MiR-144 protects the heart from hyperglycemia-induced injury by regulating mitochondrial biogenesis and cardiomyocyte apoptosis

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Abstract
Several lines of evidence have revealed the potential of microRNAs (miRNAs, miRs) as biomarkers for detecting diabetic cardiomyopathy, although their functions in hyperglycemic cardiac dysfunction are still lacking. In this study, mitochondrial biogenesis was markedly impaired induced by high glucose (HG), as evidenced by dysregulated mitochondrial structure, reduced mitochondrial DNA contents, and biogenesis-related mRNA levels, accompanied by increased cell apoptosis. MiR-144 was identified to be decreased in HG-induced cardiomyocytes and in streptozotocin (STZ)-challenged heart samples. Forced miR-144 expression enhanced mitochondrial biogenesis and suppressed cell apoptosis, while miR-144 inhibition exhibited the opposite results. Rac-1 was identified as a target gene of miR-144. Decreased Rac-1 levels activated AMPK phosphorylation and PGC-1α deacetylation, leading to increased mitochondrial biogenesis and reduced cell apoptosis. Importantly, the systemic neutralization of miR-144 attenuated mitochondrial disorder and ventricular dysfunction following STZ treatment. Additionally, plasma miR-144 decreased markedly in diabetic patients with cardiac dysfunction. The receiver-operator characteristic curve showed that plasma miR-144 could specifically predict diabetic patients developing cardiac dysfunction. In conclusion, this study provides strong evidence for the potential of miR-144 as a novel biomarker for diabetic cardiomyopathy.
1 | INTRODUCTION

Diabetic cardiomyopathy (DCM) is a heart muscle-specific disease that increases the risk of heart failure and mortality in diabetic patients independent of hypertension, coronary artery disease (CAD), or evidence of any other structural cardiac diseases.\(^1\),\(^2\) From a cellular standpoint, cardiac energy metabolic dysfunction has been proposed to contribute to the development of DCM.\(^3\) Mitochondria are power plants that generate adenosine triphosphate (ATP) for heart contraction and concurrently produce reactive oxygen species (ROS) that, if unchecked, may cause metabolic disorders and insulin resistance-related heart diseases.\(^4\)

Proper mitochondrial function is maintained through mechanisms that mainly regulate the biogenesis and dynamics of mitochondria. Mitochondrial biogenesis is a strictly controlled process involving mitochondrial DNA (mtDNA) replication, transcription and translation of mRNA-encoding genes, and loading of phospholipids and proteins destined for the nucleus in several mitochondrial subcompartments.\(^5\),\(^6\) Enhanced mitochondrial biogenesis is not only a hallmark of skeletal and cardiac muscle adaptation to exercise but also is critical for increased exercise capacity.\(^8\) However, perturbed mitochondrial biogenesis may trigger cell death and can contribute to cardiac complications of type 2 diabetes mellitus (T2DM).\(^9\) Because of the constant demand for high ATP levels for maintaining cardiac contractile activity, impaired mitochondrial biogenesis may result in disorders of mitochondrial structure and function, and have an extremely adverse impact on cardiac muscles.\(^10\) However, only a few studies have been performed on the cellular and molecular relationships between mitochondrial biogenesis and DCM, and numerous subjects await further exploration.

MicroRNAs (miRNAs, miRs) are small noncoding RNAs that suppress gene expression by transcriptional inhibition or degradation of target mRNAs.\(^11\) Over the last two decades, miRNAs have been identified as key regulators of multiple biological processes including cell proliferation, differentiation, death, development, and metabolism.\(^12\),\(^13\) Emerging evidence has suggested that aberrant expression of miRNAs may trigger disturbances in target gene networks and signaling cascades that participate in diverse pathological phenotypes. One such example is of adverse cardiac remodeling in the diabetic myocardium.\(^14\),\(^15\) Deleterious miRNAs such as miR-208a, miR-451, miR-146a, and miR-200c and protective miRNAs such as miR-133a, miR-30c, miR-21, miR-22, and miR-181a have been found to contribute to diabetic cardiac injury.\(^16\)–\(^23\) These previous findings suggest that the manipulation of miRNAs may serve as a novel therapeutic approach for targeting heart diseases in diabetes. However, only a limited number of studies on the roles of miRNAs in mitochondrial biogenesis in DCM have been conducted.

In this study, we observed reduced miR-144 expression in left ventricular samples of STZ-induced diabetic mice. We verified the downregulation of miR-144 in H9c2 and neonatal rat cardiomyocyte (NRCM) models of high glucose (HG)-induced cell injury. In addition, miR-144 overexpression rescued impaired mitochondrial biogenesis and apoptosis induced by HG, whereas miR-144 knockdown suppressed these responses. Importantly, treatment with an miR-144 agomir via tail vein injection improved STZ-induced mitochondrial damage and cardiac dysfunction and attenuated cardiac fibrosis and apoptosis in adult mice. Collectively, miR-144 overexpression might serve as a new therapeutic strategy for treating DCM.

2 | MATERIALS AND METHODS

2.1 | Patients with DCM

All human investigations conformed to the principles outlined in the Declaration of Helsinki and were approved by institutional review committees of The Third Affiliated Hospital of Soochow University (Changzhou, China). As inclusion criteria, the patients with T2DM were hospitalized in the Endocrinology Department of the Third Affiliated Hospital of Soochow University from April 2018 to June 2019, regardless of the duration of diabetes. The medical treatment consisted of oral anti-hyperglycemic agents and/or insulin therapy. A signed informed consent was obtained...
from each participant before enrollment in the study. The exclusion criteria were: (i) A history of coronary artery disease, hypertension, valvular heart disease, or other type of cardiovascular disease; (ii) A self-reported history of symptomatic micro or macrovascular complications of diabetes (including nephropathy, neuropathy, retinopathy, peripheral vascular disease, and stroke); (iii) Pregnancy; and (iv) Other significant comorbidities including malignancy, renal failure, thyroid dysfunction, or significant psychiatric illness.

On the same day of hospital admittance, each patient gave complete history and clinical examinations including echocardiography and the second day morning consisted of gated-myocardial perfusion imaging (Gated-MPI), rest, and stress. Peripheral blood samples were collected from patients after an overnight fast the next morning after admission. The following echocardiographic parameters were determined: left ventricular (LV) mass, LV end-systolic volume, and LV end-diastolic volume. The LV ejection fraction (EF) was measured to assess systolic function. LV diastolic function parameters included the peak filling rates of the early filling phase (E), atrial contraction (A), and the E/A ratio. Early diastolic lengthening mitral valve flow velocity (‘e’) and the E/e’ ratio were also calculated. The criterion of LV diastolic dysfunction (LVDD) was classified according to the European Association of Echocardiography, American Society of Echocardiography guidelines, and Doppler echocardiographic measurements in Chinese adults (EMINCA).24,25 To exclude CAD, each patient underwent Gated-MPI after rest or stress in the presence of a cardiologist. After further exclusion of CAD, T2DM patients with cardiac dysfunction were involved in the DCM group. Then, a computerized, block-randomization program was used to allocate T2DM patients without cardiac dysfunction into an experimental group and a relatively equal number of candidates (randomization ratio, 1:1) in the control group.

### 2.2 | Primary cardiomyocyte and fibroblast isolation

Neonatal rat cardiomyocytes (NRCMs) were isolated from 1-3-day-old Sprague Dawley rats as previously described.26 For fibroblast isolation, left ventricles were finely minced and digested in trypsin buffer (60% trypsin and 40% collagenase). Then, cell suspensions were centrifuged, resuspended in Dulbecco’s modified Eagle’s medium (DMEM, Corning, CA, USA) containing 1 g/l glucose supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, CA, USA), and plated for 2 hours under standard culture conditions (37°C with 5% CO2) which allowed fibroblast attachment to the culture plates.

### 2.3 | Cell culture and treatment

Rat H9c2 cells were cultured in DMEM containing 1 g/l glucose supplemented with 10% FBS and 1% streptomycin/penicillin at 37°C with 5% CO2. All transfections and assays with NRCM and H9c2 cells were conducted in low-serum medium (1% FBS). To study the potential role of miR-144 in HG-induced cardiac injury, NRCM and H9c2 cells were exposed for 48 hours to either an miR-144 mimic or negative control (NC) mimic (50 nM), or an miR-144 inhibitor or NC inhibitor (100 nM) (RiboBio, Guangzhou, China), and then treated with glucose (40 mM) (Sigma-Aldrich) for an additional 48 hours. Small-interfering RNA (siRNA) against Rac-1, peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), and adenosine monophosphate (AMP)-activated protein kinase (AMPKα), and an NC siRNA were purchased from Sangon Biotech (Shanghai, China). siRNAs with the following sequences were used: Rac-1 siRNA (5′–3′): sense: GGAGAUAUAGACGACGUTT, antisense: AUCGUGUCUUAUACUCCCTT; PGC-1α siRNA: sense: GCACGCAGUCCUAUCAUUTT, antisense: AAUAGAAUGAGCAGCGGCTT; AMPKα siRNA: sense: GCACCCUCUAUAUAUCAATT, antisense: UUUGAUUAUGAGGGGCCTT; NC siRNA: sense: UUCUCGAACGUGUACGUTT, antisense: ACGUGACACGUUGAGGAATT. Rac-1-overexpressing and NC plasmids were purchased from Sangon Biotech. Transfections with miR mimics, miR inhibitors, siRNA (50 nM), and plasmids (50 nM) were performed using the Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, CA, USA).

### 2.4 | Animal model

All animal experiments were conducted in accordance with the guidelines for the care and use of laboratory animals published by the National Institutes of Health (No. 85-23, revised 1996) and approved by the committee on the Ethics of Animal Experiments of Soochow University (Soochow, China). Male C57BL/6 mice, 5-6 weeks old, were purchased from Cavens Lab Animal (Changzhou, China) and raised in a specific pathogen-free (SPF) laboratory animal facility at Soochow University. Mice were randomly divided into 4 groups, namely the NC-agomir group (N = 6), miR-144-agomir group (N = 6), STZ + NC-agomir group (N = 15), and STZ + miR-144-agomir group (N = 15). The sequence of miR-144 agomir was sense: 5′-3′: UACAGUAUAGAUGUACU, antisense 5′-3′: AGUACAUACUACUGUA. The sequence of NC-agomir was sense: 5′-3′: UUUGAUAUACACAAAAUGACUG, antisense 5′-3′: CAGUACUUUUGUGUAGUACA. To induce diabetes, mice were administered daily intraperitoneal
injections of STZ (Sigma-Aldrich) at a dose of 50 mg/kg dissolved in 0.1 mM sodium citrate buffer (pH 4.5) for 5 consecutive days, at the same time on each day. Control mice were treated with the same volume of citrate buffer. Beginning 7 days later, the mice were injected once per week with either an miR-144 agomir or a scrambled NC (20 mg/kg) via tail vein injection. After 8 weeks, cardiac function, heart weight, heart weight/body weight ratio, heart weight/tibia length ratio, and survival rate were examined. Body weight and fasting blood glucose levels were measured at 1, 2, 4, 6, 8, and 10 weeks.

2.5 | Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNAs were isolated from H9c2 cells, NRCM, or cardiac tissues using the TRIzol reagent (Invitrogen, CA, USA) and complementary DNA was synthesized using an iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Quantitative real-time polymerase chain reactions was performed using SYBR Green Master Mix (Bio-Rad) and an ABI 7900HT Real-Time PCR Detection System (Applied Biosystems, CA, USA). mRNA expression levels were normalized to 18 seconds rRNA expression. The sequences of the primers used in this study are shown in Table 1. To quantify miRNA expression levels, the Bulge-Loop™ miRNA qPCR Primer Set (RiboBio) was used with SYBR Green Master Mix and an ABI 7900HT Real-Time PCR Detection System (Applied Biosystems). The mRNA expression levels were normalized to U6 RNA expression. The relative expression level of miRNA or mRNA was analyzed using the 2^−ΔΔCt method.

2.6 | Mitochondrial DNA content assay

Total DNA was extracted from the hearts or H9c2 cells, NRCM using a TIANamp Genomic DNA Kit (catalog number DP304, Tiangen, Beijing, China). To quantitate the mitochondrial DNA copy number per nuclear genome, cytochrome b was used as a marker for mitochondrial DNA and β-actin served as a marker for nuclear DNA. The primer sequences are listed in Table 2.

2.7 | Mitochondrial transmembrane potential (∆Ψm) assay

The mitochondrial transmembrane potential was measured using cationic dye tetramethylrhodamine ethyl ester (TMRE, Molecular Probes, Eugene, OR, USA). NRCM and H9c2 cells were loaded with 100 nM TMRE for 20 minutes in a humidified incubator at 37°C, which induces cells to fluoresce in proportion to their ∆Ψm. Then, the cells were placed in the flow-through chamber and continuously perfused with buffered salt solution containing TMRE (10 nM). After allowing 60 minutes for equilibration, sequential digital images were obtained every 1-3 minutes by a confocal microscope (Carl Zeiss, Germany), and the average fluorescence intensity for all the cell regions and background was recorded for analysis.

2.8 | Transmission electron microscope (TEM) analysis

H9c2 cells and heart tissues were harvested and quickly fixed in 2.5% glutaraldehyde for 1 hours, exposed to 1% osmium tetroxide, and then dehydrated and embedded in Durcupan. Cell and tissue samples were then sectioned to 60 nm and mounted on Cu-grids contrasted with uranyl acetate and lead citrate, and finally evaluated using a JEM-1010 electron microscope (JEOL, Japan). For H9c2 cells, images were taken at 12 000× magnification. For heart tissues, images were taken at 1 500× and 5000× magnifications. A total of 2-3 sections/specimen from each H9c2 cell sample or heart sample were used for analyzing mitochondrial arrangements and ultrastructure. For mitochondrial morphometry measurements, mitochondrial area was analyzed using Image J to trace the outer mitochondrial membrane in 2D TEM images. Mitochondrial area was calculated accordingly.

2.9 | Flow cytometry analysis

Apoptosis was analyzed using the Annexin V-FITC/PI Apoptosis Kit (Bioworld, Nanjing, China) according to the manuscript’s instructions. Briefly, H9c2 cells were digested with trypsin and then washed twice with PBS. After adding 200 µL binding buffer and incubating with 5 µL Annexin V-FITC and 5 µL propidium iodide for 20 minutes in the dark, the cells were analyzed by flow cytometry (Beckman Coulter, CA, USA) for survival, apoptosis, and necrosis. Cell survival, apoptosis (including early and late), and necrosis were analyzed between different groups.

2.10 | Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays and wheat germ agglutinin (WGA) staining

TUNEL staining was performed to detect apoptotic nuclei by fluorescence microscopy, as described previously. Briefly, for heart samples, tissues were harvested, embedded with
| Species | Forward | Reverse |
|---------|---------|---------|
| PGC-1α  | GCCACTCCACCAAGAAAG | GATCACAAACAGCGTAG |
| NRF1    | CCAAAACCAACCCCTGTGTC | ACATTCCTCAAAGGCTCCTG |
| NRF2B   | AATCTGGAGTGGTGCAAT | TCACAAATGATGGGCTGAC |
| NRF2A   | CTTGTTCCCTATTCAGACG | TTACATCACCCTCCCCAA |
| TFAM    | CTCATCCTCAGCCAGTG | CAGTGGCGCAAGATCTCAT |
| TFB1M   | GCACCTCTTTACAACTCCA | TGAATGGCTGCTGTATCTT |
| TFB2M   | GCAAAGGGCCCGAAGTGTG | TGGCAGTGGTGAAGAAGT |
| UCP3    | GCCTTTCAAGACACAA | GGGTCAGTGCACAAAAGG |
| SSBP1   | GAAGTACGCACGTAGTGG | TCCCTCCACTTCTTCTCAT |
| TOP1MT  | TGGGAGCTGTGGCTGAAA | TTGAGGCGCTGACTAGA |
| Rac-1   | TGGACGGCCATCAAGG | AGACAGCAGGTGTATACCA |
| AMPK6 | AATACGTGGCCCAATCTT | TGTCAGAGAATCTAGCAG |
| Atg5    | GGGACCTCCTCAATCAC | CTATGCGCTGACACCTT |
| Atg7    | GCCCAAGCGCCATCTTGAG | TCTGCTGAGGAATCTCAT |
| Ulk1    | CAGCAAGGGCCATCCATT | GTACCCGGGCAATCCAA |
| Mfn1    | GCTCTGTCATCAACGGCAA | TCTCATTGACCTCCCTG |
| Mfn2    | GAAGAAGATGGTGATACTT | AGAATGGCTGAGGGGGAAG |
| mt-Nd1  | CCTCAACCTAGCATCACCATT | AAGCTCATCCCCGATCATAG |
| Cs      | GTTTGTACCTTACACCACCA | TGTCAGCAGAATGCTCAAG |
| Cycs    | GATGCAAGAAGAAGAGATGT | TGGGATTTCCTAAATACCTCAT |
| Ndufb3  | GGTCTTGGAGATAGTCAGG | GAAATACCTGCCCCCTAA |
| Ndufb7  | GCCACATACTCCTCCAG | CGTCAGAGCAGTGGCAG |
| Ckm2    | CTGGCAGGAAAGAGAG | GGGACCTGAGGCTATCAAA |
| 18s     | GCACTATCATTGGCTGTT | CGGACGAGGTCACCTC |
| α-SMA   | GCTCCCTCTTGGCCACACT | CCACGTCCTACCAGG |
| Col1a1  | GCTCCCTCTTGGCCACACT | CCACGTGACCTGAGGAAG |
| Col3a1  | CGTAAACATGGGAACTGGGGCAA | CCATAGCTGAATCTGAACCCAC |
| PGC-1α  | TTCAACAAACACATGCAC | GCATTCTAAAGCACCAG |
| NRF1    | TCTATCCTGACGCTATT | GCTATCGTCATCCAGAG |
| NRF2B   | AATCTGGAGTGGTGCAAT | TCAAAATGTGGTGAAG |
| NRF2A   | GCCAGTACCTGAGCTTCTT | TAATCGTAGTGGTGTAGC |
| TFAM    | TAAATGGCAGGGAAGCCA | CAGTGGAGAAGGGGTGTT |
| TFB1M   | CCCTTGGATAGGACCAAGA | CAGGACATCCAGTTCTT |
| TFB2M   | TGTGGGTGGAATATGTTG | TCCCTCATCCTCCTCTAA |
| UCP3    | CTTGCTCCTCGTCCCTT | GCTTGGAAGTTGTGGCTCCTG |
| SSBP1   | GCCCTTCTTGTCCGACAC | GCTCTCATGGCCAAAC |
| TOP1MT  | GACCTTGATGGAAGGGCTGA | CGCCGAGTGTAAGCTA |
| Atg5    | GAGGAGCCAGTGGATATTT | GGTTCACTATCCAGAGG |
| Atg7    | GATAGGCTACGAGCACA | GCCTCAGATACCCGG |
| Ulk1    | GGATCCATGGTCATTGCA | CAAGGGCACTTGATGTTAC |
| mt-Nd1  | AACAAATTCAAACCACACCAT | TCAATATAGCTGATGGGCTAG |
| Cyacs   | TCTCATCCACGATGCTTTC | GAAAGGCCAACACG |
| Ndufb3  | GGGAGGCTCAGATGCTTTC | TTTCCACGCTGTAACTC |
| Ckm2    | AAATGAGGAAGACCCACCA | GACAACAGTCGCAATC |
| 18s     | TCAAAGACGAAAGGTGCGG | GGAACATCAAAGGCGTAG |

**Table 1**: Primer sequences for real-time PCR analysis
paraffin, frozen, and sectioned into 5-µm slices. Then, the sections were fixed with 4% paraformaldehyde (PFA), permeabilized with phosphate buffered saline containing 0.5% Triton X-100 (PBST), and blocked with 5% bovine serum albumin. One hour after blocking, the sections were incubated with a mouse anti-α-actinin antibody (Sigma-Aldrich, A7811, 1:100 dilution) overnight at 4°C. After incubation with Cy3-AffiniPure-labeled goat anti-mouse IgG (H + L; Jackson ImmunoResearch, PA, USA), the sections were stained using the TUNEL FITC Apoptosis Detection Kit (Vazyme, Nanjing, China), according to the manufacturer’s instructions. For H9c2 cells, after fixation with 4% PFA, they were permeabilized with 0.5% PBST, and TUNEL staining was performed as per the manufacturer’s instructions. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Finally, 15-20 fields per sample were imaged under a confocal microscope (Carl Zeiss, Germany). The percentage of apoptotic cells was calculated as the ratio of TUNEL-positive/DAPI-stained cells. For WGA staining, sections were first fixed with 4% PFA and washed with PBS. Then, the sections were stained with WGA (1:100 dilution, Sigma-Aldrich) to measure the size of the cardiomyocytes. Images of WGA staining were taken using a Zeiss microscope (400× magnification), equipped with a Spot Insight camera. Image J Software (National Institutes of Health) was used to quantify the fibrotic areas in each section.

2.11 Western blot analysis

H9c2 cells, NRCM, and left ventricular tissues from heart samples were extracted and lysed with RIPA lysis buffer (Beyotime, Nantong, China) containing 1 mM phenylmethylsulfonyl fluoride (Beyotime). Equal quantities of total proteins were separated by 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. The membranes were then blocked in Tris-buffered saline containing 5% dried milk and 0.1% Tween 20 for 1 hour, and subsequently incubated overnight at 4°C with primary rabbit antibodies against Bax (catalog number 2772), Bcl2 (catalog number 2876), Rac-1 (catalog number 2465), pAMPKα (172) (catalog number 2535), AMPKα (catalog number 5831), or acetylated-lysine (catalog number 9441) (all from Cell Signaling Technology, MA, USA), or with a rabbit-anti-PGC-1α antibody (catalog number NBP1-04676) from Novus Biologicals (CA, USA). The antibodies were diluted 1:1000 in each case. The blots were then incubated with a horseradish peroxidase-conjugated anti-rabbit secondary antibody. Rabbit anti-β-actin (1:1000, catalog number 4970; Cell Signaling Technology) was used as loading control. Protein bands were visualized using an Enhanced Chemiluminescence Kit (Thermo Fisher, MA, USA) and a ChemiDoc XRS Plus luminescent image analyzer (Bio-Rad). Immunoblot band intensities were analyzed with Lab Image software.

2.12 Rac-1-activity measurements

Left ventricular samples and H9c2 cells were lysed in a buffer containing NP-40, which was provided in a kit (catalog number 17-283; EMD Millipore Corporation, CA, USA). Lysates were clarified by centrifugation at 10 000 g at 4°C for 2 minutes. The p21-binding domain of p21-activated protein kinase (PAK) bound to agarose beads was added, and active Rac-1 (with PAK1 bound) was separated by successive centrifugation and washing steps. Then, the specimens were boiled in Laemmli buffer, separated by SDS-PAGE, and Rac-1 expression was quantified by western blot analysis. Specifically, Rac-1-GTP was detected with a monoclonal antibody against Rac-1-GTPγ (1:1000, catalog number 17-283; EMD Millipore Corporation, CA, USA), and total Rac-1 was detected with a monoclonal antibody against Rac-1 (1:1000, catalog number 2465; Cell Signaling Technology). The quantity of Rac-1-GTP was normalized to that of Rac-1.

2.13 Luciferase reporter assay

A fragment of the 3′UTR of Rac-1 containing the target site of miR-144 was obtained by PCR amplification and then cloned into the pGL3-Basic Vector (Promega, WI, USA) to generate the Rac-1 wt-luciferase vector. The Rac-1 mutant-luciferase vector was generated using the Mutabest kit (Takara, Tokyo, Japan). H9c2 cells were transfected in 96-well plates using lipo-2000 according to the manufacturer’s instructions. 48 hours after transfection, luciferase activities were measured using a dual luciferase reporter assay system (Promega) following a standard procedure.

| TABLE 2 | Primer sequences for mitochondrial DNA content assay |
|----------|-----------------------------------------------|
| Species  | Forward                        | Reverse                        |
| mtDNA    | Rat GGTTCTTACTCCAGGGCCATCA       | TGATTAGACCCGTATACCATCGA        |
| β-actin  | Rat CCCAGCCATGTACGTCAGCA         | CGTCCTCCGAATCCATGCA           |
| mtDNA    | Mouse CCACCTTCATCTACCATTATTATCGC | TTTTATCTGCACTGAGTTAAATCTCTGT  |
| β-actin  | Mouse CATCCGTAAAAGACCTCTATGCCAAC | ATGGAGCCACCGATCCAC           |
Echocardiography

Cardiac function was determined 9 weeks after STZ injection using echocardiography. Echocardiography was conducted in mice anesthetized with 2% isoflurane using Vevo 2100 device (VisualSonics, Inc., Toronto, Canada) equipped with a 30-MHz central frequency scan head. The following parameters were measured from M-mode images taken along the parasternal short-axis view at the papillary muscle level: left ventricular internal dimension-diastole (LVID; d), left ventricular internal dimension-systole (LVID; s), left ventricular mass (LV mass), left ventricular fractional shortening (FS), and left ventricular ejection fraction (EF). The peak filling rates of the early filling phase (E), atrial contraction (A), and the E/A ratio were obtained in the apical four-chamber view. Early diastolic lengthening mitral valve flow velocity (e') and E/e' ratio were also calculated.

Masson’s trichrome staining

Heart samples from mice were harvested and fixed with 4% PFA and sectioned into 5-µm slices. Masson’s trichrome staining was conducted using a standard protocol. Images of the left ventricular area of each section were viewed with a Zeiss microscope (400× magnification) equipped with a Spot Insight camera. Image J Software (National Institutes of Health) was used to quantify the fibrotic areas in each section.

Plasma miR-144 levels in patients with diabetic cardiac dysfunction

Because no stable endogenous miRNA for normalizing miRNA expression levels in plasma is generally accepted, we spiked our samples with cel-miR-39, which lacks sequence homology to human miRNAs. Relative miRNA expression levels were determined using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All experimental data were analyzed using SPSS software (version 23.0) and are presented as mean ± SD (determined using GraphPad Prism 6.0), unless otherwise stated. Briefly, an independent-sample t test was used for comparing two groups. One-way analysis of variance followed by Bonferroni’s post hoc test was performed to compare more than three groups. For clinical data from diabetic patients with or without cardiac dysfunction, an independent-sample t test was used for comparisons between two groups. Correction analysis between plasma miR-144 and clinical data were performed using Pearson’s method. A receiver-operator characteristic (ROC) curve was generated to evaluate the sensitivity and specificity of using serum miR-144 levels to predict the diagnosis of DCM. $P$ value < .05 was considered to reflect a statistically significant difference.

RESULTS

miR-144 expression was reduced in a cardiac hyperglycemia model

Using a murine model of cardiac hyperglycemic injury, we first evaluated miR-144 expression in heart samples by qRT-PCR. After 10 weeks of STZ administration, miR-144 expression was decreased significantly in the left ventricular samples (Figure 1A). Then, the relative miR-144 expression levels in isolated neonatal rat fibroblasts versus cardiomyocytes were determined, and the results demonstrated higher miR-144 expression in cardiomyocytes compared to fibroblasts (Figure 1B), possibly suggesting a more prominent role of miR-144 in cardiomyocytes. Furthermore, we measured the cellular levels of miR-144 in rat neonatal cardiomyocytes (NRCM) and H9c2 cells stimulated by HG, a cellular model for mimicking hyperglycemia in vitro. The miR-144 expression was also decreased in HG-treated cardiomyocytes (Figure 1C,D). Collectively, these data revealed a strong correlation between miR-144 expression and myocardial hyperglycemic injury.

Mitochondrial biogenesis and disorder were impaired in HG-induced cardiomyocytes

Previous findings demonstrated that mitochondrial structure and function are significantly impaired in the islets and muscles of diabetic animals. Mitochondria also exhibited diminished biogenesis; defective oxidative phosphorylation (OXPHOS); and reduced expression of complexes I, III, and V of the electron transport chain in STZ-treated myocardium tissue or HG-induced cardiomyocytes, suggesting the impairment and dysfunction of mitochondria in hyperglycemic hearts. Based on TEM analysis, we found that the mitochondrial volume density was not remarkably altered, but that the mitochondria from HG-induced H9c2 cells exhibited significant morphological defects, including mitochondrial vacuolization and destroyed cristae and membranes (Figure 1E). Mitochondrial biogenesis and function are typically tightly coupled in the heart. To determine whether mitochondrial biogenesis was impaired in hyperglycemic cardiomyocytes and, therefore, contributed to mitochondrial disorder, we assessed the expression of PGC-1 α, which plays a critical role in controlling mitochondrial biogenesis and function in...
FIGURE 1 Mitochondrial biogenesis and disorder are impaired in high glucose-induced cardiomyocytes. A, The expression level of miR-144 was measured by qRT-PCR in left ventricular samples with STZ treatment (n = 5). B, The relative expression level of miR-144 was determined in isolated neonatal rat cardiomyocytes compared to fibroblasts (n = 4). C-D, The expression level of miR-144 was detected in isolated neonatal rat cardiomyocytes (NRCM) and H9c2 cells stimulated by high glucose (HG) (40 mM) (n = 6). E, Representative electron micrographs of mitochondria in H9c2 cells treated with HG. Scale bar = 12,000 magnification (n = 3). F, qRT-PCR analysis of mitochondrial biogenesis-related mRNA levels in H9c2 cells treated with HG (n = 6). G, Mitochondrial DNA content assay in H9c2 cells treated with HG (n = 3). H, TMRE analysis of mitochondrial transmembrane potential (ΔΨm) in H9c2 cells (n = 4). Scale bar = 100 um. I-J, The ratio of apoptosis in HG-treated H9c2 cells as determined by flow cytometry analysis (I) and TUNEL staining (J) (n = 5). Scale bar = 100 μm. K, qRT-PCR analysis of mitochondrial biogenesis-related mRNA levels in NRCM treated with HG (n = 4). L. Mitochondrial DNA content assay in NRCM treated with HG (n = 4). M, TMRE analysis of mitochondrial ΔΨm in NRCM (n = 4). Scale bar = 50 μm. N, The ratio of apoptosis in HG-treated NRCM as determined by TUNEL staining (n = 4). Scale bar = 100 μm. Data were presented as mean ± SD. *P < .05, **P < .01, ***P < .001, ****P < .0001 versus controls.
the heart under physiological or pathological conditions.\textsuperscript{33-35} Associated with the morphological defects observed in mitochondria, the mRNA expression levels of PGC-1α, nuclear respiratory factor 1 (NRF1), mitochondrial transcription factor A (TFAM), and other genes related to mitochondrial biogenesis decreased significantly in HG-induced H9c2 cells (Figure 1F) and NRCM (Figure 1K). Likewise, the mitochondrial DNA content and transmembrane potential (ΔΨm) also decreased in both H9c2 cells (Figure 1G,H) and NRCM (Figure 1L,M) induced by HG. In order to gain insights into the underlying mechanisms of dysregulated mitochondrial biogenesis, autophagy, mitochondrial fission-fusion dynamics, and OXPHOS were also detected in H9c2 cells and NRCM, and the results showed that HG increased mitochondrial oxidative capacity and decreased autophagy, while mitochondrial fission-fusion dynamics had no statistical difference (Supplemental Figure 1A,B). Furthermore, we observed that HG led to an increased number of apoptotic cardiomyocytes, as determined by flow cytometry analysis in H9c2 cells (Figure 1I) and TUNEL staining in both H9c2 cells (Figure 1J) and NRCM (Figure 1N). Together, these data suggest that impaired mitochondrial biogenesis and apoptosis were induced by HG in cardiomyocytes.

### 3.3 | Overexpression of miR-144 preserved mitochondrial biogenesis and attenuated apoptosis

Next, we sought to determine the possible role of miR-144 in processes associated with HG-induced injury in cardiomyocytes. An miR-144 overexpression mimic and the related NC mimic were transfected into H9c2 cells, and the transfection efficacy was confirmed by qRT-PCR (Figure 2A). Based on our model of HG-induced impaired mitochondrial biogenesis and apoptosis in H9c2 cells, we observed that miR-144 improved mitochondrial morphology, as evidenced by normalized mitochondrial architecture and cristae (Figure 2B). In both H9c2 cells and NRCM, miR-144 overexpression significantly restored the mRNA levels of mitochondrial biogenesis-related genes (Figure 2C,H), the mitochondrial DNA content (Figure 2D,I), and mitochondrial ΔΨm (Figure 2E,J). In addition, upregulation of miR-144 further partially increased mitochondrial OXPHOS and preserved autophagy (Supplemental Figure 2A,B) induced by HG. Furthermore, enhanced miR-144 expression reduced cell apoptosis, as determined by flow cytometry analysis (Figure 2F) and TUNEL staining (Figure 2G,K). To determine the effects of miR-144 knockdown, an miR-144 inhibitor was transfected to H9c2 cells, and the transfection efficacy was evaluated by qRT-PCR (Figure 3A). Contrary to the effects of miR-144 overexpression, miR-144 inhibition worsened the mitochondrial morphology, as exhibited by serious mitochondrial vacuolization and increased mitochondrial fragmentation, disarray, and degeneration (Figure 3B). Mitochondrial biogenesis (Figure 3C,H), mitochondrial DNA contents (Figure 3D,I), and ΔΨm(Figure 3E,J) were also further diminished by treatment with the miR-144 inhibitor in both H9c2 cells and NRCM. Downregulation of miR-144 further decreased the expression of mitochondrial OXPHOS and autophagy-encoding genes (Supplemental Figure 2C,D). miR-144 knockdown also significantly aggravated HG-induced apoptosis (Figure 3F,G,K). Collectively, these data indicate that miR-144 protected against impaired mitochondrial biogenesis and apoptosis in cardiomyocytes.

### 3.4 | Identification of Rac-1 as a target gene of miR-144

Rac-1, a small guanosine triphosphate-binding protein, is the predominant isoform of Rac (among Rac-1, Rac-2, and Rac-3) expressed in cardiomyocytes.\textsuperscript{36} Recent findings have suggested that Rac-1 activation can induce apoptosis in both hyperglycemic hearts and HG-stimulated cardiomyocytes through NAPDH oxidase activation and mitochondrial ROS production, thereby contributing to the development of DCM.\textsuperscript{37} Previous data confirmed that miR-144 can directly target the 3′-untranslated region of Rac-1 in H9c2 cells.\textsuperscript{38} We first performed luciferase reporter assays to confirm that miR-144 could directly target the 3′UTR of Rac-1 in H9c2 cells (Figure 4A). Next, western blot analysis showed that Rac-1 expression in H9c2 cells and NRCM was decreased by the miR-144 mimic (Figure 4B,D) but increased by the miR-144 inhibitor (Figure 4C,E), indicating that miR-144 could regulate endogenous Rac-1-expression levels. Furthermore, we observed that Rac-1 activity increased significantly in the heart of STZ-treated mice (Figure 4F) and in HG-treated H9c2 cells (Figure 4G), when compared with controls. We next used Rac-1 overexpression plasmid and Rac-1 siRNA to determine the role of Rac-1 in mediating the effects of miR-144 on mitochondrial biogenesis and apoptosis. The efficacy of the Rac-1 overexpression plasmid and Rac-1 siRNA was determined by qRT-PCR (Figure 4H,L) and western blot (Figure 4I,K,M-O) analyses. Functional analysis illustrated that Rac-1 overexpression reversed the protective effects of the miR-144 mimic against HG-induced mitochondrial biogenesis impairment (Figure 5A-D,G-I) and apoptosis (Figure 5E,F,J) in H9c2 cells and NRCM. Mitochondrial OXPHOS and autophagy were also significantly aggravated with the addition of Rac-1 overexpression on both H9c2 cells and NRCM (Supplemental Figure 3A,B). In addition, Rac-1 knockdown via siRNA counteracted the aggravated mitochondrial biogenesis impairment (Figure 6A-D,G-I), OXPHOS, autophagy (Supplemental Figure 3C,D), and apoptosis (Figure 6E-F,J), induced by miR-144 silencing,
FIGURE 2  Overexpression of miR-144 preserves mitochondrial biogenesis and attenuates apoptosis. A, The transfection efficacy of miR-144 mimic in H9c2 cells (n = 4). B, Representative electron micrographs of mitochondria after transfection with miR-144 mimic versus negative control in HG-treated H9c2 cells (n = 3). Scale bar = 12,000 magnification. C-D, Mitochondrial biogenesis-related mRNA levels (n = 6) and mitochondrial DNA content assay (n = 4) after transfection with miR-144 mimic versus negative control in HG-treated H9c2 cells. E, TMRE analysis of mitochondrial ΔΨm in H9c2 cells (n = 4). Scale bar = 100 μm. F-G, The ratio of apoptosis after transfection with miR-144 mimic versus negative control in HG-treated H9c2 cells (n = 5). Scale bar = 100 μm. H-I, Mitochondrial biogenesis-related mRNA levels and mitochondrial DNA content assay after transfection with miR-144 mimic versus negative control in NRCM (n = 4). J, TMRE analysis of mitochondrial ΔΨm in NRCM (n = 4). Scale bar = 50 μm. K, The ratio of apoptosis after transfection with miR-144 mimic versus negative control in HG-treated NRCM (n = 4). Scale bar = 100 um. Data were presented as mean ± SD. *P < .05, **P < .01, ***P < .001, ****P < .0001 versus controls.
**FIGURE 3** Downregulation of miR-144 worsens mitochondrial biogenesis and increases apoptosis. A, The transfection efficacy of miR-144 inhibitor in H9c2 cells (n = 4). B, Representative electron micrographs of mitochondria after transfection with miR-144 inhibitor versus negative control in HG-treated H9c2 cells (n = 3). Scale bar = 12,000 magnification. C-D, Mitochondrial biogenesis-related mRNA levels (n = 6) and mitochondrial DNA content assay (n = 4) after transfection with miR-144 inhibitor versus negative control in HG-treated H9c2 cells. E, TMRE analysis of mitochondrial ΔΨm in H9c2 cells (n = 4). Scale bar = 100 µm. F-G, The ratio of apoptosis after transfection with miR-144 inhibitor versus negative control in HG-treated H9c2 cells (n = 5). Scale bar = 100 µm. H-I, Mitochondrial biogenesis-related mRNA levels and mitochondrial DNA content assay after transfection with miR-144 inhibitor versus negative control in NRCM (n = 4). J, TMRE analysis of mitochondrial ΔΨm in NRCM (n = 4). Scale bar = 50 µm. K, The ratio of apoptosis after transfection with miR-144 inhibitor versus negative control in HG-treated NRCM (n = 4). Scale bar = 100 µm. Data were presented as mean ± SD. *P < .05, **P < .01, ***P < .001, ****P < .0001 versus controls.
FIGURE 4  Rac-1 is identified as a target gene of miR-144. A, Luciferase reporter assays identified Rac-1 as a direct target gene of miR-144 (n = 6). B–E, Western blot analysis for Rac-1 after treatment of miR-144 mimic (B, D) or miR-144 inhibitor (C, E) in H9c2 cells and NRCM (n = 3). F–G, Western blot analysis for Rac-1-GTP in STZ-treated heart samples and HG-treated H9c2 cells (n = 3). H–K, The transfection efficacy of Rac-1 overexpression plasmid evaluated by qRT-PCR (n = 4) and western blot analyses (n = 3) in H9c2 cells. L–O: The transfection efficacy of Rac-1 knockdown siRNA evaluated by qRT-PCR (n = 6) and western blot analyses (n = 3) in H9c2 cells. Data were presented as mean ± SD. *P < .05, **P < .01, ***P < .001, ****P < .0001 versus controls.
FIGURE 5  Forced expression of Rac-1 reverses the protective effects of miR-144 overexpression on mitochondrial biogenesis and cell apoptosis. A, Representative electron micrographs of mitochondria after transfection of HG-treated H9c2 cells with Rac-1 plasmid versus negative control plasmid, in the presence of miR-144 mimic or negative control (n = 3). Scale bar = 12,000 magnification. B-C, Mitochondrial biogenesis-related mRNA levels (n = 6) and mitochondrial DNA content assay (n = 5) after transfection of HG-treated H9c2 cells with Rac-1 plasmid versus negative control plasmid, in the presence of miR-144 mimic or negative control. D, TMRE analysis of mitochondrial ΔΨm in H9c2 cells (n = 4). Scale bar = 100 µm. E-F, The ratio of apoptosis after transfection of HG-treated H9c2 cells with Rac-1 plasmid versus negative control plasmid, in the presence of miR-144 mimic or negative control (n = 4). Scale bar = 100 µm. G-H, Mitochondrial biogenesis-related mRNA levels (n = 4) and mitochondrial DNA content assay (n = 4) after transfection of HG-treated NRCM with Rac-1 plasmid versus negative control plasmid, in the presence of miR-144 mimic or negative control. I, TMRE analysis of mitochondrial ΔΨm in NRCM (n = 4). Scale bar = 50 µm. J, The ratio of apoptosis after transfection of HG-treated NRCM with Rac-1 plasmid versus negative control plasmid, in the presence of miR-144 mimic or negative control (n = 4). Scale bar = 100 µm. Data were presented as mean ± SD. *P < .05, **P < .01, ***P < .001, ****P < .0001 versus controls.
FIGURE 6  Inhibition of Rac-1 improves mitochondrial biogenesis and attenuates cell apoptosis. A, Representative electron micrographs of mitochondria after transfection of HG-treated H9c2 cells with Rac-1 siRNA versus negative control siRNA, in the presence of miR-144 inhibitor or negative control (n = 3). Scale bar = 12 000 magnification. B-C, Mitochondrial biogenesis-related mRNA levels (n = 6) and mitochondrial DNA content assay (n = 4) after transfection of HG-treated H9c2 cells with Rac-1 siRNA versus negative control siRNA, in the presence of miR-144 inhibitor or negative control. D, TMRE analysis of mitochondrial ΔΨm in HG-treated H9c2 cells (n = 4). Scale bar = 100 µm. E-F, The ratio of apoptosis after transfection of HG-treated H9c2 cells with Rac-1 siRNA versus negative control siRNA, in the presence of miR-144 inhibitor or negative control (n = 4). Scale bar = 100 µm. G-H, Mitochondrial biogenesis-related mRNA levels (n = 4) and mitochondrial DNA content assay (n = 4) after transfection of HG-treated NRCM with Rac-1 siRNA versus negative control siRNA, in the presence of miR-144 inhibitor or negative control. I, TMRE analysis of mitochondrial ΔΨm in NRCM (n = 4). Scale bar = 50 µm. J, The ratio of apoptosis after transfection of HG-treated NRCM (n = 4). Scale bar = 100 µm. Data were presented as mean ± SD. *P < .05, **P < .01, ***P < .001, ****P < .0001 versus controls.
following HG stimulation in both H9c2 cells and NRCM. These data suggest that Rac-1 is a target gene of miR-144 that mediates hyperglycemia-induced damaged mitochondrial biogenesis and apoptosis.

### 3.5 The miR-144–Rac-1 pathway regulated mitochondrial biogenesis through AMPK–PGC-1α signaling

AMPK is an energy-sensing enzyme that conserves the AMP/ATP ratio and functions as a fuel gauge by restoring the balance between cellular ATP production and consumption. Currenty, pioglitazone and metformin are advocated to regulate diabetes by activating AMPK. AMPK activation can also stimulate the downstream regulator PGC-1α to promote mitochondrial biogenesis. Previous studies of PGC-1α demonstrated that the absence of PGC-1α lead to a decreased availability of ATP and caused contractile defects, whereas PGC-1α overexpression contributed to cardiomyopathy, implying that normal–high expression levels of active PGC-1α may benefit the heart. As expected, the protein levels of PGC-1α and AMPKα were not appreciably different in cardiac samples from STZ-treated mice or HG-stimulated H9c2 cells. However, with both in vivo and in vitro hyperglycemic models, decreased AMPKα phosphorylation (Figure 7A,B,D,E) and increased PGC-1α acetylation (Figure 7C,F) were found. AMPK has been proposed to activate Rac-1 in cultured muscle cells, and endothelial cells, and macrophages. However, recent data also implied that AMPK activation is neither sufficient nor necessary for Rac-1 activity in intact skeletal muscle or cultured muscle cells. 

In addition, the activity of Rac-1 was increased in heart tissues from patients with both type 1 and type 2 DCM; thus, it remains unknown whether increased Rac-1 activity had a negative-feedback effect on AMPK phosphorylation and downstream PGC-1α levels in hyperglycemic models. Compared to the control group, we determined that AMPKα phosphorylation was decreased and PGC-1α acetylation was increased in HG-treated H9c2 cells, following Rac-1 overexpression (Figure 7G–I). In contrast, Rac-1 knockdown with siRNA increased AMPKα phosphorylation and PGC-1α deacetylation (Figure 7J–L).

To establish a causal relationship between dysfunctional AMPKα–PGC-1α and the miR-144–Rac-1 pathway in the subsequent impairment of mitochondrial biogenesis and apoptosis induced by HG, cells were transfected with AMPKα and PGC-1α siRNAs and their efficacies were evaluated by qRT-PCR (Figure 7M,O) and western blot (Figure 7N,P) analyses. Our results implied that siRNA-mediated knockdown of AMPKα and PGC-1α significantly attenuated the protective effects of miR-144 on mitochondrial biogenesis and function (Figure 8A–C). Moreover, the protective role of miR-144 against HG-induced cell apoptosis was also abolished by administering AMPKα and PGC-1α siRNAs (Figure 8D,E). These data reveal that signaling due to AMPKα activation and PGC-1α deacetylation was involved in the protective effect of miR-144 in improving mitochondrial biogenesis and reducing apoptosis.

### 3.6 The forced in vivo expression of miR-144 preserved cardiac function and improved mitochondrial biogenesis

To evaluate the effect of miR-144 overexpression on mitochondrial biogenesis and cardiac function, we administered mice an miR-144 agomir via tail vein injection to upregulate miR-144 in vivo. Diabetes was induced in male C57BL/6 mice (5–6 weeks old) by daily intraperitoneal injections of STZ (50 mg/kg). After 7 days, the mice were injected with either a scrambled NC agomir or an miR-144 agomir (20 mg/kg) once per week for 8 weeks (Figure 9A). The mice were sacrificed at 9 weeks after the first STZ injection and the efficacy of miR-144 overexpression in the heart was confirmed by qRT-PCR (Figure 9B). To determine the time points when miR-144 started to downregulate with diabtes, we detected the expression level of miR-144 at different time points after STZ injection. Results demonstrated that miR-144 was downregulated within 3–4 weeks after STZ injection, which sustained downregulation until mice were sacrificed at 10 weeks (Figure 9C). Cardiac function, including both systolic and diastolic parameters, was evaluated by echocardiography (Figure 9D). Forced miR-144 expression preserved cardiac diastolic function, including the E/A and E/e’ ratio (Figure 9E), and reversed STZ-induced decreases in the LV mass (Table 3). Mice in the STZ + miR-144-agomir group (10/15) also exhibited significantly increased survival rates compared to those in the STZ + NC-agomir group mice (5/15). No death was observed in the NC-agomir and miR-144-agomir groups (Figure 9H). Moreover, cardiac fibrosis, a well-known feature of ventricular remodeling in DCM, was significantly attenuated in miR-144 agomir/STZ-treated mice versus NC agomir/STZ-treated mice, as assessed by Masson’s trichrome staining (Figure 9I). Consistent with the staining results, the mRNA levels of pro-fibrotic genes (α-SMA and Col1a1) were decreased by miR-144 treatment in STZ hearts (Figure 9J). However, STZ-induced aberrant systolic dysfunctions (including EF, FS, LVID; d, and LVID; s) (Figure 9E), body weight, (Figure 9F), and FBG (Figure 9G) were not remarkably altered by administering miR-144 agomir, when compared to the STZ + NC-agomir group (Figure 7D and Table 3). Cardiomyocyte sizes were evaluated by WGA staining, although no statistically significant differences were found between the four groups (Figure 9K).
FIGURE 7  Hyperglycemia inactivates AMPK phosphorylation and induces PGC-1α acetylation. A-C, Western blot analysis for AMPKα phosphorylation and PGC-1α acetylation in HG-treated H9c2 cells (n = 3). D-F, Western blot analysis for AMPKα phosphorylation and PGC-1α acetylation in left ventricular samples challenged with STZ treatment (n = 3). G-I, Western blot analysis for AMPKα phosphorylation and PGC-1α acetylation after transfection with Rac-1 overexpression plasmid versus negative control in HG-treated H9c2 cells (n = 3). J-L, Western blot analysis for AMPKα phosphorylation and PGC-1α acetylation after transfection with Rac-1 siRNA versus negative control in HG-treated H9c2 cells (n = 3). M-N, The transfection efficacy of AMPKα siRNA evaluated by qRT-PCR (n = 6) and western blot analyses (n = 3) in H9c2 cells. O-P, The transfection efficacy of PGC-1α siRNA evaluated by qRT-PCR (n = 6) and western blot analyses (n = 3) in H9c2 cells. Data were presented as mean ± SD. *P < .05, **P < .01, ***P < .001, ****P < .0001 versus controls.
FIGURE 8  Rac-1 knockdown activates AMPK phosphorylation and PGC-1α deacetylation. A, Representative electron micrographs of mitochondria after transfection of HG-treated H9c2 cells with AMPKα or PGC-1α siRNA versus negative control siRNA, in the presence of miR-144 mimic or negative control (n = 3). Scale bar = 12 000 magnification. B-C, Mitochondrial biogenesis-related mRNA levels (n = 6) and mitochondrial DNA content assay (n = 4) after transfection of HG-treated H9c2 cells with AMPKα or PGC-1α siRNA versus negative control siRNA, in the presence of miR-144 mimic or negative control. D-E, The ratio of apoptosis after transfection of HG-treated H9c2 cells with AMPKα or PGC-1α siRNA versus negative control siRNA, in the presence of miR-144 mimic or negative control (n = 4). Scale bar = 100 µm. Data were presented as mean ± SD. *P < .05, **P < .01, ***P < .001, ****P < .0001 versus controls.
FIGURE 9  In vivo forced expression of miR-144 preserves cardiac function. A, Schematic illustration of experimental design for pharmacological overexpression of miR-144 using agomir. B, qRT-PCRs for relative miR-144 expression level in left ventricular samples challenged with miR-144 mimic or NC mimic (n = 5). C, qRT-PCRs for relative miR-144 expression level at different time points with STZ treatment (n = 5). D-E, Systolic and diastolic function measured by echocardiography. F, Body weight measured every 2 weeks in STZ mice. G, Fasting blood glucose measured every 2 weeks in STZ mice. H, Percent survival analysis after transfection with miR-144 agomir versus negative control in STZ-treated mice. I, Masson staining after transfection with miR-144 agomir versus negative control in STZ-treated mice (n = 4). J, qRT-PCR analysis for α-SMA, Col1a1, and Col3a1 after transfection with miR-144 agomir versus negative control in STZ-treated mice (n = 5). Scale bar = 50 µm. K, WGA staining after transfection with miR-144 agomir versus negative control in STZ-treated mice (n = 4). Scale bar = 50 µm. Data were presented as mean ± SD. *P < .05, **P < .01, ***P < .001, ****P < .0001 versus controls. #P < .05 STZ+NC agomir versus STZ+miR-144 agomir group
Next, we explored whether miR-144 overexpression could protect against STZ-induced impairment in mitochondrial biogenesis and mitochondrial dysfunction. Compared to the STZ + NC-agomir group, heart tissues in the STZ + miR-144-agomir group displayed fewer mitochondrial vacuoles and relatively normalized mitochondrial cristae and architecture (Figure 10A). Furthermore, forced miR-144 expression increased partial mRNA levels of genes participating in mitochondrial biogenesis and mitochondrial DNA content (Figure 10B,C). The expression of mitochondrial OXPHOS and autophagy-encoding genes was partially reversed by miR-144 overexpression (Supplemental figure 4A). Mechanistically, the miR-144 agomir inhibited STZ-induced increased Rac-1 activity, decreased AMPKα phosphorylation, and enhanced PGC-1α acetylation (Figure 10D-G). Moreover, miR-144 overexpression also decreased cardiomyocyte apoptosis induced by STZ, as evidenced by decreased α-actinin/TUNEL-positive cells (Figure 10H) and an increased ratio of the anti-apoptotic protein Bcl2 to the pro-apoptotic protein BAX (Bcl2: BAX ratio) (Figure 10I).

3.7 The serum level of miR-144 was reduced in patients with DCM

To evaluate the potential clinical relevance of miR-144 in patients with DCM, we further examined circulating miR-144 levels in diabetic patients with cardiac dysfunction (n = 31) versus diabetic patients without cardiac dysfunction (n = 32). The clinical characteristics of the patients are presented in Table 4. Interestingly, miR-144 level was significantly decreased in the plasma of diabetic patients with cardiac dysfunction (Figure 10J), although these data do not establish a mechanistic link between miR-144 and diabetic cardiac dysfunction in humans. Correlation analyses between plasma miR-144 and echocardiographic data showed that plasma miR-144 levels correlated positively with EF and the E/A ratio (Table 5). The ROC curve showed that plasma miR-144 could serve as a specific predictor of diabetic patients developing cardiac dysfunction with an area under the curve of 0.783 (95% confidence interval, 0.669-0.896, P < .001, Figure 10K).

4 DISCUSSION

In this study, we made several observations that advance the current understanding of the mechanisms underlying DCM. First, we generated morphological and molecular evidence of mitochondrial biogenesis dysfunction in STZ-treated mice and HG-treated cardiomyocytes. We then obtained both in vivo and in vitro evidence that forced miR-144 expression contributed to improved mitochondrial biogenesis and reduced apoptosis by targeting Rac-1. Finally, our findings suggested that impairment of AMPK–PGC-1α signaling contributed to reduced mitochondrial biogenesis and led to hyperglycemic cardiac injury. Taken together, our results have established the functional relevance of miR-144–Rac-1–AMPK–PGC-1α signaling for mitochondrial biogenesis and apoptosis in hyperglycemia models (Figure 10L). These data highlight a novel strategy for promoting cardioprotection by improving mitochondrial biogenesis and function.
In vivo forced expression of miR-144 improves mitochondrial biogenesis and reduces cardiomyocyte apoptosis. A, Representative electron micrographs of mitochondria after transfection with miR-144 agomir versus negative control in STZ-treated mice (n = 3). Scale bar = 1500 magnification (top) and 5000 magnification (bottom). B-C, Mitochondrial biogenesis-related mRNA levels and mitochondrial DNA content assay after transfection with miR-144 agomir versus negative control in STZ-treated mice (n = 5). D-G, Western blot analysis for Rac-1-GTP, AMPKα phosphorylation, and PGC-1α acetylation after transfection with miR-144 agomir versus negative control in STZ-treated mice (n = 4). H, Myocardial apoptosis measured by TUNEL staining (n = 4). Scale bar = 50 µm. I, Western blot analysis for Bax and Bcl2 after transfection with miR-144 agomir versus negative control in STZ-treated mice (n = 4). J, miR-144 is decreased in the plasma of diabetic patients with cardiac dysfunction compared to diabetic patients without cardiac dysfunction. K, The ROC curve for plasma miR-144 as a predictor for diabetic patients developing cardiac dysfunction. L, The functional relevance of miR-144–Rac-1–AMPK–PGC-1α signaling for mitochondrial biogenesis and apoptosis in hyperglycemia models. Data were presented as mean ± SD. *P < .05, **P < .01, ***P < .001, ****P < .0001 versus controls.
accompanied by impairment in ADP-stimulated respiration and ATP synthesis, indicating that mitochondrial biogenesis is not accompanied by a coordinate increase in mitochondrial function. In contrast, several reports also demonstrated that in heart samples from ob/ob mice, mitochondrial DNA contents decreased with a corresponding downregulation of oxidative respiratory chain activity. These observations raise important questions over whether diabetes-associated changes in mitochondrial biogenesis are adaptive or maladaptive, which may provide clues for developing novel strategies to treat DCM. PGC-1α is a major regulator of mitochondrial biogenesis. In the heart, knocking out PGC-1α resulted in reduced expression of genes related to fatty acid oxidation, the tricarboxylic acid cycle, and OXPHOS, without altering the mitochondrial volume density. Interestingly, cardiomyocyte-specific PGC-1α overexpression significantly increased the cardiomyocyte mitochondrial volume density, albeit with contractile dysfunction. Therefore, determining how to sustain the normal physiological level of PGC-1α in the myocardium is of great importance. In this study, we observed significant downregulation of PGC-1α and downstream effectors, such as NRF1, TFAM, and other mitochondrial biogenesis-related targets at the mRNA level, which was associated with reduced mitochondrial DNA copies, elevated OXPHOS, and decreased mitochondrial autophagy in hyperglycemic models, both in vivo and in vitro. Therefore, impaired mitochondrial biogenesis in the myocardium may represent an important pathological feature of diabetes.

Currently, the underlying mechanisms controlling mitochondrial biogenesis and cardiac dysfunction in the diabetic heart are not well delineated. Because approximately 60% of protein-coding genes are regulated by miRNAs, they have emerged as powerful regulators in almost all vital cellular processes. However, the roles of miRNAs in the diabetic heart are unclear, especially in terms of mitochondrial biogenesis. In this study, miR-144 was downregulated both in STZ-induced mice and HG-induced cardiomyocytes, which

| TABLE 4 | Clinical characteristics of diabetic patients with or without cardiac dysfunction (no. or mean ± SD) |
|-----------------------------------------------|-------------------------------------------------|--------------------------|
| | Diabetic patients (n = 31) | DCM patients (n = 32) | P value |
|-----------------------------------------------|-------------------------------------------------|--------------------------|
| Sex (male) | 17 | 14 | .22 |
| Age (year) | 56.85 ± 8.60 | 56.41 ± 7.97 | .82 |
| Duration of diabetes (year) | 7.61 ± 6.55 | 8.57 ± 5.46 | .44 |
| BMI (cm/kg²) | 23.78 ± 2.59 | 24.96 ± 5.93 | .67 |
| BNP | 41.00 ± 27.89 | 43.03 ± 36.09 | .52 |
| CTNI | 0.004 ± 0.007 | 0.004 ± 0.012 | .46 |
| CK | 57.40 ± 24.58 | 59.07 ± 17.97 | .35 |
| CK-MB | 1.18 ± 0.40 | 1.21 ± 0.55 | .79 |
| Myoglobin | 16.53 ± 6.30 | 17.18 ± 11.90 | .81 |
| Cholesterol | 4.61 ± 0.79 | 4.63 ± 0.81 | .92 |
| TG | 1.88 ± 0.94 | 2.07 ± 0.99 | .33 |
| HDL | 1.03 ± 0.30 | 1.00 ± 0.27 | .93 |
| LDL | 2.79 ± 0.62 | 2.67 ± 0.77 | .49 |
| FBG | 9.96 ± 2.91 | 11.34 ± 4.71 | .15 |
| PBG | 13.50 ± 4.18 | 15.42 ± 4.55 | .07 |
| HbA1C | 9.92 ± 2.54 | 10.15 ± 2.02 | .67 |
| Echocardiography | | | |
| EF (%) | 65.18 ± 2.33 | 63.32 ± 2.40 | .0027 |
| LVID; d (mm) | 30.04 ± 2.25 | 30.51 ± 2.51 | .43 |
| LVID; s (mm) | 46.68 ± 3.04 | 46.57 ± 3.73 | .90 |
| E/A | 1.24 ± 0.13 | 0.81 ± 0.21 | <.0001 |
| E/e’ | 7.40 ± 0.56 | 11.09 ± 2.78 | <.0001 |

BMI, body mass index; BNP, type B natriuretic peptide; CTNI, cardiac troponin I; CK, creatine kinase; TG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein; FBG, fasting blood glucose; PBG, postprandial blood glucose; HbA1C, glycated hemoglobin.

| TABLE 5 | Correlation analyses between plasma miR-144 level and echocardiographic data |
|-----------------------------------------------|-------------------------------------------------|--------------------------|
| EF | LVID; d | LVID; s | E/A | E/e’ |
|-----------------------------------------------|-------------------------------------------------|--------------------------|
| r value | .309 | −.131 | −.057 | .586 | −.146 |
| P value | .015 | .309 | .660 | <.001 | .258 |

BMI, body mass index; BNP, type B natriuretic peptide; CTNI, cardiac troponin I; CK, creatine kinase; TG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein; FBG, fasting blood glucose; PBG, postprandial blood glucose; HbA1C, glycated hemoglobin.
was consistent with previous studies involving hyperglycemic models, including epicardial adipose tissue from STZ-induced model piglets, heart samples from STZ-induced mice, and peripheral blood myonuclear cells from patients with type 1 diabetes mellitus. Despite these concordant changes, the role of miR-144 on hyperglycemic cardiac injury found in this study conflicts with a study by Yu et al. Two reasons may explain this discrepancy. First, the degree of islet dysfunction is dependent on the age of the animals and the dose of STZ. In our study, 5-6-week-old immature mice were injected intraperitoneally with STZ (50 mg/kg) each day for 5 days, which simulated the age of onset in clinical populations. In addition, although both treatment methods have been reported in other studies, the doses of STZ and animal protocols were different between this study and that conducted by Yu et al. The disease incidence and severity at different stages may invoke different roles for miRNA in the heart. Second, with in vitro studies, the effects of miRNAs also depend on the cell types used and their characteristics, as well as the concentration of HG used for stimulation. To evaluate these possibilities, it may be necessary to generate mice with inducible cardiomyocyte-specific miR-144 knockout or overexpression, at different stages of hyperglycemic cardiac injury.

The small GTPase Rac-1 is essential for the correct assembly of nicotinamide adenine dinucleotide phosphate oxidase subunits, leading to the generation of reactive oxygen species (ROS). Mitochondria represent an important source of ROS production, which contributes to apoptosis. Recent findings have demonstrated that Rac-1 activity is dramatically increased in type 1 and type 2 diabetic hearts. However, relatively few studies have directly measured the crosstalk between Rac-1 and mitochondrial function. In our study, Rac-1 was identified as a target gene of miR-144. Rac-1 overexpression reversed the protective effects of miR-144 against hyperglycemic impairment of mitochondrial biogenesis, OXPHOS, mitochondrial autophagy, and apoptosis. In contrast, Rac-1 knockdown rescued impaired mitochondrial biogenesis and dysfunction induced by HG, suggesting that Rac-1 inhibition may provide a new approach for protecting mitochondrial function. Recent studies have provided increasing evidence suggesting that Rac-1 inhibition is important for the cardiovascular system. For example, NSC23766, a pharmacological inhibitor of Rac-1, has been shown to prevent HG-induced endothelial dysfunction by restoring endothelial NO synthase (eNOS) phosphorylation and reducing oxidative stress. Therefore, inhibiting Rac-1 by pharmacological means may be expected to provide a therapeutic effect for improving diabetic cardiac injuries.

Adenosine monophosphate-activated protein kinase is a phylogenetically conserved intracellular energy sensor that plays a central role in regulating glucose uptake and metabolism. Adenosine monophosphate-activated protein kinase activity is dependent on the phosphorylation of AMPKα (Thr 172) in an activation loop by AMPK kinases. AMPK activation can increase Rac-1 activity to stimulate glucose uptake in skeletal muscle cells and regulate the eNOS pathway in endothelial cells. However, Rac-1 activation is not completely AMPK dependent. During muscle contraction in murine and human skeletal muscles, Rac-1 is activated to regulate contraction-induced glucose uptake, which is AMPK independent. Therefore, the crosstalk between AMPK activation and Rac-1 activity is still not well elucidated. Previous in vivo and in vitro data with hyperglycemic models showed that increased Rac-1 activity and decreased AMPKα phosphorylation were accompanied by increased PGC-1α acetylation, which was regulated by acetylation at multiple sites occurring through silent information regulator 1 during fasting and DCM. We therefore speculated that forced Rac-1 activity may elicit feedback inhibition of AMPK activation and PGC-1α acetylation. Western blot analysis showed that in HG-induced H9c2 cells, Rac-1 overexpression reduced AMPKα phosphorylation and increased PGC-1α acetylation, while knockdown of Rac-1 increased AMPKα activity and PGC-1α deacetylation, suggesting that Rac-1 may inhibit AMPKα activation and downstream PGC-1α levels. However, the direct relationship between Rac-1 and AMPK–PGC-1α signaling and their functional roles in regulating cardiac mitochondrial biogenesis and function need to be further clarified by performing function-rescue assays.

In our study, we determined the expression level of miR-144 at different time points after STZ injection. Results demonstrated that miR-144 was downregulated within 3 weeks after STZ injection, which sustained downregulation until mice were sacrificed at 10 weeks. The dysregulation of miR-144 preceded cardiac dysfunction in our model of diabetic cardiomyopathy. Of note, we detected a remarkable decrease in circulating miR-144 levels in patients with diabetic cardiac dysfunction, and the plasma miR-144 levels closely correlated with cardiac function, including EF and the E/A ratio. The ROC curve showed that plasma miR-144 could serve as a specific predictor of diabetic patients developing cardiac dysfunction, although the results obtained in this small group still need to be replicated in a large, independent cohort. These results suggested that miR-144 could be used as a preventive way to attenuate cardiac damage induced by hyperglycemia. Interestingly, continuous glucose monitoring in vivo suggested that the protective effects of miR-144 against hyperglycemic cardiac dysfunction did not depend on lowering glucose. Data from the recent Veterans Affairs Diabetes Trial (VADT) suggest that other cardiovascular risk-reduction strategies should be prioritized in patients with advanced type 2 diabetes and established cardiovascular disease. In the current 15-year follow-up of the VADT, no difference was found in cardiovascular events, total mortality, or quality of
life. Therefore, the benefits of rigorous glycemic control with traditional hypoglycemic drugs are limited, although new hypoglycemic strategies (such as GLP-1 agonists and SGLT-2 inhibitors) may provide novel insight into preventing and treating diabetic complications. Targeting miR-144 may play an important role in improving the prognosis of diabetic patients.

5 | CONCLUSIONS

In conclusion, the current study shows that miR-144 was markedly decreased during hyperglycemia-induced cardiac dysfunction. Pharmacological overexpression of miR-144 alleviated cardiac dysfunction induced by STZ and promoted mitochondrial biogenesis. Rac-1 is a potential downstream target of miR-144 and participates in regulating hyperglycemic cardiac dysfunction. Our findings reveal that miR-144 might serve as a novel pharmacological target for diagnosing and treating diabetic cardiac dysfunction.

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Not applicable.

CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Animal experiments are approved by the committee on the Ethics of Animal Experiments of Soochow University. All human investigations conformed to the principles outlined in the Declaration of Helsinki and were approved by institutional review committees of The Third Affiliated Hospital of Soochow University (Changzhou, China). All the patients provided written consent for the collection and use of samples in this study.

CONSENT FOR PUBLICATION

Not applicable.

AUTHOR CONTRIBUTIONS

Lichan Tao designed the study, collected patients’ data, performed histological staining and transmission electron microscopy, analyzed the data, and drafted the manuscript. Fei Hua participated in the design of the study and coordination of the whole work. Xiaoli Huang constructed animal models, prepared heart samples, and performed qRT-PCR and western blot experiments. Min Xu performed echocardiography of mice and patients.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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