

Resveratrol Ameliorates Cardiac Hypertrophy by Down-regulation of miR-155 Through Activation of Breast Cancer Type 1 Susceptibility Protein

Yuhua Fan, PhD;* Li Liu, MD, PhD;* Kun Fang, PhD; Tao Huang, MD, PhD; Lin Wan, PhD; Youbin Liu, MD; Sen Zhang, MD; Dongxia Yan, MD; Guangnan Li, MD; Yanhui Gao, MD; Yanjie Lv, PhD; Yanjun Chen, MD, PhD; Yingfeng Tu, MD, PhD

Background—The polyphenol resveratrol (Rev) has been reported to exhibit cardioprotective effects, such as inhibition of TAC (transverse aortic constriction) or isoprenaline (ISO)-induced hypertrophy. MicroRNA-155 (miR-155) was found to be decreased in hypertrophic myocardium, which could be further reduced by pretreatment of Rev. The study was designed to investigate the molecular effects of miR-155 on cardiac hypertrophy, focusing on the role of breast cancer type 1 susceptibility protein (BRCA1).

Methods and Results—We demonstrated that Rev alleviated severity of hypertrophic myocardium in a mice model of cardiac hypertrophy by TAC treatment. Down-regulation of miR-155 was observed in pressure overload– or ISO-induced hypertrophic cardiomyocytes. Interestingly, administration of Rev substantially attenuated miR-155 level in cardiomyocytes. In agreement with its miR-155 reducing effect, Rev relieved cardiac hypertrophy and restored cardiac function by activation of BRCA1 in cardiomyocytes. Our results further revealed that forkhead box O3a (FoxO3a) was a miR-155 target in the heart. And miR-155 directly repressed FoxO3a, whose expression was mitigated in miR-155 agomir and mimic treatment in vivo and in vitro.

Conclusions—We conclude that BRCA1 inactivation can increase expression of miR-155, contributing to cardiac hypertrophy. And Rev produces their beneficial effects partially by down-regulating miR-155 expression, which might be a novel strategy for treatment of cardiac hypertrophy. (*J Am Heart Assoc.* 2016;5:e002648 doi: 10.1161/JAHA.115.002648)

Key Words: BRCA1 • FoxO3a • miR-155 • resveratrol

Cardiac hypertrophy is an important physiological compensatory mechanism in response to injury and hemodynamic overload by promoting myocyte hypertrophy, enhancing protein synthesis, remodeling of the extracellular matrix, and

re-expressing a fetal gene program.¹ Cardiac hypertrophy is a stage that precedes overt heart failure and its therapeutic reversal is associated with decreased mortality.² To date, many pharmacological treatment strategies for cardiac hypertrophy, such as angiotensin-converting enzyme inhibitors, β -receptor-blockers, angiotensin receptor blockers, and diuretics, have been widely used to treat cardiac hypertrophy and early stages of heart failure.³ These therapies have been proven to be very effective in recent studies. However, the incidence of mortality attributed to chronic heart failure is still on rise. Consequently, there is an important need for alternative therapeutic strategies to prevent or reverse cardiac hypertrophy before it develops into severe chronic heart failure.

In the past decades, some antioxidants have also been reported to render beneficial effects against the deleterious effects of cardiac hypertrophy in different experimental models.^{4,5} Furthermore, gene manipulation researches have also verified the effects of antioxidants, which can be used as potential antihypertrophic treatment strategies. In this regard, resveratrol (Rev), as a polyphenol found in red wine, has been shown to inhibit cardiac hypertrophy because of its antioxidant roles. A recent study showed that Rev prevented the right ventricular hypertrophy induced by monocrotaline in

From the College of Pharmacy, Harbin Medical University-Daqing, Daqing, China (Y.F., K.F.); Department of Anesthesiology, the Third Hospital of Harbin Medical University, Harbin, Heilongjiang, China (L.L.); Radiology Department and Molecular Imaging Center, The Fourth Hospital of Harbin Medical University, Harbin, Heilongjiang, China (T.H., L.W.); Department of Cardiology, The Second Hospital of Harbin Medical University, Harbin, Heilongjiang, China (Y.L.); Department of Cardiology, The Fourth Hospital of Harbin Medical University, Nangang District, Harbin, Heilongjiang, China (S.Z., D.Y., G.L., Y.G., Y.C., Y.T.); Key Laboratory of Cardiovascular Medicine Research (Harbin Medical University), Ministry of Education, Harbin, China (L.L., Y.L., Y.T.); College of Pharmacy, Harbin Medical University, Harbin, China (Y.L.).

*Dr Fan and Dr Li Liu contributed equally to this work.

Correspondence to: Yingfeng Tu, MD, PhD, or Yanjun Chen, MD, PhD, Department of Cardiology, The Fourth Hospital of Harbin Medical University, Harbin, Heilongjiang 150001, China. E-mail: tydoctor@163.com, yanjunchen@163.com

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rats, and this effect was mediated by both an indirect effect by a reduction in pulmonary hypertension and a direct effect of Rev on cardiomyocytes.⁶ And Jason et al. have demonstrated that Rev can prevent pathological, but not physiological, cardiac hypertrophy because Rev plays less of a role in regulating nuclear factor of activated T cells (NFAT)-mediated transcription during physiological left ventricular (LV) hypertrophy.⁷ However, the exact antihypertrophic molecular mechanisms of Rev have not been fully disclosed until now.

Breast cancer type 1 susceptibility protein (BRCA1), a well-known tumor suppressor with multiple interacting partners, is predicted to have diverse biological functions.^{8–10} However, the role of BRCA1 in protecting cardiac tissue from DNA damage has not been fully explored to date. In a recent study, Gerd Hasenfuss et al.¹¹ described, for the first time, a potentially novel signaling pathway (BRAP2/BRCA1) that was involved in the process of myocardial hypertrophy. And Subodh Verma et al.¹² further reported the essential role of BRCA1 to prevent cardiomyocytes apoptosis, and markedly improve cardiac function in response to genotoxic and oxidative stress. They also disclosed that specific knockdown of BRCA1 in the heart could induce severe systolic dysfunction and limit animal model survival. Additionally, Liviana Catalano et al.¹³ observed that the antihypertrophic action of propranolol was accompanied by a significant overexpression of 2 genes, namely, BRCA1 and Cdkn2a. Furthermore, recent studies showed that Rev could prevent epigenetic silencing of BRCA1 by the aromatic hydrocarbon receptor in human breast cancer cells. Though these findings suggest BRCA1 to be a potential target for antihypertrophic therapy, whether increasing BRCA1 by Rev can offer antihypertrophic outcomes has not been fully explored in vitro and in vivo to date.

MicroRNAs (miRNAs) are a novel class of endogenous, short, noncoding, post-transcriptional RNAs, which play important roles in regulating lots of crucial biological functions by either translational repression or mRNA degradation.¹⁴ The evidences from pioneer studies have showed that dysregulation of miRNAs is involved in cardiac hypertrophy and heart failure, making it possible to target regulating of miRNAs as a novel therapy.^{15,16} To date, several miRNAs have been identified to affect the biological process of cardiac hypertrophy. For example, miR-9 and miR-133 negatively regulate cardiac hypertrophy, whereas miR-195 induces pathological hypertrophy and heart failure.^{17,18} A recent study reported that miR-27a regulated beta cardiac myosin heavy chain (β -MHC) gene expression by targeting thyroid hormone receptor in neonatal rat ventricular myocytes. In a previous study, we elucidated the feasibility of using our constructed miRNA reporter imaging system to monitor the location and magnitude of expression levels of miR-22 in cardiac hypertrophy in vitro and in vivo.¹⁹ Most recently, miR-155 was reported to be expressed in atherosclerotic plaques and proinflammatory macrophages and

the in vivo function of miR-155 in cardiomyocyte hypertrophy was also manifested.^{20–22} Another excellent study in this research area by Shyam's group demonstrated a previously unknown role for BRCA1 in epigenetic control of miR-155.²³

However, despite the fact that miR-155 has been found to show abnormal expression in diverse cardiac conditions, including those associated with prominent changes of cardiac contractility, the consequences of regulation are poorly studied with our current understanding limited to only the prohypertrophic actions of miR-155. Here, we studied the function of miR-155 in the mouse heart, and we reported that miR-155 was expressed in cardiomyocytes and its expression was dynamically regulated during cardiac hypertrophy. We also showed that Rev could further promote genetic deletion of miR-155 by simulating BRCA1 and prevent cardiac hypertrophy induced by pressure overload or agomiR-155 transfection in vivo. Furthermore, we identified that forkhead box O3a (FoxO3a) was a miRNA-155 target in the hypertrophy signaling pathway.

Materials and Methods

Animals

Eight-week-old male Kunming mice were supplied by the Medical Experimental Animal Center of Harbin Medical University (Harbin, China). Mice were fed under standard animal room conditions (temperature $21 \pm 1^\circ\text{C}$; humidity 55–60%). Food and water were freely available throughout the experiments. All experimental protocols were preapproved by the Experimental Animal Ethic Committee of Harbin Medical University (Animal Experimental Ethical Inspection Protocol No. 2009104).

Cardiomyocytes Culture and Cell Models for Hypertrophy

Neonatal mouse cardiomyocytes were isolated and cultured from ventricles of 2-day-old mice. In brief, ventricles were excised, washed, and cut into small pieces without serum in DMEM medium, then digested with a 0.25% solution of trypsin in a carbon dioxide incubator to keep the reaction temperature at 37°C . After dissociation, cells were subjected to centrifugation, followed by differential preplating to enrich cardiomyocytes and deplete nonmyocytes. Then, purified cardiomyocytes were diluted to 1×10^6 cells/mL and plated on flasks in DMEM supplemented with 10% FBS, penicillin (100 U/mL; Gibco, Grand Island, NY), streptomycin (100 μg /mL; Gibco), and 0.1 mmol/L of bromodeoxyuridine. Having been cultured for 48 hours, cells were cultured with a serum-free maintenance medium and kept for another 24 hours. To prevent hypertrophic response, different concentrations of Rev (1, 3, 5, and 10 $\mu\text{mol/L}$) were used in different treatment groups for 1 hour before being coincubated with isoprenaline

(ISO) at 10 $\mu\text{mol/L}$ for 48 hours, which has been proven to induce stable cardiomyocyte hypertrophy.

Cardiomyocytes Immunocytochemistry and Cell Surface Area Analysis

Cardiomyocytes were plated at a density of 5×10^4 cells/mL to obtain individual cells. After ISO treatment for 48 hours, cells were fixed with 4% PFA for 15 minutes and permeabilized with 0.1% Triton X-100 in PBS, followed by blocking with 5% goat serum in PBS for 1 hour at room temperature. Cells were incubated with antiactinin antibody (1:200 dilution; Sigma-Aldrich, St. Louis, MO) at 4°C overnight; after washing with PBS 3 times, the secondary antibody coupled with Alexa Fluor 594 (1:1000 dilution, Mobictech, Cambridge, MA) was added and incubated for 1 hour at room temperature. After cleaning with PBS, nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). The cell picture was captured with laser scanning confocal microscopy (FV300; Olympus, Tokyo, Japan), and the cell surface area was determined with ImagePro Plus software (version 6.0; Media Cybernetics Inc., Rockville, MD). Approximately 100 cells from randomly selected fields were calculated for each experimental group.

Pressure-Overload–Induced Cardiac Hypertrophy

Male mice were gavaged with Rev (150 mg/kg) for 1 day before transverse aortic constriction (TAC) was conducted for 4 weeks. Pressure-overload heart hypertrophy models were obtained by subjecting animals to TAC. Adult male mice (20–25 g body weight) were anesthetized and placed in the supine position and a midline cervical incision was exposed to trachea. After endotracheal intubation, the cannula was connected to a volume-cycled rodent ventilator (Ugo Basile S.R.L., Monvalle VA, Italy). The chest was carefully opened and the thoracic aorta was found. A 7-0 silk suture was placed around the transverse aorta and tied around a 26-gauge blunt needle, which was subsequently removed. This maneuver increased systolic pressure around 1.5-folds and induced cardiac hypertrophy. The chest was sutured and animals were kept until recovery of autonomic breath.²⁴ After 4 weeks, LV tissue was separated and weighted and LV tissue was then rapidly frozen in liquid nitrogen and stored at -80°C for subsequent experiments. All procedures involving animals and their care were approved by the Institutional Animal Care and Use Committee of Harbin Medical University.

Quantitative Reverse-Transcription Polymerase Chain Reaction

Total RNA samples from cultured cardiomyocytes and cardiac tissues were isolated using Trizol reagent (Invitrogen, Carlsbad,

CA), according to the manufacturer's protocols. Total RNA (1 μg) was then reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) to obtain cDNA. The SYBR Green PCR Master Mix Kit (Applied Biosystems) was used in quantitative reverse-transcription polymerase chain reaction (qRT-PCR) to quantify RNA levels of A-type natriuretic peptide (ANP), A-type natriuretic peptide (BNP), and β -MHC. GAPDH was used as an internal control. Level of miR-155 was tested also using SYBR Green PCR Master Mix Kit, with U6 as an internal control. qRT-PCR was performed on the 7500 FAST Real-Time PCR System (Applied Biosystems) for 40 cycles. For miRNA analysis, the reverse transcription primer for miR-155 was 5'-GTCGTATCCAGTGCAGGGTCC GAGGTATTCGACTGGATACGACCCCTA-3'. The sequences of reverse transcription primers for quantitative PCR (q-PCR) primers were as follows: miR-155: 5'-CTCGTGGTAATGCTAATTGTGA-3' (forward) and 5'-GTGCAGGGT CCGAGGT-3' (reverse); mouse ANP: 5'-GAACCTGCTAGACCACCT-3' (forward) and 5'-CCTAGTCCACTCTGGGCT-3' (reverse); mouse BNP: 5'-AAGCTGCTGG AGCTGATAAGA-3' (forward) and 5'-GT TACAGCCAAACGACTGAC-3' (reverse); mouse β -MHC: 5'-CAGCAGCCCAGTACCTCCGA-3' (forward) and 5'-TGTCATCAG GCACGAAGCAC-3' (reverse); and GAPDH: 5'-AACGACCCCTTC ATTGACCTC-3' (forward) and 5'-CCTTGACTGTCCGTTGA-3' (reverse).

Echocardiography

Four weeks after TAC treatment, mice from different treatment groups were anesthetized with 2.5% (vol/vol) isoflurane and placed on the experimental platform. Transthoracic echocardiography was performed using a high-resolution in vivo ultrasound imaging system with a 40-MHz phased-array transducer (Panoview β 1500; Cold Spring Biotech Corp, Hsichih City, Taiwan). Two-dimensional guided M-mode tracings were recorded from the parasternal long-axis view at the midpapillary muscle level. When the picture was stabilized, left ventricular end-diastolic dimensions (LVEDD), left ventricular end-systolic dimensions (LVESD), diastolic interventricular septal thickness (IVSTD), interventricular septal thickness in systole (IVSTS), posterior wall thickness in diastole (PWTD), posterior wall thickness in systole (PWTS), left ventricular fractional shortening (LVFS), and left ventricular ejection fraction (LVEF) were measured. All measurements were made from more than 3 beats and averaged. After functional measurement, mice were killed and hearts were collected in 4% PFA or liquid nitrogen for use.

MiRNA Inhibitor and Mimic

MiR-155 inhibitor and mimic were commercially synthesized from GenePharma (Shanghai, China) and were used for

cardiomyocytes. Cardiomyocytes were transfected with RNAs at a final concentration of 20 nmol/L for 24 hours. For miR-155 knockdown, cardiomyocytes were pretreated with Rev (10 μ mol/L) before transfection. Culture medium was replaced 6 hours post-transfection with the regular culture medium for another 24 hours. MiRNA transfection efficiency was proved by qRT-PCR.

AgomiR-155 Transfection In Vivo

Agomirs have been demonstrated as powerful tools to mimic specific miRNAs in vivo and may represent a therapeutic strategy for overexpression of miRNAs in diseases. In the current study, agomiR-155 was used to mimic the cardiac miR-155 expression in vivo. MiR-155 agomir was purchased from GenePharma (Shanghai, China). For in vivo administration of agomiR-155, mice were infused with agomiR-155 (40 nmol/L), when these mice were treated with TAC. Infusions were executed with an implanted micro-osmotic pump (1007D; DURECT Corporation, Cupertino, CA).

siRNA Transfection

Cells were cultured in a 6-well plate to 60% confluence and immediately washed with serum-free medium before transfection, and 800 μ L of serum-free medium were added every well. For each well, 200 nmol/L of siRNA was mixed with 5 μ L of Oligofectamine (Invitrogen) in 200 μ L of serum-free medium. Mixtures were cultured for 20 minutes at room temperature and then added to cells. Serum was added 4 hours later to a final concentration of 10%. Twenty-four hours after the initial transfection, a second transfection was performed in the same way as the previous one. Forty-eight hours after initial transfection, cells were treated and harvested as indicated. The siRNA sequence GAGACGUAA-CUAAGCCAG for mice BRCA1 was purchased from Shanghai GenePharma Co, Ltd. (Shanghai, China).

Western Blot Analysis

Briefly, protein concentrations were determined with a BCA protein assay kit using BSA as the standard. Equal amounts of protein (100 μ g) were fractionated by SDS-PAGE and blotted to a PVDF membrane (Millipore, Bedford, MA). Membranes were blocked for 1 hour in using 5% nonfat milk in Tris-buffered saline with Tween 20 (TBST), then probed overnight at 4°C with the following primary antibodies: BRCA1 (1:1000 dilution; Abcam, Cambridge, MA), FoxO3a and anti-GAPDH (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CAQ), all in 5% milk TBST. After incubation with the primary antibodies, membranes were incubated with secondary antibody (1:8000 dilution; Alexa Fluor 700 goat anti-mouse IgG

[H+L] or Alexa Fluor[®] 800 goat anti-rabbit IgG [H+L]; Invitrogen) in PBS at room temperature for 1 hour. Western blot bands were captured by using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) and quantified with Odyssey software (v1.2; LI-COR Biosciences) by measuring the band intensity (area \times OD) in each group and normalizing to GAPDH as an internal control. Unless otherwise stated, western blot experiments were repeated 3 times.

Statistical Analysis

All quantitative data were expressed as mean \pm SEM. Statistical analyses were performed using 1-way ANOVA for multigroup comparisons. Significance was set at $P<0.05$.

Results

Prevention of Cardiac Hypertrophy by Rev

Consistent with previous studies,^{7,25} Rev substantially decreased the size of cardiomyocytes. Results from the current study showed that the dosage (10 μ mol/L of Rev) markedly reduced the size of cardiomyocytes in vitro (Figure 1). The representative cardiomyocytes were incubated with monoclonal antibody against sarcomeric α -actinin

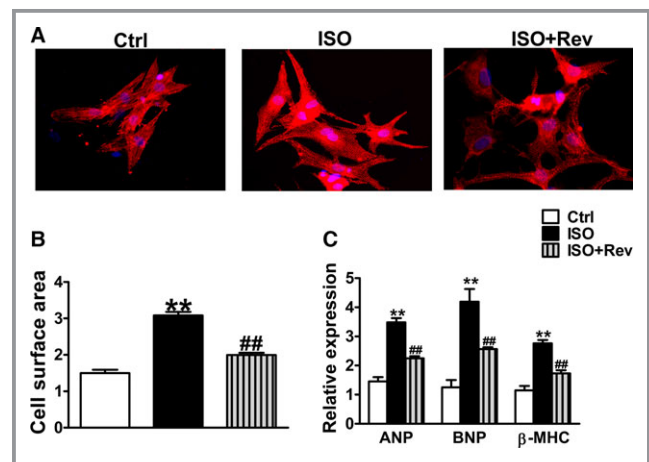


Figure 1. ISO-induced cardiomyocytes hypertrophy was reversed by Rev. A, Cardiomyocytes were stained for α -actinin protein and DAPI (original magnification, $\times 200$). B, Analysis of cell surface area; 100 cells were qualified in each group from 4 individual experiments. Data are expressed as mean \pm SEM; ** $P<0.01$ vs Ctrl; ### $P<0.01$ vs ISO-treated group. C, ISO-induced cardiomyocyte hypertrophy markers were significantly increased, whereas mRNA level of ANP, BNP, and β -MHC could be mitigated by coinubation with Rev; n=6 independent experiments. Data are expressed as mean \pm SEM; ** $P<0.01$ vs Ctrl; ### $P<0.01$ vs ISO group. ANP indicates A-type natriuretic peptide; BNP B-type natriuretic peptide; Ctrl, control; DAPI, 4',6-diamidino-2-phenylindole; ISO, isoproterenol; and β -MHC, beta-myosin heavy chain; Rev, resveratrol.

(Figure 1A). Accordingly, the surface area of ISO-stimulated cardiomyocytes significantly increased relative to normal cardiomyocytes (3.08 ± 0.10 vs 1.50 ± 0.09), whereas Rev obviously reversed the change at a dosage of $10 \mu\text{mol/L}$ (1.99 ± 0.06 vs 3.08 ± 0.10 ; Figure 1B). Concomitantly, production of ANP, BNP, and β -MHC was robustly elevated by ISO treatment and Rev could significantly reduce these proteins expression in vitro (Figure 1C).

Regulation of miR-155 Expression and Attenuation Cardiac Hypertrophy by Rev In Vitro

Numerous studies have manifested that miR-155 is ubiquitously expressed in adult cardiac tissue and plays an important role as adverse mediator of cardiac injury.^{22,26} Therefore, we verified whether miR-155 was involved in

Rev-mediated cardiac protective action. As shown in Figure 2A, expression of miR-155 was dominantly decreased in a dose-dependent manner in Rev-treated cardiomyocytes in vitro. As expected, expression of miR-155 was also obviously abrogated by treatment with Rev ($10 \mu\text{mol/L}$) in ISO-stimulated cardiomyocytes (Figure 2B). To further identify whether miR-155 was involved in cardiac protection action mediated by Rev, qRT-PCR technology was performed. We found that transient transfection with miR-155 mimic was dominantly reversed by incubation with Rev for 48 hours (Figure 2C). Consistent with our observations, Rev could also significantly reduce surface area of cardiomyocytes treated with exogenous miR-155 in vitro. Representative images are showed in Figure 2D. The cell surface area from different treatment groups is quantified in Figure 2E. Furthermore, production of ANP, BNP, and β -MHC was markedly increased

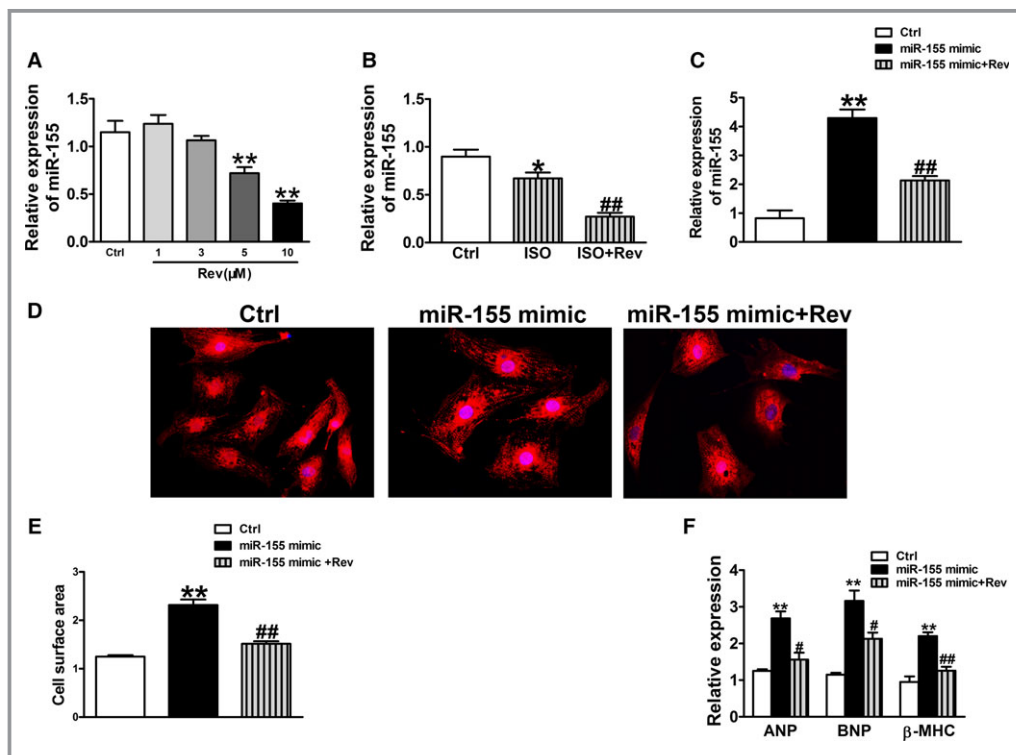


Figure 2. Rev controlled expression of miR-155 and retarded cardiac hypertrophy in vitro. A, Rev obviously decreased the level of miR-155 in a dose-dependent manner in ISO-stimulated groups; $n=6$ independent experiments. Data are expressed as mean \pm SEM; $*P<0.01$ vs Ctrl group. B, Relative to ISO-treated group, expression of miR-155 was further reduced by Rev; $n=6$ independent experiments. Data are expressed as mean \pm SEM; $*P<0.05$ vs Ctrl; $###P<0.01$ vs ISO treated group. C, Rev significantly reversed overexpression of miR-155; $n=6$ independent experiments. Data are expressed as mean \pm SEM; $*P<0.01$ vs Ctrl; $###P<0.01$ vs miR-155 mimic group. D, Cardiomyocytes were stained for α -actinin protein and DAPI (original magnification, $\times 200$). E, Analysis of cell surface area; 100 cells were qualified in each group from 4 individual experiments. Data are expressed as mean \pm SEM; $*P<0.01$ vs Ctrl; $###P<0.01$ vs miR-155 mimic group. F, ANP, BNP, and β -MHC expression level quantified by qRT-PCR in different treated groups; $n=6$ independent experiments. Data are expressed as mean \pm SEM; $*P<0.01$ vs Ctrl; $\#P<0.05$ or $###P<0.01$ vs miR-155 mimic group. ANP indicates A-type natriuretic peptide; BNP B-type natriuretic peptide; Ctrl, control; DAPI, 4',6-diamidino-2-phenylindole; ISO, isoproterenol; β -MHC, beta-myosin heavy chain; miR-155, microRNA 155; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; Rev, resveratrol.

by treatment with agomiR-155 relative to the control group, whereas Rev could obviously mitigate mRNA expression of ANP, BNP, and β -MHC in cardiomyocytes (Figure 2F).

Rev Substantially Retarded Cardiac Hypertrophy Induced by Up-regulation of miR-155 In Vivo

To further determine whether overexpression of miR-155 caused cardiac hypertrophy, mouse hearts were injected with agomiR-155. Subsequently, we found that miR-155 was strongly increased in the agomiR-155-injected group, whereas Rev (150 mg/kg) significantly down-regulated expression of miR-155 (Figure 3B). Moreover, data from Figure 3A show that agomiR-155 markedly promoted cardiac hypertrophy, whereas this pathological process was obviously reversed by Rev (Figure 3C and 3D). Next, we analyzed the ratio of heart weight (HW)/body weight (BW) in Figure 3E. The results suggested that Rev obviously mitigated the cardiac hypertrophy induced by agomiR-155 in vivo.

Rev Obviously Reversed the Impaired Cardiac Function Induced by agomiR-155 Stimulation

Cardiac function was measured using echocardiography 2 weeks after agomiR-155 transfection in vivo. Analysis of cardiac function by M-mode echocardiography suggested that hearts treated with agomiR-155 displayed thickening of the ventricular walls, whereas Rev could reverse this change (Figure 4A). Echocardiography measurement showed that LVEDD, LVESD, LVFS, and LVEF were substantially reduced in agomiR-155 treatment mouse hearts, whereas IVSTD, IVSTS, PWTD, and PWTS were markedly up-regulated, indicating the cardiac hypertrophy. However, Rev was demonstrated to possess an antihypertrophic effect in vivo and ameliorate agomiR-155-mediated cardiac hypertrophy (Figure 4B through 4G).

Potential Role of BRCA1-Signaling Pathway in miR-155 Reduction by Rev Both In Vivo and In Vitro

Because BRCA1 is an essential regulator of cardiac function and up-regulated under ischemic stress,¹² which could attenuate the elevated miR-155 in hearts.²³ Therefore, we clarified that whether BRCA1 was involved in protective action mediated by Rev in cardiac hypertrophy. Western blot technology was used in our current study. Consistent with previous reports, upon stimulation of Rev, BRCA1 level was significantly increased both in vivo and in vitro (Figure 5A and 5C). The results indicated that Rev protected against cardiac hypertrophy and was, at least in part, associated with BRCA1. To further investigate whether BRCA1 regulated expression of

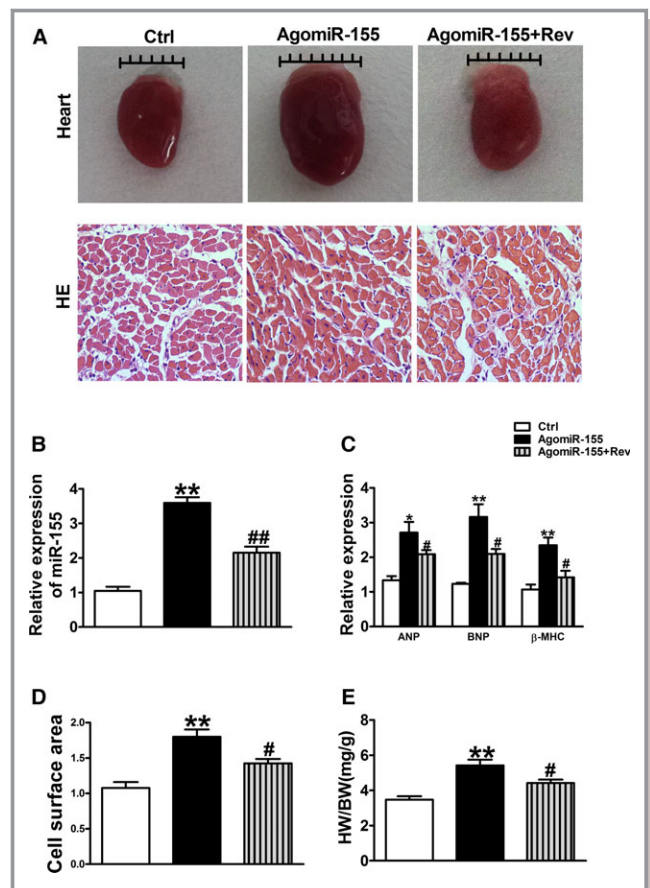


Figure 3. Rev retarded cardiac hypertrophy through attenuating level of miR-155 in the agomiR-155-treated group. A, Representative images of the heart and HE staining showing the changes of cardiac hypertrophy in different treated groups. B, Rev could also dominantly reverse expression of miR-155 in the agomiR-155-treated group; $n=6$ independent experiments. Data are expressed as mean \pm SEM; ** $P<0.01$ vs Ctrl group; ## $P<0.01$ vs agomiR-155-treated group. C, ANP, BNP, and β -MHC expression level quantified by qRT-PCR in different groups; $n=6$ independent experiments. Data are expressed as mean \pm SEM; * $P<0.05$ or ** $P<0.01$ vs Ctrl group; # $P<0.05$ vs agomiR-155-treated group. D, Cell surface area was calculated in different treated groups; $n=6$ independent experiments. Data are expressed as mean \pm SEM; ** $P<0.01$ vs Ctrl group; # $P<0.05$ vs agomiR-155-treated group. E, Heart weight to body weight ratios (HW/BW) was analyzed in different groups; $n=6$ independent experiments. Data are expressed as mean \pm SEM; ** $P<0.01$ vs Ctrl group; # $P<0.05$ vs agomiR-155-treated group. agomiR-155 indicates microRNA-155 agonist; ANP, A-type natriuretic peptide; BNP B-type natriuretic peptide; Ctrl, control; HE, hematoxylin and eosin; ISO, isoproterenol; β -MHC, beta-myosin heavy chain; miR-155, microRNA 155; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; Rev, resveratrol.

miR-155 in cardiac protective action, cardiomyocytes were transfected with siRNA BRCA1. The data from Figure 5B show that protein expression of BRCA1 was markedly reduced by treatment with siRNA BRCA1 in cardiomyocytes. As shown in Figure 5D, siRNA BRCA1 treatment obviously elevated the

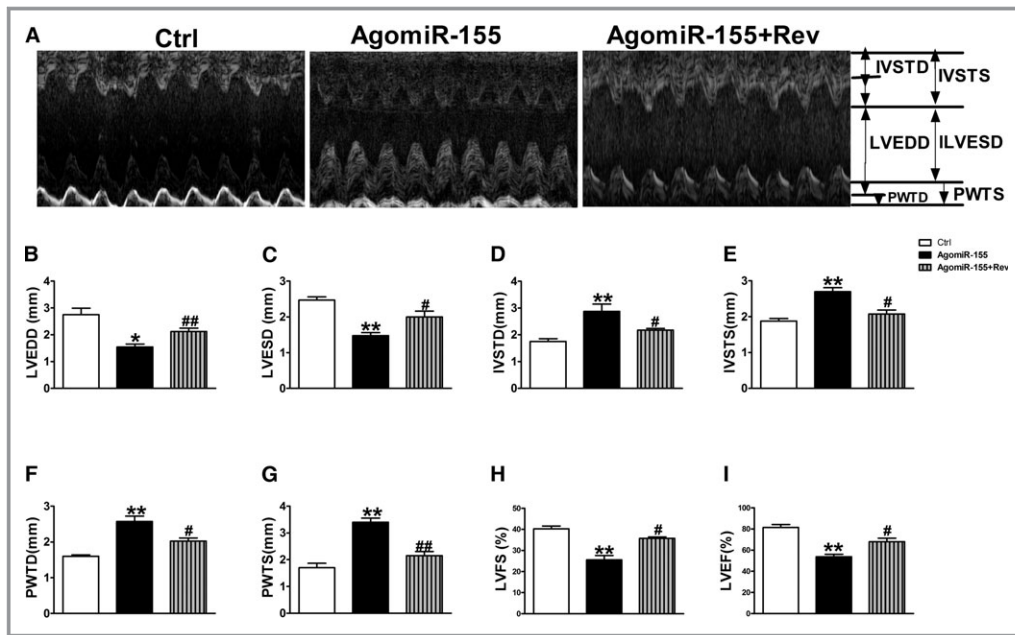


Figure 4. Rev reversed the harmful effect of agomiR-155 on cardiac function. A, Representative M-mode echocardiographs from Ctrl, agomiR-155, agomiR-155+Rev. B, LVEDD, left ventricular end-diastolic dimension. C, LVESD, left ventricular end-systolic dimension. D, IVSTD, interventricular septal thickness in diastole. E, IVSTS, interventricular septal thickness in systole. F, PWTD, posterior wall thickness in diastole. G, PWTS, posterior wall thickness in systole. H, LVFS, left ventricular fractional shortening. I, LVEF, left ventricular ejection fraction; n=6 independent experiments. Data are expressed as mean±SEM; * $P<0.05$; ** $P<0.01$ vs Ctrl; # $P<0.05$, ## $P<0.01$ vs agomiR-155-treated group. agomiR-155 indicates microRNA-155 agonist; Ctrl, control; Rev, resveratrol.

level of miR-155, but treatment with negative control (NC) had no significant effect. In addition, protein expression of BRCA1 was dominantly increased in the Rev-treated group alone in cardiomyocytes (Figure 5E). The above results indicated that BRCA1 played a beneficial role in antihypertrophic effect, which partially, but at the least, controlled the elevated miR-155. To further demonstrate our assumption, cardiomyocytes were transfected with miR-155 both in vivo and in vitro. As shown in Figure 6A and 6B, transfection with agomiR-155 or miR-155 mimic alone had no influence on protein level of BRCA1 both in vivo and in vitro, whereas coincubation with Rev could substantially elevate protein expression of BRCA1. In addition, miR-155 mimic and miR-155 inhibitor were successfully transfected in cardiomyocytes (Figure 6C). Interestingly, we found that exogenous miR-155 or miR-155 inhibitor had no influence on protein expression of BRCA1 (Figure 6D).

Regulation of FoxO3a Expression by miR-155 and Reversed its Effect by Rev

It has been indicated that FoxO3a could retard cardiac hypertrophy.²⁷ We next turned to explore whether expression of FoxO3a was altered by Rev. Results from the current study showed that protein expression of FoxO3a was robustly

increased when treated with Rev both in vivo and in vitro (Figure 7A and 7B). The down-regulated FoxO3a was verified to be associated with knockdown of BRCA1 (Figure 7C). Recently, there were several reports suggesting that FoxO3a was closely controlled by miR-155.^{28,29} We set out to speculate whether miR-155 targeted the FoxO3a gene to influence the pathological process in cardiac hypertrophy. Our results indeed provided the evidence that transient transfection with miR-155 obviously reduced the protein level of FoxO3a. However, Rev could significantly reverse expression of FoxO3a (Figure 7D and 7E). A further experiment was performed to demonstrate overexpression of miR-155 could reduce FoxO3a protein level. In contrast, protein expression of FoxO3a was obviously mitigated by treatment with miR-155 inhibitor (Figure 7F). Moreover, the protein level of FoxO3a was significantly increased in Rev treatment alone in cardiomyocytes (Figure 7G). Taken together, these results manifested that FoxO3a was involved in an antihypertrophic effect, which was controlled by miR-155.

Discussion

Several studies have demonstrated that Rev possesses various cardioprotections, including anti-inflammatory, antioxidant, antiproliferative, promoting differentiation, and

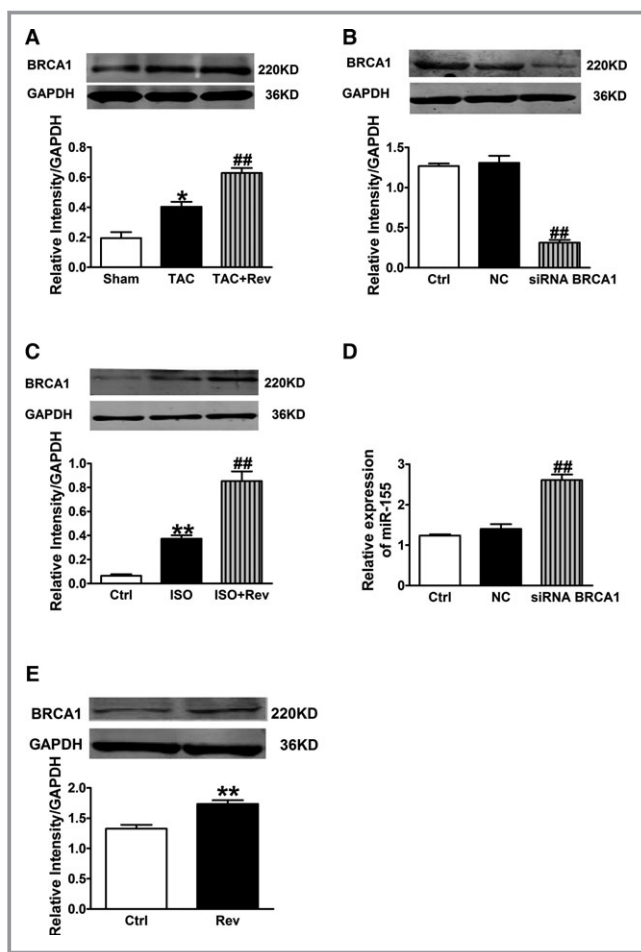


Figure 5. BRCA1 was involved in controlling cardiac hypertrophy and regulated the elevated miR-155. Western blotting suggesting Rev significantly upregulated protein expression of BRCA1 (A and C); n=3 independent experiments. Data are expressed as mean ±SEM; *P<0.05 vs sham group; **P<0.01 vs Ctrl group; ##P<0.01 vs TAC- or ISO-treated group. B, Cardiomyocytes transfected with siRNA BRCA1 dominantly inhibited protein level of BRCA1; n=3 independent experiments. Data are expressed as mean±SEM; ##P<0.01 vs NC group. D, Transfected with siRNA, BRCA1 significantly increased the level of miR-155; n=6 independent experiments. Data are expressed as mean±SEM; ##P<0.01 vs NC group. E, Protein expression of BRCA1 treated with Rev alone; n=3 independent experiments. Data are expressed as mean±SEM; **P<0.01 vs Ctrl group. BRCA1 indicates breast cancer type 1 susceptibility protein; Ctrl, control; ISO, isoproterenol; miR-155, microRNA 155; NC, negative control; Rev, resveratrol; TAC, transverse aortic constriction.

chemopreventive effects, in cardiovascular diseases. In a recent study, Jason et al. disclosed that Rev exerted antihypertrophic effects by activating AMPK by LKB1 and inhibiting Akt, thus suppressing protein synthesis and gene transcription in pathological cardiac hypertrophy.²⁵ The present study yielded several novel findings about the antihypertrophic effects of Rev. BRCA1 has been shown to have variable effects in functional, biochemical, and biophysical studies,

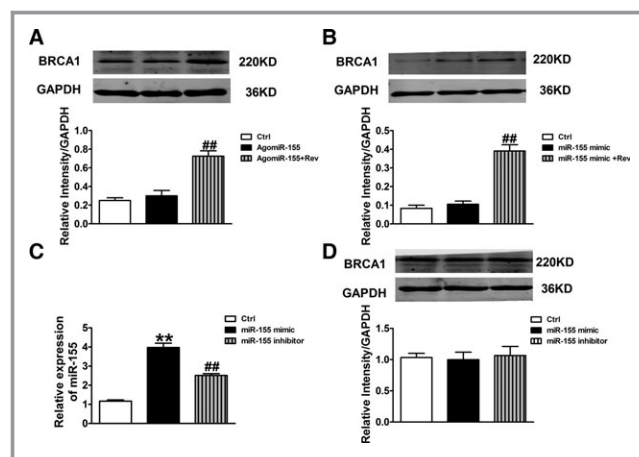


Figure 6. miR-155 had no influence on protein expression of BRCA1. A, Results showed that transfection with agomiR-155 had no effect on protein level of BRCA1, whereas Rev dominantly increased its expression; n=3 independent experiments. Data are expressed as mean±SEM; ##P<0.01 vs agomiR-155-treated group. B, Similarly, it showed that transfection with miR-155 mimic produced no change on protein level of BRCA1, whereas protein expression of BRCA1 was obviously elevated by treatment with Rev; n=3 independent experiments. Data are expressed as mean±SEM; ##P<0.01 vs miR-155-mimic-treated group. C, Quantification of miR-155 after transfection with miR-155 mimic or its inhibitor; n=6 independent experiments. Data are expressed as mean±SEM; **P<0.01 vs Ctrl; ##P<0.01 vs miR-155-mimic-treated group. D, Relative protein expression of BRCA1 was calculated in different treated groups; n=3 independent experiments. Data are expressed as mean±SEM. agomiR-155 indicates microRNA-155 agonist; BRCA1, breast cancer type 1 susceptibility protein; Ctrl, control; miR-155, microRNA 155.

and its precise effects on cardiac hypertrophy are still fully unknown. First, we demonstrated, for the first time, that up-regulation of BRCA1 level in mice cardiac hypertrophy model could be further enhanced by Rev pretreatment. Second, Rev produced protective effects against cardiac hypertrophy, possibly partially by reducing miR-155 level by increasing the BRCA1. Third, FoxO3a was involved in the antihypertrophic effect of Rev, which was targeted by miR-155 in hypertrophic cardiomyocytes. Together, the results of our study provide critical functional and mechanistic insights into the antihypertrophic effect of Rev, but also conceptually advance our view of miRNAs that may serve as potential therapeutic and drug targets.

Rev is a naturally occurring polyphenol present in many plant-based foods, which is beneficial for the cardiovascular system. The data from a recent famous study showed that Rev had a markedly inhibitory role on NFAT-dependent transcription in cardiomyocytes, which suggested that Rev could prevent pathological cardiac hypertrophy by blocking NFAT-mediated regulation of gene transcription.⁷ Furthermore, they also showed that Rev could activate AMPK and

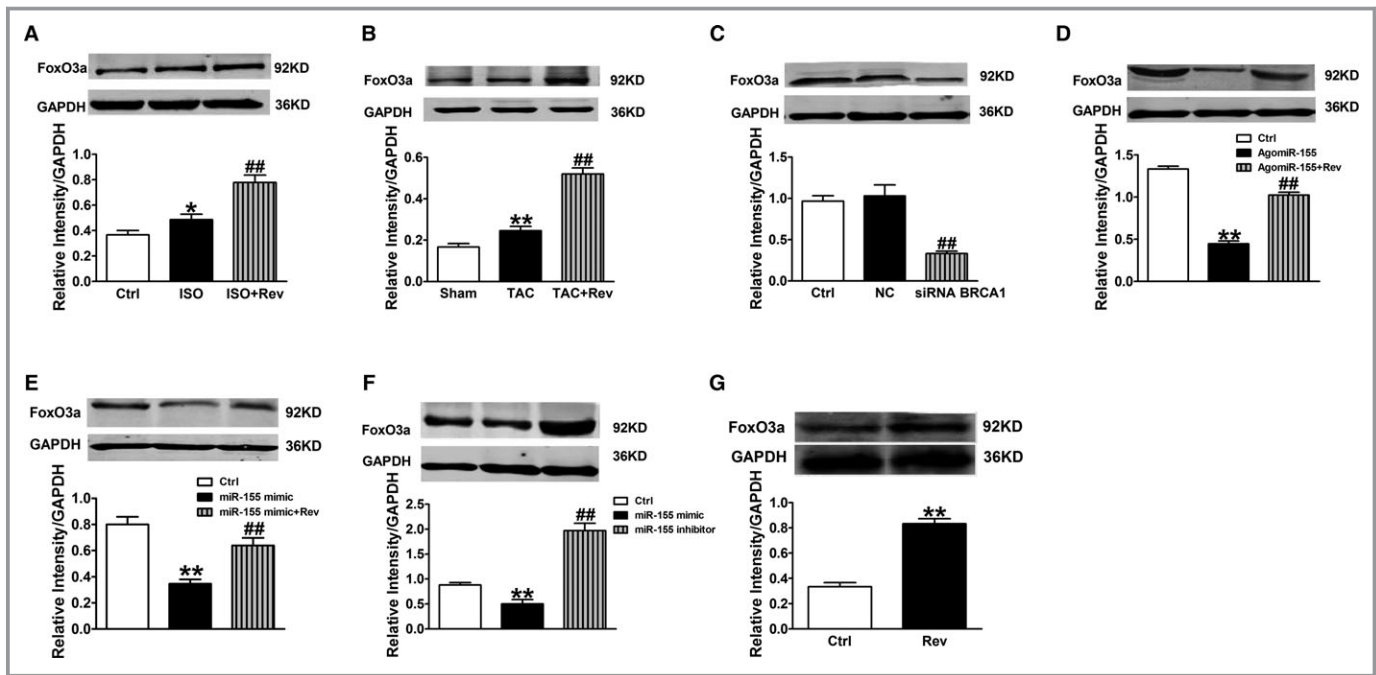


Figure 7. miR-155 inhibited the level of FoxO3a in cardiac hypertrophy. FoxO3a was dominantly increased by treatment with Rev (A and B); $n=3$ independent experiments. Data are expressed as mean \pm SEM; * $P<0.05$ vs Ctrl; ** $P<0.01$ vs sham; ### $P<0.01$ vs ISO- or TAC-treated groups. C, Protein expression of FoxO3a was inhibited by transient transfection with siRNA BRCA1; $n=3$ independent experiments. Data are expressed as mean \pm SEM; ### $P<0.01$ vs NC group. D and E, Transfection with agomiR-155 or miR-155 mimic significantly decreased protein expression of FoxO3a, whereas Rev reversed its effect both in vivo and in vitro; $n=3$ independent experiments. Data are expressed as mean \pm SEM; ** $P<0.01$ vs Ctrl; ### $P<0.01$ vs agomiR-155 or miR-155 mimic group. F, Transfected with miR-155 inhibitor alone elevated protein expression of FoxO3a; $n=3$ independent experiments. Data are expressed as mean \pm SEM; ** $P<0.01$ vs Ctrl; ### $P<0.01$ vs miR-155 mimic group. G, Protein expression of FoxO3a was increased by Rev; $n=3$ independent experiments. Data are expressed as mean \pm SEM; ** $P<0.01$ vs Ctrl. agomiR-155 indicates microRNA-155 agonist; BRCA1, breast cancer type 1 susceptibility protein; Ctrl, control; FoxO3a, forkhead box O3a; ISO, isoproterenol; miR-155, microRNA 155; NC, negative control; Rev, resveratrol; TAC, transverse aortic constriction.

inhibit Akt signaling in the cardiac myocyte, and these Rev-mediated effects could inhibit cardiac myocyte hypertrophy. In this study, administration of Rev significantly alleviated cardiac myocyte hypertrophy in vitro and in vivo, which corresponded to the results from previous studies. Recent findings suggest BRCA1 to be a potential target for antihypertrophic therapy; whether BRCA1 is involved in the antihypertrophic effect mediated by Rev has not been fully disclosed until now. To test this hypothesis, cardiomyocytes were incubated with Rev in the ISO-stimulated group. Consistent with our expectation, upon stimulation of Rev there was substantially increased protein expression of BRCA1 compared with ISO-stimulated alone. Similarly, Rev also dominantly up-regulated the protein level of BRCA1 in TAC group. It indicated that BRCA1 was indeed involved in the Rev-mediated antihypertrophic effect. In addition, we also found that Rev obviously reversed the impaired cardiac function induced by agomiR-155 stimulation. However, it should be noted that the cardiac function data were obtained by transthoracic echocardiograms under the condition of anesthesia. So, the data of cardiac function could be affected

by the narcotic condition in the mouse, which was hard to avoid in an in vivo study.

MiR-155 expression is elevated in a lot of inflammatory diseases, including rheumatoid arthritis and multiple sclerosis.^{30,31} A famous study showed that miR-155 expression in macrophages promoted cardiac inflammation, hypertrophy, and failure in response to pressure overload. And these effects were, at least partly, mediated by the direct miR-155 target, suppressor of cytokine signaling 1.²¹ Another important finding of this study is that the BRCA1-signaling pathway is involved in the regulation of expression of miR-155 in cardiac hypertrophy. To further determine whether overexpression of miR-155 caused cardiac hypertrophy, hearts were injected with agomiR-155. Subsequently, we found that miR-155 was strongly increased in the agomiR-155-injected group, whereas Rev significantly down-regulated expression of miR-155. Moreover, we also disclosed that agomiR-155 dominantly promoted cardiac hypertrophy, whereas this pathological process was partially reversed by Rev. In addition, we found that siRNA BRCA1 treatment obviously elevated the level of miR-155 in vitro and in vivo, but treatment with NC had no

significant effect. Taken together, the data from our study indicated that Rev ameliorated cardiac hypertrophy by down-regulation of miR-155 through activation of BRCA1. In addition, we should point out that nonmyocyte cells, such as cardiac fibroblasts, were not studied in our current study. And the agomiR-155 may increase expression of miR-155 in noncardiac tissues, which needs to be further explored in the future.

The FoxO subgroup contains 4 members, including Foxo1, Foxo3a, Foxo4, and Foxo6.³² It has been demonstrated that FoxO3a is expressed in the heart and skeletal muscle.^{33,34} And recent studies have showed that FoxO3a activation can induce skeletal muscle atrophy by causing transcription of the ubiquitin ligase atrogin-1 promoter.³³ Li et al. demonstrated that myocardin expression can be stimulated by ROS, and FoxO3a negatively regulates myocardin expression through controlling ROS levels.²⁷ Recently, there were several reports suggesting that FoxO3a was closely controlled by miR-155.^{28,29} In this current study, we provide the evidence that transient transfection with miR-155 obviously reduced the protein level of FoxO3a. However, Rev could significantly reverse the expression of FoxO3a. The data manifested that FoxO3a was involved in an antihypertrophic effect, which was controlled by miR-155.

The present study revealed the ability of Rev to mitigate cardiac hypertrophy that was involved in regulation of miR-155 expression through inhibiting BRCA1. These findings may be a novel molecular mechanism underlying the antihypertrophic effects of the Rev. It thus expanded our understanding of antihypertrophic therapy, reconsolidating the view that miRNAs likely have important functions and may be novel therapeutic and drug targets. However, the role of the BRCA1/miR-155 signaling axis in the hypertrophic pathway has not been deeply studied in our present work. Nevertheless, our results may lead to future studies to explore not only the implications of this axis in hypertrophy, but also the beneficial effect of this axis as a biological target for the potential novel therapy for maladaptive cardiac hypertrophy. In addition, it should be noted that our studies were performed in animal models and the findings may not be extrapolated directly to humans. This calls for a further precaution applying the results of this study to patients. Nevertheless, the findings open the door for further studies to investigate whether the miR-155 mechanism involved in Rev action also operates in the clinical setting.

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Disclosures

None.

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