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MicroRNA-27a Regulates Beta Cardiac Myosin Heavy Chain Gene Expression by Targeting Thyroid Hormone Receptor β1 in Neonatal Rat Ventricular Myocytes▽

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MicroRNAs (miRNAs), small noncoding RNAs, are negative regulators of gene expression and play important roles in gene regulation in the heart. To examine the role of miRNAs in the expression of the two isoforms of the cardiac myosin heavy chain (MHC) gene, α- and β-MHC, which regulate cardiac contractility, endogenous miRNAs were downregulated in neonatal rat ventricular myocytes (NRVMs) using lentivirus-mediated small interfering RNA (siRNA) against Dicer, an essential enzyme for miRNA biosynthesis, and MHC expression levels were examined. As a result, Dicer siRNA could downregulate endogenous miRNAs simultaneously and the β-MHC gene but not α-MHC, which implied that specific miRNAs could upregulate the β-MHC gene. Among 19 selected miRNAs, miR-27a was found to most strongly upregulate the β-MHC gene but not α-MHC. Moreover, β-MHC protein was downregulated by silencing of endogenous miR-27a. Through a bioinformatics screening using TargetScan, we identified thyroid hormone receptor β1 (TRβ1), which negatively regulates β-MHC transcription, as a target of miR-27a. Moreover, miR-27a was demonstrated to modulate β-MHC gene regulation via thyroid hormone signaling and to be upregulated during the differentiation of mouse embryonic stem (ES) cells or in hypertrophic hearts in association with β-MHC gene upregulation. These findings suggested that miR-27a regulates β-MHC gene expression by targeting TRβ1 in cardiomyocytes.

MicroRNAs (miRNAs) are negative regulators of gene expression that inhibit the translation or promote the degradation of target mRNAs (45). Mature miRNAs (10 to 24 nucleotides long) are the result of sequential processing of primary transcripts (primary miRNAs) mediated by two RNase III enzymes, Drosha and Dicer (6). Recently, it has been reported that cardiac cell-specific Dicer-deficient mice presented with cardiac dysfunction, such as cardiac sudden death, dilated cardiomyopathy, and heart failure (4, 7), suggesting an essential role of the miRNA-processing machinery in the maintenance of cardiac function. A series of microarray analyses in rodent and human hearts has revealed the profile of miRNA expression under various pathological conditions, such as cardiac hypertrophy, heart failure, and myocardial infarction, which indicated the involvement of miRNAs in cardiac pathophysiology (5, 34, 36, 39, 40, 42, 44, 46). Moreover, genetically modified mice have revealed the effect of specific miRNAs in the heart. For example, mice lacking miR-1-2 suffer from cardiac arrhythmia and congenital malformation, and transgenic mice that overexpress miR-195 in the heart or mice lacking miR-133a, a muscle-specific miRNA, present with cardiac dilatation and heart failure (25, 44, 49). These previous studies have revealed the novel role of miRNAs in cardiac development and pathophysiology.

Cardiac contractility depends on the expression of two cardiac myosin heavy chain (MHC) genes, α- and β-MHC, which are regulated in an antithetical manner by developmental, physiological, and pathological signals (47). Moreover, the β-MHC gene is upregulated in response to stress signals causing cardiac hypertrophy and heart failure. Therefore, a search for factors, including miRNAs, that can regulate β-MHC gene expression may give new findings for heart disease and therapy.

Recently, two groups have revealed that cardiac cell-specific miR-208a, encoded by an intron of the α-MHC gene, is important for the regulation of β-MHC gene expression using miR-208a-deficient mice and transgenic mice that overexpress miR-208a under the control of the α-MHC promoter (1, 45). Thus, genetically modified mice are a powerful tool for elucidating the final outcome derived from specific miRNAs. However, *in vitro* screening analyses are still needed to detect the direct effects of individual miRNAs because neurohormonal and hemodynamic effects, among others, are considered to strongly influence the gene regulation of β-MHC (30, 32) and possibly blur many direct effects of miRNAs *in vivo*.

Thyroid hormone has a fundamental role in cardiovascular homeostasis under both physiological and pathological conditions, influencing cardiac contractility, heart rate, diastolic function, and systemic vascular resistance through genomic

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and nongenomic effects (12). These multiple effects are largely mediated by the action of nuclear-based thyroid hormone receptors (TRs) (8). In particular, α-MHC expression is increased by tri-iodothyronine (T3), an active form of thyroid hormone, and β-MHC expression is decreased (8). Moreover, the alterations in thyroid hormone signaling are associated with cardiac pathophysiology, such as hypertrophy and heart failure (8, 12).

In the present study, Dicer small interfering RNA (siRNA) and 19 selected miRNAs, which have been reported previously to be muscle specific or upregulated in cardiac hypertrophy or heart failure (9), were individually transduced into neonatal rat ventricular myocytes (NRVMs) using a lentiviral vector, and MHC gene expression was evaluated. We showed (i) that downregulation of Dicer, an essential enzyme for miRNA biosynthesis, globally reduced endogenous miRNAs, which resulted in downregulation of the β-MHC gene, (ii) that miR-27a was a novel factor that could regulate β-MHC gene expression via thyroid hormone receptor β1 (TRβ1) in NRVMs, and (iii) that miR-27a was upregulated during the differentiation of mouse embryonic stem (ES) cells or in hypertrophic hearts of rodents in association with β-MHC gene upregulation.

MATERIALS AND METHODS

Cell culture. NRVMs were isolated from 1-day-old Sprague-Dawley rats as described previously (16). 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 Multiwell Primaria six-well plates (Becton Dickinson) at 37°C in a 5% CO_2 incubator. DNA transduction was carried out 48 h after the cells were plated. Cultures were treated with tri-iodothyronine (T5516; Sigma).

The 129/Ola-derived mouse ES cell line ht7 was maintained as described previously (17). For DNA transduction, undifferentiated ES cells (defined as day 0) were plated on gelatinized dishes with lentivirus-containing medium and cultured for 24 h. The promoter of the lentiviral vectors was replaced with a phosphoglycerate kinase (PGK) promoter to avoid gene silencing effects in ES cells. For differentiation induction, 3×10^4 ES cells/well were seeded into gelatinized six-well plates and cultured two-dimensionally without leukemia-inhibitory factor, feeder cells, or the formation of embryoid bodies for 8 days (38).

Animals. Male 10-week-old C57BL/6 mice were treated with a transverse aortic constriction (TAC) procedure as described previously (33). Fetal ICR mouse heart samples were obtained at 16 days after the vaginal plugs of female mice were checked. Samples were mixed and used for quantitative reverse transcription-PCR (qRT-PCR). The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (28) and was approved by the Institutional Animal Research Committee of Kyoto University.

Plasmids. MicroRNA (miRNA)-expressing vectors were constructed using a BLOCK-iT Pol II miR RNAi Expression Vector kit (Invitrogen) in accordance with the manufacturer's instructions. A control miRNA-expressing vector (Cont. miR) was obtained from the kit. For the construction of anti-miR-27a, double-stranded oligonucleotides containing three or six sequences that were completely complementary to miR-27a were inserted into a pMIR-REPORT vector (Ambion) at the PmeI site (miR-27a decoy) in accordance with previous studies (3, 10, 29). siRNA vectors were constructed from pSINsi-mU6 DNA (Takara Bio.). Double-stranded oligonucleotides were inserted into pSINsi-mU6 DNA at the BamHI/ClaI sites. Oligonucleotides targeting specific genes and the control siRNA were as follows: Dicer siRNA, 5′-GGAATGGACTCTGAGCTTA-3′; TRβ1 siRNA1, 5′-GGAATGTCGCTTTAAGAAA-3′; TRβ1 siRNA2, 5′-GGAAGCTGAAGAGAA-3′; retinoid X receptor α (RXRα) siRNA1, 5′-CAAGAGGACAGTACGCAAA-3′; RXRα siRNA2, 5′-CCAAGACTGAGACATACGT-3′; control siRNA, 5′-AATAATAATGGGGGGATCC-3′. All of these constructs were inserted into a pLenti6/V5-D-TOPO vector (Invitrogen). The rat TRβ1 gene was amplified and cloned into a pLenti6/V5-D-TOPO vector using the following primers: forward, 5′-ATGACAGAAAATGGCCTTCCAGCCT-3′; reverse, 5′-TCAGTCCTCAAAGACTTCCAAGAA-3′. The following primers were used to amplify and clone a part of the 3′ untranslated region (UTR) of the rat TRβ1 and thyroid hormone receptor-associated protein 1 (THRAP1) and the human TRβ1 and THRAP2 into a pMIR-REPORT luciferase vector in accordance with the manufacturer's instructions: rat TRβ1 3′ UTR, 5′-GGACTAGTCAGACCATGCATAGGAAACACCAT-3′ (forward) and 5′-CCCAAGCTTCACCCACATGCATTCCGTTTCCGAA-3′ (reverse); human TRβ1 3′ UTR, 5′-ACAAGCCCTGGCCCCTCCTCGACA-3′ (forward) and 5′-GCACAGTAAAATTCTGTGATAAG-3′ (reverse); THRAP1 3′ UTR, 5′-GACTTACTAATGTACTGTCACAGA-3′ (forward) and 5′-ATACAGTAATCTGTGCCATACTGA-3′ (reverse); THRAP2, 5′-CCGGGAAGCGCTTGCCCTCTGCCT-3′ (forward) and 5′-AGCCCCAGTGCTAGATCCTGTACT-3′ (reverse). The rat β-MHC luciferase promoter construct consisted of the firefly luciferase cDNA driven by a 333-bp rat β-MHC promoter sequence (15).

Lentivirus production and DNA transduction. Lentiviral stocks were produced in 293FT cells in accordance with the manufacturer's protocol (Invitrogen). In brief, virus-containing medium was collected 48 h posttransfection and filtered through a 0.45-μm-pore-size filter. One round of lentiviral infection was performed by replacing the medium with virus-containing medium supplemented with 8 μg/ml of Polybrene, followed by centrifugation at 2,500 rpm for 30 min at 32°C.

RNA extraction and qRT-PCR. Total RNA was isolated and purified from NRVMs, C57BL/6, or ICR mouse hearts using TRIzol reagent (Invitrogen), and cDNA was synthesized from 5 μg of total RNA using SuperScriptII reverse transcriptase (Invitrogen) in accordance with the manufacturer's instructions. For qRT-PCR, specific genes were amplified by 40 cycles using SYBR Green PCR Master Mix (Applied Biosystems). Expression was normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used were as follows: GAPDH forward, 5′-TTGCCATCAACGACCCCTTC-3′; GAPDH reverse, 5′-TTGTCATGGATGACCTTGGC-3′; Dicer forward 5′-ATGCGATTTTGGACTACCTCATAAC-3′; Dicer reverse, 5′-TCAGCTGTTAGGAACCTGAGGCTGG-3′; atrial natriuretic factor (ANF) forward, 5′-TTCCTCGTCTTGGCCTTTTG-3′; ANF reverse, 5′-CCTCATCTTCTACCGGCATCTTC-3′; rat brain natriuretic peptide (BNP) forward, 5′-TTCCGGATCCAGGAGAGACTT-3′; BNP reverse, 5′-CCTAAAACAACCTCAGCCCGT-3′; mouse BNP forward, 5′-GCCAGTCTCCAGAGCAATTCA-3′; BNP reverse, 5′-TGTTCTTTTGTGAGGCCTTGG-3′; rat α-MHC forward, 5′-GACACCAGCGCCCACCTG-3′; rat α-MHC reverse, 5′-ATAGCAACAGCGAGGCTCTTTCTG-3′; rat β-MHC forward, 5′-GGAGCTCACCTACCAGACAGA-3′; rat β-MHC reverse, 5′-CTCAGGGCTTCACAGGCATCC-3′; mouse α-MHC forward, 5′-GAGATTTCTCCAACCCAG-3′; mouse α-MHC reverse, 5′-TCTGACTTTCGGAGGTACT-3′; mouse β-MHC forward, 5′-CTACAGGCCTGGGCTTACCT-3′; mouse β-MHC reverse 5′-TCTCCTTCTCAGACTTCCGC-3′; Nkx2.5 forward, 5′-CAAGTGCTCTCCTGCTTTCC-3′; Nkx2.5 reverse, 5′-GGCTTTGTCCAGCTCCACT-3′; rat TRβ1 forward, 5′-AGCCAGCCACAGCACAGTGA-3′; rat TRβ1 reverse, 5′-CGCCAGAAGACTGAAGCTTGC-3′; mouse TRβ1 forward, 5′-AAGCCACAGGGTACCACTATGG-3′; mouse TRβ1 reverse, 5′-GGAGACTTTTCTGAATGGTTCTTCTAA-3′; DDR2 forward, 5′-AGTCAGTGGTCAGAGTCCACAGC-3′; DDR2 reverse, 5′-CAGGGCACCAGGCTCCATC-3′. We used TaqMan MicroRNA Assays (Applied Biosystems) to determine the expression levels of miRNAs in accordance with the manufacturer's instructions.

Northern blotting analysis. Northern blotting analysis was performed as described previously (31). In brief, small RNA fractions were isolated from total RNA using a *mir*Vana miRNA isolation kit (Ambion). The small RNA fractions (5 μg) were separated by electrophoresis using a 15% polyacrylamide (19:1) denaturing gel and transferred to nylon hybridization membrane (Hybond-NX; Amersham) using a semidry electroblotter (Bio-Rad, Hercules, CA) at 20 V for 30 min at 4°C. Cross-linking of RNA was performed using 0.16 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; Sigma) in 0.13 M 1-methylimidazole (Sigma) at pH 8.0 for 2 h at 60°C. Templates to make probes for miR-16, miR-133b, and U6 were prepared using a *mir*Vana miRNA Probe Construction Kit (Ambion) with the following oligonucleotides: miR-16 5′-TAGCAGCACGTAAATATTGGCGCCTGTCTC-3′; miR-133b 5′-TTGGTCCCCTTCAACCAGACCTGTCTC-3′; U6, 5′-CGATACAGAGAAGATTAGCATGGCCCCTGCCCTGTCTC-3′.

Luciferase assay. For luciferase reporter assays, constructs were transiently transfected using Fugene 6 (Roche) into 293FT cells or using Lipofectamine 2000 (Invitrogen) into NRVMs at the following concentrations: 0.1 μg of firefly luciferase reporter gene, 0.01 μg of pRL-TK *Renilla reniformis* luciferase control plasmid (Promega), and 0.1 μg of BLOCK-iT Pol II miR RNAi Expression Vector encoding the appropriate miRNA or the control. At 24 h after transfection, both luciferase activities were measured using a dual luciferase reporter

assay system (Toyo Ink Co.). Firefly luciferase activity was normalized for transfection efficiency by measuring that of *Renilla reniformis* control activity in accordance with the manufacturer's instructions.

Western immunoblot analysis. Immunoblot analysis was performed using standard procedures as described previously (48). Cultured cells and C57BL/6 mouse hearts 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), 0.5 mM NaF, and 10 μM Na_3VO_4 just prior to use. The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Bio-Rad). A total of 2 or 10 μg of protein was fractionated using NuPAGE 4 to 12% Bis-Tris (Invitrogen) gels and transferred to a Protran nitrocellulose transfer membrane (Whatman). The membrane was blocked using 1× phosphate-buffered saline (PBS) containing 5% nonfat milk for 1 h and incubated with the primary antibody overnight at 4°C. Following a washing step in 1× PBS–0.05% Tween 20 (0.05% T-PBS), the membrane was incubated with the secondary antibody for 1 h at 4°C. After the membrane was washed in 0.05% T-PBS, the immunocomplexes were detected using ECL Western Blotting Detection Reagent (Amersham Biosciences). The following primary antibodies were used: anti-GAPDH (Cell Signaling Technology) at a dilution of 1:1,000, anti-Dicer (sc-25117; Santa Cruz Biotechnology, Inc.) at 1:500, anti-α-MHC (ab50967; Abcam) at 1:20,000, anti-skeletal slow myosin (M8421; Sigma) for detecting β-MHC at 1:20,000, anti-TRβ1 (sc-738; Santa Cruz Biotechnology, Inc.) at 1:500, and anti-RXRα (sc-553; Santa Cruz Biotechnology, Inc.) at 1:500. As secondary antibodies, anti-rabbit, anti-mouse, and anti-goat IgG (GE Healthcare) were used at a dilution of 1:2,000. Immunoblots were detected using an LAS-1000 system (Fuji Film).

Statistics. Data are presented as means ± standard errors (SE). Statistical comparisons were performed using unpaired two-tailed Student's *t* tests or a one-way analysis of variance and Bonferroni's posthoc test, where appropriate, with a probability value of <0.05 taken to indicate significance.

RESULTS

Dicer siRNA can downregulate endogenous miRNAs and the β-MHC gene in NRVMs. To investigate the effects of miRNAs on cardiac MHC gene expression, we first tried to suppress the function of endogenous miRNAs in NRVMs. Because Dicer is an essential enzyme for miRNA biosynthesis, it was hypothesized that the downregulation of Dicer could result in a global reduction of miRNAs, which equates to a loss of function of miRNAs. Dicer siRNA was transduced into NRVMs using a lentiviral vector, which resulted in the downregulation of both Dicer mRNA and protein under serum-containing conditions (Fig. 1A and B). Moreover, Dicer siRNA could downregulate ubiquitously expressed miR-16 and muscle-specific miR-133b at the same time (Fig. 1C), suggesting the global reduction of endogenous miRNAs in NRVMs. The MHC gene has two isoforms, α- and β-MHC, and the gene expression of both isoforms was assessed in NRVMs into which Dicer siRNA was transduced. As a result, Dicer siRNA decreased both the mRNA and protein levels of β-MHC but not those of α-MHC (Fig. 1D and E). These results indicated that a loss of function of miRNAs could suppress β-MHC gene expression specifically in NRVMs under serum-containing conditions, and there were specific miRNAs that can upregulate β-MHC gene expression.

Next, to identify miRNAs that can upregulate β-MHC gene expression, 19 selected miRNAs were individually transduced into NRVMs using a lentiviral vector, and mRNA levels of β-MHC were examined under serum-containing conditions. These miRNAs contained six muscle-specific miRNAs: miR-1, -133a, -133b, -208a, -208b, and -499 (1, 25, 38, 43, 45). Thirteen other miRNAs were included because they were upregulated in diseased human hearts or in *in vivo* and *in vitro* models mimicking cardiac hypertrophy or heart failure, and were reported by more than 4 out of 10 miRNA microarray analyses performed by six independent groups (9). miR-23a, -125b, and -214 were identified in seven analyses; miR-21 was detected in six; let 7c and miR-24, -27a, and -195 were identified in five; and let 7b and miR-27b, -103, -140*, and -199* were reported in four. As a result, miR-27a was found to increase β-MHC mRNA levels most strongly among the miRNAs assessed, while several miRNAs could increase β-MHC levels to a lesser extent (Fig. 1F). The lentiviral vector used in this assay could express miR-27a, -27b, and -499 at abundant levels, which exceeded those of endogenous miRNAs in NRVMs (Fig. 1G). Thus, it was considered that these lentiviral vectors could express each miRNA sufficiently to suppress target gene expression to minimal levels, and the expression of each target gene could be suppressed equivalently even if the absolute expression levels of the overexpressed miRNAs were different (29). According to previous reports (1, 43, 45), miR-208a, -208b, and -499 can induce β-MHC gene expression. Therefore, it was examined whether these miRNAs could upregulate β-MHC gene expression in this assay system. As a result, miR-208a, -208b, and -499 could increase β-MHC protein levels (Fig. 1H). However, the reduction of these miRNAs in NRVMs transduced with Dicer siRNA was smaller than that of miR-27a (Fig. 1I), suggesting a stronger involvement of miR-27a in β-MHC gene regulation under these experimental conditions.

miR-27a can upregulate the β-MHC gene in NRVMs. To confirm whether miR-27a was actually upregulated under cardiac-pathological conditions in which β-MHC gene expression was upregulated, miR-27a levels were examined in Dahl salt-sensitive rat hearts, in which a high-salt diet can induce hypertensive hypertrophy at 11 weeks, followed by heart failure at 17 weeks. In pathological hearts of Dahl salt-sensitive rats fed a high-salt diet for 11 or 17 weeks, β-MHC gene expression was increased in association with the upregulation of ANF and BNP mRNA levels, which are markers of cardiac-pathological conditions (Fig. 2A and B). Moreover, miR-27a was also upregulated under both pathological conditions in Dahl salt-sensitive rat hearts (Fig. 2C), implicating an association between miR-27a and β-MHC gene expression.

Next, to study whether endogenous miR-27a was expressed in cardiac myocytes, miR-27a levels were examined both in NRVMs and cardiac fibroblasts. The appropriate separation between both types of cells was confirmed by detecting the mRNA of β-MHC, a myocyte-specific marker, or collagen receptor discoidin domain receptor 2 (DDR2), a cardiac fibroblast-specific marker (2) (Fig. 2D). As a result, miR-27a was found to be expressed more highly in cardiac myocytes than in cardiac fibroblasts (Fig. 2E). Further examination of miR-27a confirmed that overexpression of miR-27a in NRVMs could increase β-MHC gene expression at both mRNA and protein levels under serum-containing conditions but did not affect the level of α-MHC (Fig. 2F and G). miR-27a could also increase BNP mRNA levels but not ANF levels (Fig. 2H). These findings suggested that the upregulation of the β-MHC gene by miR-27a might result from the direct effects of miR-27a rather than from a secondary change to cardiac pathology induced by miR-27a.

Loss of function of miR-27a decreases β-MHC protein levels in NRVMs. To examine the direct effect of miR-27a on β-MHC gene regulation, β-MHC protein expression was also

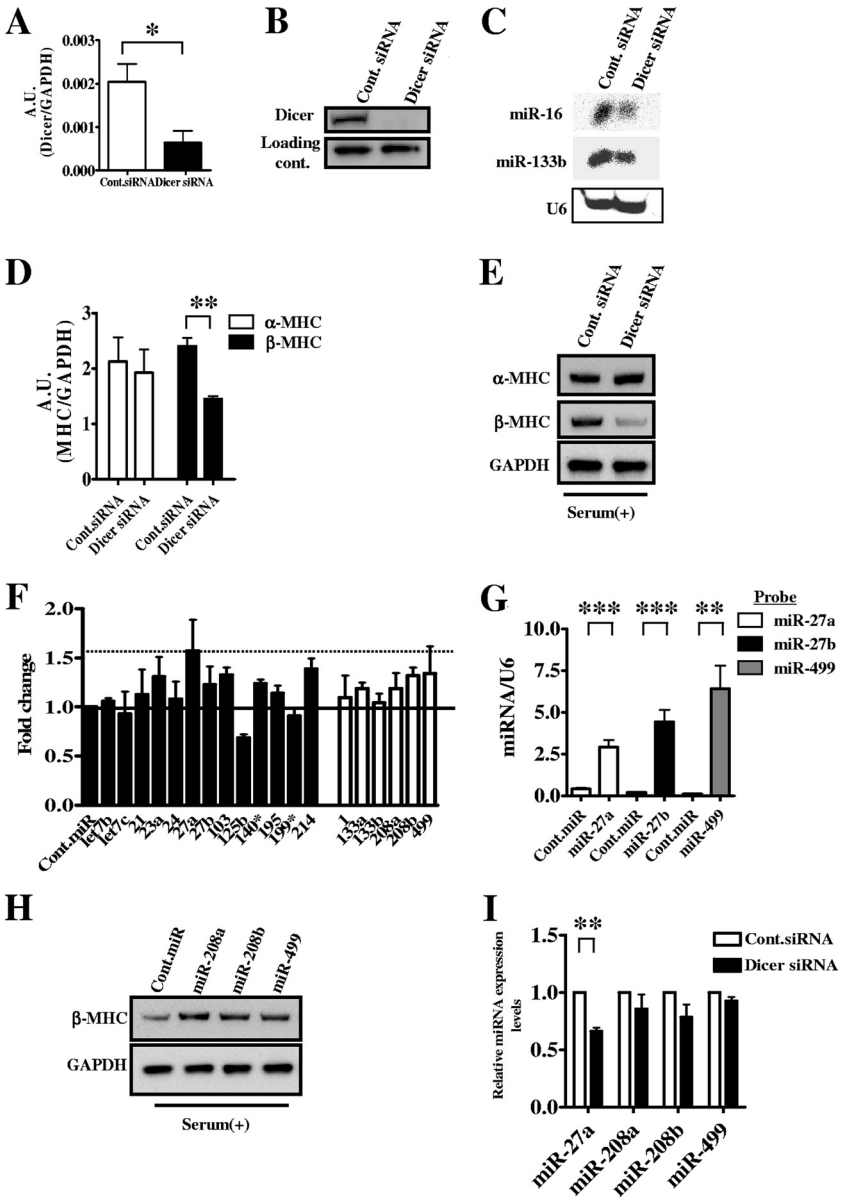

FIG. 1. Dicer siRNA downregulates endogenous miRNAs and β-MHC gene expression levels. Assays were performed 72 h (A to D and F to I) or 96 h (E) after transduction with Dicer siRNA or control siRNA (Cont.siRNA) and individual miRNAs or miR-control (Cont.miR) into NRVMs using a lentiviral vector. NRVMs were cultured under serum-containing medium. (A and B) mRNA (A) and protein (B) levels of Dicer. (C) Endogenous miR-16 and miR-133b were detected by Northern blotting analysis. (D and E) mRNA and protein levels of α-MHC and β-MHC were detected by qRT-PCR (D) and immunoblotting (E). (F) Data for β-MHC mRNA levels in NRVMs transduced with the indicated miRNAs are relative to values of Cont.miR. β-MHC mRNA was detected by qRT-PCR. (black bar, upregulated miRNAs reported previously; white bar, muscle-specific miRNAs). (G) Expression levels of miR-27a, -27b, or -499 detected by qRT-PCR in NRVMs transduced with each miRNA or Cont.miR using a lentiviral vector. (H) β-MHC was detected by immunoblotting in NRVMs transduced with the indicated miRNAs. (I) Expression levels of the indicated miRNAs in NRVMs transduced with Dicer siRNA relative to that with Cont.siRNA. Representative data are presented as means ± SE for three independent experiment (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). AU, arbitrary units.

assessed in NRVMs in which miR-27a function was suppressed. NRVMs infected with a lentiviral vector were used in which a 3′ UTR with three or six tandem miR-27a decoy sequences complementary to miR-27a sequence was linked to a luciferase reporter gene in accordance with previous studies (Fig. 3A) (3, 10, 29). The complementary sequences acted as decoys, sequestering endogenous miR-27a. When miR-27a was transfected into 293FT cells along with an miR-27a decoy, the luciferase activity of the decoy was reduced significantly (Fig. 3B). On the other hand, miR-1, -21, -133b, and -208a did not affect luciferase activity (Fig. 3B). Next, miR-27a decoys were transfected into NRVMs, which resulted in the reduction of luciferase activity (Fig. 3C). These results indicated that miR-27a decoys could specifically bind to endogenous miR-27a. When miR-27a decoys were transduced into NRVMs using a lentiviral vector, β-MHC protein levels decreased, but the level

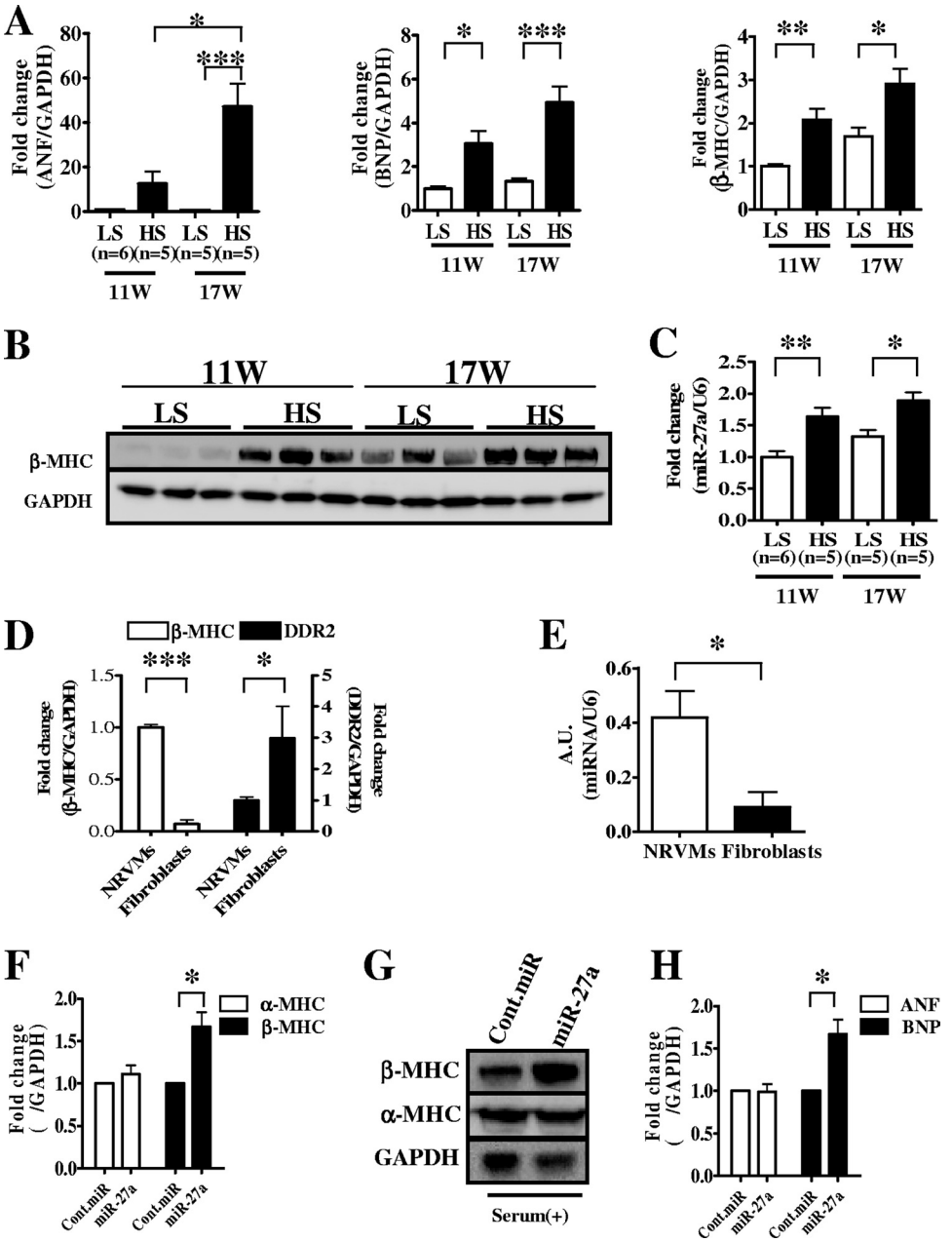

FIG. 2. miR-27a upregulates β-MHC gene expression. β-MHC, ANF, and BNP levels (A) and miR-27a expression levels (C) in Dahl salt-sensitive rat hearts were detected by qRT-PCR at 11 and 17 weeks (W). β-MHC was also detected by immunoblotting (B). LS, low-salt diet; HS, high-salt diet. (D) mRNA levels of myocyte-specific β-MHC (white bar) and cardiac fibroblast-specific DDR2 (black bar) both in NRVMs and cardiac fibroblasts. Values for β-MHC (left *y* axis) and DDR2 (right *y* axis) mRNA levels are relative to those of untreated NRVMs. (E) Endogenous miR-27a levels in NRVMs and cardiac fibroblasts were detected by qRT-PCR. (F to H) Assays were performed 72 h after transduction with miR-27a or Cont.miR. mRNA levels of α- and β-MHC (F) and ANF and BNP (H) were detected by qRT-PCR. Data are presented as means ± SE for three independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). A total of 2 μg of protein was used for immunoblotting of α- and β-MHC (G). Data are representative of three independent experiments. In panels A, C, D, F, and H, values for mRNA levels are relative to the Cont.miR mRNA level. AU, arbitrary units.

of α-MHC did not change under serum-containing conditions (Fig. 3D, E, and F). These results suggested that miR-27a is directly involved in β-MHC gene regulation in NRVMs. To assess the involvement of miR-208a, -208b, and -499 in β-MHC gene regulation in this assay system, the same study for miR-27a was performed using an miR-208a decoy. The miR-208a decoy could bind specifically to endogenous miR-208a, -208b, and -499 (Fig. 3B and C). Although a decoy construct acts by binding and sequestering a specific miRNA that is fully complementary to the sequence in the decoy, miR-208b or miR-499, which has the same or similar seed sequence, respectively, could also bind the miR-208a decoy. This result was the same

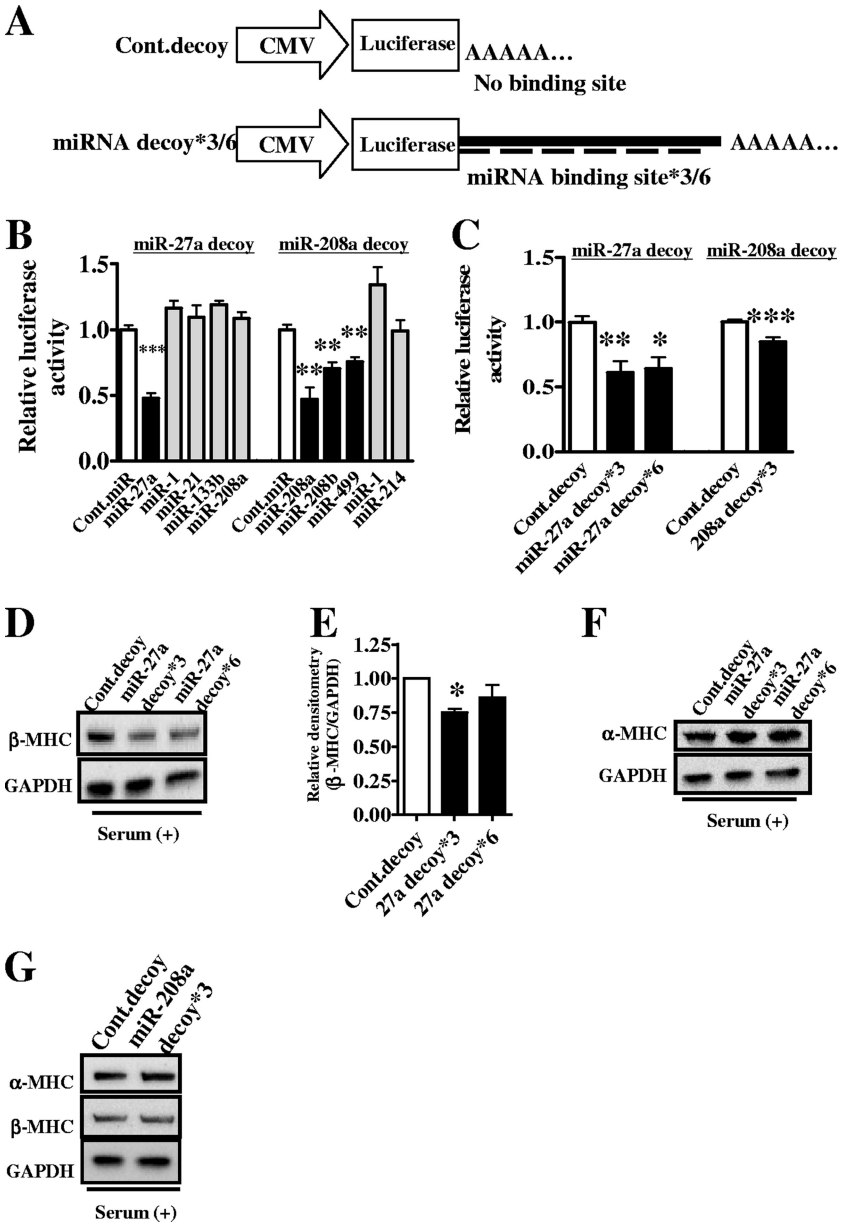

FIG. 3. miR-27a decoys decrease β-MHC protein levels. (A) Structure of the miRNA decoy. (B) 293FT cells were transfected with a luciferase decoy construct (miR-27a/-208a decoy or control decoy) along with an expression plasmid for the indicated miRNAs or miR-control (Cont.miR). (C) miR-27a and -208a decoys and the control decoy (Cont.decoy) were transfected into NRVMs. Values for luciferase activity are expressed relative to the value for the control. (D to G) Assays were performed 72 h after transduction. A total of 2 μg of protein was used for immunoblotting. NRVMs were transduced with miR-27a decoys (D and F) or an miR-208a decoy (G) using a lentiviral vector in serum-containing medium. Immunoblots were semiquantified using ImageJ densitometry software (E). Data are presented as means ± SE of three independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus control). In panels D, F, and G, data are representative of three independent experiments.

as that in a previous report (10, 29). Therefore, these miRNAs could be blocked by the miR-208a decoy. When the miR-208a decoy was transduced into NRVMs using a lentiviral vector, neither the α- nor β-MHC protein level changed under serum-containing conditions (Fig. 3G).

Target prediction of miR-27a. To find targets of miR-27a that can regulate β-MHC gene expression, a search was made for putative target genes of miR-27a using Target Scan, a bioinformatics tool for miRNA target prediction. Because miRNAs suppress gene expression, it was supposed that overexpression of miR-27a could downregulate target genes that can negatively regulate β-MHC expression, which might result in upregulation of the β-MHC gene. Transcription factors that can negatively regulate β-MHC gene transcription or related coregulators were expected to be involved in this upregulation of the β-MHC gene. There are several transcription factors that bind to the rat β-MHC promoter, and one of negative regulators of β-MHC, thyroid hormone receptor β1 (TRβ1)

(14), was predicted as one of targets of miR-27a according to Target Scan (Fig. 4A). TRβ1 binds to a negative thyroid hormone response element (TRE) in the β-MHC promoter and negatively regulates β-MHC transcription (23, 27). To test whether the putative target sequence in the 3′ UTR of TRβ1 could mediate translational repression, the 3′ UTR of TRβ1 was inserted into a luciferase reporter construct, which was transfected into 293FT cells, and cytomegalovirus (CMV)-driven miR-27a consequently decreased the luciferase activity of the construct (Fig. 4A). The human TRβ1 mRNA was predicted to have other binding sites for miR-27a, and the luciferase activity of a human TRβ1 3′ UTR reporter was decreased significantly by miR-27a (Fig. 4B). However, the luciferase activity of mutated reporters was not decreased by miR-27a (Fig. 4A and B). These findings suggested that the binding sites of miR-27a in the 3′ UTR of TRβ1 could mediate translational repression by miR-27a. Thyroid hormone receptors form a homodimer or heterodimer with retinoid X receptors and associate with nuclear proteins such as thyroid hormone receptor-associated proteins (19). Retinoid X receptor α (RXRα) and thyroid hormone receptor-associated proteins 1 and 2 were predicted as targets of miR-27a according to Target Scan. A recent study revealed that miR-27a can target RXRα in rat hepatic cell lines (20) while thyroid hormone receptor-associated proteins 1 and 2 could not be demonstrated as targets of miR-27a (Fig. 4C and D).

miR-27a targets thyroid hormone receptor beta 1. To test whether miR-27a could actually target TRβ1, TRβ1 gene expression was examined in NRVMs or human 293FT cells transduced with miR-27a or an miR-27a decoy. As a result, miR-27a decreased TRβ1 protein levels without affecting TRβ1 mRNA levels in NRVMs, and the miR-27a decoy increased TRβ1 protein levels (Fig. 5A and B). The same results were obtained in human 293 FT cells (Fig. 5C). These findings indicated that TRβ1 is a target of miR-27a both in rat and human cells. Next, it was examined whether TRβ1 siRNA could increase β-MHC gene expression in NRVMs. TRβ1 siRNAs increased β-MHC protein levels (Fig. 5D and E), whereas RXRα siRNAs did not increase β-MHC protein levels under serum-containing conditions (Fig. 5F). Because miR-27a could upregulate the β-MHC gene under serum-containing conditions, these results indicated that the upregulation of β-MHC by miR-27a resulted mainly from the downregulation of TRβ1 rather than RXRα.

miR-27a regulates β-MHC protein expression via TRβ1. To examine whether miR-27a regulates β-MHC gene expression via TRβ1, miR-27a- or miR-27a decoy-transduced NRVMs were treated with tri-iodothyronine (T3), a ligand of thyroid hormone receptors, and β-MHC protein expression was assessed. Treatment of NRVMs with 10 nM T3 could significantly upregulate α-MHC and downregulate β-MHC protein levels (Fig. 6A), which were the same results as those reported in previous studies (11, 23, 26). Overexpression of miR-27a attenuated the effect of T3, which resulted in an increase of β-MHC protein levels but did not affect those of α-MHC (Fig. 6A). The same result was obtained as that with TRβ1 siRNAs (Fig. 6B). RXRα siRNAs downregulated β-MHC protein in the absence of T3, whereas in the presence of T3, the β-MHC protein level did not change (Fig. 6C). β-MHC protein levels were examined in NRVMs transduced with miR-27a decoys. As a result, miR-27a decoys facilitated the downregulation of β-MHC protein by 10 nM T3 treatment (Fig. 6D), which was the opposite result produced by miR-27a overexpression. Next, to elucidate the effects of upregulation of TRβ1 on β-MHC gene expression, the rat TRβ1 gene was transduced into NRVMs using a lentiviral vector (Fig. 6E), and β-MHC protein expression was assessed. Overexpression of TRβ1 using our construct could decrease the luciferase activity of the β-MHC promoter construct, which contained a 333-bp rat β-MHC promoter (Fig. 6F). These results suggested that this exogenous TRβ1 could regulate β-MHC gene transcription. Overexpression of TRβ1 promoted the downregulation of β-MHC protein in the presence of 10 nM T3 while in the absence of T3, β-MHC gene expression did not change significantly (Fig. 6G and H), which was the same result as that with miR-27a decoys (Fig. 6D). Under serum-containing conditions, TRβ1 also downregulated β-MHC protein levels but did not change α-MHC protein levels (data not shown), which was the same result produced with miR-27a decoys (Fig. 5D and F). These findings indicated that miR-27a regulates β-MHC gene expression via TRβ1.

Overexpression of miR-27a upregulates the β-MHC gene in mouse ES cells. To investigate the physiological roles of miR-27a on β-MHC gene regulation via TRβ1 *in vivo*, expression profiles of endogenous miR-27a and cardiac MHC were examined in mouse ES cells during differentiation into cardiomyocytes. Undifferentiated ES cells (day 0) were two-dimensionally cultured on gelatinized dishes and induced to differentiate for 8 days. β-MHC mRNA levels in mouse ES cells increased dramatically between day 5 and day 8 (Fig. 7A), as reported previously (21), while endogenous miR-27a levels were unchanged until day 5 but increased >1.5-fold between day 5 and day 8 (Fig. 7B). On the other hand, miR-15b levels increased gradually in a time-dependent manner (Fig. 7B). Furthermore, overexpression of miR-27a in mouse ES cells could upregulate β-MHC mRNA, but did not change that of α-MHC or Nkx2.5, a marker of cardiac cell differentiation, and decreased TRβ1 protein levels (Fig. 7C and D). These findings suggested that the upregulation of miR-27a could increase β-MHC mRNA levels via TRβ1 but did not affect cardiac cell differentiation in mouse ES cells.

To study the physiological roles of miR-27a in heart development, miR-27a, β-MHC and TRβ1 gene expression levels were analyzed. As a result, during heart development, miR-27a and TRβ1 mRNA levels increased continuously while β-MHC levels decreased (Fig. 7E).

miR-27a is upregulated in mouse hearts treated with TAC. To elucidate the pathological roles of miR-27a *in vivo*, gene expression of miR-27a, β-MHC, and TRβ1 was examined in mouse hearts treated with TAC, which results in after-load-induced cardiac hypertrophy. As a result, β-MHC gene expression was upregulated, and TRβ1 gene expression was downregulated during the 3- to 4-week TAC treatment (Fig. 8A and B). Moreover, miR-27a was upregulated during 1- and 4-week TAC treatments (Fig. 8D). These findings suggested that miR-27a could be associated with the development of cardiac hypertrophy in terms of β-MHC gene regulation via TRβ1 *in vivo*.

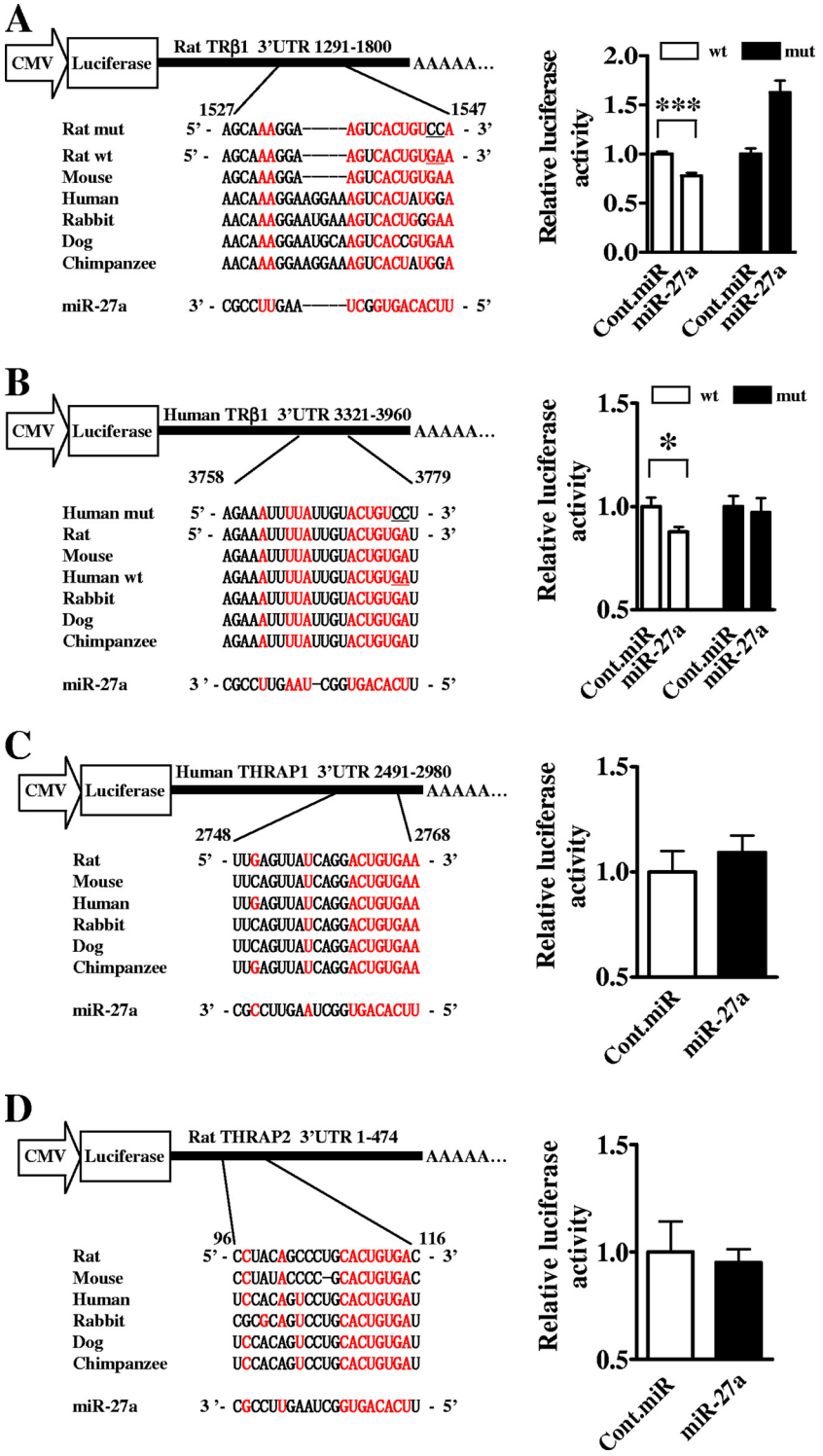

FIG. 4. Target prediction of miR-27a associated with negative TRE in the β-MHC promoter. The predicted binding sites of miR-27a in the 3′ UTR of predicted target genes are shown. 293FT cells were transfected with each of the 3′ UTR luciferase constructs and an expression plasmid for miR-27a or miR-control (Cont.miR): rat wild-type (wt) or mutated (mut) rat TRβ1 (A), human wt or mut TRβ1 (B), human thyroid hormone receptor-associated protein 1 (THRAP1) (C), and rat THRAP2 (D). Data are presented as means ± SE of three independent experiments (*, $P < 0.05$; ***, $P < 0.001$).

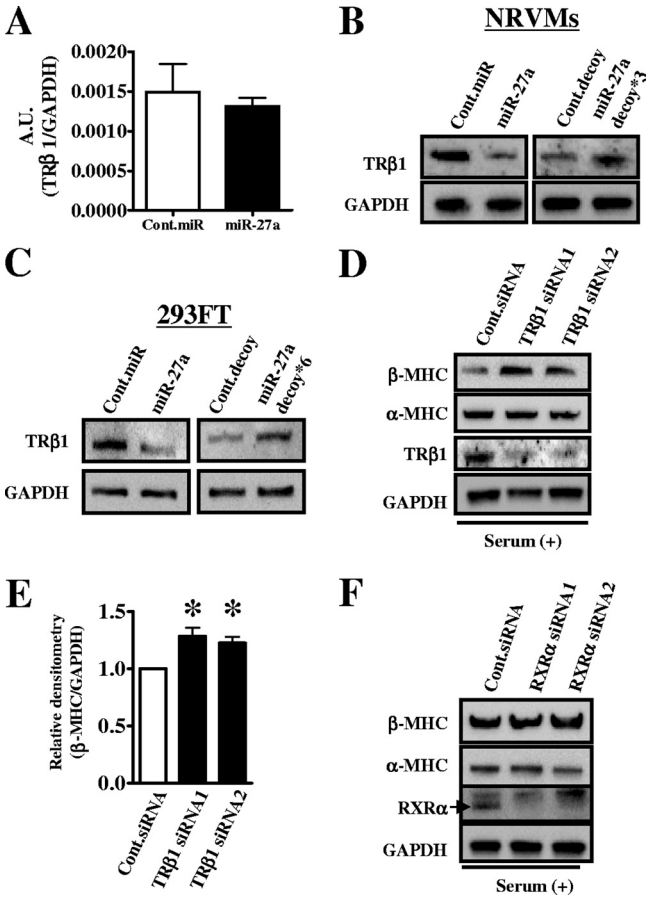

FIG. 5. miR-27a targets TRβ1. (A to C) Assays were performed 72 h after transduction using a lentiviral vector into NRVMs or 293FT cells under serum-free conditions. TRβ1 mRNA and protein levels were detected by qRT-PCR (A) and immunoblotting (B and C), respectively. A total of 10 μg of protein was used for immunoblotting. MHC (D and F), RXRα (F), and TRβ1 (D) proteins were detected by immunoblotting in NRVMs transduced with TRβ1 (D) or RXRα siRNAs (F) under serum-containing conditions. A total of 2 μg of protein was used for immunoblotting. Immunoblots were semiquantified using ImageJ densitometry software (E). In panels A and E, data are presented as means ± SE of three independent experiments (*, $P < 0.05$ versus Cont.siRNA). In panels B, C, D, and F, data are representative of three independent experiments. AU, arbitrary units.

DISCUSSION

Extensive research into the functions of miRNAs in the cardiovascular system has been performed. However, it is challenging to distinguish the direct effects of a specific miRNA from a range of indirect consequences because an miRNA may downregulate several hundred target genes at the same time. In *in vivo* studies, many environmental factors, such as cytokines, neurohormones, and hemodynamics, or interactions with interstitial components are considered to strongly affect the gene regulation of cardiac myocytes, which makes it more difficult to evaluate the direct effects of individual miRNAs. Therefore, in the present study, we attempted to examine the direct effects of miRNAs on cardiac gene regulation using primary cultures of NRVMs, in which the environmental factors or the interactions with interstitial components were limited.

We first tried to suppress global miRNA function in NRVMs by siRNA against Dicer, an essential enzyme for miRNA biosynthesis, and elucidate the effects on cardiac MHC gene expression.

Targeted Dicer deletion through use of a tamoxifen-inducible Cre recombinase in the hearts of 8-week-old mice can result in the upregulation of β-MHC mRNA levels (7), whereas β-MHC protein levels in cardiac cell-specific Dicer-deficient mice, which die shortly after birth, are unchanged (4). The present data demonstrated that Dicer siRNA could downregulate β-MHC gene expression. These differences in results might be derived from differences in β-MHC gene expression between perinatal and adult mice or from the neurohormonal and hemodynamic effects *in vivo*, which are considered to strongly influence the regulation of genes such as β-MHC (30, 32).

The present study showed a novel function of miR-27a as an activator of the β-MHC gene. Although miR-208a, -208b, and -499 are known as activators of β-MHC gene expression (1, 43, 45) and were also shown in this study to increase β-MHC gene expression, the reduction of miR-27a levels was larger than reductions of miR-208a, -208b, and -499 levels in NRVMs transduced with Dicer siRNA. Moreover, miR-27a decoys could decrease β-MHC protein levels, whereas the miR-208a decoy could not. Thus, the effects of miR-27a were stronger than those of miR-208a, -208b, and −499, which are muscle specific and play a critical role in β-MHC gene regulation (43).

Here, we demonstrated that miR-27a could modulate thyroid hormone signaling in β-MHC gene expression and that MHC gene expression is strongly regulated by thyroid hormone and receptors. Thyroid hormone receptors have four main isoforms, α1, α2, β1, and β2 (24). Isoforms α1, α2, and β1 are expressed differentially but rather ubiquitously while isoform β2 exhibits restricted tissue distribution, being found mainly in the pituitary gland (18, 24, 35). TRβ1 regulates β-MHC transcription in NRVMs but not α-MHC (23). The present data also revealed that α-MHC protein levels were not affected by TRβ1 in NRVMs. Moreover, TRα1 and TRα2 cannot be involved directly in the regulation of β-MHC transcription in NRVMs (23). These findings suggested that miR-27a can modulate thyroid hormone signaling specifically in β-MHC gene regulation via TRβ1 in NRVMs. In the absence of T3, overexpression of TRβ1 or the miR-27a decoy did not significantly decrease either β-MHC mRNA or protein levels. It has been reported that β-MHC transcription can be suppressed by TRβ1 overexpression even in the absence of T3 in NRVMs (23), and unliganded TRβ can suppress basal transcription (41). In these previous studies, β-MHC transcription was evaluated using a chloramphenicol acetyltransferase (CAT) reporter assay with a 3,300-bp rat β-MHC promoter sequence (23) or by a cell-free transcription assay using a minimal promoter, which contained a TATA box and two copies of palindromic TRE (41). The present study also showed that even in the absence of T3, TRβ1 overexpression could decrease the luciferase activity of the β-MHC promoter construct, which contained a 333-bp rat β-MHC promoter sequence, but not β-MHC mRNA or protein levels. It was supposed that there may be other consensus sequences or

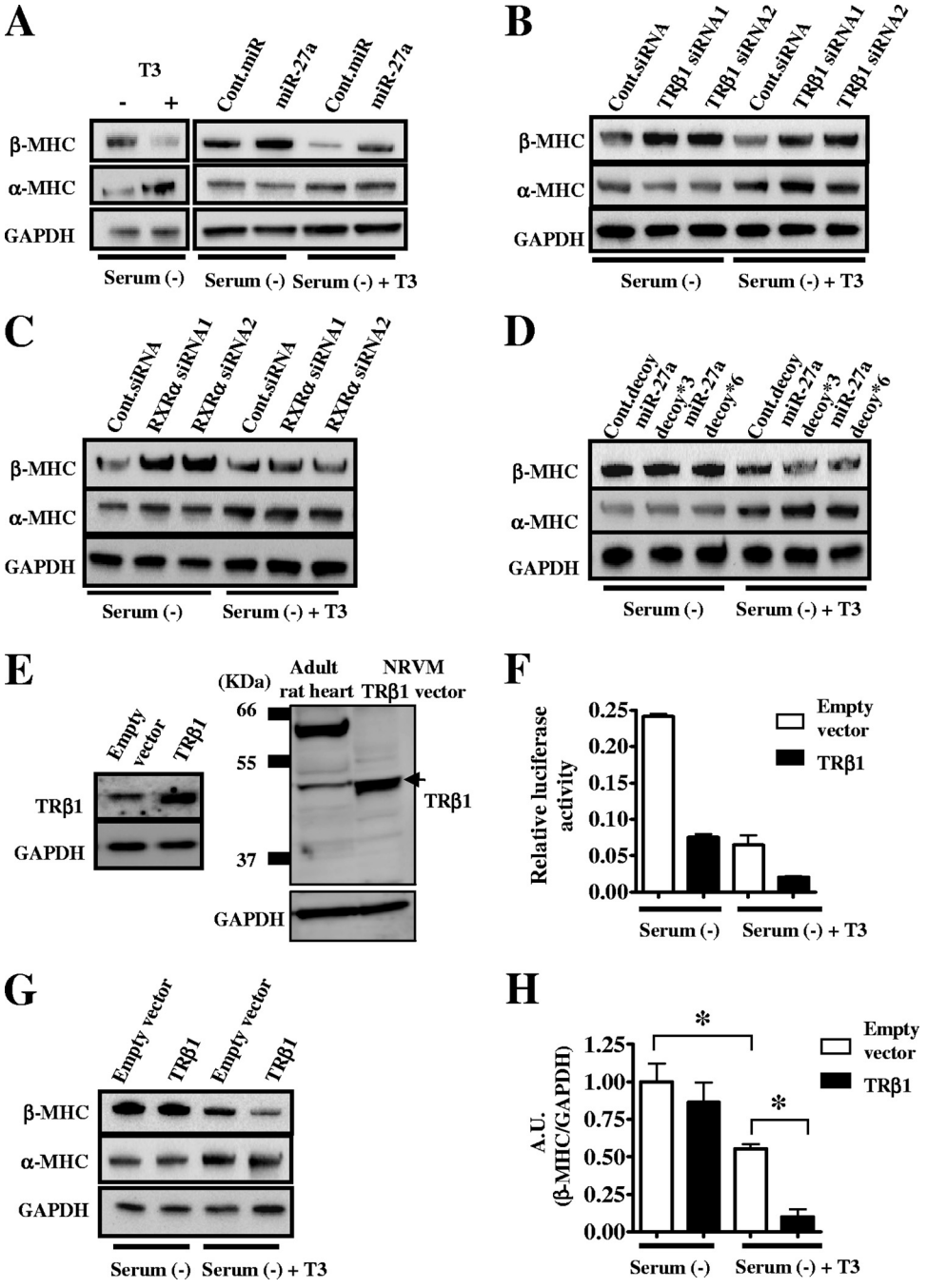

FIG. 6. miR-27a regulates β-MHC protein expression via TRβ1. (A to D) Treatment with 10 nM T3 was started 24 h after transduction and continued for 48 h. A total of 2 μg of protein was used for immunoblotting of MHC in NRVMs transduced with miR-27a (A), TRβ1 siRNAs (B), RXRα siRNAs (C), or miR-27a decoys (D). (E) A total of 10 μg of protein was used for immunoblotting of TRβ1 in NRVMs transduced with TRβ1 or an empty vector using a lentiviral vector or adult rat heart. (F) The luciferase construct contained the 333-bp sequence upstream of the translation initiation site of rat β-MHC. 293FT cells were transfected with the luciferase construct and an expression plasmid for rat TRβ1 or an empty plasmid with or without 10 nM T3. T3 treatment was started 24 h after transfection, followed by assays 24 h after T3 treatment. Values for luciferase activity are expressed relative to the value for an empty plasmid without T3 treatment. (G) A total of 2 μg of protein was used for immunoblotting of MHC. (H) β-MHC mRNA was detected by qRT-PCR in NRVMs transduced with TRβ1 or empty vector using a lentiviral vector with or without 10 nM T3. T3 treatment was started 24 h after transduction, followed by RNA extraction 72 h after treatment. In panels A to E and G, data are representative of three independent experiments. In panels F and H, data are presented as means ± SE of three and four independent experiments, respectively (*, $P < 0.05$). AU, arbitrary units.

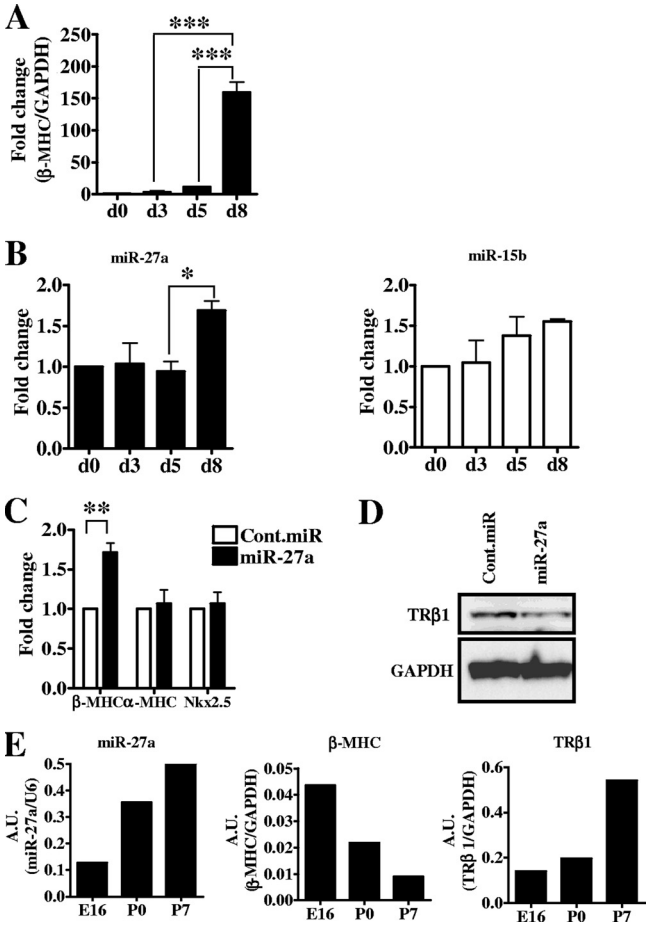

FIG. 7. Overexpression of miR-27a upregulates β-MHC gene in mouse ES cells. β-MHC mRNA (A) or endogenous miR-27a and -15b (B) were detected during mouse ES cell differentiation by qRT-PCR. β-MHC mRNA and endogenous miRNA levels are expressed relative to the respective levels at day 0. MHC and Nkx2.5 mRNAs (C) or TRβ1 protein (D) was detected by qRT-PCR or immunoblotting, respectively, in mouse ES cells at day 8 after transduction with miR-27a or Cont.miR. (E) miR-27a or β-MHC and TRβ1 mRNAs were detected by qRT-PCR in ICR mouse hearts at embryonic day 16 (E16), birth (P0), and postnatal day 7 (P7). In panels A to C, data are presented as means ± SE of three independent experiments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). In panel D, data are representative of three independent experiments. AU, arbitrary units.

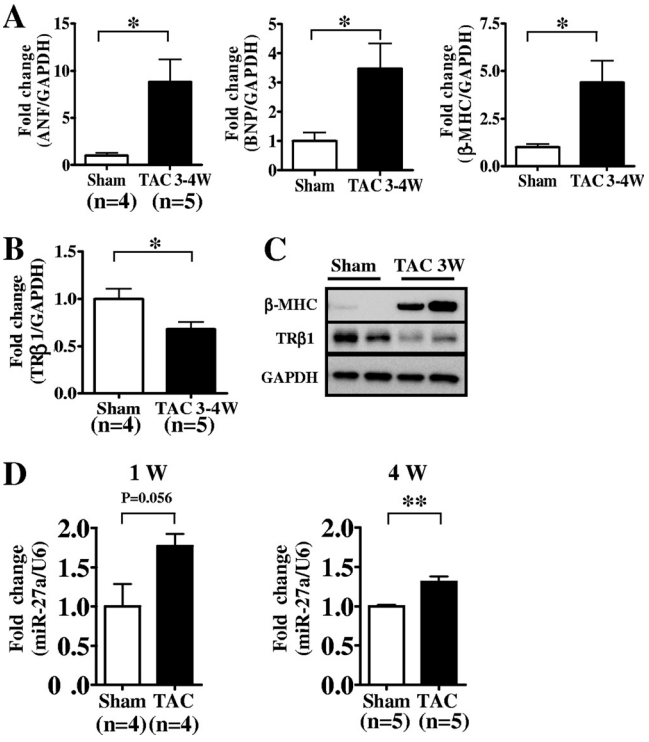

FIG. 8. miR-27a is upregulated in mouse hearts treated with transverse aortic constriction. ANF and BNP mRNAs (A) and β-MHC and TRβ1 mRNAs (B) and proteins (C) in C57BL/6 mouse hearts at 3 or 4 weeks after transverse aortic constriction (TAC) were detected by qRT-PCR (A and B) or immunoblotting (B). (C) Endogenous miR-27a levels in C57BL/6 mouse hearts at 1 or 4 weeks after TAC treatment were detected by qRT-PCR. Data are presented as means ± SE (*, $P < 0.05$; **, $P < 0.01$).

other factors that upregulate β-MHC gene expression in the absence of T3 outside the β-MHC promoter region used in this study. Under serum-free conditions or T3-free conditions in this study, the effect of TRβ1 on β-MHC gene regulation was very weak, whereas TRβ1 significantly changed β-MHC expression levels under serum-containing conditions, which suggested that factors in the serum could modulate β-MHC gene regulation by TRβ1. Serum-containing medium used in this study was found to contain T3 at a concentration of 1.12 ± 0.12 nM (our unpublished data). According to a previous report, both 1 and 10 nM T3 can decrease the β-MHC mRNA level to a minimum in cardiomyocytes (23). These findings suggested that T3 in the serum might modulate β-MHC gene regulation by miR-27a although there was a possibility that other factors might also be involved in this gene regulation.

In this study, the pathophysiologic roles of miR-27a *in vivo* were elucidated. During mouse ES cell differentiation, miR-27a was upregulated together with β-MHC mRNA, and overexpression of miR-27a in mouse ES cells increased the β-MHC mRNA level associated with the downregulation of TRβ1 protein. Moreover, miR-27a was highly expressed in adult mouse hearts (22) and upregulated during heart development. These findings imply that miR-27a could be involved in β-MHC gene regulation during ES cell differentiation and that it might play a role in heart development. However, TRβ1 protein levels were not significantly altered during ES cell differentiation (data not shown) and increased along with β-MHC downregulation during heart development, suggesting the involvement of factors other than miR-27a in TRβ1 gene regulation during ES cell differentiation to cardiac cells or heart development.

According to five miRNA microarray analyses performed by four groups (9), miR-27a was upregulated under cardiac-pathological conditions. In this study using TAC-treated mice, both miR-27a and β-MHC gene were upregulated along with TRβ1 downregulation. Because overexpression of miR-27a in NRVMs seemed to increase β-MHC gene expression directly rather than secondarily by inducing cardiac-pathological conditions, these findings suggested that miR-27a was upregulated by stress signals, inducing cardiac hypertrophy, and contributed to increasing β-MHC gene expression via TRβ1. How-

ever, it was controversial whether the dysregulation of TRβ1 had an impact on cardiac pathophysiology because mice deficient in TRβ1 have a normal contractile performance under baseline conditions (13). However, dominant negative TRβ1 mutant mice generated by a knock-in approach of a mutated TRβ1 that does not bind T3 present with decreased contractile function (37). Thus, T3 signaling via TRβ1 may be important under stressed conditions, and the upregulation of miR-27a in the TAC mouse model might affect cardiac functions. Further study is needed to elucidate precise function of TRβ1 in cardiac pathophysiology.

In summary, miR-27a can regulate β-MHC gene expression by targeting TRβ1 in NRVMs, and its upregulation in cardiac hypertrophy appears to contribute to increasing β-MHC gene expression via TRβ1.

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