Lack of miR-133a Decreases Contractility of Diabetic Hearts: A Role for Novel Cross Talk Between Tyrosine Aminotransferase and Tyrosine Hydroxylase

MicroRNAs (miRNAs) have a fundamental role in diabetic heart failure. The cardioprotective miRNA-133a (miR-133a) is downregulated, and contractility is decreased in diabetic hearts. Norepinephrine (NE) is a key catecholamine that stimulates contractility by activating β-adrenergic receptors (β-AR). NE is synthesized from tyrosine by the rate-limiting enzyme, tyrosine hydroxylase (TH), and tyrosine is catabolized by tyrosine aminotransferase (TAT). However, the cross talk/link between TAT and TH in the heart is unclear. To determine whether miR-133a plays a role in the cross talk between TH and TAT and regulates contractility by influencing NE biosynthesis and/or β-AR levels in diabetic hearts, Sprague-Dawley rats and miR-133a transgenic (miR-133aTg) mice were injected with streptozotocin to induce diabetes. The diabetic rats were then treated with miR-133a mimic or scrambled miRNA. Our results revealed that miR-133a mimic treatment improved the contractility of the diabetic rat’s heart concomitant with upregulation of TH, cardiac NE, β-AR, and downregulation of TAT and plasma levels of NE. In miR-133aTg mice, cardiac-specific miR-133a overexpression prevented upregulation of TAT and suppression of TH in the heart after streptozotocin was administered. Moreover, miR-133a overexpression in CATH.a neuronal cells suppressed TAT with concomitant upregulation of TH, whereas knockdown and overexpression of TAT demonstrated that TAT inhibited TH. Luciferase reporter assay confirmed that miR-133a targets TAT. In conclusion, miR-133a controls the contractility of diabetic hearts by targeting TAT, regulating NE biosynthesis, and consequently, β-AR and cardiac function.

MicroRNAs (miRNAs) are noncoding, regulatory RNAs that play a crucial role in the pathophysiology of several diseases, including heart failure and diabetic cardiomyopathy (1). A number of cardioprotective miRNAs are downregulated in the failing heart, which contributes to pathological cardiac remodeling (2). miRNA-133a (miR-133a) is one of the most abundant miRNAs in the heart (3). It is shared between the central nervous system and the heart (4) and has a multifaceted cardioprotective role (5). miR-133a is downregulated in failing hearts in humans and mice (6). On the one hand, downregulation of miR-133a is associated with upregulation of cardiac autophagy in humans with diabetic heart failure (7). On the other hand, transgenic overexpression of miR-133a in mice protects the diabetic heart from cardiac fibrosis (8). Albeit the cardioprotective role for miR-133a has been demonstrated at the myocardial level, its role in catecholamine biosynthesis and action via adrenergic receptors that is required for neurohumoral stimulation of cardiac contractility in diabetic hearts is poorly understood.

Diabetes mellitus (DM) is a complex disease caused due to insufficient insulin secretion from pancreatic β-cells (type 1 DM) and/or insulin resistance (type 2 DM) that results in an increased blood glucose level leading to morbidity and death (9). The number of patients with DM is
increasing at an alarming rate in the world (10, 11); however, the causes for the increased prevalence of DM and DM-mediated cardiomyopathy are poorly understood. DM is a miRNA-associated disease (12) that causes heart failure independent of coronary artery disease, hypertension, or valvular disease (13). In DM hearts, miR-133a is downregulated (7, 8) and contractility is decreased (14). Decreased contractility is caused primarily by inactivation/reduction of β-adrenergic receptors (β-ARs) (15). β-ARs are G-protein–coupled receptors, and β1-AR and β2-AR, which are the predominant subtypes in the heart, are present in the ratio of 70:30 in the left ventricle (LV), respectively, and increase contractility of the heart (16, 17). β-AR activation augments calcium uptake and increases sarcoplasmic reticulum activity by upregulating sarcoplasmic reticulum ATPase-2a (SERCA-2a), which increases contractility of the cardiomyocytes (18). In diabetic hearts, SERCA-2a is decreased (18), and β1-AR and β2-AR are downregulated (19).

The activation of β-AR depends on the release of neuronal norepinephrine (NE), a key catecholamine of the sympathetic nervous system (20), into the synaptic cleft, where it binds to β-AR on the cardiomyocyte membrane (21). Decreased contractility caused by β-AR inactivity/reduction may be a consequence of increased sympathoexcitation (22). The biosynthesis of NE is achieved through a cascade of reactions beginning with the rate-limiting enzyme tyrosine hydroxylase (TH), which converts tyrosine to dihydroxyphenylalanine, and TH is decreased in diabetic hearts (23). Tyrosine, which is a precursor for NE biosynthesis (20), is catabolized by the enzyme tyrosine aminotransferase (TAT). TAT catalyzes transamination of tyrosine in the liver, and deficiency of this enzyme causes tyrosinemia (24). In addition to the liver, TH is present in the heart, brain, and kidney (25). However, the interaction between TH and TAT for the regulation of NE biosynthesis in the heart under normal and diseased conditions, such as DM, is unknown.

The purpose of the current study was to determine the role of miR-133a in the regulation of TAT, cross talk between TH and TAT, and contractility by influencing NE biosynthesis and/or β-AR levels in diabetic hearts.

**RESEARCH DESIGN AND METHODS**

**Ethics Statement**

All animal studies were performed following the guidelines of the National Institutes of Health and protocol approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee.

**Animal Model and Treatment**

Male Sprague-Dawley rats were obtained from the Charles River Laboratories. The rats were caged individually in the University of Nebraska Medical Center animal care facility and were kept in an ambient environment with the temperature maintained at 22°C and humidity at 30–40% with diurnal cycle of 12 h dark and 12 h light. Laboratory chow and water was made available to the rats ad libitum. DM was induced in 8-week-old male rats (~225 g) by streptozotocin (STZ) injection (45 mg/kg i.v.; cat #S0130, Sigma-Aldrich, St. Louis, MO). Control animals were given citrate buffer in which STZ was dissolved. Blood glucose was measured 4 weeks after STZ administration to ensure rats developed the diabetic phenotype (blood glucose >350 mg/dL). To assess the effect of miR-133a overexpression on diabetic hearts, these diabetic rats were treated with lentivirus containing miR-133a mimic or scrambled miRNA and sacrificed at the age of 14 weeks (Supplementary Fig. 1A).

miR-133a transgenic (miR-133aTg) mice were a gift from Dr. Scot Matkovich (Washington University of St. Louis, St. Louis, MO). C57BL/6J wild-type (WT) mice were procured from The Jackson Laboratory. Both mice strains were maintained in the University of Nebraska Medical Center animal facility. STZ (65 mg/kg, i.p.) was administered to 8-week-old male WT and miR-133aTg mice, following a previously published protocol (8), for 5 consecutive days. The blood glucose level was measured at 10 weeks to ensure diabetic phenotype (blood glucose >500 mg/dL). Hemodynamic measurements were performed at 14 weeks, mice were sacrificed, and heart tissue was collected (Supplementary Fig. 1B). No exogenous insulin was administered at any time to the rats or mice.

**Assessment of Cardiac Function: In Vivo Hemodynamics**

LV pressure and rate of change of LV pressure (±dP/dt) was evaluated in the controls and diabetic rats treated with scrambled miRNA or miR-133a mimic. Rats were anesthetized with α-chloralose (70 mg/kg, i.p.) and urethane (0.75 g/kg, i.p.), and a Millar catheter (Millar Instruments, Houston, TX) containing a pressure transducer was introduced into the LV via the right carotid artery. Another catheter was inserted via the right femoral vein to assess the responsiveness of the heart to β-AR stimulation. A PowerLab data-acquisition system (AD Instruments, Colorado Springs, CO) was used for acquiring data.

**Plasmids and Constructs**

miR-133a (cat #MmiR3445-MR03), scrambled miRNA (cat #CmiR0001-MR03), anti–miR-133a (cat #MmriR-AN0880-AM04), and TAT 3′-untranslated region (UTR) clones (WT 3′UTR: cat #RmiT048999-MT01; mutant 3′UTR: CS-RmiT048999-MT01-01) were purchased from GeneCopoeia, Rockville, MD. TAT mouse cDNA clone (cat #MC204147) was purchased from OriGene Technologies, Inc., Rockville, MD. TAT small interfering (si)RNA and negative control siRNA oligonucleotides (cat #4390771 and cat #4390843) were purchased from Life Technologies, Carlsbad, CA.
In Vitro Model, Cell Culture, and Transfection

In vitro studies were performed on a CATH.a neuronal (dopaminergic) cell line using standard protocol and RPMI medium (cat #R8758, Sigma-Aldrich) with 8% horse serum and 4% FBS (Life Technologies). In brief, cells were cultured and differentiated by treating N^6,2'-O-dibutyryladenosine cyclic AMP (DB-cAMP; cat #D0627, Sigma-Aldrich) for 48 h and were transfected with plasmid or siRNA oligonucleotides for 24 h using Lipofectamine 2000 (cat #11668-019, Life Technologies). Transfected cells were processed for immunochemistry or harvested for protein isolation at 48 h after transfection.

Lentiviral Packaging

The 293T cells were cultured on a 10 cm^2 plate up to ~90% confluence. Opti-MEM media and Lipofectamine 2000 (cat #31985-070 and cat #11668-019, Life Technologies) were used to cotransfect 20 μg of the vector (miR-133a or scrambled) and 10 μg of each of RSV-REV, VSVG, and pMDLg/pRRE (plasmids for virus packaging). Viral supernatant was collected at 48 and 72 h after transfection and precipitated with sterile polyethylene glycol (PEG) solution (cat #81280, Sigma-Aldrich; 1 volume PEG to 4 volume viral supernatant collection). The virus pellets were collected by centrifuging the PEG-precipitated viral supernatant solution at 2,300 rpm for 2 h at 4°C. The virus pellets were resuspended in PBS, aliquoted in PCR tubes, and kept at −80°C until used. The virus titer was calculated by infecting virus aliquots (2, 4, 8, 16 μL) in a six-well plate seeded with 293T cells. After 2 days, GFP-tagged (miR-133a–GFP and scrambled GFP-positive) cells were counted at magnification 400× in four different fields of view for each well and the average number of GFP-positive cells per well was counted. The titer was calculated with the formula: total number of virus particle/μL (volume) = average number of GFP-positive cells in 400× field of view × 4,900/ the volume of virus added in the cell culture well.

miR-133a Assay

miRNA was isolated from the heart tissue (LV) using the mirVana miRNA Isolation Kit (cat #AM1560, Life Technologies). The purity of RNA was determined by NanoDrop 2000c (Thermo Fisher Scientific Inc., Waltham, MA), and highly pure RNA (ratio of 260:280 ≥1.8 and 260:230 ≥1.8) was used for the assays. Individual miR-133a assay was performed using miRNA-133a primers (Assay ID 002246, Life Technologies) specific for RT and TaqMan quantitative (q)PCR. U6 snRNA primer (Assay ID 001973, Life Technologies) was used for the endogenous control. miRNA amplification was performed following the manufacturer’s instructions using TaqMan Universal PCR Master Mix (cat #4427788, Life Technologies). RT-qPCR was performed in a Bio-Rad CFX qPCR System, and the results were analyzed by using BioRad CFX Manager3.0 software (Bio-Rad Laboratories, Hercules, CA).

RT-qPCR

High-quality RNA was used for RT-qPCR. cDNA was synthesized from an aliquot of 1 μg of RNA using the iScript cDNA Synthesis Kit (cat #170-8841, Bio-Rad Laboratories). The reaction was performed with priming at 25°C for 5 min, RT at 42°C for 30 min, and RT inactivation at 85°C for 5 min in a C1000 Touch thermal cycler (Bio-Rad Laboratories).

RT-qPCR

The RT-qPCR was performed using gene-specific oligonucleotide primers (Supplementary Table 1). The assay was performed using 2× iTaq Universal SYBR Green Supermix (cat #172-5121, Bio-Rad Laboratories) according to the manufacturer’s instructions. In brief, the PCR program was 95°C for 3 min and then 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 45 s. The qPCR reaction was performed in duplicate and included 4.5 μL cDNA template (100 ng), 5 μL iTaq Universal SYBR Green Supermix, and 0.5 μL gene-specific primer (10 pm). The forward and reverse primer sequences of the different genes are listed in Supplementary Table 1. The Bio-Rad CFX qPCR System was used for RT-qPCR, and data were analyzed using BioRad CFX Manager 3.0 software (Bio-Rad Laboratories).

Western Blotting

The standard Western blotting protocol was followed after protein estimation by Pierce BCA Protein Assay Kit (cat #23227, Pierce Biotechnology, Rockford, IL). Radioimmunoprecipitation assay buffer (cat #BP-115, Boston BioProducts, Worcester, MA) supplemented with protease inhibitor cocktail (cat #MSSAFE, Sigma-Aldrich) was used for whole-protein extraction from rat hearts. For protein quantification, 40 μg of protein extracts was subjected to 10% SDS-PAGE and transferred onto nitrocellulose membrane (cat #1620115, Bio-Rad Laboratories). The transferred membrane was blocked 1 h at room temperature with 5% blocking solution (Tris-buffered saline with 5% nonfat dried milk). The primary antibodies were used: β₁-AR, β₂-AR, β-actin, β-tubulin (cat #sc-568, sc-570, and sc-47778, respectively, from Santa Cruz Biotechnology, Santa Cruz, CA, and cat #MA5-16308 from Thermo Fisher Scientific, Waltham, MA), TH (cat #22941, ImmunoStar, Inc., Hudson, WI), and TAT (for rat study: cat #EP6R12, GeneTex Inc., Irvine, CA; for mouse study: cat #ab125000, Abcam, Cambridge, MA). SERCA-2a, β-MHC, and TH antibodies were raised in rabbit and used for multiplex Western blotting (cat #ab2861, ab172967, and ab112, respectively, Abcam), with GAPDH (cat #MAB374, Millipore, Temecula, CA) as the loading control. Antibodies were diluted in the ratio of 1:1,000 and incubated overnight at 4°C. Respective secondary antibodies with horseradish peroxidase (HRP) conjugates (anti–rabbit-HRP: cat #sc-2054; anti–mouse-HRP: cat #sc-2005, Santa Cruz Biotechnology) and fluorophore conjugates (anti-rabbit Alexa Fluor 488: cat #A-21441; anti-mouse Alexa Fluor 594: cat #A-21201, Life Technologies) were diluted at 1:5,000 and incubated 2 h at room temperature. The blots were developed using enhanced chemiluminescence substrate (cat #170-5061, PerkinElmer, Waltham, MA).
Bio-Rad Laboratories) or by multichannel fluorescence imaging using Molecular Imager ChemiDoc XRS System with Image Lab 4.1 software (Bio-Rad Laboratories). The band intensity was measured using Image Lab software.

**Immunocytochemistry**

Immunocytochemistry staining was performed on CATH.a neuronal cells differentiated with DB-CAMP in a six-well cell culture plate. Cells were transfected with 2 μg of scrambled miRNA or miR-133a mimic plasmid per well. After treatment and transfection, the medium was removed and cells were washed in PBS (pH 7.4) and then fixed in 4% paraformaldehyde (cat #158127, Sigma-Aldrich) for 30 min. After fixation, cells were washed in PBS three times for 5 min and then permeabilized in 0.02% Triton-X-100 (cat #215682500, Acros Organics) in PBS for 20 min. They were blocked in 1% BSA in PBS for 1 h, washed in PBS three times for 5 min, and incubated with diluted primary antibodies in PBS with 0.1% BSA at 4°C overnight. The primary antibodies used were: 1:400 dilution of anti-TH (cat #22941, ImmunoStar, Inc.); 1:200 dilution of anti-TAT (cat #EPR6121, GeneTex); the glyoxylic acid condensation reaction was used for fixation, cells were washed in PBS three times for 5 min and then permeabilized in 0.02% Triton-X-100 (cat #158127, Sigma-Aldrich) for 30 min. The secondary antibody was removed, and cells were washed in PBS three times for 5 min and incubated with diluted secondary antibodies in PBS with 0.1% BSA at 4°C overnight. The primary antibodies used were: 1:400 dilution of anti-TH (cat #22941, ImmunoStar, Inc.); 1:200 dilution of anti-TAT (cat #EPR6121, GeneTex). The primary antibody was removed the next day, and cells were washed in PBS three times for 5 min and incubated with anti-mouse Alexa Fluor 594 (cat #A21201, Life Technologies) or anti-rabbit Alexa Fluor 488 (cat #A21441, Life Technologies) for 1 h in the dark. The secondary antibody was removed, and cells were washed with PBS three times for 5 min and incubated with 1 μg/mL DAPI in PBS (cat #A1001, AppliedChem, St. Louis, MO) for 20 min. After that, cells were washed twice in PBS and mounted using the Fluoromount-G mounting medium (cat #0100-01, SouthernBiotech, Birmingham, AL). Images were captured by EVOS Cell Imaging Systems (Life Technologies) and analyzed by ImageJ software (National Institutes of Health, Bethesda, MD).

**Bioinformatics Analyses of TAT 3’ UTR**

In silico analysis predicts TAT as a putative target of miR-133a. The search Web sites were TargetScan (www.targetscan.org) and miRDB (www.mirdb.org).

**NE Assay**

NE content was measured by Norepinephrine ELISA Assay Kit (cat #NOR31-K01, Eagle Biosciences, Inc., Nashua, NH). Blood plasma and LV tissue lysate from rats administered STZ were used for NE assay. Plasma was isolated by collecting arterial blood by heart puncture and allowed a previously described protocol (26). In brief, frozen histological sections were adhered onto glass slides and immersed promptly in sucrose–phosphate–glyoxylic acid (SPG) solution (1% glyoxylic acid, 0.23 mol/L monobasic KH2PO4, and 0.2 mol/L sucrose, pH 7.4) for 5 s. Slides were dried entirely under a cool stream of air and heated to 95°C for 2.5 min after a thin layer of mineral oil was applied on the tissue surface. Slides were cooled to room temperature and sealed with a cover slip. Bluish-white fluorescence of catecholamine on tissue section was captured by fluorescence microscopy with an ultraviolet filter (Life Technologies), and images were quantified by ImageJ software.

**3,3’-Diaminobenzidine–HRP Immunohistochemistry**

Immunohistochemistry was performed on 5-μm transverse cryosections. In brief, the histological section was dipped in PBS to dissolve the freezing medium, processed for citrate antigen retrieval (heating-cooling), and fixed in 4% paraformaldehyde for 30 min at room temperature. Endogenous peroxidase activity was quenched by incubating slides in peroxidase suppressor solution (3% H2O2). After quenching, slides were blocked in 1% serum (goat or horse) in PBS for 20 min. Slides were washed twice for 3 min with PBS and incubated with primary antibodies for 3 h at 4°C. Primary antibodies used were TH, 1:400 (cat #22941, ImmunoStar, Inc.); TAT, 1:200 (cat #EPR6121, GeneTex); and anti-GFP HRP conjugate, 1:500 (cat #A10260, Life Technologies). Sections were washed in PBS three times for 5 min and incubated with respective HRP-conjugated secondary antibodies, anti–rabbit-HRP (cat #sc-2054, Santa Cruz Biotechnology) or anti–mouse-HRP (cat #sc-2005, Santa Cruz Biotechnology), as applicable, for 1 h at room temperature in a humidified chamber. Secondary antibody was removed, and sections were washed with PBS for three times for 5 min and incubated with fresh SigmaFAST 3,3’-diaminobenzidine (DAB) tablet solution (cat #D4293, Sigma-Aldrich). Sections were then washed twice in PBS, counterstained with Harris modified hematoxylin (cat #1859352, Pierce Biotechnology), and mounted with a coverslip using Permount Mounting Media (cat #SP15-100, Thermo Fisher Scientific Inc.). Images were captured by bright field microscope (Leica Microsystems, Buffalo Grove, IL) with the Image Pro 7.0 software.

**Fluorescence Immunohistochemistry**

Fluorescence immunohistochemistry was performed on 5-μm transverse cryosections following standard protocol. Primary antibodies used were anti–sarcomeric-α actin (cat #1:100, cat #ab9465, Abcam), and anti-GFP, 1:500 (cat #ab13970, Abcam). Secondary antibodies used were anti–mouse Alexa Fluor 488 (cat #A21200, Life Technologies) and anti–chicken Alexa Fluor 594 (cat #ab150176, Abcam).

**Wheat Germ Agglutinin Staining**

Wheat germ agglutinin staining was performed to stain cardiomyocyte cell boundaries and was used for measurement of cell area and hypertrophy. Frozen histological sections of the heart were kept in PBS for 5 min to dissolve the freezing medium. Hydrated sections were fixed with

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**Sucrose–Phosphate–Glyoxylic Acid**

Chemifluorescence

The glyoxylic acid condensation reaction was used for fluorescence detection and distribution of catecholaminergic nerves on 15-μm histological cryosections. The staining followed a previously described protocol (26). In brief, frozen histological sections were adhered onto glass slides and immersed promptly in sucrose–phosphate–glyoxylic acid (SPG) solution (1% glyoxylic acid, 0.23 mol/L monobasic KH2PO4, and 0.2 mol/L sucrose, pH 7.4) for 5 s. Slides were dried entirely under a cool stream of air and heated to 95°C for 2.5 min after a thin layer of mineral oil was applied on the tissue surface. Slides were cooled to room temperature and sealed with a cover slip. Bluish-white fluorescence of catecholamine on tissue section was captured by fluorescence microscopy with an ultraviolet filter (Life Technologies), and images were quantified by ImageJ software.

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freshly prepared 4% paraformaldehyde for 15 min at 37°C and then washed three times for 5 min with PBS. Next, 100–200 μL of 5 μg/mL wheat germ agglutinin (cat #W834, Life Technologies) conjugate solution was applied onto the sections and incubated for 10 min at room temperature. The sections were counterstained with DAPI. Slides were mounted with a coverslip and observed under a microscope. The images were captured by EVOS Cell Imaging Systems (Life Technologies) and analyzed by ImageJ software. 

**Luciferase Reporter Assay**

To measure luciferase activity, CATH.a cells were plated (60% confluence) in incomplete RPMI medium. Cells were cotransfected with 1 μg of 3’UTR clone with 0, 1, and 2 μg of scrambled miRNA or miR-133a mimic plasmid per well in a six-well cell culture plate. Luciferase reporter assay was performed after 48 h of transfection using the Dual-Glow Luciferase Assay Kit (cat #E2920, Promega Corp., Madison, WI), following the manufacturer’s instructions, in a GloMax-Multi+ Detection System (Promega).

**Statistical Analyses**

The statistical values are expressed as mean ± SEM. The RT, qPCR, and Western blotting experiments were repeated at least thrice in three independent samples, unless otherwise represented. Statistical analysis was performed by paired Student t test, and one-way ANOVA was used to compare among groups. P < 0.05 values are considered as statistically significant.

**RESULTS**

**Decreased Contractility of the Diabetic Heart Is Normalized by miR-133a Mimic Treatment**

Previous studies have shown that miR-133a has a role in the contractility of the pressure-overload heart (27) and that miR-133a is downregulated in diabetic hearts (28). To determine whether the lack of miR-133a decreased the contractility of the diabetic heart, we overexpressed miR-133a in the diabetic hearts by miR-133a mimic treatment and validated the upregulated miR-133a in the diabetic hearts by individual miR-133a assay (Supplementary Fig. 2A–C). We measured LV pressure and rate of contraction and relaxation (±dP/dt) of the miR-133a mimic–treated diabetic hearts at the basal level and after isoproterenol (a β-AR agonist) infusion using a Millar catheter. Our results showed that miR-133a mimic treatment normalized the relatively decreased LV pressure and ±dP/dt in the diabetic hearts (Fig. 1A and B), suggesting that a reduced level of miR-133a may contribute to decreased contractility of the diabetic hearts. Interestingly, the rate of contraction (dP/dt) was significantly improved by miR-133a mimic after 0.1 μg/kg isoproterenol treatment (Fig. 1B), suggesting that β-AR may be an important player in miR-133a–mediated improvement in the contractility of the diabetic hearts.

**miR-133a Mimic Treatment Upregulates β-AR and SERCA-2a in Diabetic Hearts**

To investigate whether miR-133a influenced β-AR expression in diabetic hearts, we measured β1-AR and β2-AR mRNA and protein levels in diabetic hearts treated with scrambled miRNA or miR-133a mimic. The mRNA and protein levels of β1-AR and β2-AR were increased in hearts treated with miR-133a mimic (Fig. 2A–D), suggesting that miR-133a overexpression has upregulated β-AR in the diabetic hearts. β-AR activation normally upregulates calcium influx in the cytoplasm, which triggers sarcoplasmic reticulum activity for sarcomeric contraction that increases the contractility of the heart. Decreased sarcoplasmic reticulum activity contributes to decreased contractility of diabetic hearts (14,18).

To determine whether miR-133a improved the contractility of the diabetic heart by influencing sarcoplasmic reticulum activity, we measured the protein level of SERCA-2a, a calcium-handling enzyme in the sarcoplasmic reticulum, in the heart. Our results showed that miR-133a mimic treatment upregulated SERCA-2a in the diabetic hearts (Fig. 2E). Overall, these results suggest that miR-133a improved the contractility of the diabetic hearts by upregulating β-AR and SERCA-2a.

To support that miR-133a mimic treatment had a similar effect on the diabetic heart as previously reported, we investigated cardiac hypertrophy in miR-133a mimic–treated diabetic hearts because the antihypertrophic effect of miR-133a, possibly by suppression of GLUT4 via targeting KLF15 (29), was documented in the nondiabetic (6) and diabetic (30) hearts. To determine cardiac hypertrophy, we measured the level of β-myosin heavy chain (β-MHC), a molecular marker for hypertrophy (Supplementary Fig. 3A), and the cross-sectional area of cardiomyocytes in histological sections of the heart (Supplementary Fig. 3B). Our results showed that miR-133a mimic treatment mitigated cardiac hypertrophy in the diabetic hearts (Supplementary Fig. 3), demonstrating that miR-133a had a similar effect on the diabetic hearts as previously reported.

**miR-133a Upregulates NE in Diabetic Hearts**

To determine the role of miR-133a on upstream signaling molecules that might have activated β-AR in the diabetic heart, we measured NE levels because NE activates β-AR and cardiac NE is decreased in the failing heart (31). Notably, miRNAs are associated with a reduced NE level in chronic heart failure (32). We therefore sought to determine the effect of miR-133a overexpression on NE level in diabetic hearts. Before measuring the cardiac NE level, we measured the plasma NE level because it is documented that the plasma NE level is increased in most forms of heart failure (33). The plasma NE level was increased in the scrambled miRNA–treated diabetic rats but was decreased in miR-133a mimic–treated diabetic rats (Fig. 3A), suggesting that miR-133a mimic treatment decreases the plasma NE level in diabetic rats. We then measured the cardiac tissue level of NE in miR-133a mimic–treated diabetic hearts. Contrary to the plasma NE level, the cardiac NE level was lower in the scrambled miRNA–treated diabetic rats and was restored after
miR-133a mimic treatment (Fig. 3B). These findings suggest that miR-133a has a crucial role in regulating the cardiac and plasma NE levels in diabetic rats.

The endogenously released NE is stored in the nerve endings, and intraneuronal storage of NE is decreased in diabetic hearts (34). To determine the role of miR-133a in intraneuronal storage of NE in diabetic hearts, we stained cryosections of the heart with SPG. The SPG binds to catecholamines and detects catecholamine storage in sympathetic neuron terminals in the heart by chemiluminescence (Fig. 3C [i]). Our results showed that miR-133a mimic treatment increased cardiac NE storage in the diabetic hearts (Fig. 3C [ii and iii]), which further support that miR-133a overexpression upregulated cardiac NE in the diabetic hearts. Overall, these findings demonstrated that miR-133a has a pivotal role in maintaining plasma and cardiac NE levels in diabetic rats; however, how miR-133a normalized the NE levels was unclear.

miR-133a Mimic Treatment Upregulates TH in Diabetic Hearts

To understand the underlying mechanism of miR-133a-mediated regulation of NE levels, it was imperative to investigate the biosynthesis of NE. Because NE is synthesized from tyrosine by TH, we determined TH levels in miR-133a mimic–treated diabetic hearts. The mRNA and protein levels of TH were upregulated in miR-133a mimic–treated
diabetic hearts (Fig. 4A and B), suggesting that miR-133a induces TH gene expression. To determine the expression of neuronal TH in diabetic hearts, we performed immunohistochemistry of TH in cryosections of the diabetic hearts. Our results showed increased intensity of neuronal TH in miR-133a mimic–treated diabetic hearts (Fig. 4C), corroborating that miR-133a mimic treatment increased the neuronal TH in the diabetic hearts.

To validate that the increase in the TH level was neuronal, we stained the heart sections with TH and a neuronal marker, microtubule-associated protein 2 (MAP2). The imaging of the heart sections showed colocalization of MAP2 with TH (Supplementary Fig. 4A), demonstrating that increases in the TH level in the miR-133a mimic–treated diabetic hearts was indeed neuronal. Further, we used the same antibody that stains neuronal TH in the heart (Supplementary Fig. 4B) to assess the expression of TH in CATH.a cells, a validated catecholaminergic neuronal cell line (Supplementary Fig. 4D), which corroborated that TH expressed in the heart was neuronal.

To determine the specific role of miR-133a in the regulation of neuronal TH level, we treated CATH.a neuronal cells that express miR-133a (Supplementary Fig. 4C) with scrambled, miR-133a mimic, and anti–miR-133a, and determined the protein level of TH in these three groups. Our results demonstrated that miR-133a mimic treatment upregulated TH (Fig. 4D and E). However, the TH level in neurons treated with anti–miR-133a did not change (Fig. 4E), suggesting that miR-133a may not have a direct or causative role in the regulation of the TH level in neurons. Because miRNA mostly inhibits genes, we infer that miR-133a mimic treatment might have upregulated TH by inhibiting another gene that normally suppresses TH.

**TAT Inhibits TH in Neuronal Cells**

Because tyrosine is a substrate for TH and is catabolized by TAT (24), we suspected that TAT might have an influence on the TH level. Although the presence of TAT in the heart has been reported (25), its role in the heart remains unclear. To determine the specific effect of TAT on the TH level, we first validated whether TAT was present in the neurons. For that, CATH.a neuronal cells were stained with anti-TAT antibody, and the cellular level of TAT

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**Figure 2**—miR-133a mimic treatment upregulates β1-AR, β2-AR, and SERCA-2a in the diabetic heart. RT-qPCR and Western blot analyses were performed to assess the levels of β1-AR, β2-AR, and SERCA-2a in diabetic hearts treated with scrambled miRNA (DM+scm) and miR-133a mimic (DM+miR). A: Bar graph represents relative fold change of β1-AR mRNA. 18sRNA was used as an internal control. B: Representative Western blot bands of β1-AR and actin (a loading control) (top), and the bar graph represents densitometric quantification of band intensity in fold change (bottom). C: Bar graph represents relative fold change of β2-AR mRNA. 18sRNA was used as an internal control. D: Representative Western blot bands of β2-AR and actin (loading control) (top), and the bar graph represents densitometric quantification of band intensity in fold change (bottom). E: Representative Western blot bands of SERCA-2a and tubulin (a loading control) (top), and the bar graph represents densitometric quantification of band intensity in fold change (bottom). The values are mean ± SEM (n = 6).
was observed. As speculated, TAT was present in the neuronal cells (Supplementary Fig. 4E). Then, we either overexpressed or inhibited TAT in CATH.a neuronal cells (Supplementary Fig. 4F and G) and measured the levels of TH. Our results revealed that suppression of TAT upregulated TH (Fig. 5A and B), whereas overexpression of TAT downregulated TH in neuronal cells (Fig. 5A and C). These findings suggest that TAT has an inhibitory effect on TH. Because TH is the rate-limiting enzyme in NE biosynthesis, TAT might have an indirect influence on NE biosynthesis.

miR-133a Modulates TAT in Diabetic Hearts

Our results demonstrated that miR-133a mimic treatment increased the level of TH in diabetic hearts (Fig. 4), but whether miR-133a upregulated TH by downregulating TAT in the heart was unclear. We determined the role of miR-133a on TAT level by measuring mRNA and protein levels of TAT in diabetic hearts treated with scrambled miRNA or miR-133a mimic. Our results revealed that miR-133a mimic treatment decreased TAT expression in diabetic hearts (Fig. 6A and B), suggesting that miR-133a suppresses TAT. Because TAT inhibited neuronal TH, we sought to determine whether miR-133a decreased neuronal TAT in diabetic hearts. For that, neuronal TAT was stained in diabetic heart sections and observed under a microscope. Our results showed that miR-133a mimic treatment reduced the levels of neuronal TAT in diabetic hearts (Fig. 6C).

We determined whether miR-133a had a direct role in the inhibition of neuronal TAT by treating CATH.a neuronal cells with scrambled miRNA, miR-133a mimic, and anti–miR-133a and then measured the levels of TAT. Our results demonstrated that miR-133a mimic treatment...
Figure 4—miR-133a mimic treatment increases TH in diabetic hearts. A: Evaluation of TH mRNA in diabetic hearts treated with scrambled miRNA (DM+scm) and miR-133a mimic (DM+miR) by RT-qPCR. 18sRNA was used as an endogenous control. The mRNA expression is presented as fold change. The values are mean ± SEM (n = 3). B: Western blot analysis (top) of TH protein in the hearts from the two groups in A shows representative bands of TH and GAPDH (a loading control), and the bar graph (bottom) shows densitometric analyses of relative TH protein in the heart represented as fold change. The values are mean ± SEM (n = 6). C: The left panels show schematic drawings of the area of cryosections of the heart and sympathetic innervations in the heart (i) and a transverse section of the heart as observed for sympathetic innervations (ii). RV, right ventricle. The right panels show representative DAB immunohistochemical staining of TH and counterstaining of hematoxylin in the heart sections of the two groups. Scale bars: 200 μm. D: miR-133a mimic treatment induces TH in CATH.a neuron cells.
downregulated TAT expression at cellular and protein levels (Fig. 6D and E), whereas anti-miR-133a treatment upregulated TAT expression (Fig. 6E). These findings suggest that miR-133a might have a direct role in regulating TAT expression in neuronal cells.

Cardiac-Specific Overexpression of miR-133a in Mice Prevents STZ-Induced Upregulation of TAT and Downregulation of TH in the Heart

To rule out the systemic effect of miR-133a mimic delivery and to validate the cross-species role of miR-133a on TAT and TH, we administered STZ to miR-133aTg mice (Supplementary Fig. 1B). We also genotyped (Supplementary Fig. 2D) and measured the cardiac levels of miR-133a (Supplementary Fig. 2E) in these mice before measuring the TAT and TH levels in the heart. The comparison of TH levels in WT and miR-133aTg mice, with and without STZ administration, demonstrated that TH level was decreased in STZ-administered WT mice but remained upregulated in STZ-administered miR-133aTg mice (Fig. 7A [i and ii]). However, TAT protein levels...
Figure 6—miR-133a mimic treatment decreases TH in the diabetic heart. RT-qPCR and Western blotting were performed for measuring TAT mRNA and protein levels, respectively, in the diabetic hearts treated with scrambled miRNA (DM+scm) and miR-133a mimic (DM+miR).

A: The bar graph shows the TAT mRNA level represented as fold change. 18sRNA is an endogenous control. The values are mean ± SEM (n = 3).

B: The top panel shows representative Western blot bands of TAT and actin (loading control), and the bottom panel shows densitometric analyses of the bands of TAT and the relative expression of TAT represented as fold change. The values are mean ± SEM (n = 3).

C: Schematics showing the area of heart section used for staining of TAT (left) and representative DAB immunohistochemical staining of TAT in the heart sections of DM+scm and DM+miR (right). Sections were counterstained with hematoxylin. RV, right
were elevated in STZ-administered WT mice but remained decreased in STZ-administered miR-133aTg mice (Fig. 7A [i and iii]). We infer from these results that cardiac-specific overexpression of miR-133a prevents DM-mediated upregulation of TAT and downregulation of TH in the heart. Moreover, we also performed immunohistochemistry for TAT and TH in the heart sections of WT and miR-133aTg mice with or without STZ administration. We observed that STZ administration decreased the number of neurons expressing TH in WT mice, but the number of TH-expressing neurons remained comparatively high in miR-133aTg mice after they were given STZ (Fig. 7B [i]). Then we measured the levels of TAT-expressing neurons. In contrast to TH, the number of TAT-expressing neurons was increased in STZ-administered WT hearts but remained downregulated in STZ-administered miR-133aTg hearts (Fig. 7B [ii]). Overall, these results revealed that the inhibitory effect of miR-133a on TAT is common in both mice and rat species and that overexpression of miR-133a suppresses TAT and induces TH in diabetic hearts of mice and rats.

**miR-133a Targets TAT**

miRNA modulates gene expression by targeting the 3’UTR of mRNA. We therefore performed in silico analyses for miR-133a–predicted targets and found that TAT was a target for miR-133a (Fig. 8A). The binding site for miR-133a on TAT 3’UTR was conserved in mouse and rat; however, mouse had three binding sites whereas rat had a single binding site (Supplementary Fig. 5). To determine whether the TAT was a direct target for miR-133a, we used TAT 3’UTR and the miR-133a binding sequence mutant TAT 3’UTR (Fig. 8B) and performed luciferase reporter assay on CATH.a neuronal cells. Our results showed that miR-133a downregulated luciferase activity of 3’UTR of TAT, which was nullified in the mutant 3’UTR of TAT (Fig. 8C and D). These findings revealed that miR-133a targets TAT.

Overall, our results demonstrate that miR-133a has a direct role in suppressing TAT. Because TAT inhibited TH, miR-133a mimic might be indirectly upregulating TH and thus regulates NE biosynthesis, which consequently leads to upregulation of β-AR and improved myocardial contractility in diabetic hearts.

**DISCUSSION**

In the current study, we demonstrate that lack of miR-133a contributes to decreased contractility of diabetic hearts and that miR-133a mimic treatment can improve the contractility of diabetic hearts. We propose that downregulation of miR-133a in the diabetic heart increases the TAT level, which in turn suppresses TH and decreases cardiac NE level, thus compromising β-AR activation and the contractility of the diabetic hearts. miR-133a mimic treatment normalizes TAT and restores the levels of TH, NE, β-AR, and the contractility of the diabetic hearts (Fig. 8E). In this study, we reveal several novel regulatory mechanisms such as miR-133a acting as an inducer of β-AR by regulating upstream activators of β-AR in diabetic hearts. Lack of miR-133a decreases the contractility of diabetic hearts. TAT is present in the neurons of diabetic hearts and inhibits TH, which may influence NE biosynthesis and β-AR in diabetic hearts. miR-133a directly modulates TAT expression in diabetic hearts.

miRNAs play a crucial role in regulating the contractility of the heart. Several miRNAs decrease the contractility, whereas others increase the contractility of the heart. In human heart failure, upregulation of miR-765 decreases contractility by regulating protein phosphatase inhibitor 1 (35). In mice and humans, SERCA-2a is regulated by miR-25. The inhibition of miR-25 improves the contractility of the failing heart by upregulating SERCA-2a (36). In a rabbit model of congestive heart failure, pacing improves contractility concomitant with upregulation of SERCA-2a and miR-133a (37). In heart failure and diabetic cardiomyopathy, SERCA-2a is downregulated (38). Our results demonstrate that miR-133a mimic treatment upregulates SERCA-2a in diabetic hearts (Fig. 2E) and improves contractility (Fig. 1A and B), which further support that miRNAs have a pivotal role in SERCA-2a regulation and cardiac contractility. Calcium influx also plays an important role during contractility of cardiomyocytes and is regulated by the sodium/calcium exchanger 1 (NCX1). miR-214 regulates NCX1 and improves the contractility of the ischemic heart (39). In the pressure-overload model of heart failure, miR-133a modulates β1-AR and its downstream signaling molecules that regulate the contractility of the heart (27). In the abdominal aortic constriction–induced pressure-overload model in mice, deficiency of IGF-1 upregulates miR-133a to alleviate myocardial contractile dysfunction (40). However, the role of miRNAs in regulation of the contractility of diabetic hearts is poorly understood. In this study, we reveal that...
miR-133a, which is antihypertrophic (30) and antifibrotic (8) in diabetic hearts, regulates contractility of the heart. Although miR-133a has been reported to modulate β-AR and its downstream signaling cascade in pressure-overload heart failure (27), the role of miRNAs in the regulation of upstream activators of β-AR have not been elucidated. Our data show for the first time that miR-133a has a crucial role in controlling the upstream activators of β-AR, especially NE biosynthesis, in diabetic hearts (Fig. 3). Our results also support a previous report indicating that miRNA expression is associated with catecholamine sensitivity (32). However, miR-133a-mediated activation of β-AR in diabetic hearts (Fig. 2A–D) differs from miR-133a-mediated inhibition of β1-AR in the pressure-overload heart (27). The different roles of miR-133a in β-AR activation in diabetic and pressure-overload hearts suggest that miR-133a may normalize the contractility of diabetic hearts by targeting other upstream signaling molecules that activate β-AR in diabetic hearts. Another reason could be that the metabolic conditions in the diabetic heart differ from those in other failing hearts (9). Further, we overexpressed miR-133a in rats by delivering miR-133a mimic, whereas miR-133aTg mice were used in the pressure-overload model (27). In the miR-133aTg mice, there are discrepancies in the results on cardiac functions depending on the α-MHC or β-MHC promoter being used (41,42). Our studies on miR-133aTg mice with the α-MHC promoter revealed that cardiac-specific overexpression of miR-133a prevented downregulation of TH in the diabetic condition (Fig. 7), which is similar to our results with diabetic rats (Figs. 4A–C and 6A–C). Further studies on other models of heart failure will provide insight on the miR-133a-mediated regulation of TAT, NE, and β-AR in the heart.

Increased plasma NE level is common in all forms of heart failure, including diabetic heart failure (33,34,43,44). Our results show that miR-133a mimic treatment decreases the plasma NE level (Fig. 3A). There is a possibility that miR-133a either mitigates the conditions that increase the plasma NE level or stimulates the conditions that decrease plasma NE level, or does both. Because miR-133a increases the cardiac NE storage in diabetic hearts (Fig. 3B and C), it is possible that miR-133a may decrease/influence NE release/spillover from nerve terminals in the heart that reduce the plasma NE level. Although these processes remain to be elucidated, it was interesting to observe that miR-133a mimic treatment decreased the plasma NE level in diabetic rats. The cardiac NE level may not be same in heart failure (45) and diabetic cardiomyopathy (22). The level of cardiac NE depends on NE storage in the nerve endings and its release in the myocardium. A study that assessed uptake of [3H]NE reported that intraneuronal storage granules of NE in the diabetic myocardium is defective (34), suggesting that storage of cardiac NE may be compromised in diabetic hearts. SPG-induced histofluorescence has been used to visualize the nerve profile in the heart and to demonstrate...
Figure 8—Regulatory role of miR-133a in diabetic hearts. miR-133a targets 3'UTR of TAT. A: The binding sequence of miR-133a with TAT 3'UTR in rat. B: The plasmid clone of TAT 3'UTR used for luciferase (Luc) reporter assay. The mutant plasmid is identical except the miR-133a binding site is deleted. CMV, cytomegalovirus; hLuc, humanized firefly luciferase; hRLuc, humanized renilla luciferase; SV40, simian vacuolating virus 40. The luciferase reporter assay results with TAT 3'UTR (C) and mutant TAT 3'UTR (D). miR, miR-133a; scm, scrambled. The relative luciferase activity is measured in CATHa cells treated with 3'UTR and increasing doses of miR-133a. The values are mean ± SEM (n = 6). E: miR-133a regulates contractility by targeting TAT in diabetic hearts. Schematic shows that the reduced level of miR-133a upregulates TAT in diabetic hearts. Elevated TAT inhibits TH, which decreases the cardiac NE (c-NE) level. It results in inactivation of β₁-AR and β₂-AR that decreases contractility in diabetic hearts. However, treatment with miR-133a mimic increases the miR-133a level in the diabetic heart that decreases TAT by binding to its 3'UTR. Decreased TAT increases the level of TH, which induces c-NE biosynthesis. The elevated level of c-NE induces β-AR and improves the contractility of diabetic hearts.
that loss of noradrenergic nerve terminal contributes to right heart failure (45). We also used SPG to assess nerve profile in miR-133a mimic–treated diabetic hearts. Our results reveal that miR-133a mimic treatment increases the number of nerves containing NE in the diabetic heart (Fig. 3C), suggesting that miR-133a mimic treatment protects the diabetic heart from noradrenergic nerve loss. Moreover, our results are consistent with the previous report that nerve density is reduced in diabetic hearts (23) and also support that miRNAs are involved in the regulation of catecholamine level in heart failure patients (32).

The biosynthesis of NE is increased by upregulation of TH (20). The TH gene has a tetranucleotide repeat in intron 1 that is associated with regulation of NE level and hypertension (46). The cardiac level of TH is decreased in diabetic (23,47) and failing hearts (31). Our results show that miR-133a mimic treatment increased TH level in diabetic hearts (Fig. 4). However, the regulation of TH in the diabetic heart has not been completely understood, and the effect of TAT on NE biosynthesis was unknown. For the first time, we elucidate that neuronal TH is regulated by TAT (Fig. 5). Our results therefore provide a novel insight into a possibility of TAT-mediated indirect regulation of NE biosynthesis and open a new window to understanding the regulation of TH in different disease conditions, including hypertension and heart failure.

TAT has a crucial role in tyrosinemia type II, hepatitis, and hepatic carcinoma (24). It regulates glucocorticoids in the liver of diabetic rodents (48). Although TAT is present in the heart (49), its functional role in the heart is yet unclear. In the current study, we reveal that TAT is a regulator of TH and that an elevated level of TAT downregulates TH in nerve terminals in the diabetic heart (Fig. 5). Interestingly, TAT is a target for miR-133a (Fig. 8A–D) and miR-133a mimic treatment decreases the level of TAT in nerve terminals (Fig. 6D and E). The miR-133a mimic-treated diabetic hearts also show decreased levels of TAT (Fig. 6A–C) indicating a novel regulatory role for miR-133a on TAT expression. Our results also open an avenue for exploring the role of miR-133a in TAT-regulated diseases such as tyrosinemia type II, hepatitis, and hepatic carcinoma.

DM is a multifactorial disease that increases the risk of heart failure (50) and ultimately causes multigorgan failure. Despite the advancement of medical research, the prevalence of DM is increasing at an alarming rate (10,11), which warrants novel therapeutic strategies to combat its complications. miRNA-based therapy offers a novel and advanced approach for developing the treatment strategy for several diseases (51), including cancer, where miR-34 is currently in clinical trial (ClinicalTrials.gov Identifier: NCT01829971). Therefore, miRNA-based therapy could be a promising approach to normalizing DM-mediated complications in the heart. Considering the multifaceted cardioprotective role of miR-133a (5) and the results of the current study, we suggest that miR-133a could be a novel candidate for exploring future therapies for diabetic heart failure.

In conclusion, we demonstrate a novel, cardioprotective role of miR-133a in the diabetic heart. We show that miR-133a protects the heart of STZ-administered diabetic rats/mice by directly targeting TAT and TAT-TH cross talk. TH and TAT enzymes are critically involved in the biosynthesis of NE, a key catecholamine stimulating the contractility of the heart muscle. Our results also demonstrate that miR-133a mimic treatment decreased plasma NE levels. These findings, by elucidating the cardioneurovascular cross talk, may help us to understand the molecular mechanisms underlying diabetic and non-diabetic forms of heart failure.

Limitations

The role of miR-133a in the pathophysiology of diabetic hearts and effect of hyperglycemia on miR-133a functions are poorly understood. Further, the results obtained from mice or rat data may vary from humans with diabetic heart failure. Our results in the current study are focused on a type 1 DM model induced by STZ that needs to be further validated with other models of type 1 and type 2 DM.

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