Overexpression of miR-223 Tips the Balance of Pro- and Anti-hypertrophic Signaling Cascades toward Physiologic Cardiac Hypertrophy

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Liwan Yang 1,5, Yutian Li 1, Xiaohong Wang 1, Xingji Mu 5, Dongze Qin 5, Wei Huang 5, Saeed Alshahrani 5, Michelle Nieman 5, Jiangtong Peng 1,5, Kobina Essadon 5, Tianqi Peng 1,5, Yigang Wang 1, John Lorenz 5, Manoorcher Soleimani 1, Zhi-Qing Zhao 5, and Guo-Chang Fan 5

From the 1Shanxi Medical University, Taiyuan 030001, China, 6Department of Pharmacology and Cell Biophysics, 6Department of Pathology and Laboratory Medicine, 7Research Services, Veterans Affairs Hospital and Department of Medicine, and 8Department of Molecular and Cellular Physiology, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0575, 9Department of Cardiology, Union Hospital Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China, and 8Critical Illness Research, Lawson Health Research Institute, Ontario N6A 4G5, Canada

MicroRNAs (miRNAs) have been extensively examined in pathological cardiac hypertrophy. However, few studies focused on profiling the miRNA alterations in physiological hypertrophic hearts. In this study we generated a transgenic mouse model with cardiac-specific overexpression of miR-223. Our results showed that elevation of miR-223 caused physiological cardiac hypertrophy with enhanced cardiac function but no fibrosis. Using the next generation RNA sequencing, we observed that most of dys-regulated genes (e.g. Atf3/5, Egr1/3, Sfrp2, Itgb1, Ndrg4, Akp1, Postn, Rxfp1, and Egln3) in miR-223-transgenic hearts were associated with cell growth, but they were not directly targeted by miR-223. Interestingly, these dys-regulated genes are known to regulate the Akt signaling pathway. We further identified that miR-223 directly interacted with 3′-UTRs of FBXW7 and Acrv2a, two negative regulators of the Akt signaling. However, we also validated that miR-223 directly inhibited the expression of IGF-1R and β1-integrin, two positive regulators of the Akt signaling. Lastly, Western blotting revealed that Akt was activated in miR-223-overexpressing hearts. Adenovirus-mediated overexpression of miR-223 in neonatal rat cardiomyocytes induced cell hypertrophy, which was blocked by the addition of MK2206, a specific inhibitor of Akt. Taken together, these data represent the first piece of work showing that miR-223 tips the balance of promotion and inactivation of Akt signaling cascades toward activation of Akt, a key regulator of physiological cardiac hypertrophy. Thus, our study suggests that the ultimate phenotype outcome of a miRNA may be decided by the secondary net effects of the whole target network rather than by several primary direct targets in an organ/tissue.

Cardiac hypertrophy is an adaptive mechanism of cardiomyocytes to different forms of injury or stress, such as myocardial infarction, hypertension, and valve disease (pathological) or chronic exercise training (physiological) (1). Both pathological and physiological cardiac hypertrophy types are featured with increased myocyte size, but they have distinct molecular and functional phenotypes (2). Pathological cardiac hypertrophy is often associated with interstitial fibrosis and increased myocyte necrosis/apoptosis, leading to cardiac dys-function (1, 2). By contrast, exercise training-induced physiological cardiac hypertrophy is characterized by overall normal cardiac structure and function, presenting an adaptive beneficial response (1, 2). Indeed, exercise training is clinically recommended as the most effective non-pharmacological intervention to reduce cardiovascular disease (2). Thus, elucidating the molecular mechanisms underlying physiological cardiac hypertrophy would help us identify novel therapies for the treatment of cardiovascular disease.

MicroRNAs (miRNAs)3 are small, endogenous, non-coding RNAs of ~22 to 26 nucleotides in length that function primarily as post-transcriptional regulators (3). Importantly, a single miRNA does not only regulate one gene expression but often fine-tunes 10s (even 100s) of gene expression simultaneously, as post-transcriptional regulators (3). Importantly, a single miRNA does not only regulate one gene expression but often fine-tunes 10s (even 100s) of gene expression simultaneously, yielding complex gene regulatory networks (3). Thus, miRNAs may play a critical role in the development of cardiac hypertrophy. Indeed, numerous miRNAs (i.e. miR-1, miR-133, miR-19a/b, miR-21, miR-23, miR-26, miR-29, miR-9, miR-98, and

3 The abbreviations used are: miRNA, microRNA; TG, transgenic; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; β-MHC, β-myosin heavy chain; SKA, skeletal α actin; IVSd, diastolic interventricular septal; LVd5s, systolic interventricular septal; LVpWd, diastolic left ventricular posterior wall; LVPWs, systolic left ventricular posterior wall; AIF3, activating transcription factor 3; Egr1, Early growth response 1; Akp1, A kinase interacting protein 1; Ndrg4, NDRG4 family member 4, or smooth muscle-associated protein 8; Postn, peristin; Itgb1, integrin β-1; Rxfp1, Relaxin/insulin-like family peptide receptor 1; Mthfd2, 1-methyltetrahydrofolate dehydrogenase 2; Celrs2, cadherin, EGF LAG seven-pass G-type receptor 2; Egln3, Egl-9 family hypoxia-inducible factor 3; Csq2, calsequestrin 2; FBXW7, F-Box and WD repeat domain containing 7; Acrv2a, activin A receptor, type II; Hif-1α, hypoxia-inducible factor 1-α; IGF-1R, insulin-like growth factor-1 receptor; α-MHCp, α-myosin heavy chain promoter; HA, heart weight; BW, body weight.
miR-223 has been shown to exert either a positive or negative influence on pathological cardiac hypertrophy (4–6). Whereas several studies have recently profiled microRNA expression signatures in rat physiological hypertrophic hearts induced by swimming or treadmill training, it still remains unknown how dys-regulated miRNAs contribute to physiological hypertrophy (7–10).

One specific microRNA, miR-223, has recently garnered attention. It was initially identified to be highly expressed in the myeloid compartment and controlled hematopoietic lineage differentiation by targeting Mef2c (11). Later on extensive studies have indicated that miR-223 negatively modulates infection and cancer development by affecting multiple inflammatory factors (e.g. granzyme B, IKKα, Roquin, STAT3, IL-6, and NLRP3) (12–14). Nonetheless, recent work has implicated that several bona fide targets of miR-223 (e.g. IGF-1R and C/EBPβ) (15, 16) are associated with physiological cardiac hypertrophy, but their functional consequences appear to be counteracted. For example, up-regulation or activation of IGF-1R has been widely proposed to facilitate developmental growth and physiological hypertrophy of the heart (17, 18). By contrast, reduction of C/EBPβ results in a phenocopy of endurance exercise with cardiomyocyte hypertrophy and proliferation (19). Therefore, it would be necessary to determine the net outcome of miR-223-overexpressing in heart.

In this study we generated a transgenic (TG) mouse model with cardiac-specific overexpression of miR-223. Our results showed that elevation of miR-223 caused physiological cardiac hypertrophy with enhanced cardiac function but no fibrosis. Ex vivo studies using neonatal rat cardiomyocytes further revealed that overexpression of miR-223 could induce cell hypertrophy. Mechanistically, we identified that miR-223 tips the balance of promotion and inactivation of the Akt signaling cascades in cardiomyocytes toward activation of Akt, a key regulator of physiological cardiac hypertrophy.

Results

Generation of miR-223 Transgenic Mouse Model—Although several targets of miR-223 (i.e. IGF-1R and C/EBPβ) are associated with the regulation of cardiac hypertrophy (17–19), the in vivo consequence of increased miR-223 levels on cardiac hypertrophy has never been investigated. To this end, we created a miR-223 TG mouse model in which miR-223 was overexpressed under the control of the cardiac-specific α-myosin heavy chain (α-MHC) promoter (Fig. 1A). Our initial genotype-screening revealed three positive lines (6, 10, and 15, Fig. 1B); however, miR-223 in line 15 could not be passed to next generation. Therefore, TG lines 6 and 10 were used for this study. Quantitative reverse transcription PCR (RT-PCR) showed that the values of quantitation PCR cycle (Cq) were significantly lower in TG-hearts (line 6, 15.07 ± 0.17; line 10, 16.13 ± 0.24) compared with WTs (25.5 ± 0.44) (Fig. 1C), indicating higher copy numbers of miR-223 in TGs than WTs. Accordingly, the 2−ΔΔCq values were 1300-fold (line 6) and 600-fold (line 10) higher in TGs than WT hearts (Fig. 1D). In addition, 3% agarose gel electrophoresis of semiquantitative RT-PCR also showed that miR-223 was dramatically overexpressed in TG hearts (Fig. 1E). All these measurements consistently indicate that the miR-223 TG mouse model was successfully generated.

Cardiac-specific Overexpression of miR-223 Induces Physiological Hypertrophy—Next, we observed that both lines of TG mice appeared to develop cardiac physiological but not pathological hypertrophy, which was evidenced. First, at 3-month of age, TG mouse hearts exhibited larger hearts (Fig. 2A), and the LV wall was thicker (Fig. 2C) than WTs; consistently, the ratios of heart weight to body weight (HW/BW) in TGs were significantly higher than sex- and age-matched WT controls (Fig. 2B), but the ratios of lung weight to body weight showed no difference (data not shown). Second, using fluorescent Oregon

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FIGURE 1. Generation of miR-223 TG mice in which miR-223 is overexpressed specifically in the heart. A, a schematic diagram depicting the miR-223 TG construct. A 357-bp DNA fragment of mouse miR-223 was inserted downstream of α-MHCp, a cardiac-specific promoter. B, genotypic analysis of RT-PCR results showed decreased values of quantitation cycle (Cq) in mouse hearts of two TG lines (6 and 10). Small nuclear RNA 6 (U6) was used as an internal control. n = 3 hearts, 3 wells of RT-PCR for each heart RNA sample; *, p < 0.05 versus WTs. D, the 2−ΔΔCq values for RT-PCR of miR-223 were remarkably higher in TGs than WT-hearts. n = 3 hearts, 3 wells of RT-PCR for each heart RNA sample; *, p < 0.05 versus WTs. E, semiquantitative RT-PCR products were run on 3% agarose gel revealed a stronger miR-223 band in TG sample than WTs. n = 3 hearts; *, p < 0.05.
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(A) WT  TG #6  TG #10

(B) HW/BW (mg/g)

(C)

(D)

(E) Cell Cross Section Area

(F)

(G) mRNA Levels (vs. GAPDH)

(H) mRNA Levels (vs. GAPDH)

(I) Blood Pressure (mmHg)

(J)

(K) Ejection Fraction (%)
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Green 488-labeled wheat germ agglutinin staining, we observed that cardiomyocyte size in cross-sections was increased in miR-223 TG mice (line 6, 1.58 ± 0.13; line 10, 1.61 ± 0.09) compared with WT controls (Fig. 2, D and E); however, the result of Masson trichrome staining revealed no detectable fibrosis in either TG hearts or WT (Fig. 2F), which was further validated by RT-PCR (Fig. 2G), showing no alterations in the expression of fibrosis gene markers Collagen I (Col I), Col III, and CTGF (connective tissue growth factor). Lastly, the levels of pathological cardiac hypertrophy markers (ANP, BNP, β-MHC, and α-SKA) also displayed no difference between WT and TG hearts (Fig. 2H). Importantly, these phenotypes persisted as mice reached 6 and 12 months of age. HW/BW ratios were higher in TG mice, but fibrosis was not evident by Masson Trichrome staining (data not shown).

Considering that hypertension is a major contributor to cardiac remodeling (20), we next tested whether the observed hypertrophy is the consequence of hypertension. The results of blood pressure measurement showed no significant variation among WT and two TG lines (Fig. 2I). Using echocardiography, we measured cardiac contractile parameters of TG mice. We observed that diastolic interventricular septal (IVSd) and systolic interventricular septal (IVSs) were significantly increased in TG hearts, comparable with WT (Fig. 2J). Both ejection fraction (EF) and fraction shortening (FS) were augmented by 20 and 16%, respectively (Fig. 2K). Please note here, in histological sections of hearts, TGs displayed the remarkable increases of both interventricular septal thickness and left ventricular wall thickness, compared with WTs (Fig. 2C). However, the diastolic left ventricular posterior wall (LVPWd) and systolic left ventricular posterior wall (LVPWs) did not differ between WTs and TGs (Fig. 2J). This inconsistency may be due to the echocardiography measurement with long axis-generated parameters (Fig. 2F). Put together, our data suggest that overexpression of miR-223 appears to induce healthy physiological cardiac hypertrophy.

Gene Expression Profiles in miR-223 Transgenic Hearts—To identify potential mechanisms underlying the miR-223-induced physiological cardiac hypertrophy, we conducted next generation RNA sequencing (RNA-Seq) in miR-223-overexpressing hearts. The results showed that there were a total of 81 up-regulated (Fig. 3A and supplemental Table S3) and 27 down-regulated (Fig. 3B and supplement Table S4) genes in miR-223 TG hearts compared with WT hearts. The complete heat map of dys-regulated genes is attached as supplemental Fig. S1. Interestingly, most of dys-regulated genes are known to directly or indirectly modulate cardiac remodeling. Therefore, we selected eight up-regulated (Atf3, Egr1, Akip1, Ndrg4, Postn, Itgb1, Rfxp1, and Mthfd2) and 3 down-regulated (celsr2, Egln3, and Csq2) genes for validation. The data of RT-PCR analysis showed that the alterations of all these selected genes except Atf3 in two lines of miR-223TG hearts were consistent with the RNA-Seq results (Fig. 3, B and D).

Overexpression of miR-223 Differentially Regulates Gene Expression in Neonatal Rat Cardiomyocytes—Given that chronic overexpression of miR-223 in hearts may induce a complex and compensatory effect on the gene expression profile, we therefore performed ex vivo experiments to elevate miR-223 levels in neonatal rat cardiomyocytes by transfection with Ad.miR-223. Ad.miR-223 and control Ad.GFP were constructed as shown in Fig. 4A. Neonatal rat cardiomyocytes were isolated and infected with Ad.miR-223 or Ad.GFP for 24 h or 48 h. We observed that the expression level of miR-223 was increased by 3.4-fold at 24 h post-transfection compared with Ad.GFP-cells, and this did not lead to any change in cardiomyocyte size. At the 48-h time point, miR-223 expression level was further increased by about 71-fold compared with GFP-cells, which significantly enlarged the cardiomyocyte size by 1.5-fold, comparing to Ad.GFP-infected cells (Fig. 4, B–D).

We next validated expression levels of those dys-regulated genes observed in TG hearts. Interestingly, at the 24-h time point when cardiomyocyte hypertrophy was absent, expression levels of Atf3, Egr1, Akip1, and Itgb1 were down-regulated, which are opposite with the alterations observed in miR-223-TG hearts. However, Postn and Rfxp1 were up-regulated, and Egln3 was down-regulated at 24h post-transfection, which are consistent with the alterations observed in miR-223-TG hearts (Fig. 4E). Nevertheless, at the 48-h time point when hypertrophy was evident in miR-223-overexpressing cells, the altered pattern of those dys-regulated genes was similar to the results from TG hearts (levels of Atf3, Akip1, Ndrg4, and Postn were higher, whereas levels of Egln3 and Csq2 were lower in Ad.miR-223 cells than Ad.GFP-cells) (Fig. 4F). Altogether, these data suggest that miR-223-induced cardiomyocyte hypertrophy may be initiated at 24 h, and with the enlargement of cell size, some gene expressions might be changed in a compensatory manner.

Although we observed that overexpression of miR-223 dynamically altered the gene expression pattern, it remains unclear how miR-223 triggers cardiomyocyte hypertrophy. Intriguingly, it has been shown that reduced Akt3/Csn2, overexpressing miR-223 improved cardiac function, determined by echocardiography. EF, ejection fraction; FS, fraction shortening. n = 5 for WT, n = 7 for both TG lines, * p < 0.05 versus WT.

FIGURE 2. Characterization of miR-223 TG mice. A, gross hearts revealed that overexpression of miR-223-induced cardiac hypertrophy. B, increased ratios of heart weight/body weight in miR-223 TG mice, compared with WT mice. n = 10, * p < 0.05. C, histological sections of hearts consistently displayed an increased interventricular septal thickness and left ventricular wall thickness, compared with WTs (Fig. 2C). However, the diastolic left ventricular posterior wall (LVPWd) and systolic left ventricular posterior wall (LVPWs) did not differ between WTs and TGs (Fig. 2D). This inconsistency may be due to the echocardiography measurement with long axis-generated parameters (Fig. 2F). Put together, our data suggest that overexpression of miR-223 appears to induce healthy physiological cardiac hypertrophy.

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(A) [Heatmap showing gene expression]

(B) [Bar chart showing mRNA expression levels]

(C) [Heatmap showing gene expression]

(D) [Bar chart showing mRNA expression levels]
**miR-223 Induces Physiological Cardiac Hypertrophy**

A renowned determinant of physiological cardiac hypertrophy (27–30), it is necessary to clarify the net effect of miR-223 overexpression on the Akt activity. To this end we measured the Akt phosphorylation levels in Ad.miR-223-infected neonatal rat cardiomyocytes at 24-h (Fig. 4, H and I) and 48-h (Fig. 4, J and K) time points. Results of Western blotting analysis revealed that phosphorylation levels of Akt at both Thr-308 and Ser-473 sites were significantly higher in Ad.miR-223-neonatal cardiomyocytes than GFP-cells (24 h and 48 h post-infection) (Fig. 4, H–K). Put together, these data indicate that elevation of miR-223 could either negatively or positively regulate the expression levels of multiple upstream factors of Akt, but the net outcome would sway toward Akt activation and, consequently, lead to cell hypertrophy.

![Diagram](image)

**FIGURE 3.** Gene expression profile in miR-223-TG hearts determined by high-throughput RNA-Seq. A and B, heat map of up-regulated genes in TG hearts; a total of 8 up-regulated genes were selected for validation using RT-PCR. C and D, heat map of down-regulated genes in TGs; three were selected for validation using RT-PCR. The original whole heat-map and all dys-regulated genes were shown in supplemental Fig. S1 and supplemental Tables S3 and S4. n = 3; *, p < 0.05 versus WT. Atf3, activating transcription factor 3; Egr1, early growth response 1; Akp1, A kinase interacting protein 1; Ndr4, NDRG4 family member 4 (or smooth muscle-associated protein 8); Postn, peristin; Itgb1, integrin β-1; Rxfp1, Relaxin/insulin-like family peptide receptor 1; Mthfd2, methylenetetrahydrofolate dehydrogenase 2.

![Diagram](image)

**FIGURE 4.** Overexpression of miR-223 in neonatal rat cardiomyocytes promotes cell hypertrophy. A, diagrams of recombinant adenoviral vectors (Ad.GFP or Ad.miR-223). B, representative images showed Ad.GFP- or Ad.miR-223-infected neonatal cardiomyocytes, and miR-223-cells displayed hypertrophy at 48 h post-infection. Similar results were observed in three additional, independent experiments. C, miR-223 expression levels were measured using RT-PCR at both time points. Small nuclear RNA 6 (U6) was used as internal control. n = 3; *, p < 0.05 versus Ad.GFP-infected cells. D, measurement of cell cross-section area indicated that cardiomyocyte size does not change at 24-h time point, but it was increased by 49 ± 3.4% at 48 h upon Ad.miR-223 infection. n = 35–40 cells per plates, 3 plates for each group; *, p < 0.05 versus Ad.GFP-infected cells. Similar results were observed in three additional, independent experiments. E and F, expression levels of those genes validated in miR-223-TG hearts were measured in Ad.miR-223-infected neonatal cardiomyocytes at 24 h and 48 h post-infection. n = 3 plates for isolation of total RNA; *, p < 0.05 versus Ad.GFP-infected cells. Similar results were observed in two additional, independent experiments.
**miR-223 Induces Physiological Cardiac Hypertrophy**

**FIGURE 5.** Hypertrophic effects of miR-223 overexpression is attenuated by MK2206. A and B, treatment of neonatal rat cardiomyocytes with MK2206 (10 nM) 1 h before Ad.miR-223 infection significantly inhibited Akt phosphorylation. n = 4 plates for isolation of total protein. *, p < 0.05 versus GFP-cells. Representative images of immunofluorescence staining with α-actinin (C) and quantitative results of cell cross section area (D) are shown. n = 25–30 cells per plates, three plates for each group. *, p < 0.05 versus PBS-treated GFP-cells. Similar results were observed in three additional, independent experiments.

of Akt levels were remarkably inhibited by the addition of MK2206 (Fig. 5, A and B). Accordingly, the results of immunofluorescence staining with α-actinin showed that miR-223-triggered enlargement of neonatal cardiomyocytes was greatly limited by treatment with MK2206 (Fig. 5, C and D). Therefore, the Akt activation appears to be an essential mediator of cell hypertrophy induced by miR-223 overexpression.

miR-223 Directly Targets FBXW7-Hif-1α-Postn and ACVR2a-Egln3-Hif-1α Signaling Pathways—As described above, it is obvious that miR-223 does not directly activate Akt, and it raises up a question; Which signaling pathway is influenced by miR-223 to activate Akt in cardiomyocytes? According to our RT-PCR results in *ex vivo* neonatal cardiomyocytes (Fig. 4, F and G) and *in vivo* TG hearts (Fig. 3, B and D), we found that only the pattern of *Postn* (periostin) and *Egln3* (Egl-9 family hypoxia-inducible factor 3, also named PHD3) alterations was consistent in miR-223-overexpressing cells and TG hearts. This suggests that miR-223 may directly regulate *Postn* and *Egln3* expression. However, these two genes are not assumed to be targets of miR-223 listed in any bioinformatics databases (miRBase, miRDB, and TargetScan). We then searched for upstream mediators of *Postn* and *Egln3* and found that there are two pathways: **FBXW7-Hif-1α-Postn** and **ACVR2a** (activin receptor type IIA)-*Egln3-Hif-1α*, which could be directly regulated by miR-223 (Fig. 6A). As shown in Fig. 6B, the 3' UTR of **FBXW7** (F-Box and WD repeat domain containing 7) contains a highly conserved miR-223-interacting region. Importantly, **FBXW7** is an E3 ubiquitin protein ligase that can ubiquitinate and degrade the Hif-1α protein under normoxia condition (31). Recent studies also indicate that reduced **FBXW7** expression causes an elevation of Hif-1α levels in ovarian cancer cells (32).

Furthermore, **Hif-1α** has been shown to positively regulate *Postn* expression in human periodontal ligament (PDL) cells (33). As for another pathway, miR-223 is predicted to interact with 3'UTR of **ACVR2a** (Fig. 6C). **ACVR2a** as a receptor of activin A is able to positively modulate Egln3 (PHD3) expression (34), whereas PHD3 regulates degradation of Hif-1α under normoxia (35). Given that Hif-1α and Postn both are well appreciated as upstream activators of Akt (24, 36), we therefore hypothesized that miR-223-induced activation of Akt is mainly through the down-regulation of **FBXW7** and **ACVR2a** (Fig. 6A).

To test this hypothesis, we first performed luciferase reporter assays to examine whether miR-223 directly recognizes 3'-UTRs of **ACVR2a** and **FBXW7**. These luciferase reporter constructs were co-transfected into H9c2 cells with either miR-223 or miR control. The luciferase activity was measured at 48 h post-transfection, and we observed that co-transfection of miR-223 strongly reduced the luciferase activity from the reporter construct containing the **ACVR2a** and **FBXW7** 3'-UTR fragments, whereas activities of the mutant 3'-UTRs were not affected (Fig. 6, D and E). These results indicate that **ACVR2a** and **FBXW7** transcripts contain binding motifs, which represent *bona fide* targets of miR-223.

Using Western blotting analysis, we next determined the protein levels of **ACVR2a** and **FBXW7** in miR-223-overexpressing neonatal rat cardiomyocytes where Akt was activated (Fig. 4, H–K). As shown in Fig. 6, F and G, Ad.miR-223-transfected neonatal cardiomyocytes displayed lower levels of **FBXW7** and **ACVR2a** than Ad.GFP-cells. Accordingly, the expression levels of Egln3 (PHD3), a downstream molecule target of **ACVR2a**, were remarkably decreased by 36% in miR-223 cells compared with control GFP-cells. Given that both **FBXW7** and **Egln3** are able to degrade Hif-1α under normoxia (31, 35), as a result, higher levels of Hif-1α were observed in Ad.miR-223-infected cells than control Ad.GFP-cells. Consequently, the expression levels of **Postn** were increased by 4.6-fold in miR-223-cells compared with GFP-cells (Fig. 6, F and Hs). Taken together, these data indicate that miR-223-induced activation of Akt in neonatal cardiomyocytes is associated with direct regulation of **FBXW7-Hif-1α-Postn** and **ACVR2a-Egln3-Hif-1α** signaling cascades. Nonetheless, considering that IGF-1R and β1-integrin are well validated authentic targets of miR-223 (15, 37) and, importantly, IGF-1R and β1-integrin, two upstream activators of Akt, are known to play critical roles in cardiac remodeling (18, 38), it thus would be argued whether these two protein levels are also reduced in neonatal cardiomyocytes transfect...
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In the present study we uncovered that cardiac-specific overexpression of miR-223 in vivo could induce physiological

![Diagram](image-url)

**Figure 6.** miR-223 directly targets the FBXW7-Hif-1α-Postn and the Acvr2a-Egln3-Hif-1α pathways. **A**, a diagram shows how miR-223 directly regulates FBXW7 and ACVR2a as well as their downstream molecules. **B** and **C**, the putative miR-223 binding sites within 3-UTRs of FBXW7 and ACVR2a are conserved among mammalian species (human, mouse, and rat), where the crucial seed-sequence regions are highlighted. **D** and **E**, relative luciferase activity of ACVR2a and FBXW7 constructs were decreased in H9c2 cells upon mimic miR-223 transfection compared with control and mutant constructs. **F** and **G**, protein levels of FBXW7, ACVR2a, and Egln3 were decreased, whereas protein levels of Hif-1α and Postn were increased in Ad.miR-223-infected neonatal cardiomyocytes at 48 h post-infection. GAPDH was used as internal control. **H**, protein levels of FBXW7, ACVR2a, and Egln3 were decreased, whereas protein levels of Hif-1α and Postn were increased in Ad.miR-223-infected neonatal cardiomyocytes at 48 h post-infection. GAPDH was used as internal control.

with Ad.miR-223. As shown in Fig. 6, G and H, acutely overexpressing miR-223 in neonatal cardiomyocyte for 48 h did significantly decrease the expression of both proteins. However, such an effect (Akt inactivation) was overcome by other pathways, leading to net outcome of Akt activation (Fig. 4, H–K).

Overexpression of miR-223 Promotes the Akt Activation in Transgenic Mouse Hearts—Lastly, we tested whether miR-223 transgenic hearts exhibited a similar change pattern as observed above in ex vivo myocytes. The results of Western-blotting analysis showed that overexpression of miR-223 significantly reduced the protein levels of FBXW7, ACVR2a, and Egln3 and increased the protein levels of Hif-1α and Postn in two lines of transgenic mouse hearts (Fig. 7, A and C). Meanwhile, protein levels of IGF-1R and β1-integrin in miR-223 TG hearts were reduced significantly compared with WTs. These data are consistent with our ex vivo findings presented in Fig. 6, F–H. However, levels of Akt phosphorylation at both Thr-308 and Ser-473 sites were higher in miR-223-TGs than WTs (Fig. 7, B and C). This suggests that overexpression of miR-223 tips the balance of Akt activity toward overall activation, leading to physiological hypertrophy (Fig. 7D).

miR-223 Is Up-regulated in Treadmill-trained Mouse Hearts—To confirm whether miR-223 was involved in exercise training-induced cardiac physiological hypertrophy, we trained WT mice on treadmill 5 days a week for 4 weeks (Fig. 8A). Heart tissues were collected at the end of weeks 1, 2, and 4. As expected, treadmill-trained mice displayed enlarged hearts after 4 weeks (Fig. 8B) with a significant higher ratio of heart weight to body weight at the end of weeks 1, 2, and 4 post-training (Fig. 8C). Consistent with our hypothesis, miR-223 level was significantly increased in the heart after treadmill training (Fig. 8D), yet the -fold change (1 week, 2.4 ± 0.05; 2 weeks, 2.8 ± 0.19; 4 weeks, 2.0 ± 0.07; n = 4, p < 0.05 versus controls) was not as dramatic as observed in miR-223 TG mouse model. Importantly, miR-223 TG mice with no prior treadmill training showed better performance when compared with WT, as evidenced by longer duration on treadmill running experiment (Fig. 8C). Lastly, we measured the expression of proteins of interest and observed increased levels of IGF-1R in treadmill-trained group, which differs from the miR-223 TG mouse model. This may due to the dynamic nature of exercise-induced cardiac hypertrophy, where many other miRNAs were altered and elicited different effects (4–10). Nonetheless, as the key regulator of physiological hypertrophy, levels of Akt phosphorylation were increased, which is consistent with miR-223 mouse hearts. Moreover, as a predicted direct target of miR-223 that was previously identified as downstream effector of Akt (19), the C/EBPβ expression level was remarkably decreased, which is consistent with our miR-223 TG model, where C/EBPβ was reduced in both lines of TG mice (data not shown), thus contributing to the induction of cardiac physiological hypertrophy (19). Meanwhile, Acvr2a and FBXW7 protein levels were also decreased in treadmill-trained mouse hearts (Fig. 8, F–G). Taken together, these data further confirm that up-regulation of miR-223 mediates cardiac physiological hypertrophy through reduced protein levels of Acvr2a and FBXW7, leading to net effect of Akt activation.

Discussion

In the present study we uncovered that cardiac-specific overexpression of miR-223 in vivo could induce physiological
hypertrophy as evidenced by 1) higher ratio of heart weight to body weight, 2) increased wall thickness and cardiomyocyte size, and 3) enhanced myocardial contractile function without cardiac fibrosis and increased expression of pathological hypertrophy markers (i.e. ANP, BNP, β-MHC, and α-SKA) compared with WT controls. Using RNA-Seq, RT-PCR, Western blotting, and luciferase-reporter assays, we identified that miR-223 directly targeted FBXW7 and ACVR2a-Egln3, which propagated secondary effects via up-regulation of Hif-1α and Postn, two upstream factors known to activate Akt (Fig. 7D). We also validated that miR-223 directly targeted IGF-1R and β1-integrin, which may lead to inactivation of Akt. However, the overall net effect strongly favors Akt activation, resulting in the development of physiological hypertrophy (Fig. 7D). As a matter of fact, using ex
vivo neonatal cardiomyocytes, we further observed that miR-223-induced myocyte hypertrophy was greatly limited by preaddition of MK2206, a specific inhibitor of Akt. This suggests that physiological cardiac hypertrophy triggered by elevation of miR-223 is largely dependent on the activation of Akt.

Currently, it is well recognized that a miRNA can affect 10s, even 100s of gene expression (3–6), which makes the miRNA study complicated. In fact, our RNA sequencing results revealed that overexpression of miR-223 in mouse hearts affected >100 gene expressions. Interestingly, most of those dys-regulated genes (e.g., Mthfd2, Dkk3, Egr3, Sfrp2, Atf5, Egr1, Atf3, Ndrg4, Nt5e, Postn, Akip1, Rsp1, Egln3, Fgf9, Celsr2, Casq2, and Ogdh; see supplemental Tables S3 and S4) are associated with cell growth and are consistent with the cardiac phenotype of hypertrophy. However, according to the miRNA seed-sequence match rule, such dys-regulated genes are not predicted to be targets of miR-223. This triggered us to explore the upstream factors, which may affect the expression of those dys-regulated genes. We then first selected 11 dys-regulated genes for validation using miR-223-TG hearts and miR-223-

FIGURE 8. Alterations of miR-223 expression and its associated proteins of interest in exercise-trained mouse hearts. A, scheme of treadmill training protocol. B, gross hearts revealed that 4 weeks of treadmill exercise training induced cardiac hypertrophy. C, increased ratios of heart weight/body weight in treadmill-trained mice compared with sedentary control mice. n = 4. *, p < 0.05. D, expression of miR-223 was significantly increased in treadmill-trained mouse hearts. n = 4. *, p < 0.05. E, total duration of treadmill exercise was significantly longer in miR-223 TG group compared with WT mice. n = 4. *, p < 0.05. F and G, protein levels of IGF-1R and phosphorylated Akt (Thr(P)308-Akt and Ser-(P)473-Akt) were increased in treadmill-trained mouse hearts, whereas levels of C/EBPβ, Acvr2a, and FBXW7 expression were reduced. n = 4; *, p < 0.05.
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overexpressing neonatal cardiomyocytes (Figs. 3 and 4). Of interest, we observed that miR-223 dynamically influenced gene expression in \textit{ex vivo} cardiomyocytes (Fig. 4, E and F), and only the alteration of \textit{Postn} and \textit{Egln3} expression levels were consistent both \textit{in vivo} and \textit{ex vivo}. This may suggest that \textit{Postn} and \textit{Egln3} could be primarily affected by miR-223, and other gene alterations may be ascribed to secondary or compensatory effects. Once the target range of miR-223 was narrowed, we next dissected which genes could regulate \textit{Postn} and \textit{Egln3} expression in hearts. Finally, we identified Hif-1α as the hub to connect up- and down-signaling cascades (FBXW7-Hif-1α-Postn and ACVR2a-Egln3-Hif-1α). Nonetheless, the mRNA levels of Hif-1α were not altered in miR-223-overexpressing cells, but its protein levels were remarkably increased in both miR-223-TG hearts and miR-223-myocytes. Indeed, it is well recognized that the Hif-1α transcription is affected only by hypoxia conditions, but under normoxia, the Hif-1α protein can be degraded by PHD3 (Egln3), FBXW7, and others via the proteasome (31–34). Therefore, miR-223-induced direct reduction of FBXW7 and Egln3 could relieve degradation of the Hif-1α protein, leading to dramatic accumulation of Hif-1α protein in cells (Figs. 6, A and F, and 7A). Hif-1α as a transcription factor is known to regulate the \textit{Postn} expression (33), and meanwhile, both Hif-1α and \textit{Postn} have been shown to positively activate Akt (23, 35), which are consistent with our observations (Figs. 3, 4, 6, and 7). Moreover, to date neither bioinformatics prediction nor previous publication has suggested that miR-223 directly targets any kinase (i.e. PP1, PP2) that is involved in Akt activation. Therefore, it appears that miR-223-induced Akt activation is mediated by secondary net effects of several interrelated pathways.

Notably, previous work by Thackaberry \textit{et al.} (39) has indicated that increased cardiac Hif-1α protein contributed to cardiac hypertrophy in AhR−/− mice. Similarly, a recent study by Montano \textit{et al.} (40) showed that inducible re-expression of \textit{HEXIM1} caused physiological cardiac hypertrophy, which was attributed to elevation of Hif-1α levels in adult mice. Chu \textit{et al.} (41) recently also reported that overexpression of Hif-1α directly triggered neonatal cardiomyocyte hypertrophy. More importantly, Zolk \textit{et al.} (42) observed that the levels of Hif-1α were significantly decreased, whereas PHD3 (Egln3) was up-regulated in human end-stage failing hearts. Hence, these data together with our findings suggest that miR-223 may be utilized as a potential agent to elevate Hif-1α levels and, consequently, rescue heart failure.

Although we identified FBXW7-Hif-1α-Postn and ACVR2a-Egln3-Hif-1α signaling cascades as major pathways involved in miR-223-elicited physiological hypertrophy, it is impossible to exclude contributions from other miR-223 targets. For example, a recent work by Zhou \textit{et al.} (16) demonstrated that C/EBPβ, a transcription factor that controls the expression of genes associated with cell proliferation, was a direct target of miR-223 in mouse macrophages and dendritic cells. Notably, Boström \textit{et al.} (19) reported that C/EBPβ-deficient mice mimic the phenotype of physiological cardiac hypertrophy in response to exercise. Indeed, we confirmed that protein levels of C/EBPβ were significantly reduced in miR-223-hearts compared with WTs (data not shown). Interestingly, treadmill exercise training also down-regulated the C/EBPβ expression in hearts (Fig. 8F), which is consistent with our miR-223 TG mouse model as well as previous publication (16). Thus, miR-223-induced physiological hypertrophy also may be attributed to the reduction of C/EBPβ.

There are several limitations in our study presented here. First, the transgenic mouse model we created was not inducible, although it was cardiac-specific overexpression. Although we performed a series of \textit{ex vivo} neonatal cardiomyocyte studies to confirm \textit{in vivo} findings, it would be better to generate an inducible model to test whether elevation of miR-223 can cause physiological cardiac hypertrophy in adult mice. Second, we showed that blockade of Akt activation significantly dampened miR-223-induced cell hypertrophy \textit{ex vivo}. One question would be argued whether the Akt inhibitor could be applied to \textit{in vivo} miR-223-TG mice. Considering that our mouse model has already exhibited hypertrophy and our \textit{ex vivo} data revealed that Akt was activated acutely by overexpression of miR-223, it would thus not be feasible to apply Akt inhibitor into this mouse model. However, it should be applicable in miR-223-inducible transgenic mouse model. Finally, although in this study we demonstrated that overexpression of miR-223 in the heart could induce physiological hypertrophy, it remains inconclusive whether miR-223 can also repress pathological hypertrophy. Recently, an \textit{in vitro} work by Wang \textit{et al.} (43) observed that the expression of miR-223 was decreased in endothelin-1-triggered hypertrophic neonatal cardiomyocytes and hypertrophic hearts. Transfection of neonatal cardiomyocytes with mimic miR-223 attenuated endothelin-1-induced cell hypertrophy by directly down-regulation of \textit{TNNI3K} (cardiac troponin I-interacting kinase) (43). However, \textit{TNNI3K} was reported to improve cardiac function and promote physiological hypertrophy without increase in interstitial fibrosis (44). Therefore, future studies are needed to clarify the effect of miR-223 on pathological cardiac hypertrophy.

In summary, our present findings suggest that elevation of miR-223 could integrate various pro-hypertrophic (i.e. increased Hif-1α and \textit{Postn}, and reduced C/EBPβ) and anti-hypertrophic signaling pathways (i.e. reduced IGF-1R and β1-integrin) but eventually promote the net increase of Akt activation (Fig. 7D). Thus, this work may unveil a previously unrecognized network involved in the development of physiological cardiac hypertrophy. Given the beneficial effects of physiological hypertrophy and pluripotent functions of miR-223, this piece of work presented here may help us better understand the impact of miR-223 in hearts and thereby provide a significant clinical value in the treatment of cardiac diseases.

\section*{Experimental Procedures}

\textit{Generation of miR-223 Transgenic Mice}—Given that the upstream DNA sequence of pre-miR-223 contains a start codon, ATG, (positioned at −103 to −105 nucleotides; see \textit{supplemental data}), we cloned a 357-bp DNA fragment of murine primary miR-223 starting at −99 nucleotides and inserted it into the downstream of the cardiac-specific α-myosin heavy chain promoter (α-MHC promoter) (Fig. 1A). This DNA vector was submitted to the Cyagen Biosciences Inc. to generate a transgenic mouse model (FVB/N background) with heart-specific overexpression of miR-223. Routine genotyping was performed by polymerase chain reaction (PCR) with the use of an upper primer from the α-MHC promoter (5’-CACATAGAAGCCTAGCCCACAC-3’) and a lower
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primer from the miR-223 DNA (5’-GTAAGCTTCCCTA TGTATGTC-3’) to amplify a 400-bp fragment spanning the junction between the α-MHCp and miR-223 DNA. The endogenous mouse Oct2 gene was used as an internal control with the forward primer (5’-TCTTAGCTCTTGCTCCGGT-3’) and reverse primer (5’-CAGTGTTGCTGGAAGAGAC-3’). Quantitative and semiquantitative RT-PCR were carried out to determine the expression levels of miR-223 in TG hearts. Relative -fold change of target genes expression was calculated using $2^{-\Delta\Delta C\text{t}}$ method. Small nuclear RNA 6 (U6) was used as a RT-PCR loading control.

**Measurement of Cardiac Function and Hypertrophy**—Cardiac function and remodeling were assessed on 3-month-old male WT and TG mice by echocardiography using a SONOS-7500 echocardiography system (Philips Medical Systems) equipped with a 15-MHz transducer. IVSd wall thickness, IVSs wall thickness, LVPWd thickness, and LVPWs thickness were measured. Left ventricular fractional shortening and ejection fraction were calculated by the system as direct indicators of cardiac function. Age and weight-matched wild-type mice were used as controls. Ratios of heart weight to body weight (HW/ BW) or to tibia length (HW/TL, data not shown) were determined as markers of hypertrophy.

**Blood Pressure Assay**—The Kent Scientific CODA 8-channel non-invasive blood pressure recording system was employed to record blood pressure in conscious WT and TG mice following the manufacturer’s protocol. Briefly, at age of 3 months, mice ($n = 4$ per group) were first allowed to adapt to the experimental setting, then blood pressure data (systolic and diastolic blood pressure) were recorded for 5 consecutive days.

**Heart Histological Analysis**—Mouse (3 months old) hearts were excised, fixed with 10% formalin, and then embedded in paraffin. Heart sections (5 μm in thickness) were made and stained with Masson’s Trichrome 2000TM kit (American MasterTech, Lodi, CA) following the manufacturer’s procedure. Wheat germ agglutinin staining was performed as described previously (45). Images were taken under the microscope (Nikon, Inteslight), and cell sizes were analyzed using the image processing program ImageJ.

**Next Generation RNA Sequencing**—RNA-Seq was performed by Genomics, Epigenetics, and Sequencing Core (GESC) at the University of Cincinnati. Total RNA was extracted from mouse hearts using miRNeasy Mini kit (Qiagen) followed by processing with WaferGen (Pleasanton, CA) Apollo 324 system and Ribo-Depletion Script. Next, an automatic RNA-Seq library was prepared using PrepX mRNA Library kit (WaferGen) and Apollo 324 NGS automatic library prep system, and cDNA was generated using Superscript III reverse transcriptase (Life Technologies, Inc.) and purified by Agencourt AMPure beads (Beckman Coulter, Indianapolis IN). A 1-μl library was quality-checked and quantified using DNA high sensitivity chip by the Bioanalyzer (Agilent, Santa Clara, CA). RNA sequencing was performed using Illumina HiSeq system according to the manufacturer’s protocol. Cardiac hypertrophy-related genes were selected for validation by RT-PCR. The primer sequences of these genes are listed in supplemental Table S1.

**Isolation and Culture of Primary Neonatal Rat Cardiomyocytes**—Hearts of rat neonates (1–3 days old) were removed after anesthetizing by ice-water bath, and cardiomyocytes were isolated using the Worthington Neonatal Cardiomyocytes Isolation System (Worthington Biochemical Corp., Lakewood, NJ) following the manufacturer’s protocol. Neonatal cardiomyocytes were then cultured in DMEM medium supplemented with 2% fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin).

**Construction of Adenovirus Vector miR-223 and Transfection of Cardiomyocytes**—Primary miR-223 DNA was PCR-amplified from miR-223 transgenic vector described above using high fidelity AccuPrime TaqDNA polymerase (Invitrogen). The amplified fragment (375 bp) was cloned into the AdEasy-1/Shuttle backbone (named as Ad.miR-223), similar to our previous construction of adenoviral vectors (45). For neonatal rat cardiomyocytes, plated cells on dishes/wells were infected with Ad.miR-223 at a 10 multiplicity of infection. 24 and 48 h later cells were collected for RT-PCR or Western-blotting assays. Ad.GFP-infected cells were used as controls.

**Western Blotting Assays**—Total proteins were extracted from hearts or cultured cardiomyocytes using the Nonidet P-40 lysis buffer with protease inhibitor (cOmpleteTM, Mini) according to the manufacturer’s instructions. Protein samples (10 μg) were fractionated by SDS-PAGE and followed the procedures as described in detail elsewhere (46). Primary antibodies against Acvr2a, FBXW7, and IGF-1R were purchased from Sigma; primary antibodies against B1-integrin, Hif-1α, Postn, Egln3, and RXFP1, C/EBPβ were purchased from Santa Cruz; primary antibodies against total Akt and Thr(P)-308 and Ser(P)-473 were obtained from Cell Signaling. GAPDH antibody (Cell Signaling) was used as a loading control. The bands were quantified using Multilmage II (Alpha Innotech) and ImageJ. The relative target protein levels were normalized to GAPDH. All antibodies (catalogue number and dilutions) used in this study are listed in supplemental Table S2.

**Measurement of Neonatal Rat Cardiomyocyte Size**—Neonatal cardiomyocytes were isolated and treated as described above. 48 h after infection cells were washed once with PBS and fixed with paraformaldehyde for 20 min at 4 °C followed by permeabilization with PBS-T solution (0.1% Triton X-100) for 4 °C to 10 min. After washing and blocking with PBS-B (4% BSA) solution at 37 °C for 30 min, cells were incubated with mouse anti-α-actinin antibody (Sigma, 1:500 dilution in PBS with 1% BSA) at 4 °C overnight. Cells were washed and stained with Alexa Fluor 594 goat anti-mouse IgG (Invitrogen, 1:500) at room temperature for 1 h, then cells were imaged using the confocal microscope and the cell size was analyzed with ImageJ.

**MK2206 Treatment**—Neonatal cardiomyocytes were cultured for 24 h before any treatment. MK2206 (Santa Cruz Biotechnology) were added to DMEM medium to a final concentration of 10 nM without FBS and then incubated for 1 h followed by adenovirus treatment as described above. One hour after adenovirus transfection, full DMEM medium (2% FBS and 1% antibiotics) was used for a 48-h cell culture. In the end, cells were fixed and stained with α-actinin antibody for measurement of the cell size as described above.

**Luciferase Reporter Assay for Validation of miR-223 Targets**—For luciferase reporter experiments, a 3’-UTR segment of Acvr2a or FBXW7 and their respective mutants were amplified by a footprint two-step PCR as described previously (45). These PCR fragments were inserted into the pMIR-
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REPORT™ luciferase miRNA expression reporter vector (Ambion, Inc.) at sites of Spel and HindIII followed by sequence validation. H9c2 cells were co-transfected in 12-well plates using DharmaFECT Duo Transfection Reagent (Thermo Fisher Scientific Inc) according to the protocol of the manufacturer, with 0.4 μg of the 3′-UTR luciferase reporter vector and 0.08 μg of the control vector pMIR-β-gal (Ambion). For each well, 100 nm mimic miR-223 or mimic miR control (Thermo Fisher Scientific Inc) was used. Cell lysates were prepared 48-h later. Luciferase activity was measured using a Monolight 3010 luminometer (Pharminen) and expressed as relative light units using a luciferase assay kit (Promega). β-Galactosidase activity was measured with a commercially available kit (Promega). 3′-UTR activity of each construct was expressed as the ratio of luciferase/β-galactosidase activity. All transfections were performed in triplicate from three independent experiments.

Mouse Treadmill Exercise Training—To induce cardiac hypertrophy, male WT mice (age of 7–8 weeks) were acclimatized to treadmill training twice a day for 2 days (day 1: non-moving treadmill for 5 min + 5 min at 5 m/min + 5 min at 10 m/min; day 2: 5 min for 5 min + 10 min for 5 min + 15 min for 5 min). One day after acclimation, the mice were subjected to exercise training that began with 5 min at 10 m/min followed by an increase of 1 m/min every minute to up to 25 m/min. Total duration of each exercise bout was 1 h or when the mice reached exhaustion (unresponsive to stimuli for 10s). Mice were trained 5 days a week for 4 weeks, heart samples were collected at the end of weeks 1, 2, and 4. Heart weight to body weight ratio was calculated (47). To compare the performance between miR-223 TG and WT mice, age-matched mice were acclimatized and subjected to treadmill exercise training. The same protocol was applied as above except the speed was increased by 2 m/min every minute to up to 30 m/min, and total duration was recorded when the mice reached exhaustion (unresponsive to stimuli for 10s) (47).

Statistical Analysis—Data were expressed as the means ± S.E. Significance was determined by Student’s t test, one- or two-way analysis of variance where appropriate to determine differences within groups. A p < 0.05 was considered statistically significant.

Author Contributions—L. Y., Y. L., and X. W. designed and performed the experiments and analyzed the data. X. M., D. Q., J. P., S. A., and K. E. performed the experiments. W. H. and Y. W. performed echocardiography analysis. T. P., M. S., and Z.-Q. Z. contributed reagents and reviewed and edited manuscript. M. N. and J. L. provided the equipment and guidance on treadmill training experiments. G.-C. F. conceived and designed the experiments, analyzed the data, and wrote the article. G.-C. F. is the guarantor of this work and, as such, had full access to all the data in this study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References
1. Maillet, M., van Berlo, J. H., and Molkentin, J. D. (2013) Molecular basis of physiological heart growth: fundamental concepts and new players. Nat. Rev. Mol. Cell Biol. 14, 38–48
2. Bernardo, B. C., Weeks, K. L., Pretorius, L., and McMullen, J. R. (2010) Molecular distinction between physiological and pathological cardiac hypertrophy: experimental findings and therapeutic strategies. Pharmacol. Ther. 128, 191–227
3. Bartel, D. P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281–297
4. Zhu, H., and Fan, G.-C. (2012) Role of microRNAs in the reperfu- sed myocardium towards post-infarct remodelling. Cardiovasc. Res. 94, 284–292
5. Da Costa Martins, P. A., and De Windt, L. I. (2012) MicroRNAs in control of cardiac hypertrophy. Cardiovasc. Res. 93, 563–572
6. Orenes-Piñero, E., Montoro-García, S., Patel, J. V., Valdés, M., Marín, F., and Lip, G. Y. (2013) Role of microRNAs in cardiac remodeling: new insights and future perspectives. Int. J. Cardiol. 167, 1651–1659
7. Fernandes, T., Baraúna, V. G., Negrão, C. E., Phillips, M. I., and Oliveira, E. M. (2015) Aerobic exercise training promotes physiological cardiac remodeling involving a set of microRNAs. Am. J. Physiol. Heart Circ. Physiol. 309, H543–H552
8. Ramasamy, S., Velmurugan, G., Shanmugha Rajan, K., Ramprashath, T., and Kalpana, K. (2015) microRNAs with apoptosis regulating potential are differentially expressed in chronic exercise-induced physiologically hypertrophied hearts. PLoS ONE 10, e0121401
9. Martinelli, N. C., Cohen, C. R., Santos, K. G., Castro, M. A., Biolo, A., Frick, L., Silvillo, D., Lopes, A., Schneider, S., and Andrades, M. E. (2014) An analysis of the global expression of microRNAs in an experimental model of physiological left ventricular hypertrophy. PLoS ONE 9, e93271
10. Ma, Z., Qi, J., Meng, S., Wen, B., and Zhang, J. (2013) Swimming exercise training-induced left ventricular hypertrophy involves microRNAs and sympathetic regulation of the PI3K/AKT/mTOR signaling pathway. Eur J Appl Physiol. 113, 2473–2486
11. Chen, C.-Z., Li, L., Lodish, H. F., and Bartel, D. P. (2004) MicroRNAs modulate hematopoietic lineage differentiation. Science 303, 83–86
12. Taibi, F., Metzinger-Le Meuth, V., Massy, Z. A., and Metzinger, L. (2014) miR-223: an inflammatory oncomir enters the cardiovascular field. Biochim. Biophys. Acta 1842, 1001–1009
13. Haneklaus, M., Gerlic, M., O’Neill, L. A., and Masters, S. L. (2013) miR-223: infection, inflammation, and cancer. J. Intern. Med. 274, 215–226
14. Wang, X., Huang, W., Yang, Y., Wang, Y., Peng, T., Chang, J., Caldwell, C. C., Zingarelli, B., and Fan, G.-C. (2014) Loss of duplex miR-223 (Sp and Sp) aggravates myocardial depression and mortality in polymicrobial sepsis. Biochim. Biophys. Acta 1842, 701–711
15. Jia, C. Y., Li, H. H., Zhu, X. C., Dong, Y. W., Fu, D., Zhao, Q. L., Wu, W., and Wu, X. Z. (2011) miR-223 suppresses cell proliferation by targeting IGF-1R. PLoS ONE 6, e27008
16. Zhou, H., Xiao, J., Wu, N., Liu, C., Xu, I., Liu, F., and Wu, L. (2015) MicroRNA-223 regulates the differentiation and function of intestinal dendritic cells and macrophages by targeting C/EBPB. Cell Rep. 13, 1149–1160
17. Ellison, G. M., Waring, C. D., Vicenzina, C., and Torella, D. (2012) Physiological cardiac remodelling in response to endurance exercise training: cellular and molecular mechanisms. Heart. 98, 5–10
18. Ren, J., and Anversa, P. (2015) The insulin-like growth factor I system: physiological and pathophysiological implication in cardiovascular diseases associated with metabolic syndrome. Biochem. Pharmacol. 93, 409–417
19. Bostrom, P., Mann, N., Wu, J., Quintero, P. A., Plovie, E. R., Panáková, D., Gupta, R. K., Xiao, C., MacRae, C. A., and Rosenzweig, A., and Spiegelman, B. (2010) C/EBPβ controls exercise-induced cardiac growth and protects against pathological cardiac remodeling. Cell 143, 1072–1083
20. Nakajomi, S., Suzuki, Y., Namikawa, K., Kiryu-See, S., and Kiyama, H. (2003) Expression of the activating transcription factor 3 prevents c-Jun N-terminal kinase-induced neuronal death by promoting heat shock protein 27 expression and Akt activation. J. Neurosci. 23, 5187–5196
21. Cheong, M.-W., Kuo, L.-H., Cheng, Y.-N., Tsai, P.-J., Ho, L.-C., Tai, H.-C., Chiu, W.-T., Chen, S.-H., Lu, P.-J., Shan, Y. S. Chuang, L. M., and Tsai, Y. S. (2015) Loss of Egr-1 sensitizes pancreatic β-cells to palmitate-induced ER stress and apoptosis. J. Mol. Med. (Berl.) 93, 807–818
22. Yang, J., Hou, Y., Zhou, M., Wen, S., Zhou, J., Xu, L., Tang, X., Du, Y.-e., Hu, P., and Liu, M. (2016) Twist induces epithelial-mesenchymal transition and cell motility in breast cancer via ITGB1-FAK/ILK signaling axis and its associated downstream network. Int. J. Biochem. Cell Biol. 71, 62–71
miR-223 Induces Physiological Cardiac Hypertrophy

23. Yu, H., Tiegelaar, W., Lu, B., van Gilst, W. H., de Boer, R. A., Westenbrink, B. D., and Sillijé, H. H. (2013) AKIP1, a cardiac hypertrophy induced protein that stimulates cardiomyocyte growth via the Akt pathway. Int. J. Mol. Sci. 14, 21378–21393

24. Utspan, K., Sonongbua, J., Thuwajit, P., Chau-In, S., Pairojkul, C., Wongkham, S., and Thuwajit, C. (2012) Periostin activates integrin α5β1 through a PI3K/AKT-dependent pathway in invasion of cholangiocarcinoma. Int. J. Oncol. 41, 1110–1118

25. Ahmad, N., Wang, W., Nair, R., and Kapila, S. (2012) Relaxin induces matrix-metalloproteinases-9 and-13 via RXFP1: induction of MMP-9 involves the PI3K, ERK, Akt, and PKC-ζ pathways. Mol. Cell. Endocrinol. 363, 46–61

26. Taniguchi, C. M., Finger, E. C., Krieg, A. J., Wu, C., Diep, A. N., LaGory, Z., Zhang, X., Gustafsson, Å. B., and Purcell, N. H. (2015) Physiological activation of Akt by PHLPP1 deletion protects against pathological hypertrophy. J. Biol. Chem. 280, 3882–3890

27. DeBosch, B., Treskow, I., Lupu, T. S., Weinerheim, C., Kovacs, A., Courtois, M., and Muslin, A. J. (2006) Akt1 is required for physiological cardiac growth. Circulation 113, 2097–2104

28. Kemi, O. J., Ceci, M., Wisloff, U., Grimaldi, S., Gallo, P., Smith, G. L., Condorelli, G., and Ellingsen, O. (2008) Activation or inactivation of cardiac Akt/mTOR signaling diverges physiological from pathological hypertrophy. J. Cell Physiol. 214, 316–321

29. Yeves, A. M., Villa-Abrille, M. C., Pérez, N. G., Medina, A. J., Escudero, E. M., and Ennis, I. L. (2014) Physiological cardiac hypertrophy: Critical role of Akt in the prevention of NHE-1 hyperactivity. J. Mol. Cell. Cardiol. 76, 186–195

30. Moc, C., Taylor, A. E., Chesini, G. P., Zambrano, C. M., Barlow, M. S., Zhang, X., Gustafsson, Å. B., and Purcell, N. H. (2015) Physiological activation of Akt by PHLPP1 deletion protects against pathological hypertrophy. Cardiovasc. Res. 105, 160–170

31. Flügel, D., Görlich, A., and Kietzmann, T. (2012) GSK-3β regulates cell growth, migration, and angiogenesis via Fbw7 and USP28-dependent degradation of HIF-1α. Blood 119, 1292–1301

32. Cassavaugh, J. M., Hale, S. A., Wellman, T. L., Howe, A. K., Wong, C., and Lounsbury, K. M. (2011) Negative regulation of HIF-1α by an FBW7-mediated degradation pathway during hypoxia. J. Cell. Biochem. 112, 3882–3890

33. Watanabe, T., Yasse, A., and Tanaka, E. (2014) Hypoxia-inducible factor-1α is required for transforming growth factor-β1-induced type I collagen, peristin and α-smooth muscle actin expression in human periodontal ligament cells. Arch. Oral Biol. 59, 595–600

34. Escribèse, M. M., Sierra-Filardi, E., Nieto, C., Sánchez-Torres, C., Matsuyama, T., Calderon-Goñez, E., Vega, M. A., Salas, A., and Sánchez-Mateos, P. (2012) The prolyl hydroxylase PHD3 identifies proinflammatory macrophages and its expression is regulated by activin A. J. Immunol. 189, 1946–1954

35. Appelhoff, R. J., Tian, Y.-M., Raval, R. R., Turley, H., Harris, A. L., Pugh, C. W., Ratcliffe, P. J., and Gleadle, J. M. (2004) Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. J. Biol. Chem. 279, 38465–38465

36. Riddle, R. C., Leslie, J. M., Gross, T. S., and Clemens, T. L. (2011) Hypoxia-inducible factor-1α protein negatively regulates load-induced bone formation. J. Biol. Chem. 286, 44449–44456

37. Shi, L., Fisslthaler, B., Zippel, N., Frömel, T., Hu, J., Elghazawy, A., Heide, H., Poppe, R., and Fleming, I. (2013) MicroRNA-223 antagonizes angiogenesis by targeting β1 integrin and preventing growth factor signaling in endothelial cells. Circ. Res. 113, 1320–1330

38. Velling, T., Nilsson, S., Jannasch, A., and Johansson, S. (2004) β1-Integrins induce phosphorylation of Akt on serine 473 independently of focal adhesion kinase and Src family kinases. EMBO Rep. 5, 901–905

39. Thackaberry, E. A., Gabaldon, D. M., Walker, M. K., and Smith, S. M. (2002) Aroyl hydrocarbon receptor null mice develop cardiac hypertrophy and increased hypoxia-inducible factor-1α in the absence of cardiac hypoxia. Cardiovasc. Toxicol. 2, 263–274

40. Montano, M. M., Desjardins, C. L., Doughman, Y. Q., Hsieh, Y. H., Yu, Y., Bensinger, H. M., Wang, C., Stelzer, J. E., Dick, T. E., Hoit, B. D., Chandler, M. P., Yu, X., and Watanabe, M. (2013) Inducible re-expression of HEXIM1 causes physiological cardiac hypertrophy in the adult mouse. Cardiovasc. Res. 99, 74–82

41. Chu, W., Wan, L., Zhao, D., Qu, X., Cai, F., Huo, R., Wang, N., Zhu, J., Zhang, C., Zheng, F., Cai, R., Dong, D., Lu, Y., and Yang, B. (2012) Mild hypoxia-induced cardiomyocyte hypertrophy via up-regulation of HIF-1α-mediated TRPC signalling. J. Cell. Mol. Med. 16, 2022–2034

42. Zolk, O., Solbach T. F., Eschenhagen T., Weidemann A., Fromm M. F. (2008) Activation of negative regulators of the hypoxia-inducible factor (HIF) pathway in human end-stage heart failure. Biochem. Biophys. Res. Commun. 376, 315–320

43. Wang, Y.-S., Zhou, J., Hong, K., Cheng, X.-S., and Li, Y.-G. (2015) MicroRNA-223 Displays a protective role against cardiomyocyte hypertrophy by targeting cardiac troponin I-interacting kinase. Cell Physiol. Biochem. 35, 1546–1556

44. Wang, X., Wang, J., Su, M., Wang, C., Chen, J., Wang, H., Song, L., Zou, Y., Zhang, L., and Zhang, Y. (2013) TNNI3K, a cardiac-specific kinase, promotes physiological cardiac hypertrophy in transgenic mice. PLoS ONE 6, e58570

45. Wang, X., Zhang, X., Ren, X.-P., Chen, J., Liu, H., Yang, J., Medvedovic, M., Hu, Z., and Fan, G.-C. (2010) MicroRNA-494 targeting both proapoptotic and antiapoptotic proteins protects against ischemia/reperfusion-induced cardiac injury. Circulation 122, 1308–1318

46. Fan, G.-C., Gregory, K. N., Zhao, W., Park, W. J., and Kranias, E. G. (2004) Regulation of myocardial function by histidine-rich, calcium-binding protein. Am. J. Physiol. Heart Circ. Physiol. 287, H1705–H1711

47. Shettigar, V., Zhang, B., Little, S. C., Salhi, H. E., Hansen, B. J., Li, N., Zhang, J., Roof, S. R., Ho, H. T., Brunello, L., Lerch, J. K., Weisleder, N., Fedorov, V. V., Accornero, F., Rafael-Fortney, J. A., Gyorke, S., Janssen, P. M., Biesiadecki, B. J., Ziolo, M. T., Davis, J. P. (2016) Rationally engineered troponin C modulates in vivo cardiac function and performance in health and disease. Nat. Commun. 7, 10794