Integrative Physiology

MicroRNA-33 Controls Adaptive Fibrotic Response in the Remodeling Heart by Preserving Lipid Raft Cholesterol


Rationale: Heart failure and atherosclerosis share the underlying mechanisms of chronic inflammation followed by fibrosis. A highly conserved microRNA (miR), miR-33, is considered as a potential therapeutic target for atherosclerosis because it regulates lipid metabolism and inflammation. However, the role of miR-33 in heart failure remains to be elucidated.

Objective: To clarify the role of miR-33 involved in heart failure.

Methods and Results: We first investigated the expression levels of miR-33a/b in human cardiac tissue samples with dilated cardiomyopathy. Increased expression of miR-33a was associated with improving hemodynamic parameters. To clarify the role of miR-33 in remodeling hearts, we investigated the responses to pressure overload by transverse aortic constriction in miR-33–deficient (knockout [KO]) mice. When mice were subjected to transverse aortic constriction, miR-33 expression levels were significantly upregulated in wild-type left ventricles. There was no difference in hypertrophic responses between wild-type and miR-33 KO hearts, whereas cardiac fibrosis was ameliorated in miR-33 KO hearts compared with wild-type hearts. Despite the ameliorated cardiac fibrosis, miR-33 KO mice showed impaired systolic function after transverse aortic constriction. We also found that cardiac fibroblasts were mainly responsible for miR-33 expression in the heart. Deficiency of miR-33 impaired cardiac fibroblast proliferation, which was considered to be caused by altered lipid raft cholesterol content. Moreover, cardiac fibroblast–specific miR-33–deficient mice also showed decreased cardiac fibrosis induced by transverse aortic constriction as systemic miR-33 KO mice.

Conclusion: Our results demonstrate that miR-33 is involved in cardiac remodeling, and it preserves lipid raft cholesterol content in fibroblasts and maintains adaptive fibrotic responses in the remodeling heart.

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Key Words: atherosclerosis | fibroblasts | fibrosis | heart failure | microRNAs

Despite numerous improvements in therapeutic options, heart failure (HF) is still the leading cause of death worldwide, indicating the need for an in-depth understanding of its cause and the development of innovative treatment strategies.1,2

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HF is characterized by diverse molecular, cellular, and physiological changes in the myocardium, resulting in adverse cardiac remodeling.3–5 Remodeling of the adult myocardium involves multiple processes, including myocyte hypertrophy, immune cell activation, and fibrosis.6–8 The cellular and molecular processes underlying cardiac remodeling are considered as a type of inflammation.9,10 Chronic inflammation followed by fibrosis can also be seen in other disorders, such as atherosclerosis,11 renal dysfunction,12 nonalcoholic steatohepatitis,13 and cancer.14

Both cardiac myocytes and nonmyocytes play essential roles in the processes involved in cardiac remodeling. In nonmyocytes, cardiac fibroblasts (CFs) are cells that have received the most attention15,16 because CFs produce a variety of growth

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From the Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, Japan (M.N., T.H., Y.K., O.B., T.Nakao, T.Nishino, D.H., Y.N., H.N., F.N., Y.I., S.K., M.K., R.H., T.Kimura, K.O.); Department of Cardiovascular Center, Osaka Red Cross Hospital, Japan (K.N., T.L.); Department of Pharmacology, Kansai Medical University, Hikakata, Osaka, Japan (T.Nakamura); Division of Translational Research, Clinical Research Institute, National Hospital Organization Kyoto Medical Center, Japan (K.H.); Herman B Wells Center for Pediatric Research, Department of Pediatrics, Indiana University of Medicine, Indianapolis (S.J.C.); and Kobe City Medical Center General Hospital, Japan (T.Kita).

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

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Novelty and Significance

What Is Known?
- Multiple processes, such as myocyte hypertrophy, immune cell activation, and fibrosis, are involved in heart failure.
- MicroRNAs play critical roles in heart failure by fine-tuning gene expression.
- MicroRNA-33 (miR-33) regulates lipid metabolism and inflammation.

What New Information Does This Article Contribute?
- MiR-33 is involved in cardiac remodeling, especially in cardiac fibrosis.
- MiR-33 regulates fibroblast proliferation by maintaining lipid raft cholesterol content.

Chronic inflammation followed by fibrosis is an important underlying mechanism of heart failure. The similar processes can be seen in other disorders, including atherosclerosis. Inhibition of miR-33 is a potent therapeutic strategy for atherosclerosis because recent reports, including ours, indicated that miR-33 reduces high-density lipoprotein cholesterol levels of sterol and fatty acid synthesis, and suppresses genes involved in cholesterol metabolism, such as ABCA1 and ABCG1 (ATP-binding cassette transporter A1 and G1) as target genes (primates including Homo sapiens also have miR-33b in the intron of SREBF1 in addition to miR-33a). It was also reported that miR-33 is involved in inflammatory processes in atherosclerotic lesions and fibrotic responses in the liver. However, the functions of miR-33 in cardiac hypertrophy and heart failure, which are also chronic inflammatory and fibrotic processes as mentioned above, remain to be elucidated.

MiR-33 is expressed ubiquitously, but its expression level in the heart varies depending on the report in question and varies from 0.5- to 10-fold of its expression in the liver. MiR-33 can, at least, be detected in the heart at a similar level to that in the liver or in immune cells, which have been investigated intensively. However, there are no reports regarding the function of miR-33 in the heart.

In the present study, we investigated the functions of miR-33 in the remodeling heart using clinical samples and genetic mouse models. We first investigated the correlation between expression levels of miR-33a/b in myocardial biopsy samples and hemodynamic parameters in patients with dilated cardiomyopathy. Next, we used miR-33–deficient (knockout [KO]) mice (both whole body KO and fibroblast-specific KO) to examine the role of miR-33 in cardiac hypertrophy and fibrosis. We applied transverse aortic constriction (TAC), a model of left ventricular pressure overload characterized by cardiac myocyte hypertrophy and interstitial fibrosis.

Methods

Detailed Methods are provided in the Online Data Supplement.

Myocardial Biopsy

We analyzed the expression levels of miR-33a/b in the tissues of endomyocardial biopsies from left ventricles (LVs) and examined the relationship between their expression levels and hemodynamic parameters in 33 patients who were diagnosed with dilated cardiomyopathy at Osaka Red Cross Hospital. Hemodynamic parameters were obtained by catheterization. All patients provided written
Informed consent for the procedure and gene expression analyses. The ethics committee of Osaka Red Cross Hospital approved the study protocol.

**Mice**

MiR-33 KO mice were generated as reported previously. MiR-33–floxed mice were generated by homologous recombination in a C57BL/6J background as described in the Online Data Supplement. Pn-Cre (Periostin-Cre) mice were generated by Dr Conway and colleagues. Mice were maintained in specific pathogen-free conditions at the Institute of Laboratory Animals of Kyoto University Graduate School of Medicine. This study was approved by the Kyoto University Ethics Review Board. The primer sequences for genotyping are listed in Online Table II. All the in vivo experiments were performed using male mice in a C57BL/6J background.

**Pressure Overload Model**

TAC was performed as described previously. Primary Neonatal Rat Cardiac Myocytes and Fibroblasts

Neonatal rat cardiac myocytes and CFs were isolated from 1-day-old Sprague-Dawley rats, as described previously. After enzymatic digestion with collagenase/dispase solution (Roche, 11097113001) and plated for 2 hours. We used adult CFs at passage 3 to 4. For isolation of tail-tip fibroblasts, the tails from 8-week-old mice were peeled, minced into 1 cm pieces, placed on gelatin-coated culture dishes, and incubated for 5 days. Cells that migrated out of the graft pieces were transferred to new plates (passage 1). We used tail-tip fibroblasts at passage 4 to 5. Mouse embryonic fibroblasts were isolated from wild-type (WT) and miR-33KO embryos at E14.5. We used mouse embryonic fibroblasts at passage 5 to 6.

**Quantitative Real-Time PCR**

To evaluate miRNA expression, single-strand cDNA was synthesized from 1 μg of total RNA by means of reverse transcriptase reaction and quantitative polymerase chain reaction (PCR) was performed using a LightCycler 96 (Roche Diagnostics) with THUNDERBIRD SYBR qPCR Mix (TOYOBO, QPS-201). Expression levels were normalized using housekeeping genes as indicated. The primer sequences are listed in Online Table III.

**Lipid Raft Staining**

We used fluorescence-conjugated cholera toxin subunit B (CTB) for lipid raft labeling. To obtain images of lipid raft staining, fibroblasts were plated onto chamber slides and stained with Vybrant Alexa Fluor 594 Lipid Raft Labeling Kit (Molecular Probes, V34405). To quantify the signals from CTB-labeled lipid rafts on the cell surface, flow cytometric analyses were performed. Fibroblasts were plated on 10-cm culture dishes and cultured overnight. The cells were dissociated using trypsin EDTA, and stained with 1 μg/mL fluorescein isothiocyanate–conjugated CTB (Sigma, C1655) at 4°C for 15 minutes and then analyzed on a FACSARiaII (Becton Dickinson). To make control samples with decreased lipid rafts, some samples were incubated with 5 mM 5-methyl-β-cyclodextrin for 1 hour before labeling with CTB.

**Echocardiography**

To analyze the cardiac function of mice, we performed echocardiography (Vevo 2100, VisualSonics) at the indicated time points after TAC.

**Statistical Analysis**

Measurements except for clinical biopsy data are presented as mean±SEM. Measurements for clinical samples are presented as median interquartile range or means±SD as indicated. To compare gene expression levels and the hemodynamic parameters in Figure 1A, linear regression analysis was performed using R version 3.2.3 (The R Project). For Gene Ontology analysis, the clusterProfiler package in R/Bioconductor was used for data sets with differentially expressed genes. P values were adjusted by false discovery rate correction. For other statistical comparisons, unpaired Student t test (2 groups, parametric), Mann-Whitney test (2 groups, nonparametric), or 1-way ANOVA with Sidak post hoc test (3 or more groups) were used as indicated in the figure legends. A P value of <0.05 was considered as statistically significant. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc).

**Results**

MiRNA-33a expression levels in cardiac tissue are associated with improving hemodynamic parameters in patients with dilated cardiomyopathy.

To analyze the expression pattern of miR-33a/b in human remodeling hearts, we measured their levels using TaqMan quantitative PCR in the tissues from endomyocardial biopsies of LVs and examined the relationships between miR-33a/b expression levels and hemodynamic parameters obtained by catheterization in patients with dilated cardiomyopathy.

A total of 33 patients were included in the analysis (Online Table I). The cardiac expression level of miR-33a was modestly but significantly correlated with ejection fraction (EF) and inversely correlated with pulmonary capillary wedge pressure, whereas the expression level of miR-33b was not correlated with these parameters (Figure 1A). Thus, we focused on miR-33a, which is highly conserved across species unlike miR-33b.

When the patients were categorized by Forrester classification (there were no patients with Forrester II HF in this cohort; Online Table I), miR-33a expression levels in patients with high-stage HF (Forrester III or IV) were significantly lower than its expression in patients with low-stage HF (Forrester I; Figure 1B), whereas the expression of SREBF2, whose intron 16 has the coding region of miR-33a, did not differ among the groups (Figure 1C).

These data from the clinical samples indicated that decreased miR-33a was associated with worsened cardiac function and miR-33a had function in the development of HF. MiRNA-33 plays an important role in pressure overload–induced cardiac fibrosis.

Because rodents have only miR-33a, but not miR-33b, unlike human or nonhuman primates, miR-33KO mice are good tools to analyze miR-33a functions. As we previously reported, miR-33KO mice show normal growth and displayed no obvious abnormalities in heart development. To analyze the function of miR-33 in response to pathological stimuli, mice were subjected to pressure overload–induced cardiac hypertrophy by TAC.

To analyze hypertrophic and fibrotic responses to TAC, we performed gene expression analyses in sham- or TAC-operated WT and miR-33KO LVs at 2 weeks after operation and also
histological analyses at 4 weeks after operation. The expression level of miR-33 was significantly upregulated in response to TAC in WT LVs (Figure 2A). Heart weight and cardiac myocyte size were increased similarly in WT and miR-33KO mice after TAC (Figure 2B and 2C; Online Figure IA–ID), which showed no significant difference in TAC-induced cardiac myocyte hypertrophy between WT and miR-33KO mice. However, histological analysis showed that TAC-induced cardiac fibrosis was ameliorated in miR-33KO mice (Figure 2D and 2E). Moreover, TAC-induced upregulation of fibrosis-related genes, such as \textit{Col1a1}, \textit{Postn}, and \textit{Tgfb3} in LVs, was significantly suppressed in miR-33KO mice compared with WT mice, despite the similar expression level of a hypertrophic marker, \textit{Nppb} (Figure 2F).

To analyze gene expression more comprehensively, we performed microarray analysis. The microarray data detected 114 upregulated genes and 148 downregulated genes in miR-33KO hearts versus WT hearts after TAC (fold change >2). Gene ontology analysis using the downregulated genes showed that several biological processes related to fibrosis and extracellular matrix were enriched (Online Figure II). The heatmap data showed suppressed upregulation of fibrosis-related genes by TAC in miR-33KO mice (Online Figure III; orange cluster in A, red and orange cluster in C, and red cluster in D), whereas the genes involved in cardiac muscle contraction showed only small changes (Online Figure IIIB).

These data showed that miR-33 deficiency did not affect the hypertrophic response to TAC but alleviated the fibrotic response to TAC. Therefore, miR-33 was considered to play an important role in TAC-induced cardiac fibrosis.

Deficiency of miR-33 worsened systolic function after chronic pressure overload.

As shown above, miR-33 deficiency alleviated fibrotic response in the heart after TAC. To investigate whether the alterations by miR-33 deficiency improved cardiac function in vivo, we performed echocardiography of WT and miR-33KO mice ≤8 weeks after TAC. During the follow-up, no WT mice died, and only 1 miR-33KO mouse died at 8 weeks.
Figure 2. Deficiency of microRNA (miR)-33 ameliorated cardiac fibrosis induced by pressure overload in vivo. A, MiR-33 expression levels in left ventricles (LVs) of wild-type (WT) mice at 2 weeks after sham or transverse aortic constriction (TAC) operation (n=4). Expression of U6 snRNA was used as an internal control. **P<0.01, by unpaired 2-tailed Student t test. B, Representative images of wheat germ agglutinin staining in LVs at 4 weeks after operation. White bar indicates 50 μm. C, Quantification of cardiac myocyte cross-sectional area in sham- or TAC-operated WT and miR-33–deficient (knockout [KO]) mice at 4 weeks after TAC (Continued)
after TAC. Echocardiographic parameters, including EF and fractional shortening, did not differ among WT and miR-33KO mice in the period from preoperation to 4 weeks after TAC. However, contrary to our expectations, both EF and fractional shortening were deteriorated in miR-33KO mice compared with WT mice at 8 weeks after TAC (Figure 3A, 3B, 3C, and 3E). The wall thickness stopped increasing at 4 to 8 weeks after TAC in miR-33KO mice, whereas it continued increasing in WT mice during the same time period (Figure 3D).

Figure 3. Deficiency of microRNA (miR)-33 impaired systolic function after chronic pressure overload. A, Representative images of M-mode echocardiography of wild-type (WT) and miR-33–deficient (knockout [KO]) mice at 8 weeks after transverse aortic constriction (TAC). B–E, Echocardiographic data of WT and miR-33KO ≤8 weeks after TAC. Ejection fraction (EF; B), fractional shortening (FS; C), interventricular septum (IVS; D), and LV diameters (LVD; E) were measured at the indicated time points after TAC (n=9–12). F, Global longitudinal strain (GLS) measured using speckle-tracking strain imaging (n=9–12). G, Western blotting analysis of phosphorylation of Akt (p-Akt) and extracellular signal–regulated kinase (p-ERK) in LVs. H, Densitometry of p-Akt and p-ERK in LVs 2 weeks after TAC (n=9). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. *P<0.05, **P<0.01 vs sham, #P<0.05 vs WT, n.s., not significant, by ANOVA with Sidak correction. Data are presented as mean±SEM.
Recent studies have shown that miR-33 has atherogenic and proinflammatory effects because it regulates cholesterol metabolism in the liver and immune cells via inhibition of genes involved in cholesterol efflux and transport, such as ABCA1, ABCG1, and NPC1 (Niemann-Pick C1). Among these genes, we confirmed the upregulation of ABCA1 even in miR-33KO CFs (Figure 5A and Online Figure VIIA–VIIIC).

Because of the altered proliferative capacity and altered gene expression involved in cholesterol metabolism in miR-33KO fibroblasts, we speculated that altered lipid rafts in miR-33KO fibroblasts were the underlying mechanism of the reduced fibrosis of miR-33KO hearts. To analyze lipid raft content in miR-33KO fibroblasts, we stained lipid rafts by fluorescence-conjugated CTB, which binds to ganglioside GM1 on the plasma membrane and is a marker to identify lipid rafts. Analyses by confocal laser scanning microscopy showed reduced lipid raft content in miR-33KO fibroblasts compared with WT fibroblasts (Figure 5B). To quantify the signal intensity by CTB binding that marked lipid rafts, we used flow cytometry. The mean fluorescence intensity of miR-33KO fibroblasts marked by fluorescein isothiocyanate-CTB was significantly lower than that of WT fibroblasts (Figure 5C and 5D). The altered lipid rafts in miR-33KO fibroblasts were consistent with the impaired activation of Akt because it has been reported that lipid rafts are involved in the phosphatidylinositol-3 kinase/Akt signaling pathway and that Akt is phosphorylated in lipid rafts on the plasma membrane (Figure 4G and 4H).

To investigate whether ABCA1 is involved in the impaired proliferation of miR-33KO fibroblasts, we performed siRNA-mediated knockdown of ABCA1. Both WT and miR-33KO fibroblasts showed similar proliferation in the condition of siRNA-mediated knockdown of ABCA1 (Figure 5E and Online Figure VIIID). The results indicated that both ABCA1 and miR-33 are involved in cellular proliferation.

These data indicated that miR-33 maintained lipid raft content by regulating genes involved in cholesterol metabolism. CF-specific deletion of miR-33 ameliorated cardiac fibrosis after pressure overload.

To eliminate the effect of increased HDL-C and the altered phenotype of immune cells in miR-33KO mice, we decided to analyze fibrotic responses in CF-specific miR-33–deficient mice. We generated miR-33–floxed mice, in which loxP sites flanked the pre-miR sequence of miR-33 (Online Figure VIIIA–VIIID). We confirmed that the expression of miR-33 was hardly detected in miR-33–deleted mice after crossing with Ayu1-Cre mice, which express Cre recombinase ubiquitously (Online Figure IXA), and also that the expression and splicing of Srebf2 mRNA was not altered in both miR-33–floxed mice and miR-33–deleted mice (Online Figure IXB and IXC).

To generate CF-specific knockout mice, we used a transgenic mouse line in which Cre recombinase was driven by a 3.9-kb promoter of mouse Periostin (Pn-Cre), which is not normally expressed in adult hearts but induced specifically in CFs by pathological stimuli, such as pressure overload.
Figure 4. Deficiency of microRNA (miR)-33 in fibroblasts impaired proliferative capacity. A, MiR-33 expression levels in neonatal rat cardiac myocytes and cardiac fibroblasts isolated by Percoll gradient separation (n=5). Expression of U6 snRNA was used as an internal control. **P<0.01, by unpaired 2-tailed Student t test. B, Growth curve analyses of 3 types of fibroblasts; mouse embryonic fibroblasts (MEFs), cardiac fibroblasts, and tail-tip fibroblasts (MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay; n=5–8). *P<0.05, by unpaired 2-tailed Student t test. C, Flow cytometric analysis of apoptotic cell death in wild-type (WT) and miR-33–deficient (knockout [KO]) MEFs. Cells were treated with 1 mmol/L hydrogen peroxide (H2O2) for 4 hours and stained with Annexin V and propidium iodide (PI). D, Quantification of the early apoptotic cells (Q4: Annexin V- PI-; n=4). **P<0.01 vs untreated control, ###P<0.01 vs WT, by ANOVA with Sidak correction. E, Representative images of in vivo bromodeoxyuridine (BrdU) incorporation in WT and (Continued)
When we generated CF-specific KO mice of miR-33 (FBKO [fibroblast-specific–deficient mice], Pn-Cre+miR-33floxflox), FBKO mice showed an ≈30% reduction in miR-33 in LVs compared with littermate control (miR-33 flox/flox) mice after TAC (Figure 6A). The cell type in which the expression of miR-33 increased after TAC was thought to be CFs because the expression levels of miR-33 in FBKO mice were the same as at baseline (Figures 2A and 6A). FBKO mice did not show higher HDL-C levels than littermate controls, whereas miR-33Δ/Δ mice showed higher HDL-C levels than control mice (Online Figure XA and XB). This result suggested that miR-33 in fibroblasts did not contribute to HDL-C elevation.

Heart weight after TAC did not differ among FBKO and control mice (Figure 6B), whereas histological analysis showed that the fibrotic area of FBKO LVs was smaller than control mice (Figure 6C and 6D), like whole body KO mice. Gene expression analysis showed that fibrosis-related genes tended to be downregulated in FBKO LVs (Online Figure XC). Furthermore, FBKO mice showed smaller numbers of Ki-67–positive fibroblasts at 2 weeks after TAC (Figure 6E).

Figure 5. MicroRNA (MiR)-33–deficient (knockout [KO]) fibroblasts showed reduced lipid raft content. A, Expression of Abca1 in miR-33KO cardiac fibroblasts (n=4). Expression of β-actin was used as an internal control. *P<0.05, **P<0.01, by unpaired 2-tailed Student t test. B, Representative images of lipid raft staining by cholera toxin subunit B (CTB). Tail-tip fibroblasts from wild-type [WT] and miR-33KO mice were stained by Alexa Fluor 594-conjugated CTB. White bar indicates 50 μm. C, Flow cytometric analysis of lipid raft staining. Tail-tip fibroblasts were stained using fluorescein isothiocyanate (FITC)-conjugated CTB after treatment with or without 5 mmol/L methyl-β-cyclodextrin (MβCD) for 1 hour. The gray curve indicates the unstained control. D, Quantification of lipid raft content labeled with CTB. Mean fluorescence intensity (MFI) of FITC-CTB were obtained using flow cytometry as shown in C (n=3). *P<0.05 vs MβCD(−) control, **P<0.05 vs WT, by ANOVA with Sidak correction. E, Fibroblast proliferation in the knockdown of ABCA1 (ATP-binding cassette transporter A1). WT and miR-33KO mouse embryonic fibroblasts (MEFs) were transfected with lentiviral small interfering RNA vectors against ABCA1 (siABCA1) and control (siControl). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed 1 day or 7 days after transfection (n=11). *P<0.01 vs WT, ##P<0.01 vs siControl, by ANOVA with Sidak correction. Data are presented as mean±SEM. DAPI indicates 4′,6-diamidino-2-phenylindole.
and 6F). Therefore, miR-33 was considered to contribute to CF proliferation and fibrotic responses after pathological stimuli, such as pressure overload.

**Discussion**

Here, by using genetic models, we demonstrate that deficiency of miR-33 resulted in reduced fibrotic response to pressure overload in vivo. Despite the reduction in fibrosis, cardiac function deteriorated in miR-33KO hearts. We also found that miR-33 in the heart was predominantly expressed in CFs, and deficiency of miR-33 impaired cell proliferation in fibroblasts both in vitro and in vivo, and that miR-33 preserved lipid raft cholesterol content in fibroblasts by regulating genes involved in cholesterol metabolism. Moreover, in clinical samples, miR-33a expression correlated with improving hemodynamic parameters, including EF. These data indicate that miR-33 promotes fibrotic responses in the heart by maintaining lipid raft content in fibroblasts, and the response has protective effects on cardiac function (Online Figure XIII).

It has been said that miRs play critical roles in normal heart development and also in HF by fine-tuning gene expression because cardiac-specific deletion of Dicer, a gene that is essential for miR processing, leads to poorly developed myocardium, rapidly progressive HF, and postnatal lethality.44–46 In our clinical samples, miR-33a expression was correlated with EF and inversely with mean pulmonary capillary wedge pressure, and its expression was downregulated in high-stage HF patients compared with low-stage HF patients. The results were consistent with our echocardiographic data using miR-33KO mice because EF and fractional shortening were significantly lower in miR-33KO mice compared with WT mice after chronic pressure overload. The wall thickness stopped increasing at 4 to 8 weeks after TAC in miR-33KO mice, and this indicated insufficient hypertrophic adaptation. In addition, the upregulation of miR-33 after TAC was only in the

![Figure 6. Cardiac fibroblast-specific deficiency of microRNA (miR)-33 reduced cardiac fibrosis after transverse aortic constriction (TAC).](image-url)

A. Expression levels of miR-33 in left ventricles (LVs) of cardiac fibroblast-specific-deficient mice (FBKO; Pn-Cre*miR-33^flxflo*) at 2 weeks after TAC (n=13–15). MiR-33^flxflo/littermates were used as controls. Mean expression level of 5 sham-operated miR-33^flxflo mice was defined as 1.0. Expression of U6 snRNA was used as an internal control. B. Heart weight of control and FBKO mice at 2 weeks after TAC (n=18). Heart weight was normalized by body weight. C. Representative images showing picrosirius red staining of LVs of control and FBKO mice. Black bars indicate 200 μm. D. Quantification of fibrotic area determined by picrosirius red staining in LVs of control and FBKO mice 2 weeks after TAC (n=18). E. Representative images of immunostaining of Ki-67 in control and FBKO LVs 2 weeks after TAC. White bars indicate 100 μm and 30 μm. F. Quantification of Ki-67-positive fibroblasts in LVs 2 weeks after TAC (n=15). *P<0.05, n.s., not significant, by unpaired 2-tailed Student t test. Data are presented as mean±SEM. DAPI indicates 4',6-diamidino-2-phenylindole.
early phase (Online Figure IE). These data indicated that miR-33 had protective effects on developing HF. MiR-33a (or miR-33 in rodents) is transcribed from intron 16 of SREBF2, which is a master regulator of lipid synthesis.22 Expression of SREBF2 mRNA did not differ between low-stage HF patients and high-stage HF patients, whereas expression of miR-33a was different between the groups. It seemed that this was because of post-transcriptional regulation of miR-33a, such as processing by DROSHA and Dicer.

Next, gene expression and histological analyses using miR-33KO mice after TAC showed that deficiency of miR-33 did not affect hypertrophic responses but alleviated fibrotic responses to pressure overload. We also found that the expression of miR-33 was predominant in CFs. Moreover, the phenotype of the reduced fibrotic response was also reproduced in CF-specific miR-33-deficient mice. These data were consistent with recent reports that inhibition of miR-33 reduces liver fibrosis in a nonalcoholic steatohepatitis model and that expression of miR-33 in hepatic stellate cells, which are the primary source for activated fibroblasts in liver, is involved in the fibrotic processes in nonalcoholic steatohepatitis.30,47 Therefore, miR-33 is considered as a profibrotic miR in pathological conditions. Cardiac fibrosis is a characteristic of failing hearts.5,8,15,20 Our data are paradoxical, in that deficiency of miR-33 reduced systolic function after chronic pressure overload, despite ameliorated cardiac fibrosis. Recently, however, it has been said that CFs and fibrosis have both adaptive and maladaptive sides, and some reports showed inconsistency between cardiac fibrosis and hypertrophy (or LV function) in the remodeling heart.4,20,21,48 For example, it has been reported that deficiency of Smad3, which is essential for canonical TGF-β (transforming growth factor beta) signaling, showed increased heart weight and worsened heart failure after TAC, despite decreased cardiac fibrosis in mice.48 Although persistent cardiac fibrosis is detrimental, fibroblasts also play a protective role in the heart by producing a necessary acute wound healing response. In addition, there are 2 types of cardiac fibrosis: reactive and reparative.49 Reactive fibrosis in the early phase of remodeling is different from the reparative (replacement) fibrosis that follows cardiac myocyte death. In our data, proliferation of fibroblasts was impaired in vivo in the acute phase (1 week after TAC) in miR-33KO mice (and also in vitro), and fibrotic responses in the early phase (2–4 weeks) were suppressed without affecting cardiac myocyte hypertrophy, and LV function worsened in the chronic phase (8 weeks). Therefore, we can explain that impaired proliferation of fibroblasts in miR-33KO mice was primary, and it resulted in inadequate reactive fibrosis and insufficient adaptive responses, such as protective paracrine factors from CFs (Online Figure XIII).

There may be additional cells that affect the phenotype of miR-33KO mice because the phenotype observed in FBKO mice was not so prominent as in systemic KO mice. Macrophages are also important for cardiac fibrosis and remodeling. Although the expression of Tgfβ3 was suppressed in peritoneal macrophages in vitro, we did not detect significant differences in macrophage infiltration between WT and miR-33KO mice in vivo (Online Figure IV). There are some reports that suggest miR-33 slows down the cell cycle by inhibiting the expression of genes associated with the cell cycle, such as CDK6 (cyclin-dependent kinase 6) and cyclin-D1.52 We found that CDK6 was not increased in miR-33KO fibroblasts (Online Figure XI). The result was probably because of the difference in cell types. Although CDK6 is preferentially expressed in hematopoietic cells, CDK4 is predominant over CDK6 in fibroblasts.53

The importance of miRs in cardiovascular hypertrophy and fibrosis in pathological conditions has been demonstrated.46-48,54 Notably, with regard to fibroblasts, several miRs in CFs have been investigated intensively, including miR-21, miR-29, and miR-30,55-58 Interestingly, members of the miR-30 family are involved both in lipid metabolism and in cardiac fibrosis,58,59 like miR-33 in our data. These studies screened differentially expressing miRs in HF samples and in CFs. The changes of miR-33 expression in HF are not so prominent as these CF-enriched miRs, probably because Srebf2 and miR-33 are ubiquitously expressed and cholesterol homeostasis is tightly regulated. The present study is notable, in that it showed the relationship between cellular lipid homeostasis and fibrotic responses.

Several limitations of this study should be acknowledged. There might be some variations in the quality of clinical biopsy samples. We did not use isolated cardiac myocytes from adult mice or human samples to analyze cell type-specific expression levels of miR-33, but instead used Percoll-isolated neonatal rat cardiac myocytes and fibroblasts because isolation of fresh cardiac myocytes from adult mice or human sample is technically difficult. To generate CF-specific KO mice, we used a Pn-Cre line, which express Cre recombinase only in a subset of fibroblasts in the heart, and there is still no truly specific marker of CFs.

Finally, inhibition of miR-33 is considered to be a potent therapeutic strategy for atherosclerosis because miR-33 has atherogenic effect by reducing HDL-C.23,41,60 It is considered that inhibition of miR-33 results in altered cellular cholesterol content because of the target genes involved in cholesterol efflux and transfer, such as ABCA1, ABCG1, and NPC1. In our data, deficiency of miR-33 reduced the lipid raft cholesterol content in fibroblasts. Statins also reduce cellular cholesterol content, and they are reported to have pleiotropic effects on various diseases, such as prostate cancer,63 Alzheimer disease,62,63 and HIV infection64 through modulation of lipid rafts.51 Therefore, our data implicated the possibility of pleiotropic effects of miR-33 inhibition. However, our data suggest that inhibition of miR-33 may cause cardiac dysfunction. It has also been reported that long-term silencing of miR-33 resulted in an unexpected increase in circulating triglyceride levels and lipid accumulation in the liver.65 Because our genetic KO mice were equivalent to permanent inhibition, long-term inhibition of miR-33 can cause harmful side effects. To utilize the protective effect of miR-33 inhibition, further research is necessary.

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Disclosures

None.

References


MicroRNA-33 Controls Adaptive Fibrotic Response in the Remodeling Heart by Preserving Lipid Raft Cholesterol

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**MicroRNA-33 controls adaptive fibrotic response in the remodeling heart by preserving lipid raft cholesterol**

Masataka Nishiga¹, Takahiro Horie¹, Yasuhide Kuwabara¹, Kazuya Nagao², Osamu Baba¹, Tetsushi Nakao¹, Tomohiro Nishino¹, Daihiko Hakuno¹, Yasuhiro Nakashima¹, Hitoo Nishi¹, Fumiko Nakazeki¹, Yuya Ide¹, Satoshi Koyama¹, Masahiro Kimura¹, Ritsuko Hanada¹, Tomoyuki Nakamura³, Tsukasa Inada², Koji Hasegawa⁴, Simon J Conway⁵, Toru Kita⁶, Takeshi Kimura¹, Koh Ono¹

¹: Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan
²: Department of Cardiovascular Center, Osaka Red Cross Hospital, Osaka, Japan
³: Department of Pharmacology, Kansai Medical University, Hirakata, Osaka, Japan
⁴: Division of Translational Research, Clinical Research Institute, National Hospital Organization Kyoto Medical Center, Kyoto, Japan
⁵: Herman B Wells Center for Pediatric Research, Indiana University of Medicine, Indianapolis, Indiana, USA
⁶: Kobe City Medical Center General Hospital, Kobe, Japan

Correspondence to:
Koh Ono, MD, PhD
Department of Cardiovascular Medicine
Graduate School of Medicine, Kyoto University
54 Shogoin-kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan
Tel.: (+81) 75-751-3190. Fax: (+ 81) 75-751-3203.
E-mail: kohono@kuhp.kyoto-u.ac.jp
SUPPLEMENTAL MATERIAL

Detailed methods

Myocardial biopsy

We analyzed the expression of miR-33a/b in the tissues of left ventricle (LV) endomyocardial biopsies and examined the relationships between their expression levels and hemodynamic parameters in 33 patients who were diagnosed with dilated cardiomyopathy (DCM) at Osaka Red Cross Hospital. The biopsies were performed to determine the pathogenesis of heart failure (HF) in accordance with the following institutional criteria: (i) new onset HF with clinical symptoms of dyspnea, chest pain, or palpitation, (ii) unexplained impairment of LV function and LV dilatation, (iii) no evidence of coronary artery disease or primary valvular disease. These criteria were in agreement with a guideline. Hemodynamic parameters were obtained by the catheterization. Final diagnoses were obtained on the basis of clinical history, laboratory examinations, electrocardiogram (ECG), echocardiography, other disease-specific tests such as computed tomography and nuclear imaging, biopsies from extracardiac tissues, as well as histological analysis of the biopsy samples. All patients provided written informed consent for the procedure and gene expression analyses. The Ethics Committee of Osaka Red Cross Hospital approved the study protocol.

Mice

MiR-33-deficient mice were generated as reported previously. To match genetic background between WT and miR-33KO mice, a miR-33+/− male mouse and a miR-33+/− female mouse were...
crossed to generate miR-33\(^{+/+}\) and miR-33\(^{-/-}\) mice, and the offspring mice were used for analyses. MiR-33-floxed mice were generated as described below. Periostin-Cre mice were generated by Dr. Conway and colleagues.\(^3,4\) Mice were maintained in temperature-controlled rooms with a 14:10 h light:dark cycle in specific pathogen-free conditions at the Institute of Laboratory Animals of Kyoto University Graduate School of Medicine. This study was approved by the Kyoto University Ethics Review Board. The primer sequences for genotyping are listed in Online Table II. All the in vivo experiments were performed using male mice in a C57BL/6J background.

*Generation of miR-33-floxed mice.*

We generated miR-33-floxed mice by homologous recombination. First, a targeting vector was constructed by modifying bacterial artificial chromosome RP24-291F2 (BACPAC Resources Center) using defective prophage \(\lambda\)-Red recombination system.\(^5,7\) A loxP sequence was inserted to 40 bp upstream of the miR-33 coding region, and another loxP sequence was inserted to downstream of the miR-33 coding region. As a selection marker, a neomycin resistance cassette flanked by FRT was inserted into the site between the miR-33 coding region and the downstream loxP (Online Figure VIIIA). We electroporated the targeting vector into C57BL/6J mouse embryonic stem (ES) cells (CHEMICON, CMTI-2) using Nucleofector\(^\text{TM}\) (Lonza) and a Mouse ES Cell Nucleofector\(^\text{®}\) Kit (Lonza, VPH-1001). Positive clones were selected by incubating cells with 200 \(\mu\)g/mL Geneticin\(^\text{®}\) (Thermo Fisher Scientific, 10131-035) for 7 days, and homologous recombination was confirmed by Southern blotting (Online Figure VIIIC). Successfully recombined ES cells were injected into blastocysts from ICR mice supplied by
Unitech Inc, and chimeric mice were bred with C57BL/6J mice to generate F1 mice. The genotype of F1 mice was confirmed by PCR. The neomycin resistance cassette was removed from the mouse germ line by crossing with CAG-FLPe transgenic mice, which express FLPe recombinase under the control of the CAG promoter (Riken, RBRC01834). The offspring miR-33\textsuperscript{flox/+} mice without the CAG-FLPe allele were crossed with each other to generate miR-33\textsuperscript{flox/flox} mice. The genotype of miR-33\textsuperscript{flox/flox} mice was confirmed by PCR and Southern blotting (Online Figure VIIIB and VIIID). To confirm Cre-mediated deletion of miR-33, we crossed miR-33\textsuperscript{flox/flox} mice with Ayu1-Cre mice, which express Cre-recombinase ubiquitously (B6;D2-Tg(Ayu1-Cre)8IImeg, a gift from Dr. Yamamura). We ensured that miR-33 was deleted at the genomic and transcript levels in the descendant Ayu1-Cre\textsuperscript{+} miR-33\textsuperscript{flox/flox} (miR-33\textsuperscript{Δ/Δ}) mice (Online Figure IXA), and splicing of \textit{Srebf2} mRNA was not altered (Online Figure IXB and IXC). To generate cardiac fibroblast (CF)-specific knockout mice, miR-33\textsuperscript{flox/flox} mice and Pn-Cre\textsuperscript{+} miR-33\textsuperscript{flox/flox} mice were crossed, and miR-33\textsuperscript{flox/flox} littermates were used as control mice. Primer sequences for the probe for Southern blotting and genotyping are shown in Online Table II.

\textit{Pressure-overload model}

Transverse aortic constriction (TAC) was performed as described previously.\textsuperscript{8, 9} Briefly, 10- to 12-week-old mice were anesthetized with sodium pentobarbital (64.8 mg/kg) administered intraperitoneally and the proximal portion of the sternum was cut open to visualize the aorta. A 7-0 silk suture was placed around the aortic arch distal to the brachiocephalic artery. The suture was tightened firmly around a blunt 26-gauge needle placed adjacent to the aorta. The needle
was then removed, and the chest and overlying skin were closed. Sham-operated mice underwent the identical surgical procedure without ligation of aortic arch.

**Primary neonatal rat cardiomyocytes and fibroblasts**

Neonatal rat cardiomyocytes and CFs were isolated from 1-day old Sprague-Dawley rats, as described previously.\(^{10,11}\) After enzymatic digestion with pancreatin (Sigma, P3292), cardiomyocytes and fibroblasts were purified by Percoll density gradient centrifugation (Sigma, P4937). We made two-layer density gradients consisting of red-colored 65% Percoll solution underneath transparent 45% Percoll solution in 15 mL tubes. The cell suspension was layered on top of the gradient and the tubes were centrifuged at 3,000 rpm for 30 min. The fraction of cardiomyocytes was harvested from the newly formed layer between the Percoll solutions, and the fraction of fibroblasts was harvested from the top of the transparent Percoll solution.

**Culture of mouse fibroblasts**

For isolation of adult CFs, hearts from 8-week-old mice were digested with collagenase/dispace solution (Roche, 11097113001) and plated for 2 h.\(^{12}\) Attached CFs were cultured in Dulbecco’s modified Eagle’s medium (DMEM, 1%glucose) (Nacalai Tesque) supplemented with 10% fetal bovine serum (FBS) and antibiotics (Gibco\(^{\text{TM}},\ 10378016\) and non-essential amino acids (Gibco\(^{\text{TM}},\ 11140050\)). We used adult CFs at passage 3-4. For isolation of tail-tip fibroblasts (TTFs), the tails from 8-week-old mice were peeled, minced into 1 cm pieces, placed on gelatin-coated culture dishes, and incubated in DMEM (1% glucose) supplemented with 10% FBS and antibiotics for 5 days.\(^{13}\) Cells that migrated out of the graft pieces were transferred to
new plates (passage 1). We used TTFs at passage 4-5. Mouse embryonic fibroblasts (MEFs) were isolated from WT and miR-33KO embryos at E14.5 and were maintained in DMEM (4.5% glucose) supplemented with 10% FBS and antibiotics. We used MEFs at passage 5-6. In the experiment of Akt activation, fibroblasts on 6-well plates at a density of $2 \times 10^5$ cells/well were serum-starved for 2 h and stimulated with 3% FBS for 3 min. The fibroblasts were washed with chilled 1× phosphate-buffered saline (PBS) and then collected in chilled lysis buffer. In the experiment using LXR agonist, fibroblasts were cultured on 6-well plates at a density of $2 \times 10^5$ cells/well, and stimulated with 1 μmol/L T0901317 (Cayman, 71810) for 24 h.

Culture of mouse macrophage

Peritoneal macrophages were obtained from the peritoneal cavity of WT and miR-33KO mice 4 days after intraperitoneal injection of 3 mL 3% thioglycolate. The cells were washed with RPMI1640 (Nacalai Tesque), spun at 1200 rpm for 3 min, and plated at a density of $1.0 \times 10^6$ cells/mL. Cells were washed 1 h later and incubated for 2 days, and then used for experiments.

Lentiviral transfection

Lentiviral stocks were produced in 293T packaging cells as described previously. Briefly, lentiviral vectors were transfected into 293T cells using Polyethylenimine Max (Polysciences, Inc.), and virus-containing medium was collected 24 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 containing 8 μg/mL polybrene, followed by centrifugation at 1220 g for 30 min. For overexpression of miR-33, we used lentiviral expression vectors for miR-33 and
a negative control (miR-control) generated using a BLOCK-iT™ Pol II miR RNAi Expression Vector Kit (Thermo Fisher Scientific) in accordance with the manufacturer’s protocol. The miR-control vector contained a hairpin structure just as for a regular premiRNA, but which was predicted not to target any known vertebrate gene (pcDNA6.2-GW/EmGFP-miR-neg control plasmid). For knockdown of Abca1, we used shRNA lentiviral vectors. The siRNA sequences were designed using siDirect version 2.0 (http://sidirect2.rnai.jp/), and then the designed shRNA oligonucleotides were inserted into lentiviral vectors. The siRNA target sequences were as follows: AAATGTACTGCGCGTGGAG for siControl, GAAGAATCTGACATTTCGAAG for siABCA1-1, and GAAAGAAAGTTATGTATGAAG for siABCA1-2.

Transfection of microRNA mimics

For transfection of miR-33, fibroblasts were transfected with 1 nmol/L miR-33 mimic (mirVana™ miRNA mimic, P/N 4464066, ID: MC12410) or control mimic (P/N 4464058) using Lipofectamine® 2000 Transfection Reagent (Thermo Fisher Scientific) in accordance with the manufacturer's protocol. To analyze ABCA1 expression in Online Figure VIIB, CFs were transfected with 1 nmol/L miR-33 mimic or control mimic, incubated for 24 h, and then treated with LXR agonist (1 μmol/L T0901317) for 24 h.

RNA extraction

To extract total RNA, tissue samples were homogenized using a polytron homogenizer in 1 mL TRI Reagent® (Sigma, T9424), and monolayer cells were lysed directly on culture dishes in 1 mL TRI Reagent®. Total RNA was purified in accordance with the manufacturer's protocol. The
quantity and quality of total RNA were measured by using a NanoDrop™2000 spectrophotometer.

**Quantitative real-time PCR**

To evaluate mRNA expression, single-strand cDNA was synthesized from 1 µg of total RNA by means of reverse transcriptase reaction using Verso™ cDNA Synthesis Kit (Thermo Fisher Scientific, AB1453A) in accordance with the manufacturer's protocol, and quantitative PCR was performed using a LightCycler®96 (Roche Diagnostics) with THUNDERBIRD® SYBR qPCR Mix (TOYOBO, QPS-201). Expression levels were normalized by the indicated housekeeping genes. The primer sequences are listed in Online Table III.

**Quantitative real-time PCR for microRNAs**

Expression levels of miR-33a and miR-33b were measured using TaqMan® MicroRNA Assays (Applied Biosystems) and StepOnePlus™ Real-Time PCR System (Applied Biosystems) in accordance with the manufacturer's protocol. Expression levels of miRs were normalized by U6 small nuclear RNA and calculated by the $2^{-\Delta\Delta Ct}$ method.

**Microarray and Gene Ontology analysis**

To analyze gene expression comprehensively, we performed DNA microarray analysis. Five RNA samples from each group (WT sham, WT TAC, miR-33KO sham, and miR-33KO TAC) were pooled and analyzed using a DNA microarray (3D-Gene Mouse Oligo Chip 24k, TORAY). For microarray analysis of miRs, Mouse miRNA Oligo chip (TORAY) was used. After global
normalization, MA plots were made to visualize the normalized microarray data and to identify
differentially expressed genes. Gene Ontology analyses were performed using data sets of
differentially expressed genes with the clusterProfiler package in R/Bioconductor. Heatmaps
were made using the gplots package in R/Bioconductor.

Western blotting

Western blotting was performed using standard procedures as described previously. In vitro
cell lysates were collected using cell scrapers in chilled lysis buffer consisting of 100 mM
Tris-HCl, pH 7.4, 75 mM NaCl and 1% Triton X-100 (Nacalai Tesque) supplemented with
Complete Mini protease inhibitor cocktail (Roche, 11836153001), 0.5 mM NaF and 10 mM
Na$_3$VO$_4$ just before use. In vivo tissue samples were homogenized using a polytron homogenizer
and lysed in chilled lysis buffer. The protein concentration was determined using BCA protein
assay kit (Bio-Rad, 5000006JA). All samples (10 µg of protein) were suspended in lysis buffer,
fractionated using NuPAGE 4–12% Bis-Tris Mini gels (Thermo Fisher Scientific,
NP0322BOX) and transferred to a Protran nitrocellulose transfer membrane (Whatman). The
membrane was blocked using 1× PBS containing 5% non-fat milk for 30 min and incubated
with the primary antibody (Online Table IV) overnight at 4 °C. After a washing step in
PBS-0.05% Tween-20 (PBS-T), the membrane was incubated with the secondary antibody
(Online Table IV) for 1 h at room temperature. The membrane was then washed with PBS-T
and detected using Millipore IMMOBILON Western Chemiluminescent HRP Substrate (ECL)
(Fisher Scientific Co., WBKLS0500) or Pierce Western Blotting Substrate (Thermo Fisher
Scientific, NCI3106), with an LAS-4000 Mini system (Fuji Film). For quantification of western blots, densitometric analyses were performed using Fiji software.15

**Histology**

After administration of an overdose of anesthetics, mice were perfused with 4% paraformaldehyde (PFA) before excising the heart, and the tissue samples were further fixed in 4%PFA at 4°C overnight. On the next day, the tissue samples were transferred to 70% ethanol for dehydration before embedding in paraffin. After the heart sections were deparaffinized, they were stained with hematoxylin and eosin, Masson’s trichrome, and picrosirius red staining. Images were acquired using a microscope (BZ-9000, Keyence). We measured the fibrotic area of picrosirius-red staining images using Fiji.15 To analyze the perivascular fibrosis, the fibrotic areas around main coronary arteries in Masson’s trichrome staining were measured using Fiji, and the areas were normalized using the corresponding vessel areas. For wheat germ agglutinin (WGA) staining, heart sections were incubated for 1 h at room temperature with FITC-labeled WGA (Sigma, L4895) to visualize myocyte membranes. Cardiomyocyte cross-sectional areas were measured using Fiji.15 More than 100 cells per heart were measured, and the average values were used for analysis.

**Lipid raft staining**

We used fluorescence-conjugated cholera toxin subunit B (CTB) for lipid raft labeling. To obtain images of lipid raft staining, fibroblasts were plated on chamber slides and stained with Vybrant® Alexa Fluor®594 Lipid Raft Labeling Kit (Molecular Probes™, V34405). The slides
were observed using a confocal microscope. To quantify the signals from CTB-labeled lipid rafts on the cell surface, flow cytometric analyses were performed. Fibroblasts were plated on 10-cm culture dishes and cultured overnight. The cells were dissociated with trypsin EDTA, and stained with 1 μg/mL FITC-conjugated CTB (Sigma, C1655) at 4°C for 15 min, and then analyzed on FACS AriaII™ (Becton Dickinson). To make control samples with decreased lipid rafts, some samples were incubated with 5 mmol/L methyl-β-cyclodextrin (MβCD) for 1 h before labeling with CTB.

**BrdU incorporation in vivo**

*In vivo* bromodeoxyuridine (BrdU) incorporation was performed to analyze cell proliferation in the heart. Mice were subjected to TAC, and 7 days later 100 mg/kg BrdU (Sigma, B9285) was administrated intraperitoneally 2 h before sacrifice. Hearts were fixed as described above. After fixation, the tissues were transferred to 15% sucrose solution at 4°C for 4 h and to 30% sucrose solution at 4°C overnight, and then embedded in Tissue-Tek OCT compound (Sakura, Japan). BrdU staining was performed for 10-μm frozen sections. The sections were rinsed in PBS and autoclaved (110°C, 20 min) in 10 mmol/L sodium citrate buffer (pH 6) for heat-mediated antigen retrieval. The sections were blocked with 5% donkey serum/PBS at room temperature for 15 min, and then incubated with primary antibodies at 4°C overnight. An anti-BrdU antibody (Abcam, ab6326, 1:100 dilution) was used for BrdU detection. An anti-vimentin antibody (PROGEN, GP53, 1:100 dilution) was used for double staining with a fibroblast marker, vimentin. The slides were rinsed three times and then incubated with Alexa Fluor® 488- or 594-conjugated secondary antibodies (Online Table IV) with DAPI (1 μg/mL) at room
temperature for 1 h. The slides were washed and mounted in VECTASHIELD® Mounting Medium (Vector Laboratories, H-1000), and then observed under a Leica TCS SP8 confocal microscope with a 40× objective in accordance with the manufacturer's instructions. Slides without primary antibodies were used as negative controls. More than 10 images per heart were randomly acquired from LVs. The number of BrdU⁺ vimentin⁺ cells in each image was counted using Fiji,¹⁵ and the average value of the images from each sample was used for analysis.

**Immunostaining**

Immunostaining for Ki-67 or CD68 was performed using paraffin-embedded sections. After the heart sections were deparaffinized, they were rinsed in PBS and autoclaved for heat-mediated antigen retrieval in citrate buffer pH 6.0 for CD68 staining, and in EDTA buffer pH 8.0 for Ki-67 staining. The sections were blocked with 5% donkey serum/PBS at room temperature for 15 min, and then incubated with primary antibodies at 4°C overnight (Online Table IV). The slides were rinsed three times and then incubated with Alexa Fluor® 488- or 594-conjugated secondary antibodies (Online Table IV) with DAPI (1 μg/mL) at room temperature for 1 h. The slides were washed and mounted in VECTASHIELD® Mounting Medium (Vector Laboratories, H-1000), and then observed using a Leica TCS SP8 confocal microscope with a 40× objective in accordance with the manufacturer's instructions. Slides without primary antibodies were used as negative controls. For quantification of Ki-67 positive cells, more than 10 images per heart were randomly acquired from LVs. The number of Ki-67⁺ vimentin⁺ cells in each image was counted using Fiji,¹⁵ and the average value of the images from each sample was used for analysis.
**MTT assay**

Proliferation of fibroblasts was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated on 96-well culture dishes at a density of 1,000 cells/well and incubated in 100 μL medium containing 10% FBS. At the indicated time point, 10 μL of MTT solution (5 mg/mL) was added. Cells were incubated at 37°C for 4 h and lysed by adding 100 μL acidified isopropanol (40 mmol/L HCl). The absorbance at 595 nm was measured using a plate reader (ARVO X3, PerkinElmer). The reference absorbance at 690 nm was used to correct for non-specific background values.

**Flow cytometric analysis of apoptotic cells**

MEFs were plated on 10-cm culture dishes at a density of 3.0 × 10^5 cells/dish and cultured for 24 h in DMEM supplemented with 10% FBS. On the next day, the cells were stimulated with 1 mmol/L hydrogen peroxide (H_2O_2) for 4 h. The cells were dissociated with trypsin EDTA, and rinsed with PBS and stained using a Dead Cell Apoptosis Kit with Alexa® Fluor 488 annexin V and PI (Molecular Probes™, V13241), and then analyzed on FACSARiaII™ (Becton Dickinson) in accordance with the manufacturer's instructions. The flow cytometric data were analyzed using FlowJo version 10.1 (FLOWJO, LLC).

**Southern blotting**
Southern blotting was performed using a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, 11585614910) in accordance with the manufacturer’s protocol. Genomic DNA samples were purified and digested with BamHI. Primer sequences used to amplify the probe are shown in Online Table II.

**Echocardiography**

To analyze the cardiac function of mice, we performed echocardiography (Vevo®2100, VISUALSONICS) at the indicated time points after TAC. Mice were kept under inhalation anesthesia with 2.0% isoflurane. Left ventricular wall thickness and diameters were measured in M mode of the parasternal short-axis view. To analyze speckle-tracking strain imaging, we used Vevo Strain (Vevo®2100). A parasternal long-axis view was used to measure global longitudinal strain (GLS) and longitudinal strain rate (LSR). Endocardium was traced, and GLS (negative value), LSR (negative value), and early diastolic LSR (positive value) were calculated in Vevo Strain in accordance with the manufacturer’s protocol.

**Statistical analysis**

Measurements for clinical biopsy samples are presented as median ± interquartile range, and other measurements are presented as means ± standard error of the mean (SEM). To compare gene expression levels and the hemodynamic parameters in Figure 1A, linear-regression analysis was performed on R version 3.2.3 (The R Project). P values and r values are shown. For Gene Ontology analysis, the clusterProfiler package in R/Bioconductor was used for data sets with differentially expressed genes as described above. P values were adjusted by FDR.
correction. For other statistical comparisons, unpaired Student’s t-test (two groups, parametric), Mann–Whitney test (two groups, non-parametric), or one-way ANOVA with Sidak’s post-hoc test (three or more groups) were used as indicated in the figure legends. A p value of <0.05 was considered as statistically significant. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc.).
Online Table I. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Low-stage HF</th>
<th>High-stage HF</th>
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<tr>
<td>Patients, n</td>
<td>33</td>
<td>17</td>
<td>11</td>
<td></td>
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<tr>
<td>Age, y</td>
<td>55.8 ± 12.3</td>
<td>54.8 ± 14.2</td>
<td>58.4 ± 10.8</td>
<td>0.47</td>
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<tr>
<td>Female, n (%)</td>
<td>13 (39%)</td>
<td>8 (47%)</td>
<td>2 (18%)</td>
<td>0.23</td>
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<tr>
<td>Body Mass Index, kg/m²</td>
<td>25.2 ± 4.2</td>
<td>24.3 ± 4.1</td>
<td>26.1 ± 4.1</td>
<td>0.28</td>
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<td>Diabetic mellitus, n (%)</td>
<td>4 (12%)</td>
<td>1 (5.9%)</td>
<td>3 (27%)</td>
<td>0.27</td>
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<tr>
<td>Hypertension, n(%)</td>
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<td>7 (41%)</td>
<td>4 (36%)</td>
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<tr>
<td>Dyslipidemia, n (%)</td>
<td>7 (21%)</td>
<td>3 (18%)</td>
<td>2 (18%)</td>
<td>1.0</td>
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<tr>
<td>Hemodynamic parameters</td>
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<tr>
<td>Cardiac Index, L/min/m²</td>
<td>2.7 ± 1.0</td>
<td>3.2 ± 1.1</td>
<td>1.9 ± 0.2</td>
<td>0.0004***</td>
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<tr>
<td>mean PCWP, mmHg</td>
<td>11.1 ± 7.1</td>
<td>7.9 ± 2.7</td>
<td>16.0 ± 9.3</td>
<td>0.0022**</td>
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<td>Ejection fraction, %</td>
<td>38.7 ± 15.6</td>
<td>41.4 ± 13.4</td>
<td>28.1 ± 9.8</td>
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<td>Serum lipid levels</td>
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<tr>
<td>Total cholesterol, mg/dL</td>
<td>195.2 ± 57.0</td>
<td>207.6 ± 64.2</td>
<td>164.6 ± 25.8</td>
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<tr>
<td>Triglyceride, mg/dL</td>
<td>127.0 ± 71.1</td>
<td>138.0 ± 85.8</td>
<td>122.6 ± 46.1</td>
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<td>HDL-C, mg/dL</td>
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<td>50.9 ± 12.6</td>
<td>40.5 ± 9.1</td>
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<td>LDL-C, mg/dL</td>
<td>121.0 ± 42.7</td>
<td>128.2 ± 50.3</td>
<td>102.5 ± 17.0</td>
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<tr>
<td>Medication, n (%)</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Statin</td>
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<td>12 (71%)</td>
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<td>Furosemide</td>
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<td>9 (82%)</td>
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<td>Thiazide</td>
<td>1 (3.0%)</td>
<td>0 (0%)</td>
<td>1 (9.1%)</td>
<td>0.39</td>
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</tbody>
</table>

Data for continuous variables and categorical variables are presented as mean ± SD and number of patients (%), respectively. Five patients did not have the parameters for Forrester classification. P values for continuous variables and categorical variables were calculated by two-tailed unpaired Student’s t-tests and two-tailed Fisher’s exact tests, respectively.

*P<0.05, **P<0.01, ***P<0.001
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sequence</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
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F: Forward, R: Reverse
## Online Table III. Primer sequences for quantitative real-time PCR

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**Online Table IV. Antibodies used for western blotting and immunostaining.**

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Supplemental References


Online Figure I. Heart weight was increased similarly in WT and miR-33KO mice after TAC. A and B, Heart rate (A) and blood pressure (B) of WT and miR-33KO mice at the age of 10 weeks (n=5 and 7). C and D, Changes in heart weight in 2 weeks (C) or in 4 weeks (D) after TAC (n=5 each). Heart weight (mg) was normalized with body weight (g). *P<0.05, **P<0.01 vs sham, n.s. not significant vs WT, by ANOVA with Sidak correction. E, Expression levels of miR-33 in LVs in the early phase (2 weeks after TAC, n=6) and in the chronic phase (10 weeks after TAC, n=9). *P<0.05 by unpaired two-tailed Student’s t-test. Data are presented as mean ± SEM.
Online Figure II. Gene Ontology (GO) analysis showed enriched biological processes related to extracellular matrix and collagen. A and B, Top 10 GO terms (biological process) enriched in miR-33KO left ventricles. The microarray data detected 114 up-regulated genes (KO/WT fold change>2) and 148 down-regulated genes (KO/WT fold change<0.5) in miR-33KO hearts 2 weeks after TAC. The up-regulated genes (A) and the down-regulated genes (B) were categorized by GO analysis. Adjusted p values in each GO term were shown.
Online Figure III. Heatmap data showed modulated expression of fibrosis-related genes after TAC in miR-33KO mice. A, B, C, and D, Heatmap data of genes involved in cardiac remodeling. The sets of genes were picked up from Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The up-regulation of fibrosis-related genes induced by TAC was suppressed in miR-33KO mice (orange cluster in A, red and
orange cluster in C, and red cluster in D), whereas the genes involved in cardiac muscle contraction showed only small changes (B).
Online Figure IV. Deficiency of miR-33 did not affect macrophage infiltration induced by TAC in vivo. A, Immunostaining images of macrophage infiltration. CD68+ macrophages were seen mainly in the fibrotic area both in WT and miR-33KO mice at 2 weeks after TAC. B, Quantitative real-time PCR analysis of macrophage markers *Cd68* and *Adgre1* (known as F4/80) (n=7-10). *P<0.05, **P<0.01 vs sham, n.s. not significant vs WT, by ANOVA with Sidak correction. C, Gene expression of macrophages in vitro. Peritoneal macrophages from
WT and miR-33KO were analyzed by quantitative real-time PCR. *P<0.05 by unpaired two-tailed Student’s t-test (n=4). Data are presented as mean ± SEM.
Online Figure V. Deficiency of miR-33 did not affect diastolic function. A, Longitudinal strain rate (LSR) analyzed using speckle-tracking strain imaging with Vevo2100. B, Early diastolic LSR (a parameter for diastolic function) analyzed using speckle-tracking imaging.
Online Figure VI. Isolated neonatal rat cardiomyocytes and cardiac fibroblasts were purified by Percoll gradient separation. Expression of *Nppb* and *Col1a1* in isolated cardiomyocyte and fibroblast fractions (n=5).

**P<0.01 by unpaired two-tailed Student’s t-test. Data are presented as mean ± SEM.
Online Figure VII. ABCA1 is regulated by miR-33. A, Expression levels of Npc1 mRNA in cardiac fibroblasts (CFs). B, Western blotting analysis of ABCA1 and NPC1 in cardiac fibroblasts (CFs). CFs were transfected with control mimic or miR-33 mimic. T0901317 (LXR agonist) was used at 1 μmol/L. GAPDH was
used as an internal control. *P<0.05 vs control, by unpaired two-tailed Student’s t-test. C, Western blotting analysis of ABCA1 and NPC1 in WT and miR-33KO CFs. GAPDH was used as an internal control. *P<0.05 vs WT, by unpaired two-tailed Student’s t-test. D, The efficiency of lentiviral knockdown of Abca1. The expression levels of Abca1 in WT and miR-33KO MEFs with siABCA1 were analyzed by quantitative PCR (n=4). **P<0.01 vs WT, ##p<0.01 vs siControl by ANOVA with Sidak correction. E, Impaired proliferation of fibroblasts by miR-33 overexpression. CFs were transfected with a lentiviral miR-33 expression vector or a control miR expression vector (miR-control). MTT assay was performed 1 day and 7 days after transfection. **P<0.01 vs miR-control, ##p<0.01 vs Day 1, by ANOVA with Sidak correction. Data are presented as mean ± SEM.
Online Figure VIII. Generation of miR-33-floxed mice. A, Schematic representation of the targeting vector and expected gene replacement at the miR-33 locus. White triangles, loxP sequences; white rhombuses, FRT.
sequences. **B**, Genotyping PCR using tail genomes of indicated genotypes. In miR-33^Δ/Δ^ mice, miR-33 loci were deleted by crossing with Ayu1-Cre mice. **C** and **D**, Southern blotting using the genome of ES cells (C) and tail genome (D) of indicated genotypes.
Online Figure IX. Expression and splicing of Srebf2 mRNA were not altered in miR-33-floxed or miR-33-deleted mice. A, Expression levels of Srebf2 mRNA, precursors of miR-33, and mature miR-33 in the livers of 8-week-old mice (n=4). Expression levels of Srebf2, pri-miR-33, and pre-miR-33 were normalized by

Online Figure IX
18S ribosomal RNA. Expression levels of mature miR-33 were normalized by U6. P values by unpaired Student’s t-test are shown. n.s. not significant. Data are presented as mean ± SEM. B, Sequencing alignment at the junction between exons 16 and 17 of Srebf2 mRNA in the indicated genotypes. C, RT-PCR analysis of Srebf2 in the indicated genotypes. There was no other bands except for that of the correct size.
Online Figure X. HDL-C levels and gene expression levels in fibroblast-specific miR-33 KO (FBKO) mice. A and B, HDL-C levels in miR-33Δ/Δ (Ayu1-Cre′miR-33floxflox) mice (A) (n=4) and FBKO (Pn-Cre+miR-33floxflox) mice (B) (sham, n=6; TAC, n=18). C, Quantitative real-time PCR analysis in FBKO mice 2 weeks after TAC (n=18). Mean expression levels in five sham-operated miR-33floxflox mice were defined as 1.0. Expression levels were normalized by Gapdh. P values by unpaired Student’s t-test (A and C) or by one-way ANOVA with Sidak correction (B) are shown. **P<0.01. Data are presented as mean ± SEM. Control, miR-33floxflox mice. FBKO, Pn-Cre′miR-33floxflox mice.
Online Figure XI. Western blotting analysis of cyclin-dependent kinase 6 (CDK6). CDK6 was not up-regulated in miR-33KO fibroblasts.
Online Figure XII. Deficiency of miR-33 had little impact on the expression of other miRs. A,

Differentially expressed miRs in miR-33KO heart. Total RNA samples from WT and miR-33KO hearts were analyzed using a microRNA-microarray. Only small number of miRs were differentially expressed (fold change>2). B, The list of differentially expressed miRs shown in A.
Online Figure XIII. Mechanism model of lipid raft maintenance by miR-33. A, Cholesterol is enriched in microdomains on the plasma membrane called lipid rafts, which function as platforms that concentrate and segregate proteins such as growth factor receptors. In wild-type cells, expression levels of ABCA1 and ABCG1, which are involved in cholesterol efflux, are suppressed by miR-33 post-transcriptionally. Accordingly, lipid rafts on the plasma membrane are preserved. However, in miR-33KO cells, efflux of cholesterol is accelerated because of loss of inhibition of ABCA1 and ABCG1. Therefore, lipid raft cholesterol content is decreased. As a result, signal transduction from outside the cell is impaired in miR-33KO cells. B, In pressure overload model, proliferation of cardiac fibroblasts (CFs) is inhibited in miR-33KO mice because of the decreased lipid rafts as shown in A. Accordingly, reactive fibrosis, which is not a replacement of dead cardiomyocytes but an adaptive response to pathological stimuli, is impaired. As a result, adaptive remodeling such as producing protective paracrine factors is insufficient in miR-33KO mice.