MicroRNA-451 Exacerbates Lipotoxicity in Cardiac Myocytes and High-Fat Diet-Induced Cardiac Hypertrophy in Mice Through Suppression of the LKB1/AMPK Pathway

Yasuhide Kuwabara, Takahiro Horie, Osamu Baba, Shin Watanabe, Masataka Nishiga, Shunsuke Usami, Masayasu Izuhara, Tetsushi Nakao, Tomohiro Nishino, Kinya Otsu, Toru Kita, Takeshi Kimura, Koh Ono

Rationale: In some patients with type 2 diabetes mellitus (DM) without hypertension, cardiac hypertrophy and attenuated cardiac function are observed, and this insulin is termed diabetic cardiomyopathy. To date, microRNA (miRNAs or miR) functions in diabetic cardiomyopathy remain to be elucidated.

Objective: To clarify the functions of miRNAs involved in diabetic cardiomyopathy caused by type 2 DM.

Methods and Results: C57BL/6 mice were fed a high-fat diet (HFD) for 20 weeks, which induced obesity and type 2 DM. miRNA microarray analyses and real-time polymerase chain reaction revealed that miR-451 levels were significantly increased in the type 2 DM mouse hearts. Because excess supply of saturated fatty acids is a cause of diabetic cardiomyopathy, we stimulated neonatal rat cardiac myocytes with palmitic acid and confirmed that miR-451 expression was increased in a dose- and time-dependent manner. Loss of miR-451 function ameliorated palmitate-induced lipotoxicity in neonatal rat cardiac myocytes. Calcium-binding protein 39 (Cab39) is a scaffold protein of liver kinase B1 (LKB1), an upstream kinase of AMP-activated protein kinase (AMPK). Cab39 was a direct target of miR-451 in neonatal rat cardiac myocytes and Cab39 overexpression rescued the lipotoxicity. To clarify miR-451 functions in vivo, we generated cardiomyocyte-specific miR-451 knockout mice. HFD-induced cardiac hypertrophy and contractile reserves were ameliorated in cardiomyocyte-specific miR-451 knockout mice compared with control mice. Protein levels of Cab39 and phosphorylated AMPK were increased and phosphorylated mammalian target of rapamycin (mTOR) was reduced in cardiomyocyte-specific miR-451 knockout mouse hearts compared with control mouse hearts.

Conclusions: Our results demonstrate that miR-451 is involved in diabetic cardiomyopathy through suppression of the LKB1/AMPK pathway. (Circ Res. 2015;116:279-288. DOI: 10.1161/CIRCRESAHA.116.304707.)

Key Words: AMP-activated protein kinase ■ cardiomegaly ■ microRNAs ■ type 2 diabetes mellitus

Obesity is a major burden worldwide, especially in developed countries. Obesity and comorbid type 2 diabetes mellitus (DM) induce structural and functional changes in the heart. Cardiac hypertrophy and decreased cardiac function are recognized in some patients with obesity and type 2 DM without hypertension, coronary artery disease, or valvular heart disease. This cardiac insult is termed diabetic cardiomyopathy. Because cardiac hypertrophy and dysfunction result in cardiac death, uncovering the mechanisms is important research goal.

Several mechanisms that elicit diabetic cardiomyopathy have been proposed. Cytokines produced by the expanded adipose tissue, such as leptin and resistin, and triglyceride accumulation induce cardiac hypertrophy. Another proposed mechanism is excess supply of saturated fatty acids (FAs), such as palmitic acid. Unoxidized FAs in cardiac myocytes produce oxidative stress, mitochondrial dysfunction, and ceramide accumulation, resulting in lipotoxicity.3,4

Editorial, see p 229

AMP-activated protein kinase (AMPK) is a major cellular sensor of energy availability. Liver kinase B1 (LKB1) and Ca2+/calmodulin-dependent protein kinase kinase β can act as major upstream kinases of AMPK in mammalian cells and phosphorylate AMPK at Thr172.5 LKB1 forms a heterotrimeric complex with ste20-related adaptor and calcium-binding

Original received July 1, 2014; revision received October 23, 2014; accepted October 30, 2014. In September, 2014, the average time from submission to first decision for all original research papers submitted to Circulation Research was 14.29 days.

From the Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan (Y.K., T.H., O.B., S.W., M.N., S.U., M.I., T. Nakao, T. Nishino, T. Kimura, K. Oono); Cardiovascular Division, The James Black Centre, King’s College London, London, United Kingdom (K. Otsu); and Department of Cardiovascular Medicine, Kobe City Medical Center General Hospital, Kobe, Hyogo Prefecture, Japan (T. Kita.).

The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.116.304707/-/DC1.

Correspondence to Koh Oono, MD, PhD, Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, 54 Shogoin-kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. E-mail kohono@kuhp.kyoto-u.ac.jp

© 2014 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.116.304707

279
protein 39 (Cab39, also known as MO25α). Although several lines of evidence indicate that AMPK phosphorylation is attenuated in diet-induced obesity (DIO) mouse hearts, the mechanisms by which AMPK phosphorylation is decreased are not fully understood.

MicroRNAs (miRNAs or miRs) are a large class of small noncoding RNAs. miRNAs suppress the translation of a target mRNA depending on the complementarity between the 5′ side seed sequence of a miRNA and the 3′ untranslated region (3′UTR) of the target mRNA. Although several reports indicate that miRNAs are involved in diabetic cardiomyopathy in a type 1 DM model of streptozotocin-induced diabetic mice, miRNA functions in diabetic hearts induced especially by type 2 DM remain to be elucidated.

In the present study, we used DIO mice to identify differentially regulated miRNAs in hearts. One of these miRNAs, miR-451, was upregulated in the hearts, and palmitic acid stimulation increased miR-451 levels in neonatal rat cardiac myocytes (NRCMs). miR-451 directly targeted Cab39 in NRCMs, and loss of miR-451 function partly rescued lipotoxicity in vitro. Furthermore, we revealed that high-fat diet (HFD)–induced cardiac hypertrophy was ameliorated in cardiomyocyte-specific miR-451 knockout (miR-451 cKO) mice. Finally, we showed that AMPK phosphorylation was increased and mammalian target of rapamycin (mTOR) phosphorylation was decreased in the HFD-fed miR-451 cKO mice compared with the HFD-fed control mice. These data strongly suggest that cardiac-specific inhibition of miR-451 is a strategy for treating diabetic cardiomyopathy.

### Methods

Detailed methods are provided in the Online Data Supplement.

### Results

#### miRNA-451 Expression Is Significantly Increased in DIO Mouse Hearts

To clarify miRNA expression changes in DIO mouse hearts, we used HFD-fed C57BL/6 mice. This is a well-established obesity and type 2 DM model, and the C57BL/6 mice become obese depending on the duration of HFD feeding, as shown in Figure 1A. In line with our and other reports, liver weights approximately doubled, and fasting blood sugar concentrations increased significantly by 1.5-fold after feeding 45 kcal% fat–containing HFD for 20 weeks (Figure 1B and 1C). These data suggest that this mouse model had marked insulin resistance and fatty liver and mimicked obesity with type 2 DM in humans.

In the present study, we used DIO mice to identify differentially regulated miRNAs in hearts. One of these miRNAs, miR-451, was upregulated in the hearts, and palmitic acid stimulation increased miR-451 levels in neonatal rat cardiac myocytes (NRCMs). miR-451 directly targeted Cab39 in NRCMs, and loss of miR-451 function partly rescued lipotoxicity in vitro. Furthermore, we revealed that high-fat diet (HFD)–induced cardiac hypertrophy was ameliorated in cardiomyocyte-specific miR-451 knockout (miR-451 cKO) mice. Finally, we showed that AMPK phosphorylation was increased and mammalian target of rapamycin (mTOR) phosphorylation was decreased in the HFD-fed miR-451 cKO mice compared with the HFD-fed control mice. These data strongly suggest that cardiac-specific inhibition of miR-451 is a strategy for treating diabetic cardiomyopathy.

#### Figure 1. Cardiac microRNA (miR)-451 is upregulated in high-fat diet (HFD)-induced obese mice.

A. Body weight changes in mice fed normal chow (NC) containing 10 kcal% fat or HFD containing 45 kcal% fat; 8 weeks: n=4; 20 weeks: n=5. B and C. Liver weights (B) and fasting blood sugar (FBS) concentrations (C) in mice fed NC or HFD for 20 weeks, n=4. D. miRNA expression profiling was performed using miRNA microarrays. The red diagonal line indicates the unchanged line. Two blue lines represent 2-fold changes. E. miR-451 expression levels in obese mice hearts fed NC or HFD for 8 or 20 weeks, n=4 to 5. F. Plasma concentrations of palmitic acid in NC- or HFD-fed mice. n=5. Data are presented as mean±SEM. *P<0.05; **P<0.01; ***P<0.001.
miR-451 Expression Is Induced by Palmitic Acid Stimulation, and Elevated miR-451 Causes Cell Toxicity in NRCMs

miR-451 is expressed abundantly in blood cells, such as erythrocytes and macrophages. Therefore, we sought to isolate cardiac myocytes and cardiac fibroblasts and determine whether miR-451 is expressed in these cells. Cardiac myocytes do not have cell surface–specific markers, and a report indicated that cardiac myocytes have higher mitochondrial content than non–cardiac myocytes and could be purified by mitochondria-labeled dyes. To mark cardiac fibroblasts, we used allophycocyanin-conjugated Thy-1.2 antibody. As shown in Online Figure IIA, isolated cells from neonatal mouse ventricles were analyzed and sorted by fluorescent-activated cell sorting. In line with a previous study, the mRNA levels of brain natriuretic peptide were significantly higher in the populations with high Mitotracker Green fluorescent content and negative Thy-1.2 (Mitohigh/Thy-1.2neg) than in populations with low Mitotracker Green fluorescent content and positive Thy-1.2 (Mitolow/Thy-1.2pos; Online Figure IIIB), indicating that the Mitohigh/Thy-1.2neg populations contain many cardiac myocytes. Whereas the mRNA levels of collagen type 1 α1 were significantly higher in Mitohigh/Thy-1.2pos populations than in Mitohigh/Thy-1.2neg populations (Online Figure IIC), indicating that cardiac fibroblasts comprise Mitolow/Thy-1.2pos populations. miR-451 levels were significantly higher in cardiac myocytes than in cardiac fibroblasts (Online Figure IIID), which suggested that miR-451 was expressed in cardiac myocytes. In addition, we tried to compare the miR-451 levels in cardiac myocytes and erythrocytes. We collected peripheral blood from a neonatal mouse, and fluorescent-activated cell sorting sorted erythrocytes that were labeled with an anti-Ter119 antibody, a marker of erythrocytes (Online Figure IVA). Because the total RNA mass extracted from erythrocytes was extremely low, and the U6 level was also lower than that in cardiac myocytes, we did not think that U6 was suitable for use as an internal control in erythrocytes. Thus, the miR-451 level per cell was determined from a standard curve for artificial miR-451, the total RNA mass obtained, and the numbers of cells collected by fluorescent-activated cell sorting. The miR-451 levels in cardiac myocytes were ≈40-fold higher than those in erythrocytes (Online Figure IVB). These data were consistent with those in a previous report. Thus, we attempted to investigate miR-451 functions in cardiac myocytes.

We next stimulated NRCMs with palmitate to determine whether this induced miR-451 expression in cardiac myocytes as both albumin-bound FAs and FAs incorporated within lipoproteins are used as fuel by cardiac myocytes, and the total palmitic acid concentration was significantly higher in the plasma of HFD-fed mice than in that of NC-fed mice (Figure 1F). As shown in Figure 2A and 2B, palmitate stimulation resulted in elevated miR-451 levels in a dose- and time-dependent manner. We also measured the expression levels of microRNAs expressed abundantly in muscle cells, including miR-133a, miR-208a, and miR-499, but we did not observe any significant increases in their levels (Figure 2B). In addition, we assessed the effects of oleic acid, an unsaturated FA, on miR-451 expression in NRCMs. Our data indicated that the total oleic acid level in plasma was approximately one half of the total palmitic acid level (data not shown). Thus, we stimulated NRCMs with oleic acid at 250 μmol/L. Although miR-451 was also upregulated by oleic acid, this increase was modest; and the miR-451 upregulation by oleic acid was significantly lower than that by palmitic acid at 250 μmol/L (Online Figure VA).

We then overexpressed miR-451 in NRCMs using a lentiviral system to elucidate miR-451 functions. Although the miR-451 level was high in NRCMs infected with a miR-451 expression vector (Online Figure VI), cell survival was significantly reduced after miR-451 overexpression (Figure 2C). Palmitate stimulation caused miR-451 upregulation in NRCMs, and ectopic expression of miR-451 induced cell toxicity; therefore, we hypothesized that miR-451 knockdown would ameliorate palmitate-induced cell toxicity. miR-451 decoys were induced in NRCMs, and these NRCMs were stimulated with palmitate. We found that cell injury measured by the lactate dehydrogenase assay was partly rescued (Figure 2D). To clarify the induction of miR-451 and lipotoxicity in NRCMs at physiological ratios of palmitate to albumin, the albumin concentration was fixed at 550 μmol/L, and NRCMs were stimulated with various concentrations of palmitate. miR-451 levels were significantly upregulated at palmitate to albumin ratios of 1:2 and 1:1 (Figure 2E). Palmitate-induced lipotoxicity was observed at a ratio of 1:2, and the lipotoxicity was significantly ameliorated when miR-451 was knocked down (Figure 2F). These data imply that palmitate induces miR-451, and the elevation in miR-451 levels in part exacerbates lipotoxicity in NRCMs.

Cab39 Is a Direct Target of miR-451 in NRCMs and Mice

We next sought to identify the target of miR-451 in cardiac myocytes. To date, macrophage inhibitory factor (MIF), Cab39, 14-3-3ζ, CUG triplet repeat-binding protein 2, and Ras-related C3 botulinum toxin substrate 1 have been identified as potential targets of miR-451 in other cells. To determine whether Cab39 is a direct target of miR-451 in cardiac myocytes, we performed luciferase reporter assays in NRCMs. To validate luciferase reporter assays, we first measured luciferase activities in reporter plasmids containing Cab39 3′-untranslated region (3′-UTR) subjected to miR-451 overexpression. As shown in Figure 3A, luciferase activity was significantly lower in the Cab39 3′-UTR reporter plasmid than in a negative control reporter. This result suggests that Cab39 3′-UTR is targeted by miR-451. We also observed that luciferase activity was not significantly upregulated by palmitate, which is consistent with the fact that palmitate-induced miR-451 is not upregulated by oleic acid, as mentioned above (Figure 2D).
identified as direct miR-451 targets. Of these bona fide targets, we focused on 2 proteins that have cell protective functions, MIF\(^{22}\) and Cab39\(^{22}\) because our data indicate that miR-451 knockdown ameliorates palmitate-induced lipotoxicity. To examine whether miR-451 can directly bind to the 3′UTRs of MIF and Cab39, wild-type (WT) or mutated (Mut) miR-451 luciferase plasmids were cotransfected with the miR-451 overexpression plasmid. Western blotting analysis revealed that miR-451 overexpression reduced MIF and Cab39 protein levels in NRCMs (Online Figure VIIIB; Figure 3B). These data indicate that MIF and Cab39 are direct targets of miR-451 in NRCMs. We also evaluated MIF and Cab39 protein levels in vivo. Although we did not observe a reduction in the MIF protein level, the Cab39 protein level in the hearts was decreased in the 45 kcal%-containing HFD-fed mice compared with the NC-fed mice (Online Figure VIIIC and Figure 3C). Thus, we focused on Cab39. To strengthen the finding that Cab39 is a target of miR-451 in NRCMs, we attempted to rescue miR-451–induced cell toxicity by Cab39 overexpression. As shown in Figure 3D, Cab39 overexpression rescued miR-451–induced cell toxicity. Furthermore, Cab39 overexpression ameliorated cell toxicity in palmitate-stimulated NRCMs (Figure 3E). Together, these data suggest that palmitic acid stimulation upregulates miR-451 expression, which reduces Cab39 levels, resulting in cell toxicity.

Cab39 is a scaffold protein of LKB1 that stabilizes the activity through the formation of a heterotrimeric complex with ste20-related adaptor in the cytoplasm.\(^{6}\) It is well established that LKB1 is an upstream kinase of AMPK. To verify the significance of the LKB1/AMPK pathway in NRCMs, we performed immunoblotting for AMPK. After palmitate stimulation, AMPK phosphorylation in NRCMs was significantly decreased (Figure 3F). To determine the specificity of palmitic acid on AMPK phosphorylation, we stimulated NRCMs with oleic acid. Suppression of AMPK phosphorylation was not observed even with oleic acid at 500 μmol/L (Online Figure VB and VC). Furthermore, we evaluated Cab39 protein levels and AMPK phosphorylation in vivo. Western blotting analysis revealed that AMPK phosphorylation was reduced in the hearts of mice fed 45 kcal% fat–containing HFD for 20 weeks in association with the decrease of Cab39 (Figure 3C).

**miR-451 cKO Mice Attenuated HFD-Induced Cardiac Hypertrophy**

We required miR-451 knockout mice to reveal miR-451 functions in the HFD-fed mouse hearts. miR-451 is transcribed with miR-144 as a bicistronic transcript. O’Carroll and colleagues\(^{14}\) reported that miR-144 and miR-451 double knockout (miR-144/451−/−) mice show ineffective erythropoiesis and mild anemia, and the phenotype of miR-451 single knockout mice was fairly distinguishable from that of the miR-144/451−/− mice.\(^{15}\) We thought that anemia induced by miR-451 deletion could affect the phenotype in the hearts of the HFD-fed mice. Thus, we crossed the α-myosin heavy chain (αMHC)-Cre transgenic mice and floxed miR-451 mice to generate the αMHC-Cre;miR-451\(^{−/−}\) or miR-451 cKO mice. To eliminate the direct effect of Cre-recombinase in cardiac myocytes, αMHC-Cre;miR-451\(^{−/−}\) mice were used as the control mice. We attempted to validate the manipulation of the miR-451 locus in the genome using PCR analysis. As shown in Online Figure VIII, WT miR-451 and floxed miR-451 could be detected as bands of 393 and 580 nucleotides, respectively. When we performed PCR using DNA extracted from the miR-451 cKO mouse hearts as a template, we observed ablated
Figure 3. Calcium-binding protein 39 (Cab39) is a direct target of microRNA (miR)-451 in cardiomyocytes, and lipotoxicity is partially rescued by Cab39 overexpression. A, 293T cells were transfected with an overexpression plasmid for miR-control (miR-ctrl) or miR-451 and a plasmid encoding luciferase wild-type (WT) Cab39 3′ untranslated region (UTR) or mutated (Mut) Cab39 3′UTR lacking the miR-451-binding site. Luciferase activities were determined 24 hours later, n=4. B, Western blotting for Cab39 in neonatal rat cardiac myocytes (NRCMs) infected with the miR-ctrl or miR-451 vector. C, Western blotting of Cab39, phosphorylated AMP-activated protein kinase (p-AMPK), and total AMPK (t-AMPK) in the hearts of mice fed normal chow or 45 kcal% fat–containing high-fat diet. GAPDH: loading control. D, Cell viability in NRCMs infected with the miR-ctrl or miR-451 vectors and lacZ or Cab39 expression vectors, n=3 to 5. E, Cell injury ratio of Cab39-overexpressing NRCMs. NRCMs were stimulated with palmitate for 48 hours, n=4. F, Left, Representative images of Western blotting for p-AMPK and t-AMPK in NRCMs after palmitate stimulation. Right, Quantification of p-AMPK by densitometry analysis, n=4. Data are presented as mean±SEM. *P<0.05; **P<0.01; ***P<0.001. AU indicates arbitrary unit.

miR-451 as an ≈250-nucleotide long band. A nonablated band was also observed after PCR analysis of heart DNA from the miR-451 cKO mice. We assumed the reason was that floxed miR-451 remained in non–cardiac myocytes.

The αMHC-Cre;miR-451fl/fl and αMHC-Cre;miR-451+/+ mice started feeding on NC or 60 kcal% fat–containing HFD when they were 8 weeks old and were fed on their respective diets until the age of 28 weeks. No differences were found in body weights, liver weights, or fasting blood sugar concentrations between the NC- or HFD-fed control and miR-451 cKO mice (Figure 4A; Online Figure IXA and IXB). The miR-451 levels showed a clear declining trend in the NC-fed miR-451 cKO mice (Figure 4B), and the levels in the hearts were significantly increased in the HFD-fed control mice compared with the NC-fed control mice. As expected, this miR-451 up-regulation in hearts was not observed in the HFD-fed miR-451 cKO mice.

Because it was reported that GATA4 regulated miR-451 expression in cardiac myocytes,25 we performed Western blotting for GATA4 to identify the mechanism by which miR-451 was upregulated in HFD-fed mouse hearts. As shown in Online Figure XA and XB, protein levels of GATA4 were significantly increased in HFD-fed mouse hearts. This upregulated GATA4 may have induced miR-451 in HFD-fed mouse hearts.

In agreement with a previous report,13 the heart weight and the ratio of heart weight-to-tibial length were significantly increased in the HFD-fed control mice compared with the NC-fed control mice (Online Figure IXC; Figure 4C). The ratio of heart weight-to-tibial length was significantly decreased in the HFD-fed miR-451 cKO mice compared with the HFD-fed control mice (Figure 4C). Transthoracic echocardiography also revealed that left ventricular wall thickness was significantly reduced in the HFD-fed miR-451 cKO mice compared with the HFD-fed control mice (Figure 4D; Online Figure IXD), although cardiac functions were not different between the HFD-fed control and miR-451 cKO mice at steady state (data not shown). Representative lectin staining of cross-sections of ventricles is shown in Figure 4E. Quantification of lectin staining revealed that HFD-induced cardiac hypertrophy was noticeably diminished in the miR-451 cKO mice compared with the control mice (Figure 4F). Because miR-451 overexpression caused cell toxicity in NRCMs, we
performed Western blotting for cleaved caspase-3 and single strand DNA staining to evaluate cell toxicity in vivo. We did not detect cleaved caspase-3 (Online Figure XIA) and there was no difference in single strand DNA–positive cardiac myocytes (Online Figure XIB–XIE). To evaluate fibrosis, we also performed picrosirius red staining and quantitative reverse transcription PCR. However, we did not observe a significant difference in fibrosis area and collagen type 1 α1 mRNA levels between the HFD-fed miR-451 cKO and control mice (Online Figure XII). These data suggest that HFD-induced cardiac hypertrophy is ameliorated in the miR-451 cKO mice.

AMPK Phosphorylation in the Heart Is Strengthened in HFD-Fed miR-451 cKO Mice

Western blotting analysis was performed to determine the signaling pathways activated in the control and miR-451 cKO mouse hearts. As expected, Cab39 protein levels in the hearts were significantly increased in the HFD-fed miR-451 cKO mice compared with the HFD-fed control mice (Figure 5A). AMPK phosphorylation at Thr172, a downstream target of the Cab39/LKB1 complex, was also substantially enhanced in the hearts of the HFD-fed miR-451 cKO mice compared with the HFD-fed control mice (Figure 5B). Next, we evaluated the phosphorylation of mTOR, a regulator of protein synthesis,26 and S6 ribosomal protein, which is a substrate of S6 kinase—a downstream target of mTOR. mTOR phosphorylation and S6 phosphorylation were significantly suppressed in the HFD-fed miR-451 cKO mice compared with the HFD-fed control mice (Figure 5C and 5D). Because AMPK suppresses mTOR signaling,26 these data suggested that HFD-induced cardiac hypertrophy was attenuated, at least in part, via the
upregulation of the LKB1/AMPK signaling pathway in the miR-451 cKO mice.

Accumulation of Lipid Intermediates and Reactive Oxygen Species Production
Previous reports have shown that several lipid intermediates, such as triglyceride and ceramide, may be cell toxic for cardiac myocytes. Thus, we evaluated the accumulation of neutral lipid using BODIPY 505/515 staining. We detected small green droplets in cardiac myocytes. However, we did not observe any differences in the accumulation between HFD-fed control mouse hearts and HFD-fed cKO mouse hearts (Online Figure XIIIA–XIIID). We next performed immunohistochemistry for ceramide and quantified ceramide content in the hearts. Long-term HFD feeding resulted in ceramide accumulation in control mouse hearts, whereas this accumulation was reduced in miR-451 cKO mouse hearts (Figure 6A–6C). Furthermore, we attempted to analyze the levels of reactive oxygen species (ROS). It has been reported that ROS peroxidize ω-6 unsaturated FAs and generate 4-hydroxy-2-nonenal, an aldehyde. The 4-hydroxy-2-nonenal binds to proteins and composes 4-hydroxy-2-nonenal adducts. Interestingly, Western blotting for 4-hydroxy-2-nonenal adducts revealed that ROS levels were higher in HFD-fed miR-451 cKO mouse hearts than in HFD-fed control mouse hearts (Figure 6D). We also performed dihydroethidium staining. As shown in Figure 6E and 6F, dihydroethidium fluorescence intensity was significantly increased in HFD-fed miR-451 cKO mice compared with HFD-fed control mice. These data suggest that ROS levels are significantly increased in HFD-fed miR-451 cKO mouse hearts compared with HFD-fed control mouse hearts.

Functional Changes in HFD-Fed miR-451 cKO Mouse Hearts
Finally, we sought to reveal the functional changes in the hearts. Cardiac stress was induced by dobutamine infusion and was monitored by cardiac catheterization. We found that the contractile reserve was reduced in HFD-fed control mouse hearts.

Figure 6. Accumulation of ceramides and reactive oxygen species levels. A, Representative images of ceramide staining. White bars indicate 40 μm. B, Quantitative results for ceramide positive area. n=5 to 7. C, Ceramide content in the hearts. n=3 to 5. D, Western blotting for 4-hydroxy-2-nonenal (4-HNE) adducts in normal chow (NC)–fed or high-fat diet (HFD)–fed control (wild-type [WT]) and cardiomyocyte-specific miR-451 knockout (cKO) mouse hearts. Numbers indicate standard molecular weights. GAPDH was used as a loading control. E, Representative images of dihydroethidium (DHE) staining. White bars indicate 60 μm. F, Quantitative results for DHE fluorescence intensity. n=5 to 7 for each group. Data are presented as mean±SEM. *P<0.05; **P<0.01; ***P<0.001. AU indicates arbitrary units.
heart compared with NC-fed control mouse heart, in line with a previous report (Figure 7A), although no difference in diastolic reserve was observed (Figure 7B). The reduced contractile reserve was ameliorated, at least in part, in the HFD-fed miR-451 cKO mouse hearts, as shown in Figure 7A.

Discussion

Our data suggest that miR-451 is involved in diabetic cardiomyopathy. Only miR-451 was upregulated in our type 2 DM model mice, and this was dependent on the duration of HFD feeding. Lu et al found that miR-223 is differentially regulated in the cardiac tissue of patients with type 2 DM. To date, most researchers have used streptozotocin-induced diabetic mice to investigate miRNA involvement in diabetic cardiomyopathy. Although Shen et al used real-time PCR to validate 16 miRNAs identified by microarray analysis, the number of dysregulated miRNAs was small in DIO mouse hearts, a type 2 DM model. We considered that this was the case for 2 reasons. First, changes in miRNA expression levels associated with diabetic cardiomyopathy are dependent on the type of diabetes mellitus. Secretion defects of insulin and insulin resistance may invoke different miRNA changes in the heart. Second, the duration of HFD feeding may have been relatively short. To evaluate these possibilities, C57BL/6 mice fed HFD for a longer period or genetically manipulated mice, such as ob/ob or db/db mice, may be needed.

Because miR-144 and miR-451 are transcribed as a bicistronic transcript, it was surprising that miR-144 expression was unchanged in DIO mouse hearts. Considering our microarray results, miR-451 levels in the hearts were noticeably higher than miR-144. It was reported that miR-451, we focused on MIF and Cab39, which have cell protective functions. Our results show that the LKB1/AMPK signaling pathway plays a protective role in HFD-induced cardiac hypertrophy. Our data strongly support that HFD-induced cardiac hypertrophy is partly because of decreased Cab39 levels through attenuation of palmitate-induced lipotoxicity. Thus, to clarify the direct targets of miR-451, we focused on MIF and Cab39, which have cell protective functions. The phenotypes of the miR-144/451 double knockout mice and miR-451 single knockout mice were almost indistinguishable. These results suggest that miR-451 is functionally dominant compared with miR-144, at least in bone marrow. Furthermore, it was shown that miR-451 biogenesis was Dicer independent and required Argonaute catalytic activity. Taken together, the evidence indicates that miR-451 plays more important roles in the heart than miR-144, and post-transcriptional regulation of miR-451 expression may be altered in diabetic hearts.

Our data indicate that palmitic acid induced miR-451 upregulation in NRCMs. How is miR-451 expression regulated? Many reports showed that palmitic acid enhances nuclear factor \( \kappa B \) signaling; therefore, 1 possible mechanism is that activation of inflammation signaling, including nuclear factor \( \kappa B \), induces miR-451. Others indicated that the miR-144/451 cluster was directly regulated by GATA4. Numerous reports suggest that GATA4 plays a pivotal role in cardiac hypertrophy in vitro and in vivo; therefore, GATA4 may regulate miR-451 levels in HFD-induced hypertrophic hearts. Indeed, we observed the upregulation of GATA4 in HFD-fed mouse hearts. Recent reports demonstrated that activation of farnesoid X receptor (FXR) increased miR-144 and miR-451 levels in the liver. The nuclear receptor FXR regulates genes involved in the synthesis, secretion, and resorption of bile acids. Although abundant FXR expression is observed in the liver, intestine, kidney, and adrenal gland, bile acids are endogenous ligands for FXR, and some reports suggest that bile acid levels in serum are increased in obese or type 2 DM mice and humans. Hence, another possible mechanism is that bile acid–activated FXR induces miR-451 expression. Further studies are required to elucidate the transcription factors that regulate miR-451 expression.

Clinical data indicated that obesity and type 2 DM are linked to cardiac hypertrophy independently of blood pressure. The present study and others show that long-term feeding of HFD to C57BL/6 mice results in cardiac hypertrophy. The phenotypes of HFD-induced obesity mouse hearts were similar to those of cardiomyocyte-specific LKB1 knockout (LKB1 cKO) mouse hearts. Ikeda et al reported that the ventricular weight-to-body weight ratio was significantly increased in the LKB1 cKO mice at 12 weeks of age compared with the control mice and fibrosis markers in cardiac ventricles were not different between the LKB1 cKO mice and control mice. Our data strongly support that HFD-induced cardiac hypertrophy is partly because of decreased Cab39 levels through miR-451 upregulation. Collectively, the evidence indicates that miR-451 downregulation is a therapeutic strategy to attenuate HFD-induced cardiac hypertrophy.

Our data show that miR-451 overexpression resulted in cell toxicity, and miR-451 knockdown attenuated palmitate-induced lipotoxicity. Thus, to clarify the direct targets of miR-451, we focused on MIF and Cab39, which have cell protective functions. Our results show that the LKB1/AMPK signaling pathway plays a protective role in HFD-induced cardiac hypertrophy. These data are consistent with those of another report, which indicated that AMPK deficiency exacerbates obesity-induced cardiac hypertrophy. Also, it is reported that miR-451 levels are significantly increased in the

Figure 7. Evaluation of cardiac functional reserves. Cardiac stress was induced by continuous injection of dobutamine and cardiac functional reserves were monitored by cardiac catheterization. A. Changes in maximum (max) dP/dT. B. Changes in minimum (min) dP/dT. n=5 to 7 for each group. Data are presented as mean±SEM. *P<0.01 vs high-fat diet (HFD)–fed control mice (wild-type [WT]) at the same dobutamine dose; **P<0.05 vs HFD-fed control mice at the same dobutamine dose. cKO indicates cardiomyocyte-specific miR-451 knockout mice; and NC, normal chow.
In conclusion, miR-451 levels were markedly increased in palmitate-stimulated NRCMs and DIO mouse hearts. We found that Cab39 was a direct target of miR-451 in the heart. miR-451 knockdown partly rescued lipotoxicity in vitro, and HFD-induced cardiac hypertrophy was ameliorated in miR-451 cKO mice through the LKB1/AMPK pathway. Thus, cardiac-specific inhibition of miR-451 is a promising strategy for treating diabetic cardiomyopathy.

Acknowledgments

We thank N. Sowa for providing excellent technical assistance.

Sources of Funding

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology and Japan Society for the Promotion of Science KAKENHI Grant Nos. 25293182, 20390255, 24790753, 24890094, 24116512, and 23390211 to T. Kimura, T. Kita, T. Horie, Y. Kuwabara, and K. Ono.

Disclosures

None.

References

Increasing evidence suggests that microRNAs are involved in cardiovascular diseases. However, the role of microRNAs in the diabetic cardiomyopathy induced by obesity and type 2 diabetes mellitus is not fully understood. Using a mouse model of diet-induced obesity, we identified that miR-451 was significantly up-regulated in the heart. miR-451 was induced in cardiac myocytes stimulated with palmitic acid, and Cab39 was a direct target of miR-451 in cardiac myocytes. Palmitate-induced lipotoxicity was rescued by miR-451 knockdown in cardiac myocytes. Furthermore, we generated cardiac myocyte–specific miR-451 knockout mice and found that high-fat diet (HFD)–induced cardiac hypertrophy was attenuated in these cardiac myocyte–specific knockout mouse hearts. Finally, we found that the contractile reserve was ameliorated in HFD-fed cardiac myocyte–specific miR-451 knockout mouse hearts as compared with that in HFD-fed control mouse hearts. These findings indicate that miR-451 is involved in diabetic cardiomyopathy, and that miR-451 could be a potential therapeutic target for cardiac dysfunction associated with obesity and type 2 diabetes mellitus.
MicroRNA-451 Exacerbates Lipotoxicity in Cardiac Myocytes and High-Fat Diet-Induced Cardiac Hypertrophy in Mice Through Suppression of the LKB1/AMPK Pathway

Yasuhide Kuwabara, Takahiro Horie, Osamu Baba, Shin Watanabe, Masataka Nishiga, Shunsuke Usami, Masayasu Izuara, Tetsushi Nakao, Tomohiro Nishino, Kinya Otsu, Toru Kita, Takeshi Kimura and Koh Ono

Circ Res. 2015;116:279-288; originally published online October 31, 2014;
doi: 10.1161/CIRCRESAHA.116.304707

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/116/2/279

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2014/10/31/CIRCRESAHA.116.304707.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Supplemental Material

Detailed Methods

Mice and diets

To examine miRNA expression changes in DIO mouse hearts, C57BL/6 male mice (Charles River Laboratories) were used. Floxed miR-451 mice and α myosin heavy chain promoter-driven Cre-recombinase (αMHC-Cre) transgenic mice were kind gifts of Dónal O’Carroll1 and Kinya Otsu, respectively. The floxed miR-451 mice and αMHC-Cre transgenic mice were backcrossed to C57BL/6 mice for at least 10 generations. The primer sequences for genotyping are listed in Online Table I. Specific pathogen-free animals were maintained in the animal laboratories of Kyoto University Graduate School of Medicine. This investigation was approved by the Kyoto University Ethics Review Board. Normal chow (NC), containing 10 kcal% fat (F-2), was purchased from Funabasi Farm Company. In the present study, two types of HFD were used. D12451 (45 kcal% fat) and D12492 (60 kcal% fat) were purchased from Research Diets, Inc. All mice were grown by NC feeding until they were 8 weeks old, after which they were fed NC or HFD for additional 8 or 20 weeks. Sixteen-hour fasted mice were euthanized, and blood was collected from the inferior vena cava in a heparinized syringe to measure the fasting glucose concentration. The hearts and livers were then excised. The organs were washed immediately in cold phosphate-buffered saline (PBS) and weighed. Cut hearts were frozen in liquid nitrogen and stored at −80 °C until analysis. To evaluate plasma concentrations of palmitic acid, we collected blood as described above from mice fasted for 6–8 h. Collected blood was centrifuged at 3300 ×g for 10 min, and plasma was stored at −80 °C.
When measuring palmitic acid levels in apoB precipitated serum, blood was collected with a syringe and then transferred to a tube containing a serum separating gel (Terumo). This tube was centrifuged at 1200 ×g for 10 min. Then polyethyleneglycol (PEG) was added to a serum aliquot to deplete apoB-containing lipoproteins. To measure tibial length, the lower legs were severed and rendered in 1 mol/L NaOH at 37 °C until the tibial bones were completely visible.

**RNA extraction**

To extract total RNA, organs were homogenized in 1 mL TRIzol® reagent (Invitrogen) using a homogenizer. Total RNA from cells was also isolated using 1 mL TRIzol® reagent. The pellet of total RNA was dissolved in diethylpyrocarbonate water. The quantity and quality of total RNA were determined using a spectrophotometer (GeneQuant pro, GE Healthcare).

**Microarray analysis and quantitative real-time PCR**

Each 500 ng of total RNA extracted from 4–6 mice was mixed and analyzed using an miRNA microarray analysis system (3D-Gene™, Toray). miRNAs were quantified using TaqMan® MicroRNA Assays (Applied Biosystems) and a 7900HT Fast Real-Time PCR System (Applied Biosystems) in accordance with the manufacturer’s instructions. miRNA levels were normalized by U6 small nuclear RNA and calculated by the 2⁻ΔΔCt method. To determine the miR-451 levels in erythrocytes, we prepared a standard curve using artificial miR-451 and calculated the miR-451 level in 100 ng of total RNA. Then the miR-451 level per cell was determined based on the total RNA mass obtained and the number of cells harvested by FACS sorting. To evaluate mRNA levels, complementary DNA was synthesized using the Transcriptor First
Strand cDNA Synthesis Kit (Roche) in accordance with the manufacturer’s instructions, and PCR was performed in a 7900HT Fast Real-Time PCR System (Applied Biosystems) with SYBR Green PCR master mix (Applied Biosystems), normalized to β-actin. The primer sequences are listed in Online Table I. Quantification was performed by the $2^{-\Delta\Delta Ct}$ method.

*Primary neonatal rat ventricular cardiomyocytes culture*

Neonatal rat ventricular cardiomyocytes were isolated from 1-day-old Sprague-Dawley rats, as described previously⁴. These cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and plated in multi-well™ Primaria™ 6- or 24-well plates (Becton Dickinson) at 37 °C in a 5% CO₂ incubator. Lentivirus infection was induced 48 h after plating the cells.

*Purification of neonatal mouse cardiomyocytes, cardiac fibroblasts, and erythrocytes*

Mouse ventricles were digested in the same manner as described above with slight modifications. In brief, ventricles were isolated from 1-day-old C57BL/6 mice and dispersed in digestion buffer containing 1.3 mg/mL pancreatin (Sigma, P3292) and 0.45 mg/mL collagenase type II (Gibco, 17101-015). Collected cells were washed in a 3:1 mixture of DMEM and medium 199 (Gibco) supplemented with 10% horse serum (Gibco), 10% FBS (Sigma, 172012), 100 units/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), and 292 μg/mL L-glutamine (Gibco). The cells were centrifuged at 280 ×g for 5 min, mixed in serum-free DMEM, and stained for fluorescence-activated cell sorting (FACS) analyses.

As described previously⁵ and according to the manufacturer’s instructions, cells were stained
in 200 nM MitoTracker® Green FM (Invitrogen, M7514) solution for 30 min at 37 °C, followed by blocking for Fcγ receptors using anti-mouse CD16/CD32 antibody (BD Pharmingen, 553142). To isolate cardiac fibroblasts, we used anti-mouse Thy-1.2 antibody conjugated with allophycocyanin (APC) (eBioscience, 17-0902-81). Rat IgG2a κ conjugated with APC (eBioscience, 17-4321-81) was used as an isotype control immunoglobulin. All dispersed cells were dissociated with cold PBS supplemented with 2% FBS and then analyzed on a FACS system (BD FACSAria™ II, Becton Dickinson) using 515–545 and 650–670 nm bandpass filters to detect MitoTracker® Green and APC, respectively. Before sorting, pregating for eliminating doublet fractions, in which one droplet contains more than two cells, was performed in accordance with the manufacturer’s instructions.

To collect erythrocytes, peripheral blood was obtained from the jugular vein of a neonatal mouse using a heparinized syringe. After blocking, erythrocytes were labeled with an anti-mouse Ter-119 antibody conjugated with FITC (eBioscience, 11-5921-81), and sorted by FACS.

**Fatty acid stimulation**

Palmitic acid (Sigma, P5585) was dissolved in ethanol, and a 500 mmol/L stock solution was stored at −20 °C. This stock solution was combined with 5 mmol/L of fatty acid-free bovine serum albumin (BSA) (Wako, 013-15143) at a molecular ratio of 10:1 (fatty acid:albumin) in serum-free medium. The indicated concentrations of palmitic acid were prepared in serum-free medium. To stimulate NRCMs with various concentrations of palmitic acid in a physiological concentration of albumin, 275 mmol/L of palmitic acid dissolved in ethanol was
added to serum-free medium containing 550 μmol/L of fatty acid-free BSA. Two days after infecting cardiomyocytes with lentivirus, cardiomyocytes were stimulated with palmitic acid. An oleic acid (Sigma, O1008) solution was prepared using a method similar to that for making the palmitic acid solution.

Cell viability and cell injury evaluation

Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) assay. The cells were labeled with MTT at a final concentration of 0.5 mg/mL for at least 6 h at 37 °C. We measured the absorbance at 595 nm using an Elx800 microplate reader (BioTek Instruments, Inc.). The cell injury ratio was determined using the lactate dehydrogenase (LDH) Cytotoxicity Assay Kit™ (Cayman Chemical Company) in accordance with the manufacturer’s instructions.

Plasmids

An expression vector of miR-451 was generated using the BLOCK-iT™ Pol II miR RNAi Expression Vector Kit (Invitrogen) in accordance with the manufacturer’s instructions. We generated two constructs for miR-451 knockdown, which were named “decoys.” In decoy ×3 and ×6 constructs, three or six complementary tandem sequences for miR-451 were inserted in the 3’ UTR of the pMIR-REPORT™ vector (Invitrogen), respectively, as described previously3, 8. The unmodified pMIR-REPORT™ vector was used as the control vector. The pRL-TK™ Renilla reniformis luciferase (RL) plasmid was purchased from Promega. For dual luciferase assays, a macrophage inhibitory factor (MIF) or Cab39 3’ UTR was also inserted in
the 3′ UTR of the pMIR-REPORT™ vector. Mutated (Mut) 3′ UTR constructs were created using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) in accordance with the manufacturer’s instructions. For Cab39 overexpression, the subcloned Cab39-coding sequence was inserted into the multicloning site in the lentivirus vector. The primer sequences used are described in Online Table II. The constructs transfected into NRCMs were transferred to the lentivirus vector because transfection efficacy using this vector is very high⁴,⁸.

Lentivirus production and DNA transduction

Lentiviral stocks were produced in 293T cells in accordance with the manufacturer’s instructions (Invitrogen), as described previously⁴,⁸. In brief, virus-containing medium was collected for 48 h after transfection and filtered through a 0.45-µm filter. One round of lentiviral infection was performed by replacing the medium with virus-containing medium that contained 8 µg/mL polybrene, followed by centrifugation at 1220 ×g for 30 min.

Dual luciferase reporter assay

The dual luciferase assay was performed as described previously⁴. In total, 0.02 µg firefly luciferase (FL) reporter plasmid, 0.25 µg miR expression vector, and 0.02 µg pRL-TK™ RL plasmid for normalizing transfection efficiency were transfected into 293T cells. After 2 days of incubation, both luciferase activities were measured using a dual luciferase reporter assay system (Toyo Ink).

Western blotting analysis
Immunoblotting analysis was performed using standard procedures as described previously⁸. Cultured cells were homogenized in lysis buffer consisting of 100 mM Tris-HCl (pH 7.4), 75 mM NaCl, and 1% Triton™ X-100 (Nacalai Tesque). The buffer was supplemented with Complete Mini™ protease inhibitor (Roche), aprotinin (Sigma), 50 mM NaF, and 1 mM Na₃VO₄ just prior to use. Protein concentrations were determined using the bicinchoninic acid protein assay kit (Bio-Rad). A total of 20 μg protein was fractionated using NuPAGE™ 4–12% Bis-Tris (Invitrogen) gels and transferred to a Protran™ nitrocellulose transfer membrane (Whatman). The membrane was blocked using 1× PBS containing 5% non-fat milk for 1 h and incubated with the primary antibody overnight at 4 °C. After being washed in 0.05% T-PBS (1× PBS and 0.05% Tween-20), the membrane was incubated with the secondary antibody for 1 h at 4 °C. After the membrane was washed again in 0.05% T-PBS, the immune complexes were detected using the ECL-Plus™ chemiluminescent detection reagent (Amersham Biosciences). The following primary antibodies were used: anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling, 14C10), 1:3000; anti-rat MIF (Torrey Pines Biolabs, Inc., TP234), 1:2000; anti-MO25α/Cab39 (Cell Signaling, C49D8), 1:1000; anti-phospho-AMPKα (Cell Signaling, Thr172, 2531), 1:500; anti-total AMPKα (Cell Signaling, 2532), 1:1000; anti-phospho-mTOR (Cell Signaling, Ser2448), 1:1000; anti-total mTOR (Cell Signaling, 7C10), 1:1000; anti-phospho-S6 ribosomal protein (Cell Signaling, D57.2.2.E), 1:1000; anti-total S6 ribosomal protein (Cell Signaling, 5G10), 1:1000; anti-cleaved caspase-3 (Cell Signaling, 9661), anti-GATA4 (Santa Cruz, sc-9053), 1:500; anti-4-hydroxy-2-nonenal (4-HNE) (Nikken Seil Co., Ltd, clone HNEJ-2, MHN-020P), 2 μg/mL. Anti-rabbit IgG (GE Healthcare) and anti-mouse IgG (GE Healthcare) were used as
secondary antibodies each at a dilution of 1:2000. Immunoblots were detected using LAS-3000 (Fujifilm). For quantification of western blotting, densitometric analyses were performed using ImageJ64 software (NIH).

**Transthoracic echocardiography**

Mice were anesthetized with 300 mg/kg of 2,2,2-tribromoethanol (Sigma, T48402), and heart rates were kept at 500–600 beats/min. Transthoracic echocardiography was performed using the SONOS 4500 Ultrasound Imaging System (Philips). Left ventricular wall thickness was measured in the parasternal short-axis view.

**Lectin staining, cross-sectional area measurement, and other histological analyses using paraffin embedded hearts**

We performed perfusion-fixed mice with 4% paraformaldehyde before excising the heart, which was further fixed in 4% paraformaldehyde at 4 °C overnight. The next day, the tissue was transferred to 70% ethanol for dehydration until paraffin embedding. Paraffin-embedded ventricular short-axis sections were stained with FITC-conjugated lectin (Sigma, L4895). Single strand DNA staining was performed using an anti-single strand DNA antibody (IBL, 18731). Picrosirius red staining was also performed using paraffin embedded section. Images were acquired with BZ-9000 (Keyence). Cardiomyocyte cross-sectional area measurements were made using BZ-H1M analyzing software (Keyence). Approximately 100 cells were measured per heart at ×400 magnification, and the averages were used for analysis⁹.
Ceramide, neutral lipid, and DHE staining

Hearts were fixed as described above. After fixation, the specimens were incubated in 15% sucrose/PBS at 4°C for 2-3 h and in 30% sucrose/PBS at 4 °C overnight. The next day, the tissue samples were embedded in Tissue-Tek OCT (Sakura, Japan) compound. Ceramide staining was performed for 8μm frozen section. A heart section was washed with 0.05% T-PBS, and then blocked with 5% donkey serum in PBS at room temperature for 15 min. An anti-ceramide antibody (Enzo Life Sciences, Inc. MID 15B4) was used for ceramide detection. After washing three times with 0.05% T-PBS, Alexa Fluor 594 donkey anti-mouse IgG antibody (Life technologies, A-21203) was used as a secondary antibody. For neutral lipid staining, heart sections were incubated in PBS containing 1 μmol/L of BODIPY 505/515 (Life Technologies, D-3921) dye at room temperature for 1 h. For dihydroethidium (DHE; Life Technologies, D-1168) staining, harvested hearts were washed in cold PBS, and then directly embedded in Tissue-Tek OCT (Sakura, Japan) compound. Frozen sections of 10 μm were incubated in PBS containing 10 μmol/L of DHE at room temperature for 1 h. After washing in PBS, sealed sections were observed using BZ-9000 (Keyence). Five to ten images were randomly acquired using the same exposure time for each mouse, after which fluorescence intensities were determined using ImageJ64 software (NIH).

Quantification of ceramide in hearts

Total lipids were extracted from the hearts by Folch method. Ceramide content was measured using high-performance liquid chromatography/evaporative light-scattering detection.
A standard curve was determined using commercially available ceramide (Takasago International Corporation, Ceramide TIC-001).

**Cardiac catheterization in mice**

We analyzed cardiac functional reserve by cardiac catheterization of mice that were continuously administered dobutamine via the jugular vein. Briefly, a mouse was anesthetized with isoflurane, intubated, connected to a rodent ventilator (Harvard Apparatus, Inspira ASVv), and maintained on 1.5% isoflurane. The mouse was then placed on a heat pad to maintain its body temperature and electrocardiography was monitored during this procedure. Ventilation was by positive-pressure respiration. The settings were determined according to the following formulas: tidal volume (ml) = 6.2 × \( M^{1.01} \); respiration rate (min\(^{-1}\)) = 53.3 × \( M^{-0.26} \); \( M \) = body weight (kg). Both vagal nerves were cut, and a 1.0 French mouse pressure catheter (Millar, SPR-1000) was inserted into the right carotid artery via a small incision. The catheter tip was manipulated across the aortic valve into the left ventricle. To infuse dobutamine, the left jugular vein was cannulated with a stretched polythene tube. After stabilizing hemodynamic conditions, dobutamine was administered intravenously with an infusion pump (KD Scientific, KDS100). The dobutamine dose was increased from 4 \( \mu g/kg/min \) to 16 \( \mu g/kg/min \) every 2 min. Left ventricular pressure signals obtained from 10 to 20 beats were averaged, and then analyzed using PowerLab software (ADInstruments, LabChart 5).

**Statistical analysis**

Measurements are presented as means±SEM (standard error of the mean). For statistical
comparisons, Mann–Whitney U test (two groups) and one-way ANOVA (three or more groups) with Tukey’s post-hoc test were used as appropriate. For statistical comparisons only in Online Figure II, unpaired t-test was used. To compare cardiac functional reserves, two-way ANOVA with Tukey’s post-hoc test was used. A probability value of <0.05 was considered to indicate statistical significance. Statistical analyses were performed using GraphPad Prism 6 statistical packages (GraphPad Software, Inc.).
Online Table I. Primer sequences for genotyping miR-451fl/fl mice and αMHC-Cre transgenic mice and quantitative RT-PCR

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Forward (5′ –3′)</th>
<th>Reverse (5′ –3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-451fl/fl</td>
<td>GGCTGGGATATCATCATATACT</td>
<td>AGTTAAATGACAAGTGCTCTTA</td>
</tr>
<tr>
<td>αMHC-Cre</td>
<td>GAACACACCTGGAAGATGCTCCT</td>
<td>CTGATTCTGGCAATTTCGGCAAT</td>
</tr>
<tr>
<td>Target gene</td>
<td>Forward (5′ –3′)</td>
<td>Reverse (5′ –3′)</td>
</tr>
<tr>
<td>BNP</td>
<td>GCCAGTCTCCAGAGCAATTCA</td>
<td>TGTTCCTTTTGTAGGCCCTTG</td>
</tr>
<tr>
<td>Col1A1</td>
<td>GCCAAGAAGACATCCCTGAAG</td>
<td>TCATTGCATTGCACGTCATC</td>
</tr>
<tr>
<td>β-actin</td>
<td>AGATTACTGCTCTGGCTCCTA</td>
<td>CAAAGAAAGGGTGTAACAC</td>
</tr>
</tbody>
</table>

αMHC-Cre: α myosin heavy chain promoter-driven Cre-recombinase; BNP: brain natriuretic peptide; Col1A1: collagen type 1 α1.
Online Table II. Primer sequences for subcloning mmu-Cab39

<table>
<thead>
<tr>
<th>Direction</th>
<th>Sequence (5’ –3’ )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>GCTCTAGACACCATGCGTCCCATTTGGCAAGT</td>
</tr>
<tr>
<td>Reverse</td>
<td>CCGCTCGAGCTAGGCTTCCTGCTGGGGCTCTCTTT</td>
</tr>
</tbody>
</table>

Cab39: calcium-binding protein 39. These sequences include restriction enzyme sites.
Supplemental References


Online Figure I. Expression changes in miRNAs in the hearts of mice fed normal chow (NC) or high-fat diet (HFD) for 8 weeks. miRNA expression profiling was performed using a miRNA microarray. The red diagonal line indicates the unchanged line. Two blue lines represent 2-fold changes.
Online Figure II. Total palmitic acid levels in untreated and in PEG-treated serum. Apo-B containing lipoproteins were precipitated by PEG, after which palmitic acid levels were measured. PEG: polyethyleneglycol, n=3. Data are presented as mean±SEM. *, p < 0.05.
Online Figure III. miR-451 levels in cardiomyocytes and cardiac fibroblasts.

A, Isolating cardiomyocytes and cardiac fibroblasts from neonatal mouse hearts. Fluorescence-activated cell sorting (FACS) analysis of neonatal mouse ventricle-derived cells stained with Mitotracker Green dye and APC-conjugated Thy-1.2 antibody. Blue (P2) and green (P3) populations indicate the Mitotracker\textsuperscript{low}/Thy-1.2\textsuperscript{posi} and Mitotracker\textsuperscript{high}/Thy-1.2\textsuperscript{neg} populations, respectively. B and C, mRNA levels of brain natriuretic peptide (BNP) and collagen type 1 α1 (Col1A1) in FACS-sorted cell populations. n=4. D, miR-451 levels normalized to U6 levels in cardiomyocytes and cardiac fibroblasts, n=4. A.U.: arbitrary units. Data are presented as mean±SEM. *, p < 0.05.
Online Figure IV. Isolating erythrocytes by FACS sorting, and miR-451 levels in cardiomyocytes and erythrocytes.

A, FACS analysis of neonatal mouse peripheral blood after staining with anti-mouse Ter-119 conjugated with FITC. B, miR-451 levels per cell for cardiomyocytes and erythrocytes. n=4. A.U.: arbitrary unit. Data are presented as mean±SEM. *, p < 0.05.
Online Figure V. miR-451 levels in NRCMs stimulated with oleic acid.

A, miR-451 levels in NRCMs after stimulation with 250 μmol/L of the indicated acid for 24 hours. n=4–5. Ole: oleic acid; Pal: palmitic acid. B, Representative western blotting images for p-AMPK and t-AMPK after oleic acid or palmitic acid stimulation in NRCMs for 24 hours. C, Quantitative AMPK phosphorylation results by densitometry, n=6. A.U.: arbitrary units. Data are presented as mean±SEM. *, p < 0.05; ***, p < 0.001.
Online Figure VI. miR-451 level in NRCMs infected with miR-451 overexpression vector.

miR-451 levels were measured using Taqman microRNA real-time PCR. U6 indicates U6 small nuclear RNA used as an internal control, n=4. A.U.: arbitrary units. Data are presented as mean±SEM. *, p < 0.05.
Online Figure VII. Macrophage inhibitory factor (MIF) is a direct target in neonatal rat cardiomyocytes (NRCMs).

A, Dual luciferase analysis to examine if MIF is a direct target of miR-451. 293T cells were transfected with the miR-control (miR-ctrl) or a miR-451 expression plasmid, and luciferase plasmid-harboring WT MIF 3’UTR or Mut MIF 3’UTR. n=4. B, Western blotting of MIF in NRCMs infected with the miR-ctrl or miR-451 expression vectors. C, Western blotting of MIF in the hearts of mice fed 10 kcal% fat-containing NC or 45 kcal% fat-containing HFD for 20 weeks. GAPDH: glyceraldehyde-3-phosphate dehydrogenase (as a loading control). Data are presented as mean±SEM. ***, p < 0.001.
Online Figure VIII. Verification of miR-451 recombination in the heart genome of cardiomyocyte-specific miR-451 knockout (miR-451 cKO) mice. Validation of miR-451 recombination by electrophoresis of PCR products amplified from the heart and tail genomes.
Online Figure IX. The phenotypes of cardiomyocyte-specific miR-451 knockout (miR-451 cKO) mice.

A–D, liver weights (A, n=9–10), fasting blood sugar (FBS) concentrations (B, n=10–14), heart weights (C, n=11–15), and posterior wall thickness at diastolic phase (PWTd) (D, n=6–9) of indicated mice fed NC or HFD for 20 weeks. WT and cKO indicate αMHC-Cre;miR-451+/− and αMHC-Cre;miR-451n−/n, respectively. Data are presented as mean±SEM. *, p < 0.05; **, p < 0.01.
Online Figure X. GATA4 expression in control and cKO mouse hearts fed NC or a HFD for 20 weeks.

A, Representative western blotting images for GATA4. GAPDH: glyceraldehyde-3-phosphate dehydrogenase was used as a loading control. B, Quantitative results for GATA4 levels by densitometry, n=6. A.U.: arbitrary units. Data are presented as mean±SEM. **, p < 0.01; ***, p < 0.001.
Online Figure XI. Evaluation of cell toxicity for NC or HFD-fed mouse hearts.

A, Western blotting for cleaved caspase-3. GAPDH: glyceraldehyde-3-phosphate dehydrogenase was used as a loading control. WT and cKO indicate αMHC-Cre;miR-451+/+ and αMHC-Cre;miR-451+/−, respectively. B–E, Representative images of single strand DNA staining. Control and miR-451 cKO mice were fed NC or HFD for 20 weeks. Black bars represent 100 μm.
Online Figure XII. Fibrosis in control and miR-451 cKO mice fed NC or HFD.

A–D, Representative images of control and miR-451 cKO mice that were fed NC or HFD for 20 weeks. Black bars represent 100 μm. E, Quantitative results of fibrosis areas determined using picrosirius red staining, n=3–6. F, Col1α1 mRNA levels, n=5–7. WT and cKO indicate αMHC-Cre;miR-451+/+ and αMHC-Cre;miR-451−/−, respectively. Data are presented as mean±SEM.
**Online Figure XIII.** Neutral lipid staining of control and miR-451 cKO mice fed NC or HFD using BODIPY 505/515 neutral lipid probes.

A–D, Representative images of neutral lipid staining. Control and miR-451 cKO mice were fed NC or HFD for 20 weeks. White bars represent 40 μm.