR-23a knockdown (Fig. 2G) also attenuated the hypertrophic responses induced by ET-1 (Fig. 2, H and I). In Fig. 2, E–H, there was a significant difference between PE or ET-1 alone and PE or ET-1 in the presence of miR-23a antagonist (p < 0.05). Thus, it appears that miR-23a is necessary for conveying the hypertrophic signal.

Cardiac Hypertrophy in Animal Models Can Be Attenuated by Knockdown of miR-23a—Subsequently, we tested whether miR-23a plays a role in cardiac hypertrophy in the animal model. Administration of the miR-23a antagonist was able to reduce miR-23a levels upon treatment with PE (Fig. 3A). The hypertrophic phenotype (Fig. 3B) and cross-sectional areas (Fig. 3C) were attenuated by knockdown of miR-23a. Concomitantly, the heart weight/body weight ratio (Fig. 3D), and the expression levels of hypertrophic markers including β-MHC (Fig. 3E), ANP, and BNP (Fig. 3F) were reduced by miR-23a antagonist. In Fig. 3, C–F, there was a significant difference.
between PE alone and PE in the presence of miR-23a antagomir (p < 0.05). Knockdown of miR-23a inhibited hypertrophy induced by pressure overload, compared with the group of TAC alone (p < 0.05) (Fig. 3G). These results indicate that hypertrophy can be influenced by miR-23a in the in vivo model.

Foxo3a Is a Downstream Target of miR-23a—Foxo3a has been demonstrated to be able to inhibit cardiac hypertrophy (24, 28, 32). We detected its expression levels upon hypertrophic stimulation. Both PE and ET-1 induced a decrease in Foxo3a expression (Fig. 4A). To explore the molecular mechanism by which Foxo3a is reduced, we analyzed the targets of miR-23a using a bioinformatics program. Surprisingly, the 3’ UTR of Foxo3a contains three potential binding sites of miR-23a (Fig. 4B). To understand whether Foxo3a is a target of miR-23a, we first tested whether knockdown of miR-23a could influence Foxo3a expression levels in the hypertrophic program.
The reduction of Foxo3a expression levels was attenuated by miR-23a antagomir but not its negative control (Fig. 4, C and D). Second, we tested whether miR-23a could influence the endogenous Foxo3a. Enforced expression of miR-23a induced a reduction of endogenous Foxo3a expression (Fig. 4 E). To know if the cellular event holds true in the animal model, we detected the expression levels of Foxo3a in miR-23a transgenic mice, and observed a low level of Foxo3a in these mice in comparison with the wild-type mice (Fig. 4 F). Thus, it appears that miR-23a can influence Foxo3a expression.

We explored how miR-23a controls Foxo3a expression. To this end, the 3’ UTR of Foxo3a was cloned into the luciferase construct. miR-23a induced a reduction of luciferase activity compared with the group of Foxo3a-3’ UTR alone (Fig. 4G). Because the 3’ UTR of Foxo3a has three potential binding sites, we analyzed which sites are targeted by miR-23a. The introduction of mutations in BS3 predominantly led to the failure of miR-23a to inhibit the luciferase activity, suggesting that BS3 is the targeting site of miR-23a. Taken together, Foxo3a is a direct target of miR-23a.

**Hypertrophy Regulation by miR-23a**

We tested whether miR-23a and Foxo3a are functionally related in the hypertrophic pathway. First, we tested whether exogenous Foxo3a can influence hypertrophy. Enforced expression of Foxo3a led to a significant reduction of hypertrophic responses compared with ET-1 (Fig. 5A) or PE alone (Fig. 5B). Second, we investigated whether the miR-23a antagomir can influence hypertrophy. We produced three constructs in which the oligonucleotides in the binding sites were mutated (Fig. 5C). The introduction of mutations in BS3 predominantly led to the failure of miR-23a to attenuate the inhibitory effect of miR-23a on hypertrophy.
miR-23a antagonom on hypertrophy was counteracted by the knockdown of Foxo3a (*, *p* < 0.05 compared with the group of PE plus anta-23a) (Fig. 5C), suggesting that Foxo3a is a target of miR-23a in hypertrophy.

To further test if miR-23a suppresses the function of Foxo3a, it is better to know if the downstream targets of Foxo3a can be affected by miR-23a. We chose Mn-SOD, because it is well known that Mn-SOD is one of the reported targets of Foxo3a (26, 37). ET-1 and PE induced a reduction of Mn-SOD (*, *p* < 0.05 compared with the group of the control) (Fig. 5, D and E). Mn-SOD is able to scavenge ROS (38). Thus, we analyzed the levels of ROS. Enforced expression of caFoxo3a induced a reduction of ROS levels (Fig. 5F). The miR-23a antagonist attenuated the reduction of Mn-SOD, and this was abolished by knockdown of Foxo3a (Fig. 5G). These results indicate that miR-23a can control the function of Foxo3a in the hypertrophic cascades.

**Foxo3a Counteracts the Effect of miR-23a in Animal Models**—To better understand the relationship between Foxo3a and miR-23a, one approach is to test whether these two factors have an impact in the animal model. We produced caFoxo3a transgenic mice, and furthermore, Foxo3a and miR-23a transgenic mice were back-crossed to produce miR-23a/Foxo3a bi-transgenic mice. Morphometric analysis of all transgenic mice including mir-23a, Foxo3a, and mir-23a/Foxo3a revealed no obvious pathological phenotype. All transgenic mice were born normally and appeared externally indistinguishable from wild-type littermates in terms of survival to adulthood. The gross morphology of transgenic hearts did not differ from wild-type (WT) mice (Figs. 1A and 6A). We also analyzed older animals (24 weeks of age) and again observed no difference compared with WT littermates. To determine whether all transgenic mice would affect cardiac function, we performed echocardiography. Echocardiographic studies showed that there were no significant differences in posterior wall thickness, left ventricle diastolic and end-systolic dimensions, septal wall thickness, heart rate, or fractional shortening (Table 1). These results demonstrated that all transgenic mice had normal global cardiac structure and function.

Although Foxo3a transgenic mice had no baseline phenotype, they exhibited less hypertrophic response than wild-type controls upon PE treatment (Fig. 6A). Bi-transgenic mice bearing both miR-23a and caFoxo3a transgenes exhibit a high level of Foxo3a level (Fig. 6B). They developed less obvious hypertrophic responses than single miR-23a transgenic mice as revealed by the hypertrophic phenotype (Fig. 6C), heart weight/body weight ratios, cross-sectional areas (Fig. 6D), and the expression levels of hypertrophic markers including ANP and β-MHC (Fig. 6E). The cardiac function of miR-23a/Foxo3a bi-transgenic mice also was ameliorated (Fig. 6F). In the TAC model, we observed similar results (Fig. 6, G and H). In Fig. 6, D–H, there was a significant difference between miR-23a and 23a/Foxo3a (*p* < 0.05). Apoptosis is one of the characters of pathological cardiac hypertrophy, therefore, we investigated the apoptotic rate in miR-23a and miR-23a/Foxo3a transgenic mice upon PE treatment. As shown in Fig. 6I, mir-23a/Foxo3a bi-transgenic mice had less apoptosis than miR-23a mice upon PE treatment. Thus, Foxo3a and miR-23a have a cross-talk in the in vivo animal model.

**DISCUSSION**

Heart failure is one of the leading causes of hospitalization and death worldwide. It is essential to elucidate new molecular mechanisms for discovering novel impactful therapeutic targets suppressing maladaptive hypertrophy and the consequent heart failure. Our present work demonstrated that miR-23a transgenic mice are more susceptible to undergoing hypertrophy upon stimulation with pathological insults. In searching for the downstream targets of miR-23a, we found that Foxo3a is negatively regulated by miR-23a. Strikingly, miR-23a and Foxo3a bi-transgenic mice exhibited less hypertrophy. Our data reveal a novel mechanism regulating cardiac hypertrophy.

miRNAs play pivotal roles in many physiological and pathological processes. A growing body of evidence has demonstrated that cardiac hypertrophy is negatively regulated by miRNAs. For example, overexpression of miR-98 reduces cardiomyocyte size, whereas knockdown of miR-98 augments cardiac hypertrophy induced by angiotensin II (39). Myocardin is a transcriptional cofactor expressed at a relatively low level in cardiomyocytes under physiological conditions. However, it can be up-regulated by hypertrophic stimulation and consequently mediate hypertrophic signals (40, 41). miR-9 can suppress myocardin expression thereby inhibiting hypertrophy (33). miR-133 and miR-1 are anti-hypertrophic miRNAs that belong to the same transcription unit. They are down-regulated in the mice model of cardiac hypertension as well as in left ventricular tissue of patients with cardiac hypertrophy (16). The inhibitory effect on hypertrophy warrants future studies to directly employ their mimics to tackle hypertrophy.

In contrast, a variety of miRNAs can provoke the hypertrophic program. Overexpression of miR-199b promotes hypertrophy, whereas the antagonist of miR-199b completely abrogates the classical hypertrophic phenotype induced by calcineurin or phenylephrine treatment (42). miR-208 is one of the miRNAs that are up-regulated in cardiac hypertrophy in the TAB animal model (18). It participates in the initiation of hypertrophy and is required for fibrosis and the expression of β-MHC in response to stress and hypothyroidism (19). Transgenic overexpression of miR-208 in the heart is sufficient to induce hypertrophic growth in mice, resulting in pronounced repression of the miR-208 regulatory targets thyroid hormone-associated protein 1 and myostatin, which are negative regulators of muscle growth and hypertrophy (5). miR-21 levels are increased selectively in fibroblasts of the failing heart, it augments ERK–MAP kinase activity through inhibition of sprouty homologue 1. This leads to fibroblast survival and growth factor secretion thereby controlling the extent of interstitial fibrosis and cardiac hypertrophy (43). Our present work reveals that miR-23a is up-regulated in response to treatment with PE, ET-1, and pressure overload. Loss- and gain-of-function analysis revealed that miR-23a can mediate their hypertrophic signals.

miRNAs themselves cannot directly execute hypertrophy, and they need to exert their effect through hypertrophic executioners. A variety of downstream targets of miRNAs have been
identified. Cyclin D2 is a target of miR-98, and overexpression of cyclin D2 attenuates the inhibitory effect of miR-98 on cardiac hypertrophy (39). miR-199b promotes hypertrophy by targeting the nuclear NFAT kinase dual specificity tyrosine phosphorylation regulated kinase 1a (Dyrk1a) (42). In searching for the downstream targets of miR-23a, we found that Foxo3a is regulated by miR-23a in the present study. miR-23a has been shown to be able to target the muscle-specific ring finger protein 1 (MuRF1) thereby controlling hypertrophic program (12). MuRF1 is a family member of ubiquitin ligases, and plays an important role in regulating hypertrophy (44–46), whereas Foxo3a is a transcription factor. Given the complexity of hypertrophic machinery, it necessitates the characterization of the relationship among miR-23a, MuRF1, and Foxo3a.

It is of note that Foxo3a can only partially but not completely reverse miR-23a effects. A miRNA may have many targets (47, 48). One reason accounting for this phenomenon can be the multiple targets of miR-23a. Another reason might be that

![miR-23a/Foxo3a bi-transgenic mice exhibit reduced hypertrophic responses](image.jpg)

**FIGURE 6.** miR-23a/Foxo3a bi-transgenic mice exhibit reduced hypertrophic responses. A, reduced hypertrophic responses of Foxo3a transgenic mice (Tg) to PE treatment. Gross hearts (upper panel, bar = 2 mm) and the ratio of heart/body weight and cross-sectional areas are shown; *, p < 0.05 compared with the group of PE plus WT. B, Foxo3a levels analyzed by immunoblot in wild-type mice (WT), miR-23a transgenic mice (23a), and miR-23a/Foxo3a bi-transgenic mice (23a/Foxo3a). Quantitative analysis of Foxo3a levels are shown in the upper panel. *, p < 0.05 compared with the group of WT in the presence of PE (n = 7–8). C–E, miR-23a/Foxo3a bi-transgenic mice blunts enhanced hypertrophic response. WT, miR-23a Tg mice, and miR-23a/Foxo3a bi-transgenic mice were infused with PE as described under “Experimental Procedures.” C, histological sections of hearts, gross hearts (upper panel, bar = 2 mm), heart sections stained with hematoxylin and eosin (middle panel, bar = 2 mm), cross-sectional areas analyzed by staining with FITC-conjugated wheat germ agglutinin (lower panel, bar = 20 μm). D, the ratio of the heart/body weight, and measurement of cross-sectional areas analyzed by staining with FITC-conjugated wheat germ agglutinin; *, p < 0.05 compared with the group of PE plus miR-23a. E, the expression levels of ANP and β-MHC; *, p < 0.05 compared with the group of PE plus miR-23a. F, analysis by cardiac function by echocardiography. Diastolic left ventricular posterior wall thickness (LVPWd), fractional shortening (FS), diastolic interventricular septal thickness (IVSd), systolic left ventricular internal diameters (LVIDs) (n = 6–8) are indicated. *, p < 0.05 compared with the group of PE plus miR-23a. G, Foxo3a attenuates miR-23a effects in TAC hypertrophy model. Heart and body weight ratio, and cross-sectional area measurements are shown. *, p < 0.05 compared with the group of TAC plus miR-23a. H, hypertrophic marker genes expression. Upper panel, β-MHC levels analyzed by qRT-PCR; *, p < 0.05 compared with the group of TAC plus miR-23a. Lower panel, ANP and BNP levels analyzed by immunoblot. I, apoptosis in different transgenic mice. Myocardial sections were stained by TUNEL assay, and apoptosis was analyzed; *, p < 0.05 (n = 6).
Foxo3a is a transcriptional factor and regulates other molecules that synergistically cooperate with miR-23a in the hypertrophic machinery. The detailed underlying mechanism needs to be elucidated.

According to the prediction by the bioinformatic program of Targetscan, miR-23a has a large number of potential targets. However, only a few targets of miR-23a have been validated by experiments. For example, modulation of miR-23a can affect the expression levels of Fas that is confirmed to be a target of miR-23a (49), miR-23a directly binds to the 3′ UTR of X-linked inhibitor of apoptosis mRNA levels (50). It would be interesting to identify other targets of miR-23a in the hypertrophic pathways.

To better understand the functional role of the miR-23a-Foxo3a axis in regulating hypertrophy, we for the first time produced double transgenic mice that bear both a miRNA and its target transgenes. We observed that miR-23a and Foxo3a bi-transgenic mice exhibit less hypertrophy, suggesting that their cross-talk occurs in the animal model.

Our present work revealed that Foxo3a can regulate Mn-SOD levels. Our results are consistent with previous publications indicating that antioxidant enzymes such as Mn-SOD and catalase are the downstream targets of Foxo3a (26, 32). Our data further showed that ROS levels are affected by Foxo3a in the hypertrophic model. It has been published that antioxidant treatment can attenuate cardiac hypertrophy (51–53). Thus, it appears that Foxo3a, the antioxidant enzymes, and ROS constitute an axis in the hypertrophic pathway.

In summary, our present study demonstrates that miR-23a can promote cardiac hypertrophy through suppressing Foxo3a. We employ miR-23a transgenic mice and found that these mice are more susceptible to undergoing hypertrophy. However, in miR-23a and Foxo3a double transgenic mice the hypertrophic responses are attenuated. Our results reveal that miR-23a and Foxo3a form an axis in the hypertrophic machinery, and this axis can be modulated for the development of novel therapeutic approaches for tackling hypertrophy as well as heart failure.

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