THE ROLE OF ncRNA IN CARDIAC REMODELING

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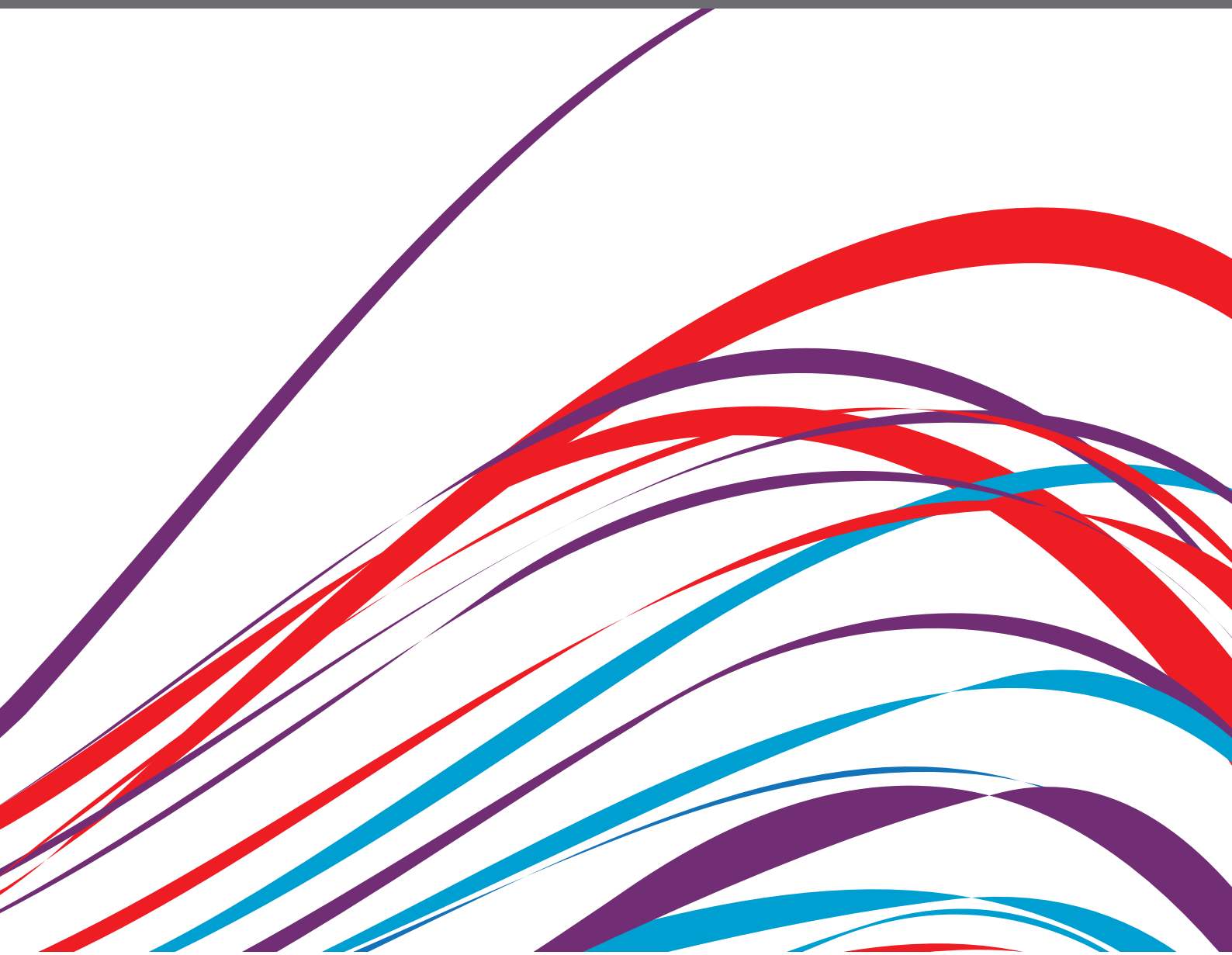

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THE ROLE OF ncRNA IN CARDIAC REMODELING

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ORIGINAL RESEARCH
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Different Expressions of Pericardial Fluid MicroRNAs in Patients With Arrhythmogenic Right Ventricular Cardiomyopathy and Ischemic Heart Disease Undergoing Ventricular Tachycardia Ablation

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Introduction: Pericardial fluid is enriched with biologically active molecules of cardiovascular origin including microRNAs. Investigation of the disease-specific extracellular microRNAs could shed light on the molecular processes underlying disease development. Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an inherited heart disease characterized by life-threatening arrhythmias and progressive heart failure development. The current data about the association between microRNAs and ARVC development are limited.

Methods and Results: We performed small RNA sequence analysis of microRNAs of pericardial fluid samples obtained during transcutaneous epicardial access for ventricular tachycardia (VT) ablation of six patients with definite ARVC and three post-infarction VT patients. Disease-associated microRNAs of pericardial fluid were identified. Five microRNAs (hsa-miR-1-3p, hsa-miR-21-5p, hsa-miR-122-5p, hsa-miR-206, and hsa-miR-3679-5p) were found to be differentially expressed between patients with ARVC and patients with post-infarction VT. Enrichment analysis of differentially expressed microRNAs revealed their close linkage to cardiac diseases.

Conclusion: Our data extend the knowledge of pericardial fluid microRNA composition and highlight five pericardial fluid microRNAs potentially linked to ARVC pathogenesis. Further studies are required to confirm the use of pericardial fluid RNA sequencing in differential diagnosis of ARVC.

Keywords: microRNA, small RNA sequencing, pericardial fluid, arrhythmogenic (right ventricular) cardiomyopathy, expression analyses

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INTRODUCTION

Pericardial fluid is a plasma ultrafiltrate found between the visceral and parietal pericardium, a double-walled sac surrounding the heart and roots of great vessels (1). Pericardial fluid is formed by the diffusion from both pericardial and epicardial vessels, as well as trans myocardial diffusion and acts as a lubricant, isolating, and protective layer for the beating heart (2). Due to a low clearance rate (3), pericardial fluid is enriched with heart-derived biologically active molecules, including cytokines, hormones, and nucleic acids (4–9). Thus, the composition of pericardial fluid potentially could influence the heart physiology and reflect certain heart conditions.

Among nucleic acids circulating in the pericardial fluid, extracellular microRNAs are of special interest. These short regulatory RNAs are extremely stable in the extracellular space due to the formation of RNA–protein complexes or packaging into vesicles, which protect them from nuclease digestion (10–14). Currently, data about pericardial microRNAs in patients with different structural heart diseases are lacking. A few studies reported on the differential expression of pericardial microRNAs in some common cardiovascular conditions. For instance, miR-423-5p was found to be enriched in pericardial fluid compared to serum; moreover, its levels differed in stable and unstable angina pectoris and aortic stenosis (15). Kuosmanen et al. (9) profiled microRNAs from the pericardial fluid of heart failure patients undergoing open-heart surgery and found no associations between microRNA profile and the clinical phenotype. In another study, exosomes from the pericardial fluid of patients were reported to contain myocardial-derived microRNAs promoting angiogenesis *in vitro* and *in vivo* (16). Taken together, these data suggest that microRNAs of pericardial fluid are biologically active molecules and could participate in cell-to-cell crosstalk.

Here, we focused on arrhythmogenic right ventricular cardiomyopathy (ARVC), an inherited disease difficult to diagnose and prognosticate (17, 18). At the early stages, ARVC is often asymptomatic or is manifested by heart rhythm abnormalities. During the ARVC progression, heart failure develops as a result of substitution of myocardium with fibrous and fat tissues (19). At the molecular level, ARVC is accompanied by significant changes in the signaling pathway activity (20–22). MicroRNAs as transcriptional regulators were reported to be involved in this pathological signaling network. Expression levels of miR-21 and miR-135b were found to be upregulated and downregulated correspondingly in the myocardium of ARVC patients (23). Transcriptome analysis of the HL-1 cells with PKP2 knockdown representing ARVC *in vitro* model revealed the downregulation of miR-184 (24). MicroRNA expression screening in plasma samples of ARVC patients and patients with idiopathic ventricular tachycardia (VT) revealed decreased expression of miR-320a associated with ARVC (25). Study performed on cardiac stromal cells disclosed three microRNAs (hsa-miR-520c-3p, hsa-miR-29b-3p, and hsa-miR-1183) differentially expressed between ARVC and control condition (26). Reduced blood plasma level of miR-320a-3p and elevated plasma levels of miR-144-3p, miR-145-5p, miR-185-5p, and miR-494-3p were reported to be associated with ARVC (27). A study involving transgenic mice carrying human DSG Q558* gene revealed different patterns of miRNA expression between the right and left ventricles: miR-217-5p and miR-708-5p were found to be upregulated and miR-499-5p was found to be downregulated specifically in the right ventricle (28). A recent study analyzing the microRNA expression profiles in blood and right ventricle tissue samples revealed that the expression of six microRNAs (miR-122-5p, miR-133a-3p, miR-133b, miR-142-3p, miR-182-5p, and miR-183-5p) was able to discriminate ARVC samples from healthy ones or other cardiomyopathy samples (29).

Although serum and myocardial microRNA expression evaluation has been reported, no common microRNA expression signature for ARVC is known. We suggested that microRNA composition of pericardial fluid in patients with ARVC might be specific, reflecting myocardial ARVC-related structural and molecular changes. We performed sequencing of microRNAs circulating in pericardial fluid of ARVC patients and patients with post-infarction VT (control group). We described the microRNA composition of pericardial fluid and performed an analysis of differentially expressed microRNAs.

MATERIALS AND METHODS

Patient Characteristics

Patients referred for epicardial VT mapping and ablation between January 2019 and November 2020 were prospectively screened for inclusion into the study. Inclusion criteria were the following: indication to VT ablation; presumably epicardial VT exit site; a definite ARVC diagnosis, or the presence of a proven post-myocardial infarction scar; signed informed consent for the study. General exclusion criteria were the following: previous cardiothoracic surgery that potentially prevented pericardial manipulations; ongoing electrical storm (multiple defibrillation shocks in a short period of time); unsuccessful epicardial access; a previous ablation procedure <3 months ago; previous epicardial ablation; inadvertent right ventricle puncture during epicardial access, and/or visible blood in the pericardial fluid sample. Specific exclusion criteria for ARVC patients were other from "definite ARVC" diagnosis according to the established criteria; predominantly left ventricle disease and/or severe left ventricle systolic dysfunction (<40%); stenotic coronary artery disease; the history of angina and/or myocardial infarction. Specific exclusion criteria for ischemic group patients were severe right ventricle systolic dysfunction; indefinite myocardial scar; a coronary artery lesion requiring intervention. Patient clinical characteristics are summarized in **Table 1**.

Pericardial Fluid Collection

Epicardial access was performed under general anesthesia *via* a subxiphoid transcutaneous puncture under fluoroscopic guidance, as described in detail earlier (30). Special attention was paid to enter the pericardial space without any damage to the right ventricle and to a minimal use of contrast media. Once a sheath was introduced into the pericardial space, pericardial fluid was aspirated into an empty sterile syringe. The fluid was visually

TABLE 1 | Patient demographics and clinical characteristics.

Patient	Age	Sex	Disease-associated genetic variants	Diagnosis	Cardiac arrest	ICD implanted	LV EF, %	RV dysfunction	Heart failure, functional class	Antiarrhythmic drugs*	Number of VT induced	Previous ablation, >3 months	Previous ablation, <3 months	Acute ablation results
1	73	M		CAD, post-MI	No	No	23%	No	II	BB	1	0	1	VT non-inducible
2	70	M		CAD, post-MI	No	Yes	40%	No	II	Amiodarone + BB	2	0	1	VT non-inducible
3	53	M		CAD, post-MI	No	Yes	30%	No	III	Sotalol	1	0	0	VT non-inducible
4	30	F	*PKP2* c.C235T, p.R79X, rs121434420	ARVC, definite	No	Yes	57%	No	I	BB	0	0	1	VT non-inducible
5	53	M	*PKP2* c.2509delA, p.S837Vfs, rs727504432, *DSG2* c.T3352A, p.S1118T	ARVC, definite	No	Yes	64%	Yes	I	Amiodarone + BB	1	0	0	VT non-inducible
6	59	F		ARVC, definite	No	Yes	67%	No	I	Amiodarone	0	0	0	VT non-inducible
7	38	M	*JUP* c.G2105A, p.R702H, rs200690479	ARVC, definite	No	Yes	60%	Yes	I	BB	0	0	0	VT non-inducible
8	20	M	*FLNC* c.G3800A, p.R1267Q, rs768767784	ARVC, definite	No	Yes	53%	No	I	BB	VF	3	1	Clinical VT non-inducible
9	24	M	*DSG2* c.G671A, p.S224N	ARVC, definite	No	No	50%	No	I	BB	0	0	0	VT non-inducible

CAD, coronary artery disease; MI, myocardial infarction; ARVC, arrhythmogenic right ventricular cardiomyopathy; ICD, implantable cardioverter-defibrillator; VT, ventricular tachycardia; BB, beta-blocker; VF, ventricular fibrillation.
**Antiarrhythmic drugs present during the ablation procedure.*

assessed for the presence of blood; blood-contaminated samples were discarded. A blood sample was collected from a femoral vein sheath immediately after the pericardial access. Collected pericardial fluid was centrifuged at 3,000 g at 4°C for 15 min, and then supernatant was collected, aliquoted, and stored at −80°C.

Genetic Testing

Genomic DNA was extracted from blood using FlexiGene DNA Kit (Qiagen). Target sequencing of 108 cardiomyopathy- and arrhythmia-associated genes was performed using Haloplex target enrichment (Agilent) with subsequent sequencing on MiSeq instrument (Illumina) as previously described (31). If disease-related genetic variants had not been identified, exome sequencing was performed as previously described (32). For the genetic variant verification, Sanger sequencing using a BigDye Terminator v3.1 kit and a 3,500 Genetic Analyzer (Applied Biosystems) was performed.

RNA Extraction

Before RNA extraction, samples were additionally centrifuged at 3,000 g at 4°C for 15 min and the obtained supernatant was used for RNA extraction. Small RNAs were extracted using SPLIT RNA Extraction Kit (Lexogen) according to manufacturer's recommendations.

Small RNA Library Preparation and Sequencing

Small RNA libraries were generated using Small RNA-Seq Kit (Lexogen) according to manufacturer's recommendations. The number of amplification cycles was 20 for all samples. Libraries were quantified using capillary gel electrophoresis using Bioanalyzer 2,100 (Agilent), then pooled in equimolar ratios based on 143-bp peak area, and purified in 6% PAAG gel using Gel Extraction Module (Lexogen). Sequencing was performed using MiSeq Reagent Kit v3 2x75bp and MiSeq equipment (Illumina) according to manufacturer's recommendations.

Bioinformatic Analyses

Obtained paired-end reads were merged using FLASH tool (33), length filtered 15–31 bp using Geneious Prime 2020.0.5 (https://www.geneious.com), and then aligned to the mature microRNA database (miRbase, http://www.mirbase.org/) using Novoalign implemented in mirPRo tool (34). Counts were normalized, and differential expression was calculated using R Studio version 1.2.5019 (35) with R version 3.0.1 (36) DESeq2 package (37). Hierarchical clustering and data visualization were performed in Phantasus version 1.5.1 (https://artyomovlab.wustl.edu/phantasus/). Tissue-specific expression profile of microRNAs was determined using human microRNA tissue atlas (38). MicroRNA set enrichment analyses were performed using TAM 2.0 tool (http://www.lirmed.com/tam2/) (39). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (40) and are accessible through GEO Series accession number GSE164490.

Quantitative PCR

Levels of selected microRNA were evaluated by qPCR. To remove heparin traces, RNA was treated with heparinase (Sigma) as was described before (41). For reverse transcription and qPCR microRNA-specific TaqMan Assays, TaqMan MicroRNA Reverse Transcription Kit and TaqMan Universal Master Mix II no UNG (all Thermo Fisher Scientific) were used according to manufacturer's recommendations. For miR-3679-5p measurement, a reverse transcription stem-loop primer and a primer pair for amplification were designed using sRNAPrimerDB online service (42). In this case, reverse transcription was performed using TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific), and real-time PCR was performed using qPCRmix-HS SYBR (Evrogen). TaqMan Assays and designed primer sequences are indicated in **Supplementary Table 1**.

Correlation Analysis

Pearson's correlation coefficient (r) and *p*-value were calculated using GraphPad Prism v.5.00 to explore the association between small RNA sequencing data and real-time PCR data. Linear regression was used to plot the line of best fit shown in each graph.

RESULTS

Patient Characteristics

Among 40 patients undergoing epicardial ablation during the study period, nine subjects were eligible according to the inclusion and exclusion criteria: six patients with definite ARVC according to 2010 ARVC Task Force Criteria (19) and three control patients with coronary artery disease and the history of previous myocardial infarction, with no suspicion toward inherited channelopathy syndromes (**Table 1**). Four out of six ARVC patients (patients 4, 5, 7, 9) carried genetic variants in genes (*PKP2*, *DSG2*, and *JUP*) coding for desmosomal proteins—plakophilin-2, desmoglein-2, and junctional plakoglobin. One ARVC patient (patient 8) carried a genetic variant in *FLNC* gene coding for actin-binding filamin C protein. Target sequencing and subsequent whole-exome sequencing did not reveal disease-linked genetic variants in patient 6.

Presence of MicroRNA in Pericardial Fluid

Sequencing of microRNA revealed its presence in all pericardial fluid samples. In each sample, 145–411 microRNAs were detected, with the average number of 269 microRNAs per sample. Here, 105 microRNAs were shared between all samples (**Table 2**).

Description of Pericardial MicroRNAs

We analyzed microRNAs found in pericardial fluid according to the following criteria: (1) affiliation to a particular microRNA family; (2) cell- and tissue-specific expression. We identified 19 microRNA families presented in pericardial fluid by two or more microRNAs (**Table 3**). The most abundant in pericardial fluid microRNA family was the ubiquitous let-7 family with 11 detected microRNAs. Since the pericardial fluid could be enriched by cardiac microRNAs, we evaluated the presence

TABLE 2 | List of microRNAs shared between all samples.

hsa-let-7a-3p	hsa-miR-26a-5p	hsa-miR-99b-5p	hsa-miR-181a-5p	hsa-miR-320c
hsa-let-7a-5p	hsa-miR-26b-3p	hsa-miR-99a-3p	hsa-miR-181b-5p	hsa-miR-335-3p
hsa-let-7b-5p	hsa-miR-26b-5p	hsa-miR-99a-5p	hsa-miR-181c-5p	hsa-miR-335-5p
hsa-let-7c-5p	hsa-miR-27a-3p	hsa-miR-100-5p	hsa-miR-181d-5p	hsa-miR-345-5p
hsa-let-7d-3p	hsa-miR-27b-3p	hsa-miR-101-3p	hsa-miR-182-5p	hsa-miR-361-3p
hsa-let-7d-5p	hsa-miR-28-3p	hsa-miR-106b-3p	hsa-miR-183-5p	hsa-miR-378a-3p
hsa-let-7e-5p	hsa-miR-28-5p	hsa-miR-122-5p	hsa-miR-186-5p	hsa-miR-421
hsa-let-7f-5p	hsa-miR-29a-3p	hsa-miR-125a-5p	hsa-miR-191-5p	hsa-miR-423-5p
hsa-let-7g-5p	hsa-miR-29c-3p	hsa-miR-125b-5p	hsa-miR-192-5p	hsa-miR-451a
hsa-let-7i-5p	hsa-miR-30a-3p	hsa-miR-130a-3p	hsa-miR-193a-5p	hsa-miR-484
hsa-miR-10a-3p	hsa-miR-30a-5p	hsa-miR-140-3p	hsa-miR-195-5p	hsa-miR-486-5p
hsa-miR-10a-5p	hsa-miR-30b-5p	hsa-miR-141-3p	hsa-miR-199a-3p	hsa-miR-497-5p
hsa-miR-10b-5p	hsa-miR-30c-5p	hsa-miR-143-3p	hsa-miR-199b-3p	hsa-miR-532-5p
hsa-miR-15b-5p	hsa-miR-30d-5p	hsa-miR-146a-5p	hsa-miR-200a-3p	hsa-miR-574-5p
hsa-miR-16-5p	hsa-miR-30e-3p	hsa-miR-146b-5p	hsa-miR-200b-3p	hsa-miR-652-3p
hsa-miR-19b-3p	hsa-miR-30e-5p	hsa-miR-148a-3p	hsa-miR-203a-3p	hsa-miR-744-5p
hsa-miR-21-5p	hsa-miR-34a-5p	hsa-miR-148b-3p	hsa-miR-204-5p	hsa-miR-769-5p
hsa-miR-22-3p	hsa-miR-92a-3p	hsa-miR-151a-3p	hsa-miR-221-3p	hsa-miR-888-5p
hsa-miR-23b-3p	hsa-miR-93-5p	hsa-miR-151a-5p	hsa-miR-222-3p	hsa-miR-1180-3p
hsa-miR-24-3p	hsa-miR-95-3p	hsa-miR-152-3p	hsa-miR-320a-3p	hsa-miR-1246
hsa-miR-25-3p	hsa-miR-98-5p	hsa-miR-181a-3p	hsa-miR-320b	hsa-miR-4286

of known cardiomyocyte-specific microRNAs: hsa-miR-1-3p, -133a-3p, -208a-3p, -208b-3p, -486-5p, and -486-3p. Hsa-miR-486-5p was detected in all samples, hsa-miR-1-3p was presented in six samples, hsa-miR-133a-3p in four samples, hsa-miR-486-3p was detected only in two samples, and hsa-miR-208a-3p and hsa-miR-208b-3p were not detected. Besides cardiomyocytes, the heart also consists of fibroblasts and endothelial cells. Thus, we identified in all pericardial fluid samples the microRNAs from miR-29 and miR-30 families, also having a high expression level in fibroblasts. Moreover, hsa-miR-21-5p, known to be expressed in cardiac fibroblasts (43), was presented in all studied samples. Two microRNAs (hsa-miR-93-5p; hsa-miR-106b-3p) from endothelial-specific family miR-17 were also detected in all pericardial fluid samples. Blood cell-derived microRNAs are also likely to contribute to pericardial fluid microRNA profile. Erythrocyte-specific hsa-miR-144-3p and hsa-miR-451a were detected in five samples and in all samples correspondingly.

Clustering and Differential Expression Analyses

Analysis of sample similarity revealed the high similarity of microRNA profiles of control group (post-infarction VT) and ARVC samples (**Figure 1A**). Principal component analysis (PCA) was performed in order to determine whether microRNA expression pattern is able to separate control and ARVC pericardial fluid samples. PCA showed no segregation of control or ARVC samples over the first two principal components (**Figure 1B**). Then, differential expression analysis was performed to reveal microRNA expression levels that significantly differ between patients with ARVC and the control group. Only microRNAs with at least one non-zero count between samples were taken into analyses. Although none of the microRNAs passed the multiple testing correction, we used non-corrected *p*-values taking into account the small group size and pilot nature of the study (**Figure 1C**). We found five differentially expressed microRNAs: two were downregulated in the ARVC group compared to the control group (hsa-miR-3679-5p and hsa-miR-21-5p), and three were upregulated in the ARVC group compared to the control group (hsa-miR-122-5p, hsa-miR-206, and hsa-miR-1-3p).

Enrichment Analysis of Differentially Expressed MicroRNAs

To provide the functional annotation of microRNAs differentially expressed between ARVC and control groups and to prove these microRNAs are associated with disease development, we performed microRNA set enrichment analysis. Comparison of differentially expressed microRNAs found in pericardial fluid with databases of disease-associated microRNAs showed overrepresentation of cardiovascular pathology terms—chronic atrial fibrillation, coronary heart disease, arrhythmia, heart diseases, and hypertension. Also, various muscular pathology terms were found including muscular dystrophy, musculoskeletal abnormalities, and distal myopathy (**Figure 2A**). MicroRNA set mapping against databases containing biological processes resulted in diverse terms including cell cycle, heart and muscle development, inflammation, hormone-mediated signaling pathway, T-helper 17 cell differentiation, muscle development, skeletal muscle cell differentiation, cell death, cell proliferation, and cardiogenesis (**Figure 2B**).

TABLE 3 | MicroRNA families with two or more microRNAs detected in all pericardial fluid samples.

MicroRNA family	Number of microRNAs	MicroRNAs
let-7	11	hsa-let-7a-3p; hsa-let-7a-5p; hsa-let-7b-5p; hsa-let-7c-5p; hsa-let-7d-3p; hsa-let-7d-5p; hsa-let-7e-5p; hsa-let-7f-5p; hsa-let-7g-5p; hsa-let-7i-5p; hsa-miR-98-5p
miR-10	9	hsa-miR-10a-3p; hsa-miR-10a-5p; hsa-miR-10b-5p; hsa-miR-99a-3p; hsa-miR-99a-5p; hsa-miR-99b-5p; hsa-miR-100-5p; hsa-miR-125a-5p; hsa-miR-125b-5p
miR-30	7	hsa-miR-30a-3p; hsa-miR-30a-5p; hsa-miR-30b-5p; hsa-miR-30c-5p; hsa-miR-30d-5p; hsa-miR-30e-3p; hsa-miR-30e-5p
miR-181	5	hsa-miR-181a-3p; hsa-miR-181a-5p; hsa-miR-181b-5p; hsa-miR-181c-5p; hsa-miR-181d-5p
miR-28	4	hsa-miR-28-3p; hsa-miR-28-5p; hsa-miR-151a-3p; hsa-miR-151a-5p
miR-8	3	hsa-miR-141-3p; hsa-miR-200a-3p; hsa-miR-200b-3p
miR-15	3	hsa-miR-15b-5p; hsa-miR-16-5p; hsa-miR-195-5p
miR-26	3	hsa-miR-26a-5p; hsa-miR-26b-3p; hsa-miR-26b-5p
miR-148	3	hsa-miR-148a-3p; hsa-miR-148b-3p; hsa-miR-152-3p
miR-320	3	hsa-miR-320a-3p; hsa-miR-320b; hsa-miR-320c
miR-17	2	hsa-miR-93-5p; hsa-miR-106b-3p
miR-25	2	hsa-miR-25-3p; hsa-miR-92a-3p
miR-27	2	hsa-miR-27a-3p; hsa-miR-27b-3p
miR-29	2	hsa-miR-29a-3p; hsa-miR-29c-3p
miR-95	2	hsa-miR-95-3p; hsa-miR-421
miR-146	2	hsa-miR-146a-5p; hsa-miR-146b-5p
miR-199	2	hsa-miR-199a-3p; hsa-miR-199b-3p
miR-221	2	hsa-miR-221-3p; hsa-miR-222-3p
miR-335	2	hsa-miR-335-3p; hsa-miR-335-5p

Quantitative PCR Validation of Differentially Expressed MicroRNAs

To validate microRNA expression data obtained by small RNA sequencing technique, we measured levels of differentially expressed microRNAs using qPCR and performed correlation analyses. Expression levels of three differentially expressed microRNAs (hsa-miR-1-3p, hsa-miR-21-5p, and hsa-miR-122-5p) measured by qPCR strongly correlated with sequencing data, whether expression levels of two other microRNAs (hsa-miR-206 and hsa-miR-3679-5p) demonstrated inconsistency between qPCR and small RNA sequencing (**Figure 3**).

DISCUSSION

In the present study, we performed profiling of microRNAs in pericardial fluid samples obtained from patients with ARVC or post-infarction VT patients. In contrast to previous studies that used open-heart surgery for pericardial fluid collection (9, 15, 16), we obtained pericardial fluid samples during transcutaneous epicardial access. This approach allowed us to obtain high-quality pericardial fluid samples from rare patient groups.

To date, qPCR is a gold standard approach to detect microRNAs including a circulating pool of microRNAs (44, 45). However, rapidly developing technologies of next-generation sequencing (NGS) allow to perform accurate quantitative and qualitative assessment of nucleic acids including small RNAs extracted from solid or liquid tissue samples (46–48). In the current study, we performed sequencing of microRNAs extracted from pericardial fluid samples, described the pericardial fluid microRNA composition, and performed the differential expression analysis with subsequent microRNA set enrichment analysis.

In line with previously published reports (9, 16), we detected microRNAs in all investigated samples and revealed a similar spectrum of pericardial fluid microRNAs, indicating the validity of NGS-based approaches for microRNA detection. Pericardial fluid is formed by the diffusion from both pericardial and epicardial vessels, as well as trans-myocardial diffusion. Consequently, pericardial fluid microRNA repertoire is at least partially formed by secretion of cardiac cells—cardiomyocytes, endothelial cells, and cardiac fibroblasts (9). Since most microRNAs are expressed in a broad spectrum of cell types and tissues, it is hardly possible to precisely determine their origin. However, we detected several microRNAs known to be expressed predominantly in fibroblasts (including cardiac fibroblasts), endothelial cells, or erythrocytes. As was previously reported (9, 15), cardiac-specific microRNAs were not present in all pericardial fluid samples and their average expression levels were low. This observation indicated the moderate release of these microRNAs from cardiomyocytes and likely the absence of acute myocardial injury in the studied patient group, as opposed to early stages of myocardial infarction, which is accompanied by the elevation of cardiac microRNA levels in serum (49).

It has been suggested that microRNA composition of pericardial fluid could reflect cellular and molecular events underlying cardiac pathologies (9). Consistent with a previous report (9), high levels of five microRNAs associated with cardiac disease (let-7b-5p, hsa-miR-16–5p, hsa-miR-21–5p, hsa-miR-125b-5p, and hsa-miR-451a) were found in pericardial

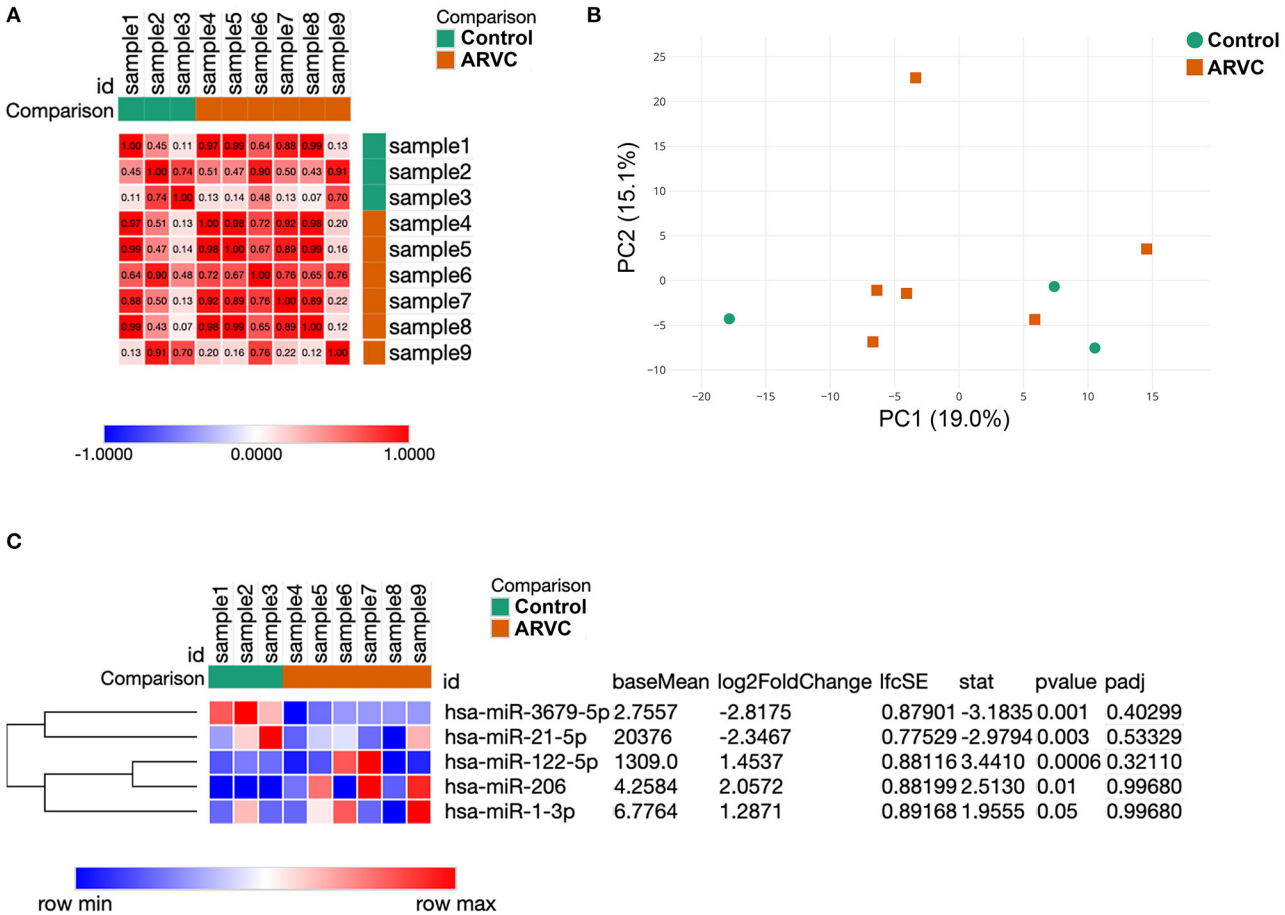

FIGURE 1 | Results of samples clustering and microRNA differential expression analysis. **(A)** Similarity matrix of pericardial fluid samples calculated based on their microRNA expression patterns. **(B)** Principal component analysis of pericardial fluid samples. **(C)** Expression heatmap and result of statistical analyses of five differentially expressed microRNAs between control and arrhythmogenic right ventricular cardiomyopathy (ARVC) pericardial fluid samples.

fluid samples (**Table 2**). Some of these microRNAs could be potentially relevant for ARVC pathogenesis even in the absence of severe cardiac pathological remodeling.

Expression analysis revealed five microRNAs differentially expressed between ARVC and control groups. Among differentially expressed microRNAs, hsa-miR-1-3p and hsa-miR-21-5p were reported to be highly expressed by cardiomyocytes and cardiac fibroblast correspondingly (50, 51). These two microRNAs are well-known to contribute to various cardiovascular diseases including ischemic heart injury, atrial fibrillation, and cardiomyopathies of different origins (52). Hsa-miR-206 was reported to be highly expressed in skeletal muscle but can also be present in the myocardium. Heart-specific overexpression of hsa-miR-206 in transgenic mice led to Cx43 downregulation and subsequently contributed to abnormal heart rate and PR interval and shortened life span. At the same time, hsa-miR-122-5p and hsa-miR-3679-5p do not reveal any specific heart-expression profile; while the first one is present in the liver in high amounts (53) and in blood cells at lower levels (53), the second one does not have any tissue-specific expression pattern (38). Hsa-miR-122-5p is essential for embryonic liver development and also was reported to regulate multiple physiological and pathological processes in the adult liver (53, 54). Intriguingly, hsa-miR-122-5p levels in heart tissue and blood samples were shown to discriminate arrhythmogenic cardiomyopathy patients from unaffected family members and patients with other cardiomyopathies (29). In contrast, little is known about hsa-miR-3679-5p, the novel player in cardiovascular biology. Originally, it was discovered in peripheral blood (55). Later, two histone demethylases, KDM7A and KDM6A (UTX), were identified as hsa-miR-3679-5p direct targets in monocytes. Downregulation of these genes by hsa-miR-3679-5p led to the reduction of adhesion molecules and regulation of monocyte adhesion to endothelial cells, which could be linked to an inflammatory response (56). Future studies are needed to prove whether these processes are relevant to ARVC. Surprisingly, a link between differentially expressed microRNAs and fibrosis-related genes encoding for proteins responsible for extracellular matrix deposition was found. Four out of five differentially expressed

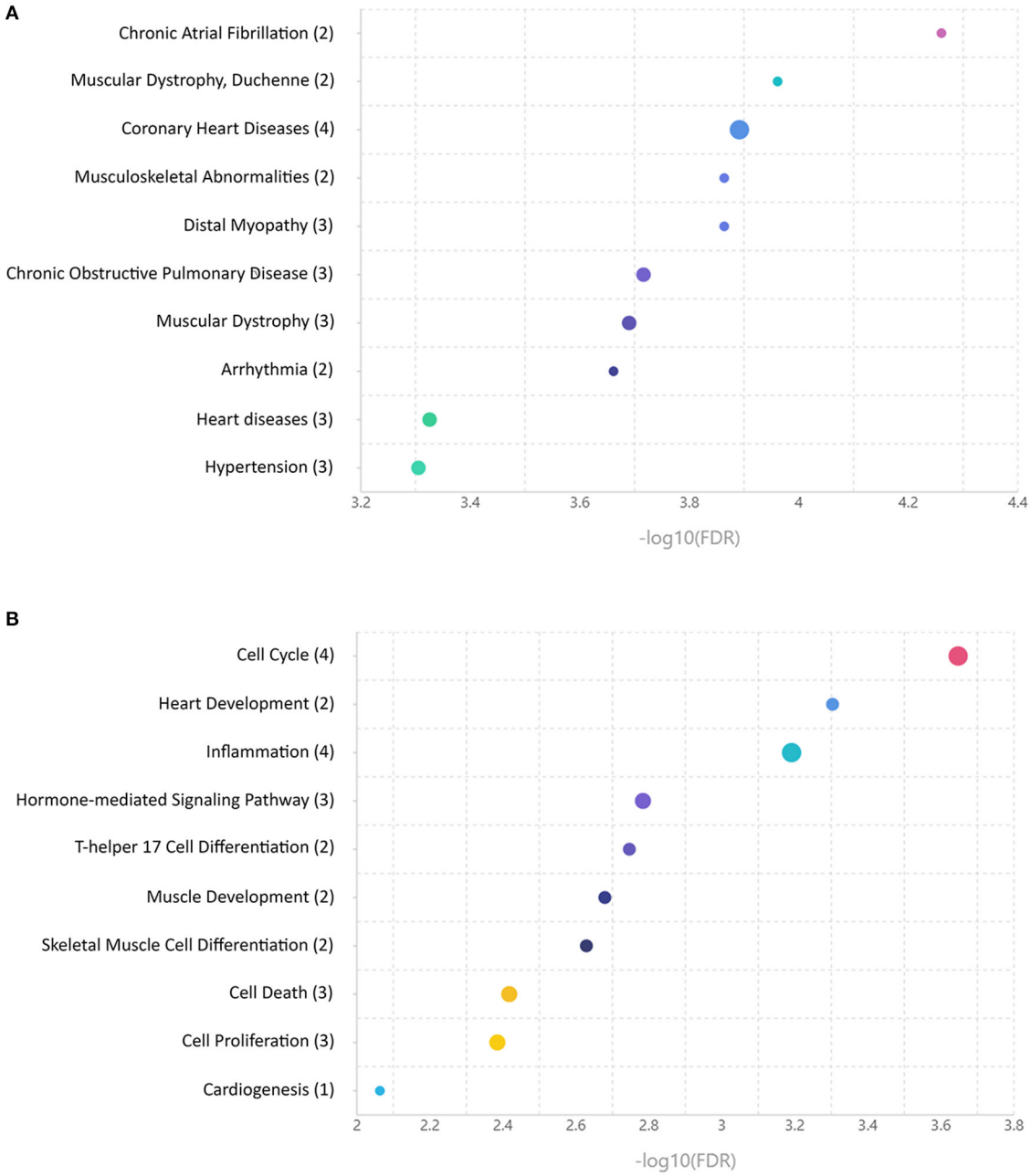

FIGURE 2 | Enrichment analyses of microRNAs differentially expressed between control and arrhythmogenic right ventricular cardiomyopathy (ARVC) pericardial fluid samples. **(A)** MicroRNA set mapping against database containing disease-associated microRNA sets. **(B)** MicroRNA set mapping against database containing biological processes-associated microRNA sets. The number of microRNAs overlapping between the datasets is indicated in round brackets.

microRNAs (hsa-miR-1-3p, hsa-miR-21-5p, hsa-miR-206, and hsa-miR-122-5p) were reported to regulate directly or indirectly matrix metalloproteinase 2 gene (43, 57–59) and genes coding for collagen isoforms (60–63). These microRNAs were also reported to regulate vimentin expression—a protein being a strong marker of mesenchymal cell- and fibroblast-specific intermediate filament (64–67).

In line with these data, the enrichment analysis found a strong association between differentially expressed microRNA set and cardiovascular diseases including persistent atrial fibrillation, coronary artery disease, unspecified heart disease, and arrhythmias. A number of associations with skeletal muscle pathologies were found likely due to the overlap of microRNA expression profiles between cardiac and skeletal muscle tissues. Analyses of associations with various biological processes found the associations with muscle development and differentiation and with basic biological processes like cell cycle, cell proliferation, and cell death. Moreover, an association with

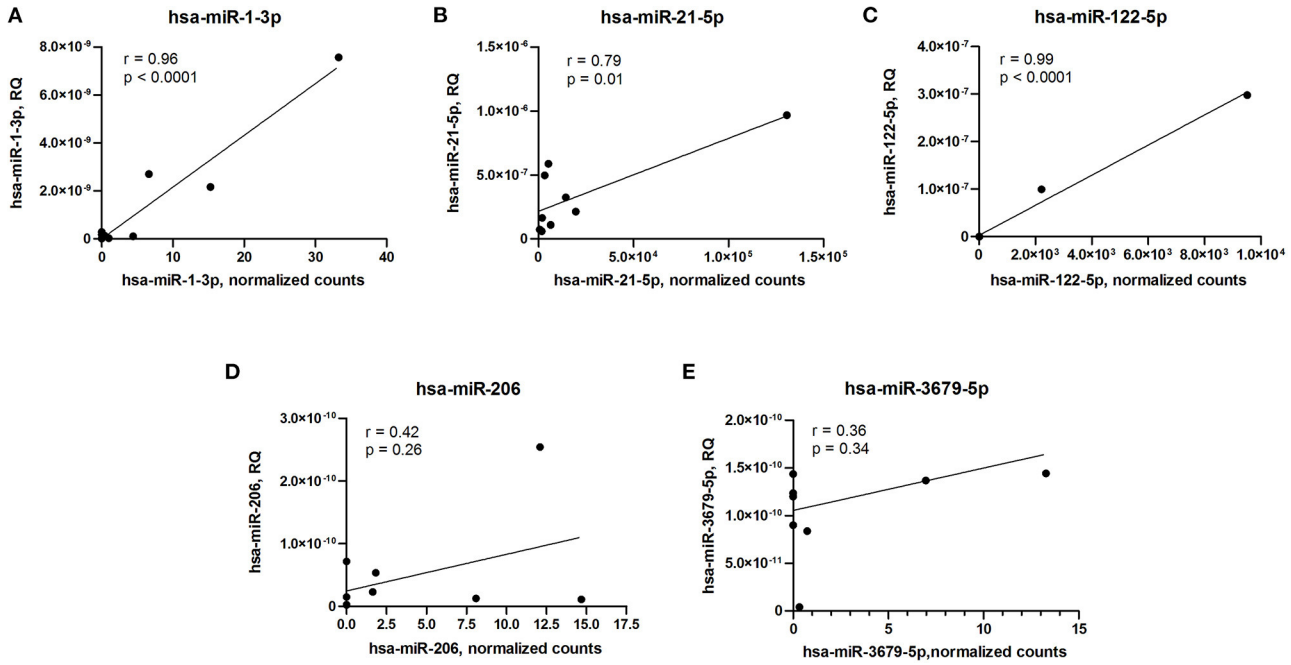

FIGURE 3 | Correlation analyses of microRNA expression between qPCR and small RNA sequencing data. **(A)** hsa-miR-1-3p. **(B)** hsa-miR-21-5p. **(C)** hsa-miR-122-5p. **(D)** hsa-miR-206. **(E)** hsa-miR-3679-5p. RQ, relative quantity. Pearson's correlation coefficient (r) and *p*-value are indicated in each plot.

inflammation, which is frequently concomitant with the heart pathology, was observed.

Despite the fact that small RNA sequencing could be used for accurate microRNA quantification, the variation of detection levels between different methods and platforms was reported (68, 69). The combination of sequencing data with subsequent qPCR analyses of selected targets allows to take advantage of both techniques and validate the results using an independent approach (69). We performed correlation analyses of five differentially expressed microRNA levels measured by small RNA sequencing and qPCR. Surprisingly, only a partial correlation between small RNA sequencing and qPCR results was observed, and a similar fact was earlier reported in other studies (68, 69). In the present study, only three out of five microRNAs differentially expressed between ARVC and control group showed a strong correlation between the two techniques used for quantification.

Our study has several limitations. First, similarly to the previous studies, we were not able to profile pericardial fluid microRNAs of healthy subjects due to the invasive technique of sample collection. Consequently, as a result, there was an age difference between the experimental groups that could introduce additional bias to the microRNA expression. In addition, the study included a very limited number of patients meeting the inclusion criteria. At last, a low number of differentially expressed genes were identified, which restrict the power of enrichment analyses.

In conclusion, we performed microRNA profiling of pericardial fluid obtained from patients with recurrent VT due to ARVC or previous myocardial infarction using small RNA sequencing technique. We described the pericardial fluid microRNA composition and revealed five differentially expressed microRNAs. Once confirmed in future studies with a larger number of patients, these microRNAs might be used in differential diagnosis of structural heart diseases in patients undergoing invasive procedures involving epicardial access.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Local ethical committee of Almazov National Medical Research Centre. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AKh collected samples, performed experiments, analyzed the data, and wrote the paper. DP performed experiments and analyzed the data. YF collected samples and performed experiments. AKn collected samples and performed experiments. KS collected samples and co-wrote the paper. DL performed

epicardial ablation, collected samples, and co-wrote the paper. EM performed epicardial ablation, collected samples, and co-wrote the paper. AKo supervised the research, acquired funding, and co-wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.647812/full#supplementary-material

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REVIEW
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The Role of Long Non-coding RNAs in Sepsis-Induced Cardiac Dysfunction

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Sepsis is a syndrome with life-threatening organ dysfunction induced by a dysregulated host response to infection. The heart is one of the most commonly involved organs during sepsis, and cardiac dysfunction, which is usually indicative of an extremely poor clinical outcome, is a leading cause of death in septic cases. Despite substantial improvements in the understanding of the mechanisms that contribute to the origin and responses to sepsis, the prognosis of sepsis-induced cardiac dysfunction (SICD) remains poor and its molecular pathophysiological changes are not well-characterized. The recently discovered group of mediators known as long non-coding RNAs (lncRNAs) have presented novel insights and opportunities to explore the mechanisms and development of SICD and may provide new targets for diagnosis and therapeutic strategies. LncRNAs are RNA transcripts of more than 200 nucleotides with limited or no protein-coding potential. Evidence has rapidly accumulated from numerous studies on how lncRNAs function in associated regulatory circuits during SICD. This review outlines the direct evidence of the effect of lncRNAs on SICD based on clinical trials and animal studies. Furthermore, potential functional lncRNAs in SICD that have been identified in sepsis studies are summarized with a proven biological function in research on other cardiovascular diseases.

Keywords: long non-coding RNA, sepsis, cardiac dysfunction, biomarker, gene therapy

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INTRODUCTION

Sepsis is a syndrome with life-threatening organ dysfunction induced by a dysregulated host response to infection (1, 2). In-hospital mortality among patients with septic shock is reported to reach up to 40% (1). Septic shock is a series of circulatory, metabolic, and cellular abnormalities and is defined by a requirement for vasopressor support and persistent hyperlactatemia in the absence of hypovolemia (3, 4). Epidemiological studies showed that ∼28.3 to 41% of all hospitalized sepsis patients died due to multiple organ failure (5), and sepsis-induced cardiac dysfunction (SICD) was identified as being closely associated with higher mortality rates (6, 7). Cardiac dysfunction is one of the major complications to sepsis, hence is predictive of a poor clinical outcome. Due to the pathophysiological changes of sepsis, cardiac lesions might be induced by a series of factors including myocardial ischemia, myocardial depressant substance, inflammation, adrenergic pathways deregulation, calcium overload, mitochondrial disorder,

coronary microvascular dysfunction, and myocardial damages (4). Animal and cell experiments with lipopolysaccharide (LPS)-induced sepsis models demonstrated a significantly higher rate of cardiomyocyte apoptosis, intracellular ROS accumulation, elevated cytoplasm cytochrome C levels, and activated inflammatory pathways (8).

The development of genome-wide association studies (GWAS) and RNA sequencing (RNA-Seq) facilitated the discovery that a large part of the nucleotide genome presents limited or no protein-coding capabilities, although these regions are still effectively transcribed. The RNAs related to these regions were named non-coding RNA (ncRNA) (9). Long non-coding RNA (lncRNA) is a type of ncRNA that is composed of more than 200 nucleotides and contributes to transcriptional and post-transcriptional regulation of RNA. According to their molecular function, lncRNAs can be classified as signal, decoy, guide, scaffold, enhancer, or sponge lncRNAs (especially circular RNAs) (10, 11) (**Figure 1**). Whether circular RNAs (circRNAs) belong to the lncRNAs is a matter of controversy. However, in consideration of their similarities in function and definition to lncRNAs, we regard circRNAs as a unique subtype of lncRNA, and consequently they are included in this review (11–13).

Modulation of lncRNA plays important roles in various stages of sepsis development and pathophysiological processes, and this may offer potential novel diagnostic and therapeutic strategies to reduce the mortality and burden of SICD. Using sequencing analysis, more than 80% of the primary genetic elements were observed to change in patients with critical sepsis (14). *In vitro*, human umbilical vein endothelial cells (HUVECs) exposed to LPS showed a 28- to 70-fold increase in the expression of lncRNAs (15). Differential expression of lncRNAs has been observed in several other cell types after exposure to the plasma of septic patients or LPS, including human tubular epithelial cells, monocytes, and cardiomyocytes, indicating a tissue-specific biological function of lncRNA (16–18). Therefore, the lncRNAs involved in SICD regulate both cardiomyocytes and non-cardiomyocytes. Current evidence indicates a role for lncRNAs in regulation of cardiomyocyte functions, such as mitochondrial homeostasis, calcium handling, contraction, and apoptosis.

Activation of inflammatory pathways mediated by Toll-like receptor (TLR) signaling in response to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) is an important mechanism of cardiomyocyte injuries caused by sepsis. These inflammatory pathways include those involving nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK), as well as some other pathways (19). The lncRNAs involved in immune responses are also likely to contribute to the origins of SICD. However, since lncRNAs present multiple modalities of action with low conservation in vertebrates, exploring the individual functions of a particular lncRNA is challenging and more difficult than similar research on microRNAs (miRNAs). Hence, several lncRNAs involved in inflammatory responses in cardiomyocytes lack associated evidence in SICD (20).

This review summarizes the direct evidence for the involvement of lncRNAs in SICD based on clinical research studies of patients with SICD and basic biology explorations using animal or cell models of SICD. Furthermore, the lncRNAs involved in both sepsis and cardiovascular diseases (CVD) among individual studies are described and their potential associations in SICD are analyzed; these studies were treated as indirect evidence for the role of lncRNAs in SICD.

THE ASSOCIATION BETWEEN LNCRNAS AND SICD

A recent study using microarray and whole genomic transcription sequencing with bioinformatics analyses on blood samples from patients with sepsis discovered 46 differentially expressed lncRNAs (DElncRNAs) (21). Additionally, 28 upregulated and 61 downregulated lncRNAs were identified in the public reported NCBI GEO dataset (22). Similar analyses based on cardiac tissue from mouse or rat sepsis models reported 74 (23) to 1,275 DElncRNAs, and revealed 14 lncRNAs that were highly correlated with 11 mitochondria-related differentially expressed mRNAs (24) and 11 differentially expressed circRNAs (25). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis indicated that upregulated lncRNAs were significantly enriched in the p53, NF-κB, and HIF-1 signaling pathways (26). Tissue-specific RNA-Seq in artificial induced inflammation revealed that some LPS-mediated lncRNAs were correlated to cardiometabolic traits (16). Thus, lncRNAs participate in regulating mitochondrial function, metabolic homeostasis, and inflammation signaling in cardiomyocytes during sepsis attacks.

Evidence in the literature linking lncRNAs and SICD can be divided into two distinct types. The first type of evidence (direct) presents clear confirmation of the involvement of lncRNAs in SICD, either from clinical samples or animal models, with definite molecular function demonstrated. The second type of evidence (indirect) describes studies where lncRNAs displayed differentiated expression in sepsis samples and were proven to have a critical role in maintaining cardiomyocyte function but lacked convincing evidence in SICD.

LNCRNA INVOLVED IN SICD AMONG VARIOUS CELL TYPES

Here, direct evidence of the involvement of lncRNAs in SICD is summarized (**Figure 2**). This evidence is based on the findings from basic molecular biological research using animal models

Abbreviations: ASOs, antisense oligonucleotides; CAD, coronary artery heart disease; circRNAs, circular RNAs; CLP, cecal ligation and puncture; CMVECs, cardiac microvascular endothelial cell; CRISPR, clustered regularly interspaced short palindromic repeats; CVD, cardiovascular disease; DAMPs, damage-associated molecular patterns; GWAS, genome-wide analyses; HCAECs, human primary coronary artery endothelial cells; HCASMC, coronary artery smooth muscle cells; HIF1α, Hypoxia-inducible factors 1α; HUVECs, human umbilical vein endothelial cells; I/R, Ischemia/Reperfusion; lncRNAs, long non-coding RNAs; LPS, lipopolysaccharide; MI, myocardial infarction; NF-κB, nuclear factor κB; PAMPs, pathogen-associated molecular patterns; PBMCs, Peripheral blood mononuclear cells; RNAi, RNA interference; SICD, sepsis-induced cardiac dysfunction; XIAP, X-chromosome-linked inhibitor of apoptosis; VSMCs, vascular smooth muscle cells.

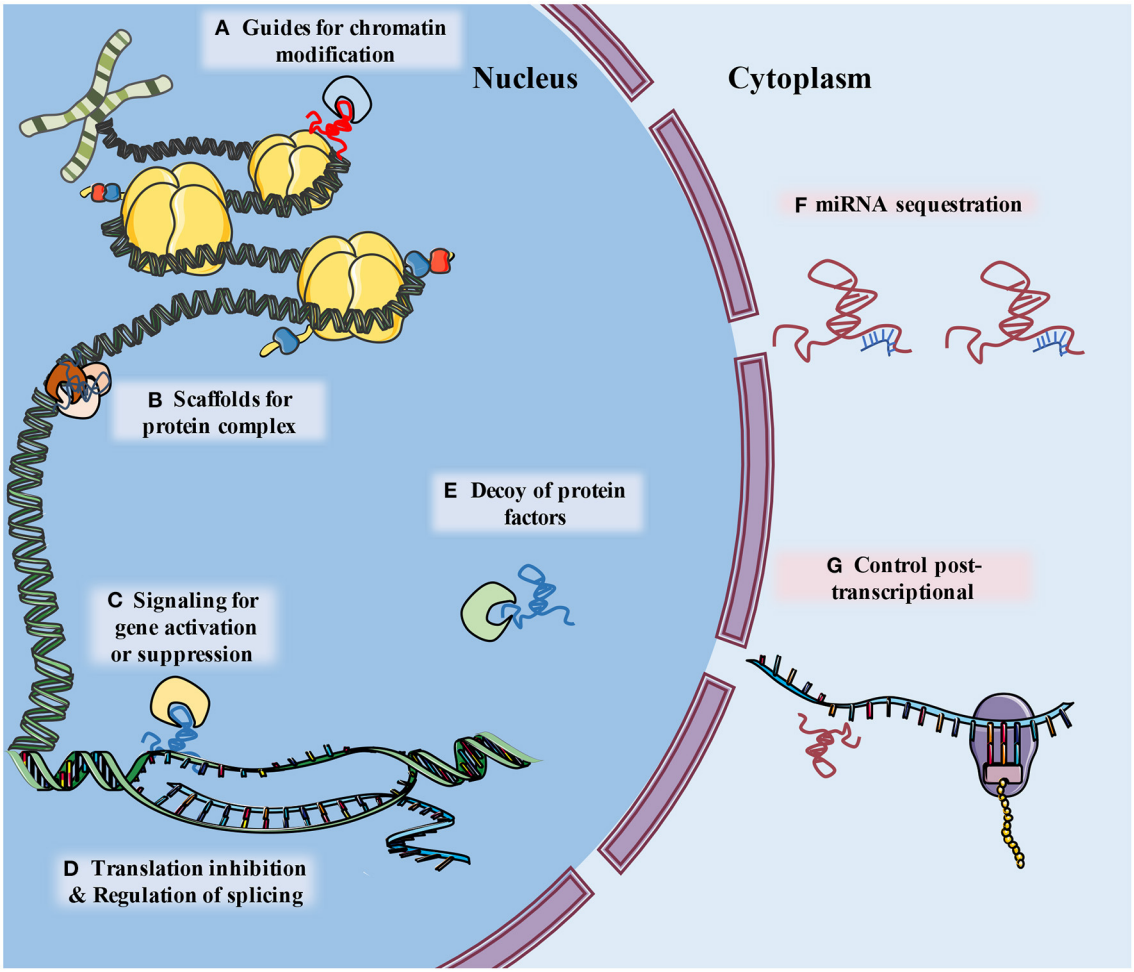

FIGURE 1 | The schematic diagram describes classification of lncRNA functions. (A) LncRNAs guide ribonucleoprotein complexes to specific location of chromatin. (B) LncRNAs support assembly of protein complex. (C) lncRNAs serve as molecular signals for tissue and temporary specific activation of transcription. (D) LncRNAs can alter splicing patterns of mRNA and suppress transcription by sequestering transcription factors. (E) LncRNAs can bind to and take away protein factors, such as transcription factors and chromatin modifiers, to influence transcriptome. (F) LncRNA can "sponge" miRNA by base pairing with their complementary base sequence and reduce their effects (G) lncRNAs may interact with a variety of RNA binding proteins (RBPs), leading to alternations of mRNA stability, splicing, protein stability and subcellular localization.

of hypodynamic septic shock induced by LPS and cecal ligation and puncture (CLP) (27); cardiac muscle cell lines (primary culture cardiomyocytes, H9C2, HL-1, and AC-16 cell lines) and microvascular cell lines exposed to serum from septic patients or administered with LPS (28); and clinical studies of sepsis patients subjected to cardiac dysfunction (**Table 1**).

Cardiomyocytes

LncRNAs participate in cardiomyocyte function through inflammatory signaling pathways, cytokine release, mitochondria homeostasis, apoptotic processes, and cell proliferation and migration during SICD.

Inflammation Signaling

LncRNAs are involved in the inflammatory process by regulating inflammation signaling, including the NF-κB, JAK/STAT, and MAPK pathways, and production of cytokines, such as IL-1, IL-6, IL-10, and TNF-α. The lncRNA MALAT1 is responsible for the septic inflammatory response under LPS administration in cardiomyocytes by downregulating miR-150-5p to increase expression of IL-6, TNF-α and the NF-κB signaling pathway (29), and TNF-α induction partly relied on serum amyloid antigen 3 (SAA3) (30). MALAT1 also interacts with p38 MAPK/NF-kB and miR-125b to aggravate cardiac inflammation and dysfunction in sepsis (31). The lncRNA NEAT1 was associated with disease severity, higher mortality risk, and unfavorable prognosis in sepsis patients (32). Furthermore, NEAT1 plays an important role in cardiomyocyte injury and apoptosis associated with miR-140-5p, miR-193a, miR-27b, miR-181b, miR-129-5p, miR-495-3p, miR-125a-5p, and their corresponding downstream regulated genes (33–40). NEAT1 knockdown can improve the outcome of LPS-induced myocardial injuries in mice by upregulating miR-144-3p (41) and downregulating expression of TLR2 and p65 and mRNA levels of inflammatory indicators to

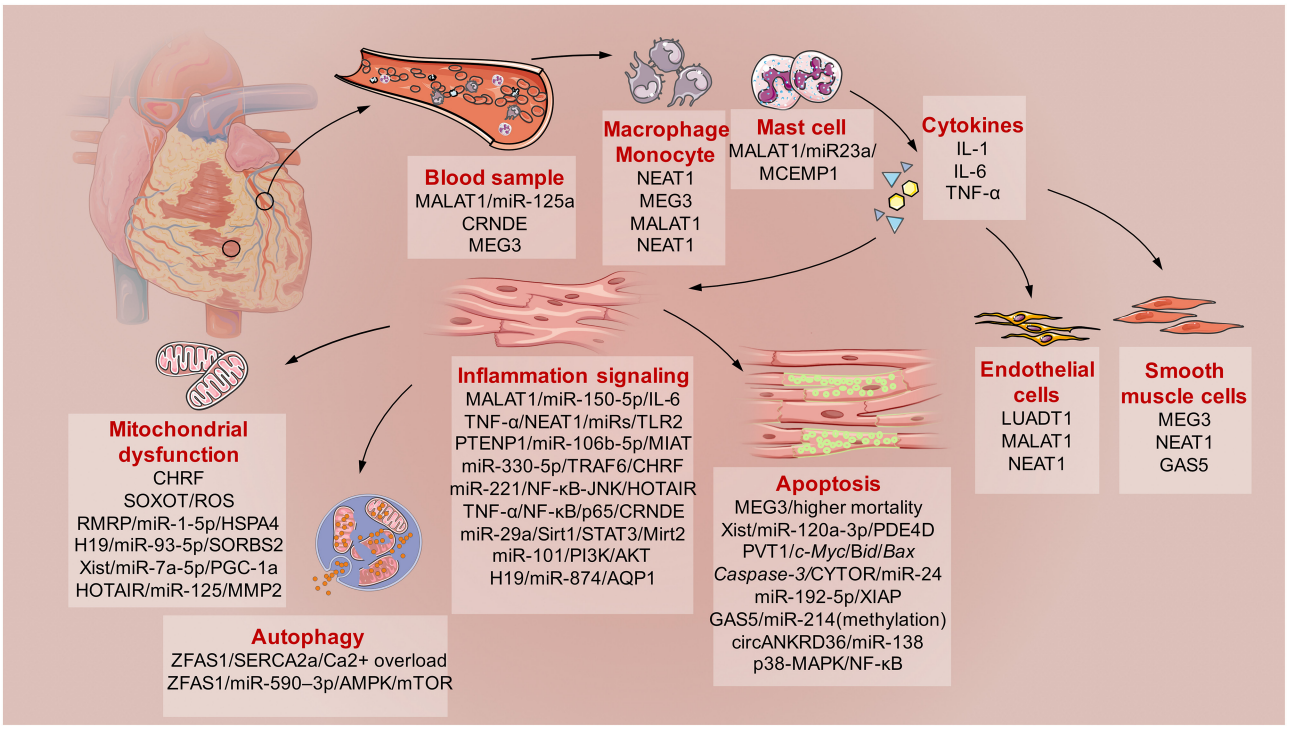

FIGURE 2 | The schematic diagram describes the involved lncRNAs in sepsis induced cardiac dysfunction (SICD). Generally, current evidences demonstrated some lncRNAs served as biomarkers for SCID. Then, monocytes, macrophage, and mast cells would be activated with kinds of cytokines secretion. After that, immune responses of cardiomyocytes would lead to mitochondrial dysfunction, apoptosis and autophagy under the regulation of specific lncRNAs. Besides, lncRNAs also participates in the regulation of endothelial cells and smooth muscle cells during SICD.

inhibit the TLR2/NF-κB signaling pathway (42). Expression of lncRNA PTENP1 was upregulated in sepsis models subjected to LPS administration, while miR-106b-5p expression was downregulated. Matrine administration could attenuate changes in expression of these two ncRNAs, and the cardioprotective effects of matrine were reversed by overexpression of PTENP1 or knockdown of miR-106b-5p (43). The lncRNA MIAT directly binds to miR-330-5p to activate TRAF6/NF-κB signaling axis and further promotes inflammatory response as well as oxidative stress in LPS-induced septic cardiomyopathy (44).

Silencing the lncRNA CHRF protected H9c2 cells against LPS-induced injury via upregulation of miR-221 and modulation of NF-κB and JNK pathways (45). In addition, silencing HOTAIR lncRNA reduced secretion of TNF-α into the circulation by inhibition of NF-κB signaling through dephosphorization of NF-κB p65 subunit, and helped preserve cardiac function in septic mice (18). Moreover, knockdown of circHIPK3 effectively alleviated LPS-induced myocarditis (46).

Beyond the lncRNAs that contribute to triggering inflammation, there is a series of lncRNAs that present a protective value of SICD. LncRNA CRNDE attenuates miRNA-29a to enhance expression of *Sirt1*, which contributes to inhibition of NF-κB and STAT3 inflammation signaling in myocardial tissue under septic attack (47). LncRNA Mirt2 silenced miR-101 and attenuated the myocardial inflammatory response in sepsis rats through the PI3K/AKT signaling pathway, and this improved cardiac remodeling and function (48). However, no human homologs of Mirt1 and Mirt2 have been described to date.

In an *in vitro* model established on cardiomyocytes subjected to LPS, there was a negative relationship between lncRNA H19 and miR-874, and a positive correlation between H19 and Aquaporin 1 (AQP1). H19 could act as AQP1 competing endogenous RNA (ceRNA) by regulating miR-874 and restoring LPS over-activated inflammatory responses and myocardial dysfunction (49, 50).

Mitochondria

Mitochondria are one of the most important organelles of cardiomyocytes, but they are quite sensitive to external and internal stimulations, resulting in mitochondrial dysfunction and leading to metabolic disorder with accumulation of reactive oxygen species (ROS). Mitochondrial dysfunction is associated with DNA damage and apoptosis. In experimental models of sepsis attacks, reduced mitochondrial membrane potential (MMP), elevated mitochondrial cytochrome C, and downregulated ROS scavenging were identified (8). LncRNAs make significant contributions to maintaining cardiac mitochondria homeostasis, hence these studies revealed a critical role of lncRNAs in response to sepsis attack.

Zhang et al. (45) demonstrated that silencing the lncRNA CHRF prevented LPS-triggered mitochondrial apoptosis

TABLE 1 | Direct evidence of lncRNAs in SICD.

LncRNA	Bindings	Downstream factors	Molecular function	Sepsis Models (*in vitro* + *in vivo*)	Outcomes
MALAT1↑	miR-150-5p↓	–	miRNA sponge	H9C2 + LPS	IL-6↑ TNF-α↑ NF-κB signaling pathway↑
↑§	–	SAA3↑	–	HL-1 + LPS Mice + LPS	TNF-α↑
↑	miR-125b↓*	–	–	H9c2 + LPS Rat +CLP	p38 MAPK/ NF-κB↑
NEAT1↓φ	miRNAs of inflammatory indicators	TLR2 and p-p65↓	–	Mice + LPS	Myocardial Pathological ↓ Injury Myocardial Apoptosis↓ Oxidative Stress↓ Inflammation↓ TLR2/NF-κB signaling pathway↓
↓	miR-144-3p↓	p-IκBα and p-p65↓	miRNA sponge	HL-1 + LPS	Myocardial Cell Injury↓ NF-κB Signaling Pathway↓
PTENP1↓	miR-106b-5p↑	–	miRNA sponge	H9c2 + LPS Mice + CLP	Cell viability↑ IL-6↓and TNF-α↓ Inflammation↓
MIAT↑	miR-330-5p↓	TRAF6↑	miRNA sponge	HL-1 cells + LPS Mice + LPS	TRAF6/NF-κB signaling axis↑
CHRF↓	miR-221↑	P65↓	miRNA sponge Protein localization	H9c2 + LPS	Mitochondrial apoptosis↓ Cell viability↓ Apoptosis rate↓ IL-6 and TNF-α↓ NF-κB↓ and JNK pathways↓
circANKRD36z↓	miR-138↑	–	miRNA sponge	H9c2 + LPS	Apoptosis↓ and inflammatory injury↓ p38MAPK/NF-κB↓
HOTAIR↑$	–	–	–	HL-1 + LPS Mice + LPS	TNF-α↑ phosphorylation of NF-κB p65 subunit↑
CRNDE↑§	miR-29a↓	SIRT1↑	miRNA sponge	H9c2 + LPS Rat + LPS	Cardiomyocyte apoptosis↓ Oxidative stress↓ phosphorylated NF-κB p65↓ and Cleaved PARP1↓ NF- κB/PARP1 signaling↓
Mirt2↑	miR-101↓	–	miRNA sponge	Rat + CLP	IL-1β↓, IL-6↓, TNF-α↓, MPO↓ IL-10↑ PI3K/AKT Signaling Pathway↓
rPVT1↓φ	Irak-2↓	c-Myc↓ Myd88↑	Protein stabilization	H9C2 + LPS	Myocardial Depression↑ Cell Apoptosis↑
Xist↓	mir-7a-5p#	PGC-1α↑ Tfam↓	–	Mouse cardiomyocytes MCM cells + LPS	Cardiomyocyte ATP levels↑ Cardiomyocyte apoptosis↓
CYTOR↑	miR-24↓	XIAP↑	miRNA sponge	H9c2 + LPS Mice + LPS	viability↑ Apoptotic↓ TNF-α↓and IL-1β↓ LDH↓
KCNQ1OT1↑	miR-192-5p↓	XIAP↑	miRNA sponge	H9c2 + LPS Rat + LPS	Proliferation↑ Apoptosis↓ TNF-a↓, IL-1b↓, and IL-6↓
CircHIPK3↓	–	–	miRNA sponge?	H9c2 + LPS Mice + CLP	Heart damage markers↓ And myocardial apoptosis↓ Oxidative stress↓ and Inflammation↓
MEG3↓	P53‡	–	–	AC16 + LPS Plasma from sepsis patients	Apoptosis↓
GAS5↑φ	miR-124↓	–	miRNA Methylation	AC16+LPS	Apoptosis↓
H19↓	miR-93-5p↑	SORBS2↓	miRNA sponge	H9C2 + LPS Sepsis patients	Cell growth inhibition↑ Mitochondrial damage↑

(Continued)

TABLE 1 | Continued

LncRNA	Bindings	Downstream factors	Molecular function	Sepsis Models (*in vitro* + *in vivo*)	Outcomes
↓	miR-874↑	AQP1↓	miRNA sponge	UL-1 + LPS Serum from peripheral blood samples of sepsis patients	TNF-α, IL-6, and IL-1β↑
CHRF↓	miR-221↑	P65↓	miRNA sponge Protein localization	H9c2 + LPS	Mitochondrial apoptosis↓cell viability↓apoptosis rate↓ IL-6 and TNF-α↓ NF-κB↓ and JNK pathways↓
RMRP↑	miR-1-5p↓	HSPA4↑	miRNA sponge	HL-1 + LPS Mice + LPS	Apoptosis↓ MMP↑ Mitochondrial damage↓
SOX2OT↓$	SOX2↑	–	Transcriptional suppression	H9c2 + LPS Mice + LPS	MMP↑ Mitochondrial reactive oxygen species↓ Mitochondrial dysfunction↓
ZSAF1↓$	miR-590–3p↑	–	Base paring	Mice + CLP	Pyroptosis↓ Autophagy↑ AMPK/mTOR signaling↓
MALAT1↓^	EZH2	EZH2	Histone modification	CMVECs isolated from rats + LPS Rat +CLP	CMVEC cell hyperpermeability and apoptosis ↓
LUAD1↑	miR-195†	Pim-1↑	Base paring	Plasma from sepsis patients HCAECs	Apoptosis of HCAECs↓

Downstream factors included proteins which are reported to be directly modulated by lncRNAs or their binding molecules and gene locus.
Rising arrow or a falling arrows of lncRNAs depend on the regulation of included studies, not on their expression change after sepsis. Direction of arrow of downstream factors and outcome relies on direction of arrows of lncRNAs.
**MiR-125b was proved to modulate MALAT1 as a upstream regulator.*
^MALAT1 was downregulated by ulinastatin.
& PVT1 upregulates Myd88 by protein stabilization but it's unknown how PVT1 downregulate c-Myc.
 # Database analyses found that Xist has a binding site of miR-7a-5p, but there is no direct modulatory relationship between these two non-coding RNAs.
$ Evidences of studies were acquired based on transgenic mouse.
§ Researchers of included studies screened lncRNAs by microarray.
φ Researchers of included studies screened lncRNAs by RNA-sequencing.
† LUADT1 and miR-195 demonstrate strong base paring between each other, but overexpression of LUADT1 and miR-195 did not significantly alter the expression of each other.
‡ lncRNA MEG3 may interact with p53 to regulate cancer cell apoptosis and it may be involved in the pathogenesis of sepsis by a similar mechanism.
? Included study did not mention mechanism of this lncRNA, but other study reported its mechanism.

and inflammation of cardiomyocytes. LncRNA SOX2 overlapping transcript (SOX2OT) is a proven mitochondrial damage factor in sepsis and contributes to mitochondrial dysfunction progression by inhibiting SOX2 expression in septic cardiomyopathy. Knockdown of SOX2OT could restore the MMP, along with reduction of ROS production induced by LPS, while overexpression of SOX2OT enhanced mitochondrial damage (51).

LncRNA RMRP acts as a sponge for miR-1-5p and provides a protective effect to mitochondria via the RMRP-miR-1-5p-HSPA4 network, which is known to play crucial roles in inflammation (8). LncRNA H19 and SORBS2 (Sorbin and SH3 domain-containing protein 2) were downregulated in H9C2 cells following administration of LPS, and miR-93-5p was simultaneously upregulated. LncRNA Xist is instrumental in X-chromosome inactivation and inhibits apoptosis in acute myocardial infarction (MI) (52). Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and adenosine triphosphate (ATP) expression was markedly reduced in sepsis leading to mitochondrial dysfunction, but mitochondrial function was restored after the inhibition of Xist and mir-7a-5p, which reduced apoptosis in response to LPS (53). Inhibition of lnc-HOTAIR aggravates oxidative stress-induced damage of H9c2 cells through the HOTAIR/miR-125/MMP2 axis (54).

LncRNA H19 is an important regulator of mammalian development and disease in that it inhibits cell proliferation (55). H19 is normally highly expressed during *in utero* development and then downregulated at birth (56), while re-expression occurs in some cardiovascular disease settings (57–59). In accordance with its inhibition function of cell proliferation, H19 was proved as precursor of miR-675, which inhibits cardiomyocyte hypertrophy and contributes to cardiac fibroblast proliferation and fibrosis through repression of DUSP5/ERK1/2 (60). Furthermore, H19 is involved in myocardial ischemic preconditioning via increasing the stability of nucleolin protein, which mitigates the damage caused by MI (61). Human GWASs demonstrated significant associations between the H19 locus and systolic or mean arterial blood pressure (9). LPS-induced cell growth inhibition and mitochondrial damage was significantly

reversed by overexpression of H19, which sponged miR-93-5p to promote SORBS2 expression (62).

Apoptosis

Cardiomyocyte apoptosis, which is a key parameter for SICD and leads to long-term myocardial dysfunction, has been proposed to occur as a result of a sequence of cellular damages (63). Several signaling pathways are involved in apoptosis regulation via nuclear and mitochondrial approaches. However, crosstalk between lncRNAs and signaling pathways has been identified, and several lncRNAs regulate the process of apoptosis.

Overexpression of lncRNA MEG3 is associated with high mortality rates in patients with sepsis, thus is indicative of poor clinical outcomes and is believed to be associated with LPS-induced renal epithelial cell and cardiomyocyte apoptosis (64). LncRNA Xist promoted apoptosis of cardiomyocytes and inhibited proliferation of these cells by downregulating miR-130a-3p and upregulating *PDE4D*, which is a direct target of miR-130a-3p (52).

LncRNA PVT1 also showed significant upregulation and a vital functional role in maintaining the myocardial contractile function in rat models of hypodynamic septic shock induced by LPS. Knockdown of PVT1 induced cell apoptosis in LPS-induced cardiomyocytes through increasing the expression of *c-Myc*, *Bid*, *Bax*, and *Caspase-3* and decreasing expression of *Myd88* and *Bcl-2* (23). LncRNA CYTOR was markedly downregulated during sepsis. This lncRNA negatively regulated expression of miR-24 and apoptosis-related proteins that were regulated by miR-24. MiR-24 directly targeted the 3′UTR of X-chromosome-linked inhibitor of apoptosis (XIAP) and suppressed its expression. Downregulation of CYTOR aggravated sepsis-induced cardiac injury via regulation of miR-24/XIAP (65). The lncRNA KCNQ1OT1 is similar in mechanism to CYTOR. It was considerably downregulated in myocardial tissues of septic rats, whereas miR-192-5p was increased in these tissues. CYTOR regulates XIAP through miR-192-5p, which pairs with the 3′UTR of XIAP, and represses its protein translation. These findings show that downregulation of KCNQ1OT1 aggravates cardiac injury through the miR-192-5p/XIAP axis during sepsis (66). LncRNA GAS5 may upregulate miR-214 through a methylation pathway to inhibit cardiomyocyte apoptosis in sepsis (67).

Fan et al. (46) demonstrated that circHIPK3 expression was significantly upregulated when exposed to LPS *in vivo* and *in vitro*, and that knockdown of circHIPK3 effectively alleviated LPS-induced myocarditis by attenuating inflammation-induced apoptosis of cardiomyocytes. Furthermore, silencing circANKRD36 exerted an anti-inflammatory and anti-apoptosis function in LPS-exposed H9c2 cells via the p38-MAPK/NF-κB pathway and upregulation of miR-138 (68). Another study confirmed an association between circANKRD36 and miR-15/MyD in regulating apoptosis due to inflammation damage (69).

Autophagy

Autophagy is an important biological process for regulating cellular homeostasis. However, there is currently limited data demonstrating the involvement of lncRNAs in regulating cardiomyocyte autophagy. One study revealed that lncRNA ZFAS1 was an endogenous SERCA2a inhibitor and induces mitochondria-mediated apoptosis via cytosolic Ca2+ overload (70). ZFAS1 is activated by the transcription factor SP1 and aggravates the progression of sepsis-induced cardiac dysfunction via miR-590–3p/AMPK/mTOR signaling-mediated autophagy and pyroptosis of cardiomyocytes (71, 72).

Immune Cells

During sepsis, the immune system is the frontier responding to harmful stimulations, and monocytes, macrophages, and neutrophils all make significant contributions to targeting organ damage. The innate immune response induces strong activation of the cytokine system, which has plethoric effects on various organs and the vasculature, leading to changes in vascular permeability, endothelial function, and activation of further mediators such as bradykinin, histamine, and the complement and coagulation systems.

The lnc-MALAT1/miR-125a axis presents excellent value in differentiating sepsis patients from healthy controls using peripheral blood samples (73). In another study using clinical blood samples, lnc-CRNDE was found to trigger inflammation through the TLR3-NF-κB-cytokine signaling pathway and the downstream release of inflammatory cytokines (74). As a major protein related to innate immune and inflammatory responses, TLR3 is known to cause cardiac dysfunction and other organ damage during sepsis (74, 75). Low expression of lnc-MEG3 might also serve as a potential biomarker for the development, progression, and prognosis prediction of sepsis (76–78). Furthermore, overexpression of MEG3 prevented LPS-induced macrophage apoptosis and secretion of inflammatory factors by inhibiting activation of the NF-κB signaling pathway (77).

Lnc-MALAT1 plays multiple roles in inflammatory stimulation in the macrophage cell line RAW264.7 (79). This lncRNA could inhibit the proliferation of LPS-stimulated RAW264.7 cells by inducing SMAD3 expression via downregulation of hsa-miR-346 (79). Lnc-MALAT1 also promotes inflammation in septic mice by binding to miR-23a to upregulate mast cell-expressed membrane protein 1 (MCEMP1) (80).

High expression of NEAT1 in peripheral blood mononuclear cells (PBMCs) can be considered as an additive marker for the diagnosis of sepsis (81), while another study confirmed that monocyte-enriched NEAT1 was suppressed in post-MI patients (82). Data from experiments with NEAT1-knockout (NEAT1-KO) mice identified NEAT1 as a novel lncRNA-type immunoregulator affecting monocyte-macrophage functions and T cell differentiation. NEAT1-KO marrow-derived macrophages (BMDMs) responded to LPS with increased ROS production and disturbed phagocytic activity (82).

Endothelial Cells

Cardiomyocytes are the dominant type of cells in the heart. However, various cell types comprise functional heart tissue. Endothelial cells contribute to form microvascular circulation in myocardia. Endothelial cell dysfunction impairs the micro-circulation function, inducing ischemic cardiac lesions. In

sepsis attacks, endothelial cells are also major targeted sites. However, few studies have drawn correlations between lncRNAs and endothelial cell damage. The lncRNA LUADT1 was downregulated in patients with sepsis and in cultured human primary coronary artery endothelial cells (HCAECs) exposed to LPS. Overexpression of LUADT1 upregulated the expression of *PIM1*, a target of miR-195. These findings indicated that overexpression of either LUADT1 or *PIM1* would reduce the damage effects of miR-195 on LPS-induced apoptosis of cardiac endothelial cells (83). Yu et al. (84) demonstrated that the drug Ulinastatin protected against LPS-induced cell hyperpermeability and apoptosis of cardiac microvascular endothelial cell (CMVECs) via downregulation of lncRNA MALAT1 and EZH2. Moreover, Liu et al. (85) reported that miR-150 could induce sepsis-induced endothelial injury by regulating endoplasmic reticulum (ER) stress and inflammation via the MALAT1-mediated NF-κB pathway. Lnc-NEAT1 also participates in the viability and survival of coronary endothelial cells (86, 87).

Smooth Muscle Cells

Smooth muscle cells also significantly contribute to maintenance of coronary vessel circulation. However, smooth muscle cells were the targets of inflammation damage due to sepsis attacks. Ahmed et al. (88) demonstrated a role of NEAT1 in regulating phenotypic switching by repressing smooth muscle-contractile gene expression through an epigenetic regulatory mechanism. Silencing lnc-NEAT1 in vascular smooth muscle cells (VSMCs) enhanced expression of smooth muscle-specific genes while attenuating proliferation and migration of the VSMCs. The lncRNA MEG3 could modulate the balance of proliferation/apoptosis in VSMCs by regulating the miR-26a/SMAD1 axis (89). In addition, the lncRNA GAS5 exacerbates hypertensive arterial remodeling by regulating VSMC phenotypic conversion, which leads to microvascular dysfunction (90). However, there is lacking convinced evidence of GAS5 on SICD.

PREDICTED LNCRNAS BASED ON AVAILABLE EVIDENCE

In addition to the above-mentioned lncRNAs with direct evidence in SICD, some other lncRNAs were reported to be involved both in sepsis and some types of CVD by other mechanisms. In view of the molecular functions of lncRNAs in regulating cardiomyocyte homeostasis and their expression during sepsis but without convincing evidence presented in a single study focusing on SICD, the most reported lncRNAs and associated mechanisms are summarized in this review to demonstrate their comprehensive impacts. **Table 2** lists the lncRNAs that we predicted might play a role in SICD although no direct evidence is available from biological experiments or clinical trials. These lncRNAs were found to express differentially or function in sepsis and participate in CVD or other cardiac psychopathological processes in individual studies.

LncRNAs That Present Similar Functions in Sepsis and CVD

The mechanisms of lncRNAs in regulating downstream signaling is complicated, although research on ncRNAs is growing. However, direct evidence of lncRNAs on SICD remain limited. Moreover, sepsis is considered as a type of syndrome that damages various organs. Hence, research on sepsis includes investigations on various damages beyond cardiac dysfunction, such as lung injuries, kidney disorders, and other damages. Based on this predicament, we selected to review the lncRNAs involved in sepsis without evidence based on SICD but which had been confirmed as having similar protective or adverse roles in other types of CVD. The lncRNAs outlined in this part of the review are highly likely to have their capabilities proven in future SICD studies.

ANRIL

Several studies demonstrated that the lnc-ANRIL/miR-125a axis could serve as a predictor for prognosis, severity, and inflammation among sepsis patients (91–93). LncRNA ANRIL is the prime candidate gene at Chr9p21 and widely recognized as a critical part of endothelial inflammation and cell proliferation (91, 94–97). Single nucleotide polymorphisms (SNPs) and splice variants of ANRIL were reported to regulate endothelial cell activities involved in coronary artery heart disease (CAD) and MI (98–103). Abnormal expression of ANRIL is associated with vascular endothelium injury and proliferation, migration, and apoptosis of VSMCs; which also contribute to mononuclear cell adhesion and proliferation (104, 105). ANRIL knockdown induced cardiomyocyte apoptosis in acute MI by regulating IL-33/ST2 or Akt (106, 107). Enhanced expression of ANRIL and suppressed expression of miR-181b, which was inhibited by ANRIL, were recorded in CAD populations and confirmed ANRIL as an independent risk factor (108).

DC

Lnc-DC, also known as whey acidic protein/four-disulfide core domain 21 (Wfdc21), was reported to be correlated with immune responses. Knockdown of lnc-DC downregulated expression of pro-inflammatory factors, such as IL-1β and TNF-α, in LPS-treated macrophages through the STAT3/TLR4 signaling pathway (109). Alikhah et al. (110) found significant correlations between expression of lnc-DC with SOCS1 and STAT3 in CAD patients.

THRIL

LncRNA THRIL is upregulated during sepsis and may serve as a sponge of miR-19a to upregulate TNF-α (111). This lncRNA is considered to play important roles in the innate immune response and inflammatory diseases in humans (112). THRIL mediates autophagy of endothelial progenitor cells via the AKT pathway and FUS (113). Knockdown of THRIL protected H9C2 cells against hypoxia-induced injuries by regulating miR-99a (114). This mechanism was further demonstrated by Sheng et al. (115) with the observation that Geniposide alleviated hypoxia-induced injury through downregulation of THRIL in H9c2 cells.

TABLE 2 | Summary of potential lncRNAs in SICD based on available evidence.

LncRNA	Disease	Expression	Samples or tissue/cell source	Downstream factors	Molecular function	Function
ANRIL	Sepsis	Up	Plasma from patients of sepsis	miR-125a↓	–	Biomarker of severity, inflammation, and prognosis
	AMI	Up	Mice myocardial tissue HL-1	Deubiquitinase USP17 IL-33 ST2	–	Apoptosis↑
	MI	Up	Ischemic hearts HUVECs	Akt phosphorylation↑	–	Cell migrations↑ and Tubulogenesis↑ Ischemia-induced Angiogenesis↑
	Inflammation-relevant CAD	Up	CAD patients HCAECs HUVECs CAD mice	miR-181b↓ EMT-specific Proteins	–	Inflammatory factors↓ and Vascular-protective factors↓
UCA1	Sepsis	Up	HMECs	–	–	Pre-inflammatory mediators↑
		Up	WI-38 cells	miR-499b-5p↓ TLR4↓	decoy	Inflammatory injury ↑apoptosis↑
	I/R§	Up	H9C2 cells	–	–	ER stress↓ and Cell apoptosis↓ Mitochondria Dysfunction↓ and Oxidative stress↓
Lnc-DC	Sepsis	Up	kidneys and liver	Stat3↑ Toll-Like Receptor 4↑	–	Pro-inflammatory factors↑
	CAD	Up	PBMCs	STAT3↑	–	JAK/STAT pathway↑
THRIL	Sepsis	Up	Blood extraction from sepsis patients HBEpCs	miR-19a↓	miRNA sponge	TNF-α↑
	CAD	Up	CAD blood samples EPC	FUS	Protein binding	Cell viability↓ cell autophagy↑ Cell proliferation↓ AKT pathway↑
	MI	Up	H9C2	miR-99a↓ Brg1↓	miRNA sponge	Cell injuries↑ PI3K/AKT and mTOR Signaling pathways↓
HULC	Sepsis	Up	HMECs	–	–	Pre-inflammatory mediators↑
	TNF-α↑	Down	HUVECs	miR-9↓	DNA methyltransferases	Apoptosis↓
	I/Rφ	Down	Rat myocardial tissue H9C2	miR- 377-5p↓	miRNA sponge	Cardiomyocyte apoptosis↓
Lnc-P21	Sepsis*	Up	–	–	–	Macrophage activation Septic shock susceptibility autophagy Cardiomyocyte adherens junctions
	CAD	Down	HA-VSMC RAW264.7 Carotid arteries	MDM2↑	Enhancer	Cell proliferation↓ Apoptosis↑ Neointima Formation↓
TUG1	Sepsis	Down	Serum samples from urosepsis patients RMC	miR-142-3p↓ sirtuin 1↑	miRNA sponge	Cell viability↑ Apoptosis↓ Cytokines production↓ Autophagy↓
	Hypertension	Up	Rat isolated VSMCs	miR-145-5p↓ FGF10↑	miRNA sponge	Proliferation↑ Migration of VSMCs↑
	Atherosclerosis	Up	RAW264.7 MOVAS Mice aorta and aortic sinuses	miR-133a ↓ FGF1↑	miRNA sponge	Cell growth↑ Inflammation↑ Apoptosis↓

(Continued)

TABLE 2 | Continued

LncRNA	Disease	Expression	Samples or tissue/cell source	Downstream factors	Molecular function	Function
SNHG16	Sepsis§	Down	Blood sample from sepsis or respiratory infection/pneumonia RAW264.7	miR-15a/16↓ TLR4↑	binding miRNAs	Inflammatory pathway↓
	CAD	Up	Peripheral blood from sepsis patients HCASMC	miR-218-5p↓	Decoy	Proliferation and migration of HCASMC cells↑ Apoptosis↓
	Cardiac hypertrophy	Up	H9c2	miR-182-5p↓ IGF1↑	miRNA sponge	Cardiac hypertrophy↑
aHIF	Sepsis*	–	–	–	–	Expression Profiling Golgi stress Acute lung injury
	End-stage heart failure	Up	Human heart tissues	HIF↓	Antisense transcript	–
	MI	Up	Peripheral blood cells	–	–	–

** These lncRNAs are involved in pathophysiologic process of sepsis or CVD but there is no direct evidence involved in SICD.*

§ Researchers of included studies screened lncRNAs by microarray.

φ Researchers of included studies screened lncRNAs by RNA-sequencing.

The direction of arrow of downstream factors indicates function of lncRNAs, not their change in status of diseases.

The direction of arrow of function indicates outcome of restored or upregulated lncRNAs.

In addition, THRIL was increased in CAD patients and proved as a biomarker to evaluate CAD risk (116).

SNHG16

The lncRNA SNHG16 can act as a ceRNA to downregulate the miR-15a/16 cluster, reducing LPS-induced inflammatory signaling (117). SNHG16 also helps regulate miR-218-5p and promotes the proliferation and migration of coronary artery VSMCs via the Wnt/β-catenin pathway, protecting against the injuries from MI (118). Furthermore, silencing of SNHG16 repressed Ang II-imposed cardiac hypertrophy by targeting the miR-182-5p/IGF1 axis (119).

lncRNAs That Present Opposite Roles in Sepsis and CVD

Selection of potential lncRNAs involved in SICD is difficult, even with meticulous attention. Some findings from different individual studies demonstrated opposing functions of lncRNAs between sepsis and CVD, either in a protective or adverse direction. However, it is possible that there may be a shared intermediate target. Here, such lncRNAs are briefly described, but further analysis is required in relation to these lncRNAs and SICD. Moreover, the long-term effects of lncRNAs on CVD also lacks convincing data and this is another area that requires further research.

UCA1

Upregulation of lncRNA UCA1 is necessary for the response of pro-inflammatory immune cells during LPS-induced sepsis (120, 121). However, UCA1 inhibits ischemia/reperfusion (I/R)-induced oxidative stress and mitochondria dysfunction via suppression of ER stress (122).

HULC

HULC could induce pro-inflammatory mediators in response to LPS exposure in endothelial cells (120). Overexpression of HULC in HUVECs promoted angiogenesis by increasing cell viability, proliferation, and tube-like structure formation through downregulation of miR-29b (123). HULC also participated in TNF-α- (124) and I/R- (125) induced cardiomyocyte apoptosis through regulation of miR-9 and miR-377-5p expression.

P21

LncRNA-p21 serves as a repressor in p53-dependent transcriptional responses (126). This lncRNA regulates neointima formation, VSMC apoptosis, and atherosclerosis by enhancing p53 activity (127). Expression of lncRNA-p21 was significantly increased in a septic model and it predominantly functioned in *cis* to activate expression of p21, its neighboring gene (128). P21 itself is involved in regulation of macrophage activation, septic shock susceptibility (129), autophagy in LPS-induced cardiac dysfunction (130), and cardiomyocyte adheres junctions in endotoxemia (131).

TUG1

The lncRNA TUG1 promoted proliferation and migration of VSMCs in the hypertensive state by activating the miR-145-5p/FGF10 axis and the Wnt/β-catenin pathway to aggregate vascular remodeling (132). Another study reported that knockdown of TUG1 ameliorated atherosclerosis via upregulation of miR-133a expression following its target gene FGF1 (133). TUG1 expression was also reported to help alleviate acute lung injuries by targeting miR-34b-5p/GAB1 (134).

Another Strategy in Searching for lncRNAs

It is theoretically possible to regulate typical molecules of signal pathways by interfering with their corresponding lncRNAs. However, in terms of the extensive functions of those pathways, this train of thought is a low priority. For example, lncRNA HIFa-AS is a natural antisense transcript of Hypoxia-inducible factor 1-α (HIF1α) and is overexpressed in the failing heart. HIFa-AS destabilizes the mRNA producing HIF1α, which regulates transcription of cellular responses to hypoxia, especially in post-ischemic angiogenesis (135). HIFa-AS was also discovered to play a role in MI (98). Huang et al. reported that lncRNAs upregulated in sepsis were significantly enriched in the HIF-1 signaling pathway via KEGG analyses (26), and two studies found that HIF-1α participated in acute lung injury after sepsis (136, 137). Nevertheless, there are no studies reporting the role of lncRNA HIFa-AS in sepsis.

lncRNAs AS BIOMARKERS AND THERAPEUTIC TARGETS

SICD is more like a functional disorder than a biochemical phenomenon. There is uncertainty as to whether SICD itself is pathogenic or is simply a reflection of the severity of the underlying disease process of sepsis. Diagnosis of SICD largely relies on ultrasonography imaging and troponin measurement. Increasing ultrasonic measurement indicators of left ventricular systolic and diastolic performances, and right ventricular dysfunction are applied to clinical practice and scientific research (4). Similar to troponin, the elevation of hormones B-type natriuretic peptide (BNP) and N-terminal pro-BNP (NT-proBNP) are determined mainly by the severity of sepsis other than specific abnormalities in cardiac function (138). To date, no ultrasonic prognostication has been demonstrated in patients with septic cardiomyopathy (139–142). Apart from lacking reliable biomarkers, the degree to which cardiac dysfunction represents cardiac structure damage and heart failure instead of a protective hibernation-type mechanism remain difficult to resolve (143). Current therapy for sepsis is predominantly focused on restoring cardiac output by inotropic agents and fluid resuscitation. The Surviving Sepsis Campaign guidelines recommend inotropic therapy in patients with persistent hypoperfusion despite adequate fluid loading (144). Limited and underperforming inotropic agent options, including dobutamine, catecholamines, and levosimendan, also contribute to SICD-related deaths (145–147). Therefore, novel biomarkers and therapeutic targets are urgently needed to improve the diagnosis and treatment of SICD.

LncRNAs as Predictive Biomarkers

As previously discussed, numerous lncRNAs are aberrantly expressed in SICD compared with normal cardiac tissue or cell lines, and this is useful to distinguish SICD patients from healthy cohorts. Although nearly all lncRNAs with direct evidence of their involvement in SICD were declared as potential biomarkers of SICD, those lncRNAs also show aberrant expression patterns in sepsis without cardiac dysfunction, especially critical patients, and in other non-sepsis situations such as MI, I/R, and acute kidney injury (21, 22, 24, 26, 110, 114, 148). This reduces the reliability of using these lncRNAs as potential biomarkers of SICD. To date, there is no study reporting the sensitivity and specificity of the diagnostic efficiency of these lncRNAs.

Compared with myocardial biopsy, blood sampling is largely non-invasive and thus is an ideal diagnostic approach. Several SICD-related lncRNAs can be present in the blood, as are the aforementioned cases. However, the circulating lncRNA differential expression profile is heterogeneous among different studies, partly due to severity, genetic background, and the pathogenic microorganism involved (149). One challenge with the clinical application of these lncRNAs is how to develop a convenient and rapid technique to detect the target lncRNAs in sepsis and thus bring the advantage of being less time-consuming than microbial culture into full play.

LncRNAs as Therapeutic Targets

To date, many studies have confirmed that lncRNAs are essential contributors to SICD progression due to the diversity of actions and cellular processes implicated. However, few practical examples of therapeutic applications of lncRNAs have been reported. The prognosis of SICD in general is poor, and this is in part due to the lack of therapeutic targets. The critical roles of lncRNAs in SICD make them promising targets for novel therapeutic interventions, and the base-pairing principle is much more straightforward than designing a specific protein-binding inhibitor. Multiple different approaches can be used in perturbing specific lncRNAs, including RNA interference (RNAi), antisense oligonucleotides (ASOs), clustered regularly interspaced short palindromic repeats (CRISPR)/Cas, CRISPR-Display, and the λN –Gal4 system.

Some lncRNAs, which regulate transcriptional outputs in *cis*, do not function in exogenous overexpression studies. Therefore, the λN –Gal4 system has been used to overcome this constraint by enhancing the overexpression of lncRNA in *cis* (150, 151). As duplex RNAs that have to be loaded into AGO2 protein to form an RNA-induced silencing complex (RISC) and interact with target lncRNA, RNAi is a reliable approach for targeting lncRNAs in the cytoplasm and inhibiting gene expression (152). Compared with RNAi, ASOs, as single-stranded DNAs, are more reliable gene silencing agents than duplex RNAs for the RNAs that are localized to cell nuclei (153, 154). ASOs with appropriate modifications have become readily available (155) and newer generation ASOs allow spatial control of target delivery (156). However, unlike RNAi (42, 53), no study to date has reported the application of ASOs in SICD. With higher efficiency, specificity, and the ability to modulate gene expression (157), the CRISPR/Cas method has dominated in recent years (158, 159) and CRISPR Display, which allows the insertion of RNA domains into DNA loci, was specially developed to modulate the expression of lncRNAs (160). Except for cell and animal models, ASOs and RNAi have already been applied in clinical trials for treatment of HBV (161).

CONCLUSION

Sepsis-induced cardiac dysfunction is challenged by a lack of uniformity in its definition of incidence, prognosis, and clinical importance. Two other core problems are whether cardiac dysfunction definitely contributes to poor outcome or prognosis, or is simply a reflection of organ failure in general, and the degree to which sepsis-induced cardiac dysfunction is adaptive or pathological (4). Construction of an ideal SICD animal model is difficult and existing research on this condition has only utilized sepsis models to investigate cardiac dysfunction, which partly accounts for the ambiguous mechanisms of SICD. The development and widespread use of GWAS and RNA-Seq has facilitated more discoveries and deeper understanding of lncRNAs, which in turn has helped exploration of SICD regulatory circuits and molecular mechanisms to make a comprehensive and clear definition of this condition, rather than it simply being based on observation of clinical patients.

Several limitations and challenges need to be solved before lncRNAs can reach clinical application. A primary concern is how to specifically target certain tissues or cell populations. As previously mentioned, nearly all identified lncRNAs in SICD or sepsis exhibit functions in other organisms or display multiple mechanisms of action. Second, it is well-established that unlike protein-coding genes, the majority of human long non-coding RNAs (lncRNAs) are considered non-conserved, suggesting variable evolutionary pressure between mRNA and lncRNAs (162). LncRNA conservation includes four dimensions: the sequence, structure, function, and expression from syntenic loci (163). However, several lncRNAs, such as HOTAIR (164) and Xist (165), exhibit clear functional roles in various mammalian species with poor sequence conservation (166). This phenomenon may be due to conserved secondary structures that do not alter with mutations in the sequence outside of structural regions (167, 168). Lnc-H19 and MALAT1 has been proved to be promising targets for cancer therapy (169, 170). Most of published studies of homolog lncRNAs were related to cancers, and now, more than forty clinical trials associated with lncRNA, including a study of lnc-NBR2 in sepsis, are in process in clinicaltrials.gov. Moreover, owing to unique secondary structure, circRNAs resist degradation by exoribonucleases, resulting in more abundant expression. Long-read sequencing technologies promise to improve current annotations and provide a novel perspective to locate homologs in human (171). However, secondary structures are more difficult to intervene in by conventional means than sequence mutation based on existing knowledge and technology. This leads to difficulties in constructing lncRNA knockout animal models. In addition, Joung et al. (172) reported that ∼50% of lncRNAs influence the expression of neighboring protein-coding genes and many lncRNAs overlap with protein-coding genes, making it difficult to specifically knockout a lncRNA without affecting neighboring genes. RNA modification, especially m6A modification, also influences lncRNA function, for example in the case of m6A of Xist (173). Furthermore, the finding that micropeptides are encoded by lncRNAs (174) means research on lncRNAs has become more complicated and confusing. Overall, lncRNA of SICD is a promising field and remains largely undiscovered.

AUTHOR CONTRIBUTIONS

DZ and YL conceived the presented idea. JL, YZ, and YL summarized the reference and drafted the manuscript. JL drafted the table. DZ organized the figure with online free material. DZ and YL supervised the project and contributed equally to the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Circle the Cardiac Remodeling With circRNAs

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Cardiac remodeling occurs after the heart is exposed to stress, which is manifested by pathological processes such as cardiomyocyte hypertrophy and apoptosis, dendritic cells activation and cytokine secretion, proliferation and activation of fibroblasts, and finally leads to heart failure. Circular RNAs (circRNAs) are recently recognized as a specific type of non-coding RNAs that are expressed in different species, in different stages of development, and in different pathological conditions. Growing evidences have implicated that circRNAs play important regulatory roles in the pathogenesis of a variety of cardiovascular diseases. In this review, we summarize the biological origin, characteristics, functional classification of circRNAs and their regulatory functions in cardiomyocytes, endothelial cells, fibroblasts, immune cells, and exosomes in the pathogenesis of cardiac remodeling.

Keywords: cardiac remodeling, non-coding RNA, circular RNA, heart disease, gene regulation

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INTRODUCTION

The heart is made up of a variety of cells, including cardiomyocytes and non-cardiomyocytes (fibroblasts, smooth muscle cells, endothelial cells, and immune cells etc.,). These cells communicate with each other in both physiological and pathological conditions through direct cell-cell interaction and paracrine signaling. Fibroblasts, the major component of connective tissue, produce the extracellular matrix (ECM) scaffold that organizes different cellular components of the heart (1). Endothelial cells (ECs) are located on the inner surface of blood vessels and lymphatics, controlling vasomotor tension and regulating angiogenesis (2, 3). Resident and recruited immune cells regulate cardiac microenvironmental homeostasis and inflammation during maladaptive remodeling (4). Pathological conditions, such as hypertension or myocardial infarction (MI), induce maladaptive reactions in cardiomyocytes and non-cardiomyocytes, leading to the deterioration of cardiac function and eventually heart failure. However, molecular and cellular mechanisms of cardiac remodeling have not been fully understood.

Other than linear splicing, the sequence of primary transcripts from gene loci is also found to be processed by back-splicing to generate circular RNAs (circRNAs). CircRNAs can be classified as reverse spliced exons (5–7) or intron-derived RNAs (8, 9). Back-splicing is generally thought as a rare event, although, mammalian circRNAs have been reported decades ago (7). The recent deep sequencing data showed evidences that an unexpectedly large number of circRNAs are in fact expressed (5, 6). And thousands of circRNAs have been identified from different cells and tissues (10–13). More importantly, emerging evidences indicated that circRNAs regulate different cellular behaviors, including proliferation, differentiation, apoptosis, and migration (12, 14).

Early report demonstrated the altered expression of circRNAs in human failing heart (15), indicating the participation of circRNAs in the regulation of the pathogenesis of cardiac diseases. Recent studies have further shown that circRNAs are involved in a variety of cardiovascular diseases, including cardiac remodeling, by regulating the pathophysiology of cardiomyocytes, fibroblasts, endothelial cells, and immune cells (16–19). However, the underlying mechanisms of circRNAs' regulatory functions are not fully understood. A more comprehensive understanding of circRNAs will promote the development of circRNA-based diagnosis and therapeutic interventions in cardiovascular disease. In this review, we will focus on the nature of circRNA and how these circular molecules regulate the pathogenesis of cardiac remodeling.

THE IDENTIFICATION, PROCESSING, AND CHARACTERIZATION OF CIRCRNAS

Identification of circRNA

In 1976, Sanger used the term "circRNA" for the first time to describe viroids, which was his identification of a single stranded, covalently closed RNA molecules that are infectious (20). Early detection of circRNA was rare and its function was poorly understood. They were believed as by-products of linear RNA and thought as "junk RNA" (21). Three decades ago, circRNAs were accidentally discovered in mammals (22, 23). More and more circRNAs have recently been identified by taking the advantage of the breakthrough of high-throughput sequencing, and the biological functions of these emerging molecules have been investigated and uncovered rapidly (6). circRNAs are currently considered as a special type of non-coding RNAs, although, some studies have suggested that circRNAs may have protein-coding capability *in vivo*.

General Characteristics of circRNAs

Most circRNAs are exonic and have some important characteristics: (I) CircRNAs are expressed in large quantities in many species, from plants to mammals (5). Multiple circRNA isoforms are often processed from a single host gene by selective splicing. Notably, more than 100 circRNA isoforms of the Ryanodine receptor 2 (RyR2) gene are expressed in human hearts (24). (II) CircRNAs are usually expressed in a cell type- and/or developmental stage-specific manner (6, 25, 26). The expression profiles of circRNAs are different in four stages of cardiac differentiation: undifferentiated stage, mesoderm stage, cardiac progenitor cell stage, and final cardiomyocyte stage (26). (III) CircRNAs are not easily degraded by RNA exonuclease because of their covalently closed circular structure. CircRNAs are more stable and have a longer half-lives than linear RNAs (27, 28). These features of circRNAs make these molecules potential candidates for disease diagnosis and prognosis biomarkers, especially the presence of circRNAs in plasma.

Categorization of circRNA

CircRNAs can be divided into three subtypes according to the mode of biogenesis: circRNA, Exon-intron circRNAs (EIciRNA), and ciRNAs. Most circRNAs are derived from exons in linear transcripts, lacking introns, and mainly present in the cytoplasm. In contrast, ciRNAs lack exon sequences, are present in the nucleus and have no obvious enrichment of miRNA binding sites (8, 29). EIciRNA sequences contain exons and introns, which are mainly located in the nucleus and form a protein-RNA complex with U1 snRNP and Polymerase II to regulate the transcription of their parent genes (30, 31). CircRNAs can also be classified as intragenic circRNAs and intergenic circRNAs based on the position of circRNA-originated locus in the genome.

Mechanisms of circRNA Formation

CircRNAs are produced by a unique splicing mechanism called backsplicing (5, 32). Classical splicing events include a typical donor (GU) at the 5 "end of the intron and an receptor (AG) at the 3" end of the intron (33). The circRNAs are formed because the splicing does not finish in the linear manner between intronic donor splicing site next to an exon to the receptor splicing site before the downstream exon, but to the receptor splicing site before the upstream exon. This process produces a covalently closed RNA molecule with or without exons. Three models have been proposed for the formation of circRNAs: (I) intron pairing-driven circularization, (II) RNA binding protein-driven circularization, and (III) lariat-driven circularization (**Figure 1**).

Intron Pairing-Driven Circularization

Compared with linear splicing, the reverse complementary sequences between introns bracketing circRNAs were significantly more abundant (9). In intron-pairing-driven circularization, cis-acting elements, hairpin structures, or complementary sequences located in the flanking introns of an exon are often used for direct base pairing (5, 9, 34). The minimal intron region required for circRNA circularization has been identified (35): even if the intron is <100 nucleotides and contains a typical splicing site sequence and a short reverse repeat sequence, it is sufficient for exon cyclization. This process appears to be more complicated than canonical base pairing, since not all reverse repeats lead to exon cyclization. It is worth noting that multiple exon cyclization events can occur in one gene locus, and exon cyclization efficiency can be modulated by RNA pairing within flanking introns or by competition for RNA pairing within a single intron (36). Alternative formation of reverse repeats of introns, such as repeated ALU pairs, and the competition between them often result in alternative circularization, which leads to the occurrence of producing multiple circRNA transcripts from a gene locus (36). However, how base pairing between introns affects the assembly of the spliceosome during back-splicing is still not fully understood and warrants further investigation.

RBP-Driven Circularization

The protein-protein interaction between RNA binding proteins (RBPs) makes the splicing sites of pre-mRNA come closer, which further facilitates the spliceosome to participate in the back-splicing reaction. Multiple RBPs have been shown to regulate the generation of circRNAs. Both the RNA-binding motif protein 20 (RBM20) (37) and splicing factor muscleblind (MBL) (38) were shown to increase the generation of circRNAs by binding

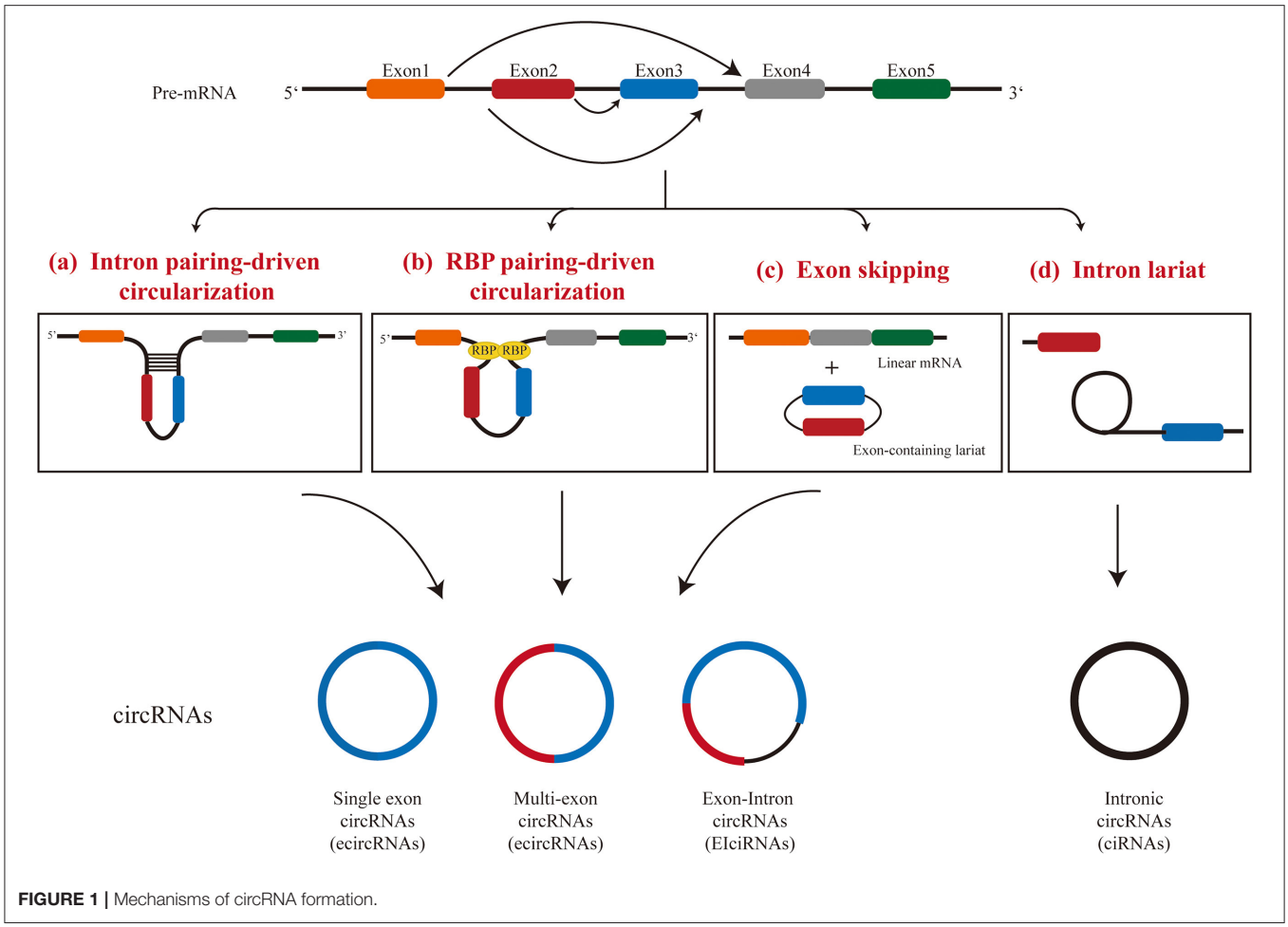

FIGURE 1 | Mechanisms of circRNA formation.

to specific intron motifs. RBM20 was identified as an important splicing factor in the heart with the function of regulating the formation of circRNAs in TTN gene locus (37).

A further study suggested that the RBP-driven circularization and the intron-pairing-driven circularization may work together to regulate the formation of circRNAs (39). Intronic repeats in flanking introns are believed to provide an opportunity for RBM20 to facilitate the circularization event. After that, a subset of proteins are recruited, which further regulate the formation of circRNA by modulating the activity of spliceosomes. Each gene locus may require a different set of protein factors for the generation of multiple circRNAs.

Lariat-Driven Circularization

Interestingly, a circRNA can also be produced during linear splicing by lariat-driven circularization, in which circRNAs may be generated during exon-skipping events (40) or intron removal in pre-mRNA splicing (8). TTN gene is an example of generating circRNAs through exon-skipping events with more than 80 circRNAs generated through this mechanism in the heart (37). Lariat RNAs are the intermediate product of splicing of pre-mRNA. Under normal circumstances, lariat RNAs released in canonical splicing undergo debranching at the 2′-5′ phosphodiester bond, and are then degraded by exonucleases (41). However, the specific structure of 7 nt GU-rich near the 5′ splice site and 11 nt C-rich near the branching site of lariats prevent the debranching event, and therefore, these RNA molecules remain circular (8, 42). These type of circular RNAs become mature after the 3′ tail of the lariat is degraded up to the branching point (43).

THE REGULATORY MECHANISMS OF CIRCRNAS

Accumulating data have shown that circRNAs exert their regulatory function through the following mechanisms: (1) functioning as miRNA sponges to sequester miRNAs and de-repress their targets; (2) functioning as scaffolds to bind RNA binding proteins and regulate the activity of downstream signaling; (3) binding to snRNP and polymerase II to regulate transcriptional activity; (4) functioning as competitors for parental gene splicing and expression; (5) functioning as templates for protein synthesis (**Figure 2**).

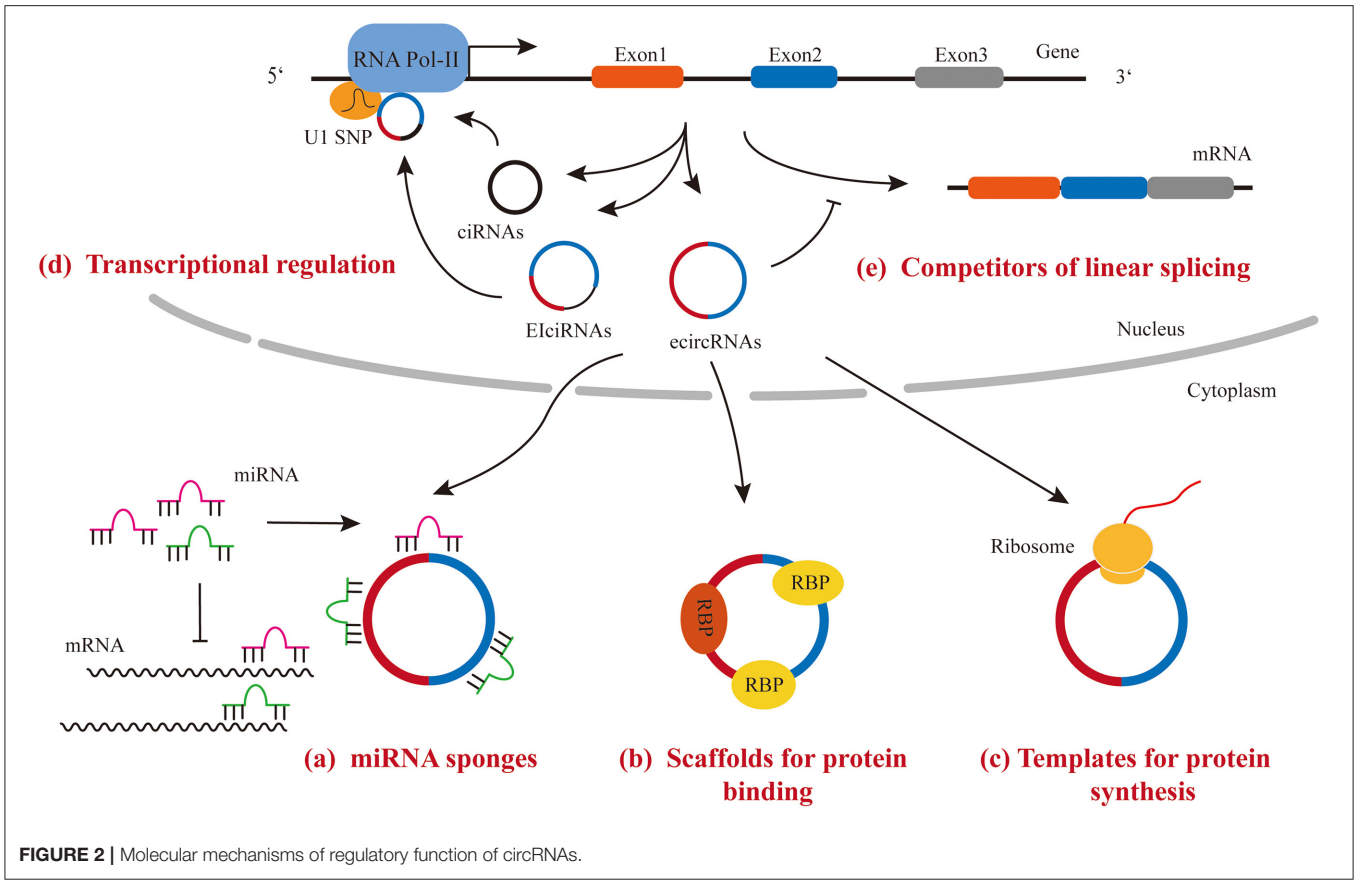

FIGURE 2 | Molecular mechanisms of regulatory function of circRNAs.

MicroRNA Sponge

MiRNAs repress gene expression post-transcriptionally by binding to the 3′ UTR of target mRNAs. CircRNAs have been demonstrated to possess multiple miRNA binding sites by both computational prediction and experimental assays. The interaction between circRNA and miRNA leads to miRNA retention and then lowering their bioactivity, which is referred to the "sponge effect." For example, a CDR1 locus-derived circRNA, CDR1as, has 63 highly conserved miR-7 binding sites (6, 44). Since no linear transcript of CDR1as were detected, the knockout strategy is simple and the removal of DNA sequence for circRNA from the genome will not affect the expression of any linear transcript from the same DNA locus. The high expression level of CDR1as and the presence of large amount miR-7 binding sites per molecules makes the circRNA a competitive inhibitor of endogenous miR-7.

Scaffold for Protein Interaction

Some circRNAs possess protein binding motifs. Therefore, these circRNAs interact with selected proteins and regulate their activity or localization. For example, the interaction between circPABPN1 and HuR prevents HuR from binding to PABPN1 mRNA and reduces its translation (45). In another example, circMBL, a circRNA derived from MBL gene locus and containing the conserved binding site of MBL, binds to MBL and regulates the splicing of its own pre-mRNA (38). However, computational analysis predicted that the density of RBP binding sites is lower in circRNAs than in 3′ UTR regions of protein-coding genes (46).

Transcriptional Regulation

Most circRNAs are presented in the cytoplasm and act as either miRNA sponges or scaffolds. However, ciRNA and EIciRNA, such as circEIF3J and circPAIP2, remain in the nucleus and interact with U1 snRNA and RNA polymerase II complex to enhance the transcriptional activity of their parent gene (31). However, the underlying mechanism of the regulatory function of EIciRNA remains unclear.

Competitors of Linear Splicing and Gene Expression

The process of circRNA formation also affects the expression of host gene. Some evidences indicated that circRNA formation competes strongly with the linear splicing of pre-mRNA, and therefore, regulates host gene expression (38, 47). For example, an increase in linear splicing efficiency in Drosophila S2 cells led to a decrease in circRNA expression (38). In another example, a decrease in spliceosome components in drosophila cells resulted in an increase in circRNA levels and a decrease in its associated linear mRNA expression (47). Since linear- and back-splicing use the same typical splicing receptor and donor, it is not surprising

that the level of circRNAs is negatively associated with the level of their linear mRNA isoforms.

Templates for Protein Synthesis

Although, circRNAs were first identified as non-coding RNAs, some of these circular RNA molecules were found to have the protein/peptide coding capability. Given that circRNAs are mostly localized in the cytoplasm and contain protein-coding exons, people wonder whether they can be loaded into ribosomes and serve as a template for protein/peptide synthesis. Interestingly, studies showed the initiation of translation of circRNAs can occur either at the internal ribosomal entry site (IRES) or at nucleotides with m6A modification in 5′ untranslated region (UTR) (48, 49), although, they lack the cap-dependent translation elements. So far, only a few endogenous circRNAs, such as circFBXW7, circMBL, and circ-ZnF609, have been shown to possess the effective open reading frame for protein/peptide translation (50–55). The function of most circRNA-derived peptides is unknown. It is worthy to note that circRNA-derived peptides were found to be expressed under different stress conditions, such as the translation of circ-ZnF609 in response to heat shock (50, 51). Although, translation of circRNAs does not appear to be a common function of circRNAs, the next important task in this field is to determine the regulatory function of circRNA-derived proteins/peptides.

PARTICIPATION OF CIRCRNAS IN THE PATHOGENESIS OF CARDIAC REMODELING

Due to the limited regenerative capacity of myocardial tissue, the heart undergoes extensive remodeling to compensate the loss of cells or response to stress. During remodeling, hypertrophic growth and limited proliferation occur in cardiomyocytes. In addition, non-cardiomyocytes such as cardiac fibroblasts, endothelial cells, smooth muscle cells and immune cells, are all shown to actively participate in this disease progress. For example, the dying cardiomyocytes secret cytokines to activate the proliferation and differentiation of cardiac fibroblast, and to recruit immune cells to clean up the dead cardiomyocytes; ischemic and oxidative stress also trigger the proliferation of endothelial cells and angiogenesis for re-establishment of blood supply. Different cell types are tightly act together and communicate with each other either through cell-cell junction or cytokines, or even through exosomes during this process.

More and more evidences demonstrated that non-coding RNAs constitute a regulatory network in almost all forms of human diseases, including cardiac remodeling and heart failure. These RNA molecules incorporate into the known protein regulatory network to orchestrate a highly complicated gene regulatory network in human diseases. Cracking the "code" of this network will provide us a roadmap to fully understand mechanisms beneath disease phenotype we observed, and eventually lead us to a better and more effective therapy. Previous studies have demonstrated that some non-coding RNAs, such as microRNAs and long non-coding RNAs, have altered expression and play important regulatory roles in cardiac remodeling (14, 56, 57). Due to the different structure of circular RNAs, this type of non-coding RNAs have not been broadly studied until recently (58). Studies showed that circular RNAs are widely presented in different mammalian cell types (5). Growing evidence shows that circRNAs play important roles in cell proliferation, apoptosis, migration, and differentiation (43, 50, 59). Importantly, RNA-sequencing data showed that a subset of circular RNAs are dysregulated in diseased heart (37), supporting the idea of circular RNAs possessing regulatory functions in cardiac remodeling. Here, we systematically review the recent study progress of circRNAs in cardiac remodeling (**Table 1** and **Figure 3**) and discuss the function of some representative circRNAs in cardiomyocytes, fibroblasts, endothelial cells, immune cells, and exosomes in detail.

Circular RNAs in Cardiomyocyte

As the main contractile cells in the beating heart, the alteration of cardiomyocytes is the central of cardiac remodeling in various disease status. In adult heart, cardiomyocytes occupied around 75% of left ventricular volume (101). Cardiomyocytes undergo hypertrophic growth, apoptosis/necrosis, and limited proliferation during cardiac remodeling. Non-coding RNAs, such as microRNAs and long non-coding RNAs, have been demonstrated to regulate the pathophysiology of cardiomyocytes in diseased heart (102). Here, we discussed some emerging examples of circRNAs in cardiomyocytes during cardiac remodeling.

HRCR

HRCR was identified as the first circRNA regulating cardiac hypertrophy (16). The expression of circRNA HRCR was shown substantially decreased in mice in response to ISO or TAC treatment. HRCR has a protective function on cardiac hypertrophy and heart failure. Forced expression of HRCR mediated with adenoviral constructs in mouse heart decreases cardiomyocyte hypertrophic growth, interstitial fibrosis and preserves the cardiac function upon ISO treatment. Mechanistically, six target sites for miR-223 were identified in HRCR. HRCR sequesters and decreases the activity of miR-223, and upregulates the expression of target of miR-223, apoptosis repressor with CARD domain (ARC), which is a known regulator of cardiomyocyte hypertrophy and apoptosis (16).

CircSLC8A1

CircSLC8A1 (also named CircNCX1, or named circSlc8a1-1 in mouse), which is enriched in cardiomyocytes, was identified as the most abundant circRNA in human and mouse heart (103), whose host gene encodes the protein of sodium-calcium exchanger (NCX). CircSLC8A1 is generated from the 2nd exon of the host gene SLC8A1. Although, the expression of CircSLC8A1 remains unaltered under some disease conditions such as cardiac hypertrophy in mouse and failing heart in human, it has been confirmed to be involved in regulation of hypertrophic growth of cardiomyocytes as an endogenous sponge for miR-133a (24, 37, 60, 104). Of note, inhibition of circSLC8A1

TABLE 1 | A list of reported regulatory circRNAs in cardiac remodeling.

CircRNAs	Host gene	Target	Mechanism	Function	References
CircRNAs in cardiomyocytes					
HRCR	HRCR	miR-223	miRNA sponge	Promoting cardiac hypertrophy and heart failure	(16)
CircSLC8A1	NCX	miR-133a-3p	miRNA sponge	Promoting cardiomyocytes apoptosis and hypertrophy	(60, 61)
Cdr1as	Cdr1as	miR-7	miRNA sponge	Promoting cardiomyocyte apoptosis	(62)
CircNfix	Nfix	miR-214/interaction between Ybx1 with Nedd4l	miRNA sponge/Scaffolds for protein interaction	Inhibiting cardiomyocyte proliferation and angiogenesis	(63)
ACR	ACR	Dnmt3B	Transcriptional regulation	Inhibiting autophagy and cell death	(64)
CircHipk3	Hipk3	N1ICD, miR-185-3p, miR-17-3p	Scaffold for protein interaction/miRNA sponge	Promoting cardiomyocyte proliferation	(65)
CircHIPK2	Hipk2	miR-485-5p	miRNA sponge	Promoting autophagy and apoptosis	(66)
CircITCH (hsa_circ_0001141)	ITCH	miR-330-5p	miRNA sponge	Inhibiting cardiomyocyte apoptosis	(67)
CircPan3	Pan3	Undetermined	Undetermined	Inhibiting cardiomyocyte apoptosis	(68)
CircFoxo3	Foxo3	Foxo3	Scaffold for protein interaction	Promoting cell apoptosis/death	(69)
CircRNA_000203	Myo9a	miR-26b-5p, miR-140-3p	miRNA sponge	Promoting cardiac hypertrophy	(70)
CircTtc3	Ttc3	miR-15b-5p	miRNA sponge	Inhibiting ATP depletion and apoptotic death	(71)
Circ_0010729	Undetermined	miR-27a-3p, miR-145-5p, miR-370-3p	miRNA sponge	Inhibiting apoptosis and glycolysis	(72–74)
MFACR	MFACR	miR-652-3p	miRNA sponge	Promoting mitochondrial fission and the apoptosis of cardiomyocytes	(75)
CircMACF1	MACF	miR-500b-5p	miRNA sponge	promoting cardiomyocyte apoptosis	(76)
Hsa_circ_0097435	Undetermined	Undetermined	Undetermined	Promoting cardiomyocyte apoptosis	(77)
Circ_0062389	PI4KA	Undetermined	Undetermined	Promoting cardiomyocyte apoptosis	(78, 79)
CircPostn	Postn	miR-96-5p	miRNA sponge	Promoting cardiomyocyte apoptosis	(80)
CircRNAs in cardiac fibroblasts					
CircRNA_000203	Myo9a	miR-26b-5p	miRNA sponge	Promoting fibrotic phenotype of CFs	(81)
Circ_0060745	Undetermined	Undetermined	Undetermined	Increasing myocardial infarct size and worsening cardiac functions after AMI and contributes to activation of NF-κB under hypoxia	(82)
CircNFIB	Nfib	miR-433	miRNA sponge	Inhibiting CFs proliferation	(17)
Circ_LAS1L	LAS1L	miR-125b	miRNA sponge	Inhibiting the activation, proliferation, migration and promotes apoptosis of CFs	(83)
CircPAN3	PAN3	miR-221	miRNA sponge	Promoting fibrotic phenotype of CFs and activation of autophagy	(84)
CircHIPK3	HIPK3	miR-29b-3p	miRNA sponge	Promoting proliferation, migration of CFs and development of cardiac fibrosis	(85, 86)
		miR-152-3p	miRNA sponge	Promoting proliferation, migration and phenotypic transformation of CFs	(87)
CircRNA_010567	Undetermined	miR-141	miRNA sponge	Promoting fibrotic phenotype of CFs	(88)
CircYap	YAP	TPM4 and ACTG	Scaffold for protein interaction	Inhibiting fibrotic phenotype and migration of CFs	(89)
Circ-Foxo3	Foxo3	ID-1, E2F1, FAK and HIF1α	Scaffold for protein interaction	Promoting senescence of CFs	(90)
CircRNAs in endothelial cells					
Circ-CCAC1	ERBB2	EZH2	Scaffold for protein interaction	Disrupting endothelial barrier integrity and promoting angiogenesis	(91)
Circ_0003204	USP36	miR-370-3p	miRNA sponge	Inhibiting proliferation, migration and tube formation of endothelial cell	(92)
CircDLPAG4	DLGAP4	miR-143	miRNA sponge	Inhibiting endothelial cell migration, without affecting cell viability, and apoptosis	(93)

(Continued)

TABLE 1 | Continued

CircRNAs	Host gene	Target	Mechanism	Function	References
CircVEGFC	VEGFC	miR-338-3p	miRNA sponge	Promoting vascular endothelial cells apoptosis	(94)
Circ-RELL1	RELL1	miR-6873-3p	miRNA sponge	Promoting inflammation in ECs	(95)
CiRS-7	LINC00632	miR-26a-5p	miRNA sponge	Promoting tube formation in microvascular endothelial cells	(96)
Circ_0003645	chr16:19656207-19663412	Undetermined	Undetermined	Promoting endothelial cell inflammation and apoptosis after silencing	(97)
CZBTB44	chro11: 130130750-130131824	miR-578	miRNA sponge	Promoting cell viability, proliferation, migration and tube formation	(98)
Hsa_circ_0030042	FOXO1	eIF4A3	Scaffold for protein interaction	Inhibiting abnormal autophagy	(99)
CircRNAs in immunocytes					
CircSnx5	Snx5	miR-544/SOCS1	miRNA sponge	Inducing immunological tolerance	(19)
CircRNAs in exosomes					
CircHIPK3	HIPK3	miR-29a	miRNA sponge	Inhibiting endothelial cell apoptosis	(100)

promotes TAC-induced hypertrophy and HF in mouse (60). Cardiac-specific overexpression of circSlc8a1 *in vivo* mediated by the AAV9 increases heart weight and results in cardiac dilatation. Different from the expression in hypertrophy, the expression of circSLC8A1 have been demonstrated abnormally increased in dilated cardiomyopathy (105, 106), and upregulated in ischemic rat cardiac cells and mouse heart (61). Through a similar mechanism of acting as miR-133a-3p sponge, the NCX-derived circRNA increased the levels of CDIP1, a target for miR-133a-3p, which promotes cardiomyocyte apoptosis. Therefore, circSLC8A1 could exert different regulatory function in cardiomyocytes depending on the type of stress. Interestingly, circSLC8A1 interacts with the mouse ribosome or rat Argonaute 2 protein, which indicates circSLC8A1 is likely involved in the regulation of mRNA translation (106). Interesting, the expression level of circSLC8A1 is increased and positively correlated with the expression of CK-MB in the pericardial fluid of acute ischemic heart disease patients, which shows the potential of using this circRNA as an auxiliary diagnostic marker for clinical acute coronary syndromes (107).

CircNfix

CircNfix was identified as a super enhancer-associated circRNA by an integrated analysis with RNA-seq data and super enhancer catalogs (63). CircNfix was shown to regulate cardiomyocyte proliferation and angiogenesis. *In vivo* knockdown of circNfix mediated by cTNT-driven shRNA expression through AAV9 viral delivery system promotes cardiomyocyte proliferation evidenced by the increased the expression of the proliferation markers and the total cardiomyocyte number in infarcted mouse hearts, which leads to an improved cardiac function after MI. Although, CircNfix functions as a miRNA sponge to decrease the promotive function of miR-214 on cardiomyocyte proliferation, unexpectedly, it also promotes Ybx1 degradation through ubiquitination by enhancing the stability of the interaction between Ybx1 with Nedd4l, an E3 ubiquitin ligase. This observation indicates that CircNfix also functions as a scaffold for protein docking. Since transcriptional factor Ybx1 activates the expression of Ccna2 and Ccnb1 (108), CircNfix further decreases the proliferation of cardiomyocyte during myocardial infarction.

ACR

A recent study demonstrated that a circRNA ARC (autophagy-related circular RNA) plays an important role in cardiomyocyte autophagy (64). The expression of circRNA ACR is markedly decreased after the heart subjected to ischemia/reperfusion. ACR attenuates the increased autophagy level upon ischemia/reperfusion injury and plays a protective role in cardiomyocytes *in vivo*. Mice with overexpression of ACR in the heart had a less cardiomyocyte death in ventricular tissue and a smaller infarction. Mechanistically, instead of a miRNA sponge, ACR acts as a regulator of chromatin modification by binding to Dnmt3B and inhibiting Dnmt3B-mediated DNA methylation of Pink1 promoter. Since Pink1 targets and phosphorylates FAM65B, which was shown to have a regulatory role in autophagy, ARC mediates cardiomyocyte autophagy through a Dnmt3B/Pink1/FAM65B signaling cascade.

CircHipk3

Similar to ACR, some of circRNAs have been demonstrated to serve as a signaling regulator by binding with protein in cardiomyocytes. A recent study found an increased expression level of circHipk3 in the fetal or neonatal mouse heart (65). AAV9-mediated overexpression of circHipk3 attenuated cardiac dysfunction and fibrosis in a mouse model of myocardial infarction. CircHipk3 was shown to interact with N1ICD protein to increase N1ICD acetylation level and stability, which was partially responsible for the beneficial effect of circHipk3 in cardiomyocytes. On the contrary, silencing circHIPK3 has a protective effect in a variety of heart diseases. The hypertrophic growth of cardiomyocytes was markedly inhibited by the knockdown of circHIPK3 in a TAC-induced cardiac hypertrophy model (109). Since CircHIPK3 is a sponge of miR-185-3p, decreased level of CircHIPK3 in the knockdown increases the inhibitory effect of miR-185-3p on CaSR. In addition,

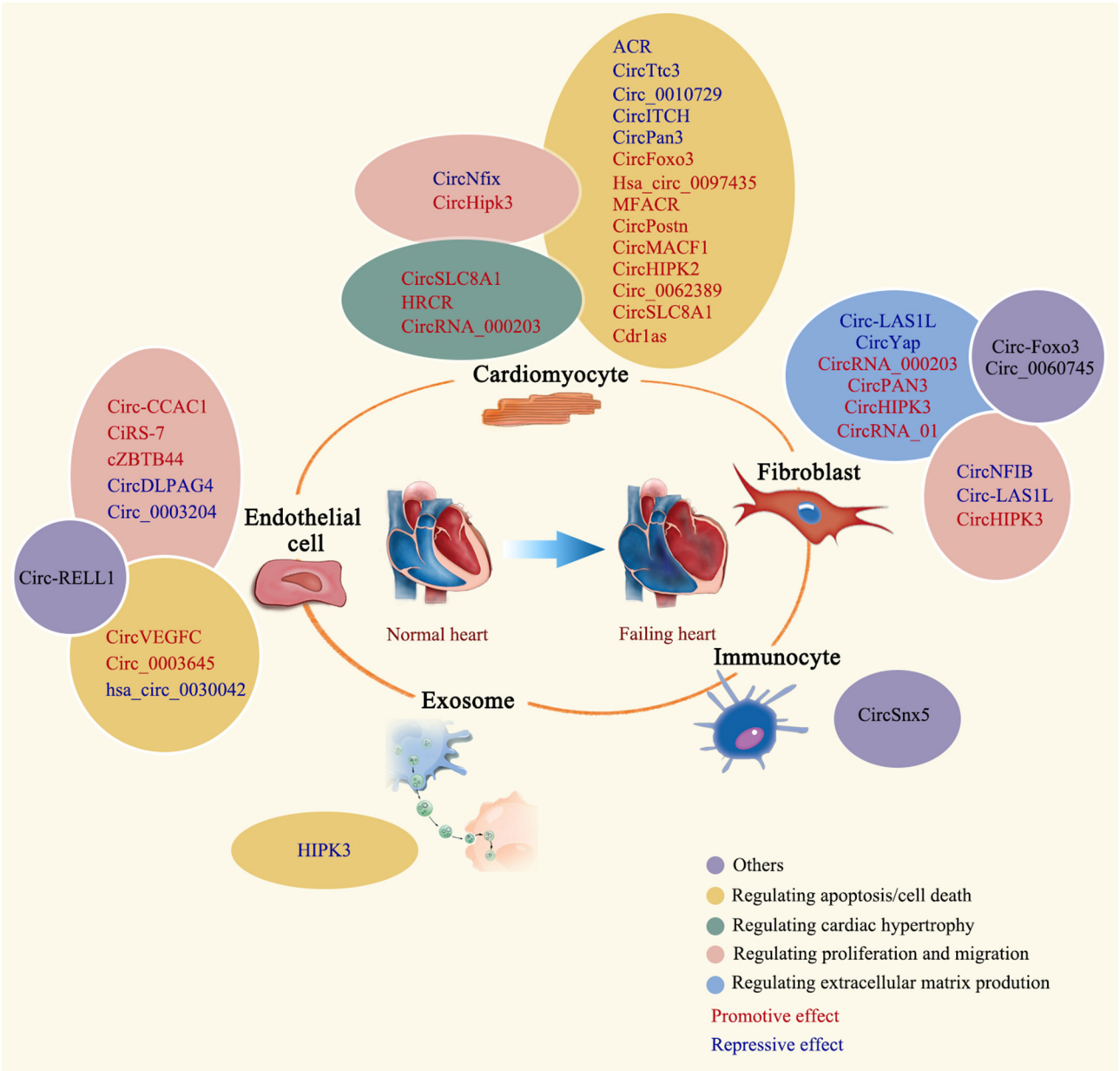

FIGURE 3 | Regulatory roles of circRNAs in cardiac remodeling. The regulatory role of summarized circRNAs is indicated by colored ovals. CircRNAs are labeled in different colors to indicate the promotive or repressive function.

knockdown of circHIPK3 benefits the heart after myocardial infarction through a circ-HIPK3/miR-17-3p/ADCY6 signaling cascade in cardiomyocytes (110). While in a model of LPS-induced myocarditis, knockdown of circHIPK3 significantly represses cardiomyocyte apoptosis and alleviates oxidative stress and inflammation in cardiac tissue (111). Interestingly, it was reported that circHIPK2, originated from the second exon of another HIPK family member HIPK2, facilitated autophagy in H2O2-caused myocardial injury *via* sponging miR-485-5p and de-repressing miR-485-5p target, ATG101 (66, 112).

Other circRNAs in Cardiomyocytes

Doxorubicin is widely used in tumor chemotherapy, however, with the dose-dependent cardiotoxicity. Doxorubicin-induced cardiotoxicity involves many molecular mechanisms, including induction of reactive oxygen species (113), inhibition of the activity of topoisomerase II (114), interruption of calcium homeostasis, induction of mitochondrial dysfunction, and destruction of sarcomere function (115). Recent studies showed that circRNAs are involved in doxorubicin-induced cardiomyopathy. A previous study identified 356 differentially

expressed circRNAs in doxorubicin-treated human hearts (67). CircITCH, a circRNA highly conserved between human and mice, was significantly downregulated in DOX-induced cardiomyopathy. AAV9-mediated overexpression of circITCH ameliorates oxidative stress and DNA damage, cell death, contractile dysfunction, and calcium handling defects in DOX-induced cardiomyopathy in a mouse model by acting as a miR-330-5p sponge to de-repress the expression of SIRT6, BIRC5, and ATP2A2. In another recent study, overexpression of circPan3 was shown to attenuate DOX-induced cardiomyocyte apoptosis with unknown mechanism (68). In another example, circ-Foxo3 interacts with the anti-senescence proteins ID1 and E2F1, and anti-stress proteins FAK and HIF1α to prevent their nuclear translocation for transcription (90). Therefore, silencing of circ-Foxo3 relieved the cardiac injury induced by doxorubicin. In addition, circFoxo3 levels were significantly higher in I/R injury resulted from 24 h of cold storage and reperfusion in heart transplantation (69). *In vivo* and *in vitro* experiments demonstrates that knockdown of circFoxo3 improves heart graft function and reduced cell apoptosis/death and mitochondrial damage.

Circular RNAs in the Activation of Cardiac Fibroblasts

Cardiac fibrosis, generally referred to an aberrant accumulation of extracellular matrix (ECM) proteins in the interstitial space of heart tissue, is closely associated with pathological cardiac remodeling. It manifests as deposition of scar, increasing stiffness, decreasing contraction and impaired heart function, which ultimately resulting in heart failure (116). Although, cardiac fibrosis is a complex process and involves many types of cells in the heart, such as cardiomyocytes, fibroblasts, lymphocytes, and pericytes (117–119), extensive studies have proved that cardiac fibroblasts (CFs) play a pivotal role in this process (120). When suffered cardiac injury, the proliferation and migration of CFs are increased. Moreover, cytokines, especially the transforming growth factor-β (TGF-β), and growth factors are also secreted, which contributes to fibroblast activation and ultimately transform CFs into myofibroblasts (121). In this situation, myofibroblasts begin to express α-smooth muscle actin (α-SMA) and enhance the secretion of ECM proteins, such as collagen type I and collagen type III, which results in the formation of scar and eventually leads to cardiac fibrosis (122). Recent studies have shown circRNAs actively participate in the pathogenesis of cardiac fibrosis, especially in the activation of cardiac fibroblasts.

CircRNA_000203

CircRNA_000203, derived from Myo9a, is upregulated in the myocardium of diabetic mouse and Ang-II-treated cardiac fibroblasts. Overexpression of circRNA_000203 in mouse CFs *in vitro* increased the expression of Col1a2, Col3a1, and α-SMA, indicating the activation of CFs and the accumulation of ECM. Moreover, RNA pull-down confirmed that circRNA_000203 is a sponge of miR-26b-5p. The targeting of CTGF and Col1a2 by miR-26b was experimentally confirmed, which was interfered by the overexpression of circRNA_000203 (81). Interestingly, circRNA_000203 is also upregulated in Ang-II-treated cardiomyocytes. Forced expression of circRNA_000203 promotes the hypertrophic growth of cardiomyocytes and transgenic mice with cardiac-specific overexpression of circRNA_000203 have an advanced phenotype in a model of Ang-II-induced cardiac hypertrophy (70).

CircNFIB

CircNFIB (mmu_circ_0011794), generated from the exon regions of Nfib, was identified as a candidate circRNA to sponge miR-433, a miRNA promoting cardiac fibrosis (123). CircNFIB has a decreased expression in both 3-week post-MI mice hearts and TGF-β-treated CFs. The proliferation of CFs, induced by TGF-β treatment, was significantly inhibited by the overexpression of circNFIB *in vitro* (17). Mechanistically, circNFIB de-represses AZIN1 and JNK1, which are targeted by miR-433. Overexpression of circNFIB increased the expression of AZIN1 and JNK1 and impaired the activation p38/ERK/Smad3, thus confirming the pivotal role of circNFIB as a competing endogenous RNA (ceRNA) in cardiac fibrosis. However, the role of circNFIB *in vivo* in cardiac remodeling is still unclear and requires for further investigation.

Circ_LAS1L

CircRNAs have been found to be involved in acute myocardial infarction (AMI) in recent years (124). Bie et al. (125) demonstrated the crucial function of miR-125b/SFRP5 axis in CF growth and activation in previous report. Further, analysis showed that miR-125b targets circ_LAS1L with two binding sites, which was confirmed by RIP and RNA Pull-down. The expression level of circ_LAS1L is significantly downregulated while miR-125b expression is increased in AMI patients. Forced expression of circ_LAS1L upregulates the expression of SFRP5 and downregulates the expression of α-SMA, collagen I, and collagen III in CFs. Interestingly, gain function of miR-125b together with overexpression of circ_LAS1L appears not to modulate CFs proliferation, apoptosis, and migration. However, SFRP5 siRNA, instead of miR-125b mimics, bypassed the counter effect of circ_LAS1L on CF proliferation and migration, indicating that circ_LAS1L functions as a sponge of miR-125b to modulate CFs proliferation and migration *in vitro* (83). Whether circ_LAS1L has a repressive function on cardiac fibrosis upon cardiac injury *in vivo* warrant investigation in the future.

CircRNA_PAN3

CircPAN3, a circRNA generated from the PAN3 locus, has been found to maintain the self-renewal of intestinal stem cells (126), to modulate drug resistance in acute myeloid leukemia (AML) (127, 128), and to recede myocardial ischaemia/reperfusion injury (129). Recently, Li et al. reported circPAN3 as a new profibrotic factor in cardiac fibrosis (84). The expression of circPAN3 increases significantly in fibrotic regions of rat heart induced by myocardial infarction (MI). Silencing of circPAN3 in MI hearts reduces the level of fibrosis, including decreased expression of fibrotic markers, and inhibits cardiac myocyte apoptosis and autophagy. Consistently, knockdown of circPAN3 represses TGF-β induced proliferation, migration and

autophagy of CFs *in vitro*. In the molecular level, circPAN3 was demonstrated to interact with miR-221 and sequester miR-221 from regulate its targets, FoxO3, as a sponge. Gain function of miR-221 decreases the expression of FoxO3 and ATG7, two known targets for miR-221 and have been proved crucial in autophagy in previous studies (130). These data indicate that circPAN3 promotes fibrosis *via* miR-221/FoxO3/ATG7 cascade-mediated autophagy.

Circ_0060745

A recent study reported that circ_0060745 expression level in CFs is increased dramatically in the myocardium of AMI mice (82). Knockdown and overexpression of circ_0060745 improved and deteriorated the cardiac function, respectively. In addition, the silencing of circ_0060745 leads to less cell apoptosis in the infarcted areas while circ_0060745 overexpression had the opposite effect. Further, analysis found that the expression of inflammatory cytokines, including IL-6, IL-12, IL-1β, and TNF-α, are decreased upon knockdown of circ_0060745, which could suppress peritoneal macrophage migration. The downregulation of the inflammatory cytokines is induced by the inhibition of NF-κB activation in the circ_0060745 knockdown.

CircHIPK3

CircHIPK3 (mmu_circ_0001052), originated from exon 2 of HIPK3, has been reported to play an important role in cancers (131). Recent studies suggested it also regulates cardiac fibrosis *via* different signaling cascades. Ni et al. found circHIPK3 promoted CF proliferation, migration and activation by modulating the activity of a known fibrosis-related microRNA, miR-29b-3p, through sponging (85). Similarly, another study showed circHIPK3 induced cardiac fibrosis through a circHIPK3/miR-29b-3p/Col1a1/Col3a1 signaling cascade in mouse diabetic cardiomyopathy model (86). Interestingly, circHIPK3 modulates CFs function in a hypoxia condition in a similar sponge manner, but through a different signaling cascade, the circHIPK3/miR-152-3p/TGF-β2 axis (87). Therefore, circHIPK3 is likely to be an important upstream node of the miRNA-mediated posttranscriptional gene regulation network in cardiac fibrosis.

CircYap

CircYap hsa_circ_0002320, generated from exons 5 and exon 6 of YAP pre-mRNA, is the highest expressed isoforms derived from Yap gene locus in human hearts. The expression level of circYap decreases significantly in hypertrophic patient hearts of patients and in pressure-overloaded mouse hearts. The forced expression of circYap alleviates the declined heart function and increased cardiac fibrosis in a TAC-induced mouse cardiac hypertrophy model. Overexpression of circYap in cardiac fibroblasts (MCF) *in vitro* suppresses the expression of fibrotic markers and migration of cardiac fibroblasts. Mechanistically, circYap interacts with both tropomyosin-4 (TMP4) and gamma-actin (ACTG), and enhances the interaction between TMP4 and ACTG, which subsequently inhibits the actin polymerization and cardiac fibrosis (89).

Circular RNAs in Endothelial Cells

Endothelial cells (ECs) play a central role in cardiac remodeling, regeneration, as well as angiogenesis in the treatment of cardiovascular diseases (132, 133). Therefore, it is of great significance to identify factors that promoting and inhibiting angiogenesis and their underlying molecular mechanisms. To date, many studies have confirmed that circRNAs are involved in regulating the proliferation, migration, apoptosis, and tubule formation of ECs, which further mediates the dynamics of ECs and regulates angiogenesis.

Circ-ZnF609

The level of circ-ZNF609 in peripheral blood leukocytes of coronary artery disease patients is significantly decreased (134). In another study of circRNAs in retinal vascular dysfunction, Circ-ZnF609 was found significantly upregulated under conditions of high glucose and hypoxia stress *in vivo* and *in vitro* (18). Silence of circ-ZnF609 increased endothelial cell migration and tube formation, and protected endothelial cells against oxidative stress and hypoxia stress *in vitro*. Circ-ZnF609 acts as an endogenous miR-615-5p sponge to sequester miR-615-5p and inhibit its function, leading to the increased expression of MEF2A. Overexpression of MEF2A rescues the endothelial cell migration, tube formation, and apoptosis mediated by the silence of circ-ZnF609, which further demonstrates the regulatory mechanism of circ-ZnF609 on ECs *via* circ-ZnF609/miR-615-5p/MEF-2A signaling cascade.

Interestingly, circ-ZnF609 was also identified as a circRNA regulating muscle differentiation in mice and humans, and its expression is altered in Duchenne muscular dystrophy (DMD) myoblasts (50). Circ-ZnF609 specifically controls the proliferation of myoblasts. It was demonstrated that circ-ZnF609 is associated with heavy polysomes and translated into a protein in a splicing-dependent and cap-independent manner, providing an example of protein encoding circRNA in eukaryotes. Whether circ-ZnF609 functions as a miRNA sponge or protein-coding circRNA, or both, or it has a preferable manner depended on cell type, needs to be studied in the future. Furthermore, the role of endothelial circ-ZnF609 in the heart upon ischemia/reperfusion *in vivo* warrants investigation.

CircFndc3b

CircFndc3b is differentially expressed in the mouse hearts after myocardial infarction (MI) and in the heart tissues of patients with ischemic cardiomyopathy (135). Overexpression of circFndc3b in cardiac endothelial cells increases the expression of vascular endothelial growth factor-A, enhances angiogenesis, and reduces the apoptosis of cardiomyocytes and endothelial cells. In post-MI hearts, adeno-associated virus-mediated overexpression of circFndc3b reduces myocardial apoptosis, enhances neovascularization, and improves left ventricular function. CircFndc3b interacts with the RNA-binding protein FUS in sarcoma to regulate VEGF expression and signal transduction. These findings highlight the physiological role of circRNA in heart repair and suggest that regulating the expression of CircFndc3b is a potential therapeutic strategy for protecting the heart from myocardial infarction.

CZNF292

Hypoxia condition is introduced in the ischemic region of the diseased heart. To identify hypoxia-related circRNAs, endothelial circRNAs were screened from human umbilical vein endothelial cells cultured under normal or hypoxia conditions (136). CZNF292 is one of candidates identified in the screen. *In vitro*, target-specific depletion of CZNF292 with siRNAs inhibits angiogenic germination and spherical germination of endothelial cells, suggesting that CZNF292 has pro-angiogenic function. The overexpression of CZNF292 further confirms its pro-proliferation effect. Interestingly, the circRNA appears not associate with Argonaute, indicating it unlikely functions as a microRNA sponge. These data suggest that endothelial circRNAs could mediate angiogenesis under hypoxia condition with undetermined mechanism. Although, the *in vivo* function of CZNF292 in the heart is still unknown, it is interesting to investigate whetherCZNF292 promotes angiogenesis *in vivo* and benefit the heart after ischemia/reperfusion injury.

Circular RNAs in the Dynamics of Cardiac Immunocytes

Inflammation and fibrosis are two key factors in cardiac remodeling. Inflammatory response is generally caused by acute cell death, for instance, the sudden loss of cardiomyocytes after myocardial infarction. Necrotic cardiomyocytes crack and release cellular contents, which activates the inflammation reaction for cleaning dead cells and matrix debris (137, 138). During the cardiac inflammatory process, immunocytes accumulate in myocardium, infiltrate surrounding tissue and further regulate inflammatory reaction (139, 140). Among immunocytes, dendritic cells (DCs), derived from bone marrow, are antigen-presenting cells and crucial in immune response. Moreover, cardiac DCs has been reported to have heart-protective effects in acute myocardial infarction (AMI), such as the deletion of DCs in mice deteriorates the cardiac remodeling (141). In addition, cardiac specific tDCs (tolerogenic dendritic cells) can evoke the generation of Tregs, which can promote a macrophage-specific repair program after AMI (142).

Recently, Yu et al. reported a novel DC-expressed circRNA, named circSnx5, has a vital function in maintaining cardiac immune homeostasis. CircSnx5, generated from the snx5 gene locus, represses the maturation of DCs when its expression is upregulated in DCs. Knockdown of circSnx5 results in an inflammatory phenotype of dendritic cells. Mechanistically, circSnx5 sponges miR-544 and de-represses the downstream target of miR-544, the suppressor of cytokine signaling 1 (SOCS1). On the other hand, circSnx5 directly influences the nuclear translocation of PU.1 to regulate the expression of downstream MHC class II, which is critical to DC's function. In addition, the injury and inflammation of cardiac tissue is decreased, and the cardiac function is improved after introducing circSnx5-overexpressing DCs into experimental autoimmune myocarditis (EAM) mice. Thus, all these results confirm that circSnx5 has a protective effect on AMI (19).

Circular RNA Messenger in Exosomes

It is well-known that exosomes are involved in the intercellular communication. Correct communication between cells has been shown critical in preserving body homeostasis and health (102). Cell-cell communication *via* exosomes is involved in the pathological processes of some chronic diseases such as cancer and heart diseases (143, 144), but the disease progression regulated by circRNAs from the shuttling exosomes in the heart were less studied (145).

A recent study has provided evidence supporting the role of exosomal circRNAs in multiple physiological processes including the regulation of the heart function. Wang et al. found that exosomal circHIPK3 is highly expressed in hypoxic exosomes secreted from cardiomyocytes. Silencing of circHIPK3 is associated with increased levels of apoptosis, ROS, MDA, and proapoptotic proteins in cardiac microvascular endothelial cells (CMVECs) (100). The upregulated circHIPK3 sponges miR-29a, an apoptosis-suppressing miRNA, to de-repress the expression of IGF-1, and subsequently regulates the oxidative damage in CMVECs.

CONCLUSION

In conclusion, circRNAs are identified as new players to participate in the process of human diseases. Although, the biogenesis and molecular mechanism of circRNAs are still not fully understood, emerging evidences have demonstrated that these circular RNA molecules are broadly present in mammalian cells with different regulatory functions. The heart is composed of a variety of cell types that interact with each other through direct contact or paracrine signaling. More and more studies have proved that circRNAs are involved in the process of cardiac remodeling and have important regulatory functions in cardiomyocytes, endothelial cells, fibroblasts and immune cells during this disease process. The regulation of circRNAs in different cell types of the heart adds a new layer of regulation to the known gene regulation network of cardiovascular disease. Currently, we are still facing challenges in the study of circRNAs. For example, most of circRNAs can not be knocked out for the loss-of-function study since targeting circRNAs using CRISPR–Cas9 or DNA recombination strategy is likely to affect the splicing or expression of linear host genes. Although, the expression of circRNAs could be knocked down by specific siRNAs, the narrow junction of back-splicing limits the design of siRNA for a portion of circRNAs. Therefore, approaches for circRNA study are urged to be improved, which will lead us to better understand the function of circRNAs in human diseases.

Overall, roles of circRNAs in the pathogenesis of cardiovascular disease still remains largely unknown. Unlike linear RNA molecules, the stability of circRNAs grants the advantage of these circular RNA molecules in therapeutic applications, such as disease diagnosis and transgene delivery in gene therapy. Given that extracellular vesicles or exosomes contain circRNAs, capturing tissue-specific and disease-specific vesicles or exosomes for

circRNAs profiling could be a good strategy to identify biomarkers for disease diagnosis. Therefore, deeper and systematic studies of circRNAs in the content of different diseases, such as cardiac hypertrophy and heart failure, is the prerequisite of moving the knowledge of circRNAs into the therapeutic applications against the deadly cardiovascular disease.

AUTHOR CONTRIBUTIONS

TY, TL, TD, and Z-PH prepared the manuscript. TY, TL, and TD wrote the main parts of the article and produced graphics. YD and YC reviewed and edited the manuscript. Z-PH drafted the final version of the manuscript. All authors read and approved the final manuscript.

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REVIEW
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Roles of MicroRNAs in Glucose and Lipid Metabolism in the Heart

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MicroRNAs (miRNAs) are small non-coding RNAs that participate in heart development and pathological processes mainly by silencing gene expression. Overwhelming evidence has suggested that miRNAs were involved in various cardiovascular pathological processes, including arrhythmias, ischemia-reperfusion injuries, dysregulation of angiogenesis, mitochondrial abnormalities, fibrosis, and maladaptive remodeling. Various miRNAs could regulate myocardial contractility, vascular proliferation, and mitochondrial function. Meanwhile, it was reported that miRNAs could manipulate nutrition metabolism, especially glucose and lipid metabolism, by regulating insulin signaling pathways, energy substrate transport/metabolism. Recently, increasing studies suggested that the abnormal glucose and lipid metabolism were closely associated with a broad spectrum of cardiovascular diseases (CVDs). Therefore, maintaining glucose and lipid metabolism homeostasis in the heart might be beneficial to CVD patients. In this review, we summarized the present knowledge of the functions of miRNAs in regulating cardiac glucose and lipid metabolism, as well as highlighted the miRNA-based therapies targeting cardiac glucose and lipid metabolism.

Keywords: microRNAs, glucose, lipid, heart, metabolism

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INTRODUCTION

Under normal physiological conditions, in order to fulfill a continuous demand for ATP, the heart can metabolize a range of substrates *via* mitochondrial oxidative phosphorylation and substrate level phosphorylation, such as fatty acids, glucose, lactate, and amino acids (1). Before feeding into glycolysis or pentose phosphate pathway, glucose in cardiomyocytes is phosphorylated to glucose-6-phosphate (G6P). Activated by acyl CoA synthetase (ACS), cytosolic free fatty acids can form fatty acyl-CoAs, then could enter mitochondria for oxidation or form ceramides, diacylglycerol (DAG) and triacylglycerol (TAG). Although, the adult hearts mainly use fatty acids for ATP production, hearts demonstrate increased reliance on other substrates such as glucose under pathological conditions (2). The glucose and lipid metabolism in the normal and diseased heart have attracted increasing attentions. Under normal circumstances, except that the sources are lactate, ketone bodies, and amino acids, more than 95% of all substrates are derived from fatty acids and glucose to use for ATP generation for maintaining the function of the heart (2). Importantly, the glucose and lipid metabolism could be changed under pathological injury condition in the heart. It is well accepted, e.g., that hypertrophic heart undergoes a reprogramming process in metabolism, characterized by the increased reliance on glucose metabolism and decreased fatty acids oxidation, which is associated with an increase in glycolysis in the hypertrophied heart (3–5). Cardiac ischemia would lead to poor oxygen supply, inadequate washout of metabolic wastes, and increased glycolytic flux, because the amount of oxygen and

metabolic substrates that delivered to the myocardium is insufficient to meet the myocardial energy requirements (6). Abnormal glucose metabolism has also been noted in patients with diabetes mellitus and has been associated with cardiac dysfunction (7). Cardiac glucose uptake in diabetic cardiomyopathy is reduced despite hyperglycemia, which could also contribute to the impaired myocardial glucose utilization in diabetes due to decreased protein level of cardiac GLUT-4 (8). MicroRNAs (miRNAs) are small conserved non-coding RNAs which typically inhibits target mRNA translation or promoting target mRNA degradation in physiological or pathological processes (9, 10). Increasing evidence also suggests nuclear or mitochondrial miRNAs could enhance target gene expression through non-canonical mechanisms (11–13). During several phases of cardiac development, many miRNAs have been detected as important regulators to maintain the formation of normal functional heart tissue (14). miR-17-92 cluster, e.g., was suggested as a critical regulator of cardiomyocyte proliferation and might be a therapeutic target for cardiac repairing and heart regeneration (15). Cardiac miRNAs, such as miR-1 (16), miR133a (17), miR-208a/b (18), and miR-499 (19) could enhance regenerative properties and contribute to the reprogramming of mature non-cardiac cells to cardiomyocytes (20). During the progression of various cardiovascular diseases (CVDs), such as hypertrophy, diabetic cardiomyopathy, and myocardial ischemia, many studies also reported that a variety of miRNAs exerted important functions (21–23). Overexpression of miR-297 was found to accelerate the progression of cardiac hypertrophy by increasing the protein expression of ATF4, Xbps1, chaperon Grp78, and calreticulin, the endoplasmic reticulum stress markers (24). Overexpression of miR-200b was reported to prevent diabetes-induced cardiac functional and structural changes by inhibiting endothelial-to-mesenchymal transition (25). Delivery of antisense microribonucleic acid (anti-miR) against miR-21 improved cardiac function, as well as reduced cardiac fibrosis and hypertrophy in a pig model of myocardial ischemia/reperfusion injury (26).

Importantly, it has been reported that miRNAs possess crucial roles in regulating the glucose and lipid metabolism in a variety of organs. miR-146a, e.g., has been found to improve lipid accumulation as well as glucose and insulin tolerance *via* promoting the oxidative metabolism of fatty acids in the liver (27). In the kidney, through blocking the TLR4/NF-κB pathway, miR-140-5p protected renal tubular epithelial cells against high glucose-induced injury (28). Furthermore, in our recent study, we found miR-320a significantly aggravated diet-induced hyperlipidemia and hepatic steatosis (29). Importantly, the roles of miRNAs in the pathological and physiological regulation of glucose and lipid metabolism in the heart have also been gradually discovered. We previously found that nuclear miR-320a caused lipotoxicity in the diabetic heart and induced cardiac dysfunction by activating transcription of fatty acid metabolic genes (13).

In this review, we focused on the current knowledge to briefly summarize and discuss the regulation of miRNAs in glucose and lipid metabolism during the pathological processes of the heart, and highlighting the potential therapeutic strategies for diseases associated with abnormal cardiac glucose and lipid metabolism.

ROLES OF miRNAs IN GLUCOSE METABOLISM IN THE HEART

In the heart, miRNAs are critical, which participate in cardiac developmental and pathological processes (30). Blocking the expression of all miRNAs in the cardiovascular system has been reported to lead to death in early pregnancy due to severe heart and vascular development defects (31). To support both electrical and mechanical activities, the heart needs a continuous energy supply which are mainly produced by mitochondrial oxidative phosphorylation under normal circumstances (31). A growing number of studies have also shown that miRNAs played crucial roles in the diseased heart by regulating glucose metabolism.

Roles of miRNAs in Glucose Transport in Heart

Glucose has been proven to be transported into cardiomyocytes by the glucose transporters, glucose transporter 4 (GLUT-4) or GLUT-1, in the sarcolemma (32). In response to various stresses, such as insulin stimulation, increased energy demand, or ischemia, GLUT-4 and GLUT-1 are transported from intracellular vesicles to the sarcomembrane to increase the rate of glucose uptake and glucose transport (33, 34). Moreover, previous study has shown that the expression of GLUT4 in cardiomyocytes could be regulated by miRNAs. Lu et al. found that overexpression of miR-223 increased glucose uptake *via* increasing the GLUT4 protein expression (35). It is worth mentioning that whether miR-223 regulate glucose uptake in cardiomyocytes only by targeting GLUT4 is not clear and remains to be further studied.

Cardiomyocyte hypertrophy, which is characterized by increased size of cardiomyocytes, is one of the compensatory mechanisms of various CVDs (36). Changes in cardiac energy metabolism and substrate utilization are hallmarks of a hypertrophied heart, including increased dependence on glucose, reduction in fatty acid oxidation rate, and decreased high-energy-phosphate content (37). The shift of substrate preference from fatty acid to glucose is therefore considered beneficial in the hypertrophied heart because glucose has a higher oxygen efficiency for ATP production (38). Moreover, many studies suggested that promoting glucose utilization in the hypertrophied heart could be beneficial (39, 40). Studies were performed to explore whether miRNAs affect glucose transport in the hypertrophic cardiomyocytes. Takahiro et al. found that miR-133 decreased the protein level of KLF15 and the level of its downstream target GLUT4, which was involved in metabolic control in the hypertrophic cardiomyocytes (41). Interestingly, Trotta et al. also found the melanocortin 5 receptor agonism reduced the ratio of GLUT1/GLUT4 glucose transporters on the cell membranes and increased the intracellular PI3K activity in the hypertrophic H9c2 cells by decreasing of the levels of miR-133a (42). The important roles of miR-133 in cardiomyocyte glucose transport were confirmed in different models of cardiac

hypertrophy. Moreover, Yang et al. found that miR-200a-5p could disturb glucose metabolism by inhibiting selenoprotein n (Seln), selenoprotein t (Selt), selenoprotein 15 (Sep15), and selenoprotein p1 (Sepp1) expression to alter glucose transport, which eventually induce cardiomyocyte hypertrophy (43).

Diabetic cardiomyopathy is a metabolism-related heart disease, which is characterized by clinical heart failure and diastolic relaxation abnormalities in the early stage in the absence of dyslipidemia, hypertension, and coronary artery disease in the advanced stage (43). Due to decreased protein level of cardiac GLUT-4, cardiac glucose uptake is reduced despite hyperglycemia which could also contribute to the impaired myocardial glucose utilization in diabetes (8). Li et al. (44) revealed that the level of let-7 was increased in the myocardium of diabetic rats compared with non-diabetic rats, whereas improved glucose uptake by inhibiting of the let-7 family miRNAs through GLUT4 pathways. Similarly, Ju et al. (45) found miR-150 reduced the glucose utilization by decreasing the translocation and expression of GLUT-4 in the insulin-resistant cardiomyocytes.

In conclusion, miRNAs play important roles in the glucose transport in cardiomyocytes under both pathological and physiological processes (**Figure 1A**).

Roles of miRNAs in Glycolysis in the Heart

After glucose transport into cardiomyocytes, the first step of glucose catabolism is glycolysis, which produces ATP (46). Although, cardiomyocytes use ATP produced by glucose through the process of glycolysis is limited under normal physiological conditions, glycolysis is thought to facilitate some glucose molecules to be diverted into macromolecular precursors required for lipid, amino acid, and nucleotide biosynthesis and the pentose phosphate pathway (47). Importantly, Mallet et al. (48) suggested miRNAs play important roles in glycolysis of normal cardiomyocytes. They found miR-378 inhibited LDHA expression whereas miR-378* indirectly activated its expression to balance between oxidative phosphorylation and glycolysis in cardiomyocytes. However, the detail mechanism that miR-378 and miR-378* regulate the glycolysis pathway of cardiomyocytes under physiological conditions remains to be further studied.

Most cancer cells rely on aerobic glycolysis, a phenomenon known as the Warburg effect, which differs from the fact that normally differentiated cells rely primarily on mitochondrial oxidative phosphorylation to generate energy for cellular process (47). Similarly, under pathological conditions of the heart, the level of glycolysis would change in contrast to normal physiological state in the heart (49).

During the early stage of myocardial ischemia, glycolysis produces ATP and maintains ionic homeostasis, providing a beneficial effect (50). However, under severe ischemia, glycolysis becomes more harmful than beneficial (51). Importantly, multiple studies have shown that miRNAs played two sides function in glycolysis to regulate cardiac function after myocardial ischemia. On one hand, by performing loss- and gain-of-function experiments and glycolysis stress test, Lei et al. (52) detected that miR-27a-3p restoration enhanced cell viability, depleted cell apoptosis, and promoted glycolysis by targeting TNFR-associated factor 5 (TRAF5) in hypoxia-induced AC16 cells. Borden et al. used AAV delivery system to deliver miR-294 in mice and measured oxygen consumption rates (OCR) and extracellular acidification rates (ECAR). They found that miR-294 could significantly promote proliferation of cardiomyocytes and enhance oxidative phosphorylation and glycolysis that lead to improved cardiac function by targeting Wee1/CyclinB-CDK1 complex after myocardial infarction (53). Bartman et al. (54) performed loss- and gain-of-function experiments and measured ECAR, which revealed that the upregulation of miR-21 facilitated glycolysis and cardioprotection through Per2-dependent mechanisms in myocardial ischemia. Pyruvate dehydrogenase kinase 1 (PDK1), a phosphorylate kinase, phosphorylates pyruvate dehydrogenase leading to elevated anaerobic glycolysis. Zhu et al. (55) observed that miR-138 promoted mitochondrial respiration and inhibited glycolysis through directly targeting PDK1 by measuring lactate product, ECAR, and glycolysis key enzyme, which protected against cardiac cell dysfunction during ischemia. On the other hand, many studies have also shown that miRNAs play a key role in glycolysis to deteriorate cardiac function after myocardial ischemia. Fan et al. showed that miR-125b abolished the beneficial effects of lncRNA-XIST in activating glucose metabolism and cardiomyocyte protection under hypoxia by directly targeting hexokinase 2 (HK2), the key enzyme of glycolysis (56). Similarly, Zhang et al. (57) found that miR-34a inhibited the restoration of glycolysis in dysfunctional cardiomyocytes during ischemia reperfusion (I/R) injury. Moreover, Rane et al. (58) detected that miR-199a was rapidly downregulated in cardiomyocytes and the expression of HK2 and pyruvate kinase-M2 (Pkm2) were enhanced during I/R injury.

It is well-known that there is a prominent metabolic shift from fatty acid oxidation to glucose utilization during cardiac hypertrophy and pathological remodeling, which is associated with an increase in glycolysis in the hypertrophied heart (59, 60). Moreover, it was suggested that the elevation of glycolysis during cardiac hypertrophy and pathological remodeling was through the activation of fructose 2,6-BP and phosphofructokinase-1 (PFK1) in response to cardiac pressure overload (59, 60). It is worth mentioning that miR-135 was found to target PFK1 and inhibit aerobic glycolysis in pancreatic cancer cell, which indicated the possible functions of miRNAs in cardiomyocyte glycolysis (61). However, the role of miRNAs in glycolysis during cardiomyocyte hypertrophy has not been fully revealed (**Figure 1B**).

Roles of miRNAs in Aerobic Oxidation of Glucose in the Heart

Glucose can be converted to pyruvate by glycolysis pathway. As the end-product of glycolysis, pyruvate is ultimately transported into mitochondria and is critical for mitochondrial ATP generation. In mitochondria, pyruvate is the main fuel input to drive several major biosynthetic pathways across the citrate cycle and enhance the carbon flux of the citrate cycle (62).

In humans, the mitochondrial pyruvate carrier (MPC), formed by two paralogous subunits, MPC1 and MPC2, is required to deliver pyruvate from the mitochondrial

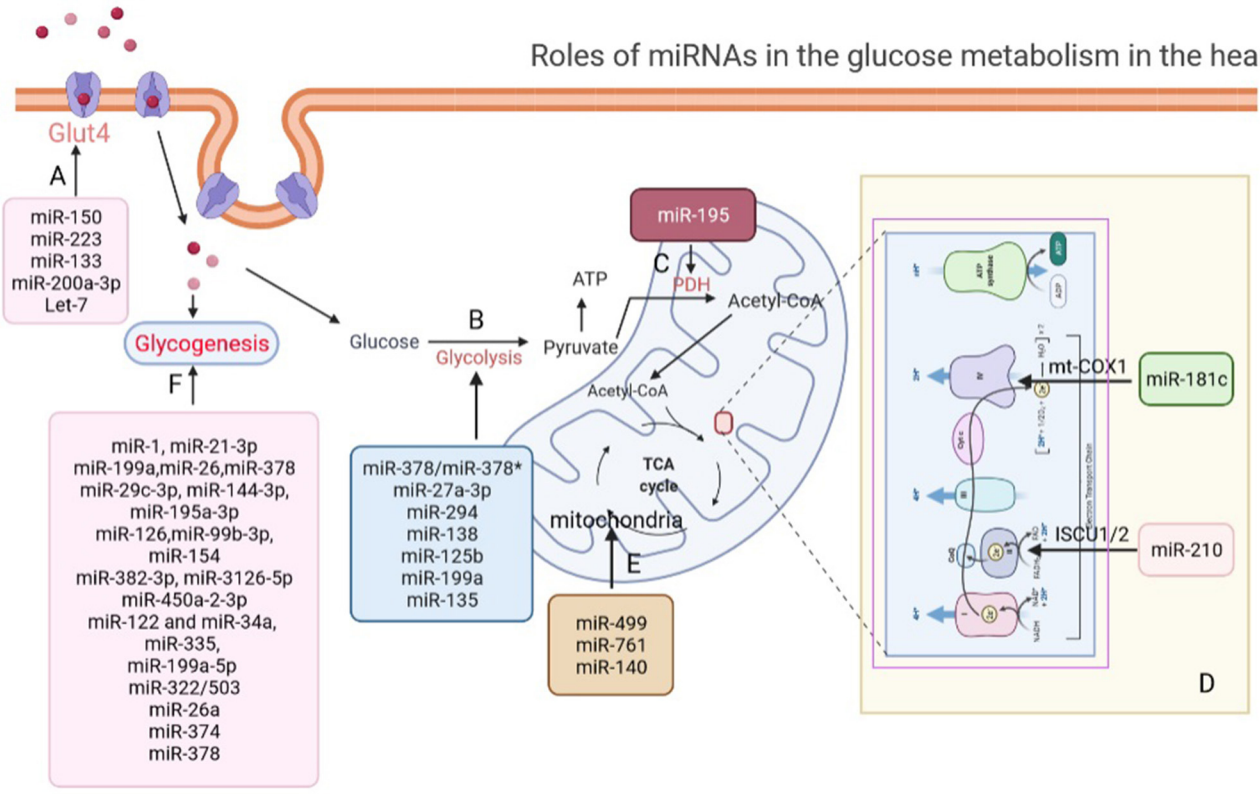

FIGURE 1 | Roles of miRNAs in glucose metabolism in the heart. **(A)** miRNAs regulate glucose transport *via* modulating the expression of GLUT4 in the heart. **(B)** miRNAs participate in glycolysis regulation in the heart. **(C)** miR-195 increased acetylation of PDH to promote pyruvate and NAD^+ convert into acetyl-CoA. **(D)** miR-181c and miR-210 involve in electron chain complex remodeling in cardiomyocytes by targeting and suppressing mt-COX1 and ISCU1/2. **(E)** miR-499, miR-761, and miR-140 regulate aerobic glucose oxidation by directly affecting mitochondrial function in the heart. **(F)** miRNAs regulate glycogenesis in the heart. GLUT4, glucose transporter type 4; PDH, pyruvate dehydrogenase complex; mt-COX1, cytochrome *c* oxidase subunit 1; ISCU1/2, iron-sulfur cluster assembly proteins.

intermembrane space to the mitochondrial matrix (63). In response to cold and heat stress of common carp by performing high-throughput sequencing, Sun et al. found that miRNAs might regulate the expression of MPC in the liver of fish (64). However, the effects of miRNAs on MPC expression and function in the heart remained to be further explored.

After passing through MPC, pyruvate will be oxidized into carbon dioxide by oxidative phosphorylation to ultimately support the generation of ATP (65). Subsequently, pyruvate and NAD^+ are irreversibly converted into acetyl-CoA, NADH, and carbon *via* the pyruvate dehydrogenase complex (PDH), which serves for bridging glycolytic metabolism in cytoplasm with oxidative phosphorylation and citric acid cycle (66). Importantly, Zhang et al. (67) detected that the expression of miR-195 was increased in failing myocardium, which downregulated the expression of SIRT3 by enhancing global protein acetylation, including PDH complex and ATP synthase directly targeting 3′-untranslated regions that were essential for cardiac energy metabolism (**Figure 1C**).

In the diseased heart, it was proven that the activity levels of oxidative respiratory chain complex would also change (68). Das et al. indicated that the expression of miR-181c was activated under hypoxic conditions of HF and suppressed cytochrome *c* oxidase subunit 1 (mt-COX1) to involve in electron chain complex IV remodeling in cardiomyocytes, which in turn increased the production of ROS in the heart (69). Similarly, miR-210 suppressed iron-sulfur cluster assembly protein ISCU1/2 expression, which is a chaperone to assemble iron-sulfur clusters and transport these clusters within the functional position in the cell, in hypoxic conditions of heart (70) (**Figure 1D**).

The tricarboxylic acid (TCA) cycle, a central route for oxidative phosphorylation in cells, depends on the oxidative respiratory chain to fulfill bioenergetic, biosynthetic, and redox balance requirements (71). The oxidative respiratory chain, containing four complexes, establishes an electrochemical gradient over the inner membrane to connect the transport of electrons to oxygen for ATP synthesis (72). In a healthy heart, the various complexes of the oxidative respiratory chain perform their respective functions to maintain the oxidative phosphorylation of glucose supporting cardiomyocytes. Moreover, it has been suggested that many miRNAs play a critical role in regulating mitochondrial function in the heart (**Figure 1E**). The downregulation of miR-140, as well as the

overexpression of miR-499 or miR-761, e.g., could prevent apoptosis and mitochondrial fission in cardiomyocytes *via* regulating mitochondrial fusion/fission-related proteins which led to cardiomyocyte apoptosis, mitochondrial fragmentation, and myocardial infarction (73, 74).

Roles of miRNAs in Glycogenesis in the Heart

In addition to the glucose consumed by normal metabolism, the excessive glucose can be converted to glycogen for storage through the glycogen synthesis pathway in the heart (75). Cardiac glycogen is an important source of glucose to support high-energy demands of a normal heart (76). Several studies have revealed that miRNAs played an important role in maintaining the balance of glycogen synthesis in the heart. Wei et al. (77), e.g., suggested that deletion of miR-1s led to a large portion in upregulated genes which associated with the cardiac fetal gene programing including glycolysis, cell proliferation, fetal sarcomere-associated genes, and glycogenesis by massively parallel sequencing. Moreover, they found that cardiac-specific overexpression of Errβ, the primary target of miR-1, could induce glycogen storage, cardiac dilation, and sudden cardiac death.

Several key enzymes such as glycogen synthase kinase-3α (GSK3α) and glycogen synthase kinase-3β (GSK3β) are involved in glycogen synthesis (78). It was reported that miRNAs could target GSK3β in some cardiac pathological processes, such as myocardial I/R injury, cardiac hypertrophy, and cardiac fibrosis, which suggested that miRNAs might be involved in glycogen synthesis. Our previous study, e.g., showed that miR-21-3p suppressed HDAC8 expression and decreased phospho-Akt and phospho-Gsk3β expression to attenuate cardiac hypertrophy (79). Moreover, miR-199a (80), miR-26 (81), miR-378 (82), miR-29c-3p, miR-144-3p, miR-195a-3p (83), and miR-126 (84) were reported to target GSK3β in direct or indirect manners during the occurrence and development of pathological cardiac hypertrophy, respectively. Meanwhile, miR-99b-3p (85), miR-154 (86), miR-382-3p, miR-3126-5p, and miR-450a-2-3p (87) were also found to target GSK3β in the pathological process of myocardial fibrosis. miR-122, miR-34a (88), miR-335 (89), miR-199a-5p (90, 91), miR-322/503 (92), miR-26a (93–95), miR-374 (96), and miR-378 (97) were found to target GSK3β in I/R injury model (**Figure 1F**). However, these studies did not explicitly indicate that these miRNAs were participated in cardiomyocyte glycogen synthesis during these cardiac pathological processes (**Table 1**).

ROLES OF miRNAs IN LIPID METABOLISM IN THE HEART

The heart uses ketone bodies, lactate, glucose, fatty acids, and amino acids as energy-providing substrates, among which more than 70% of all substrates are derived from fatty acids to generate ATP in adult heart (98). Moreover, many studies indicate that miRNAs are essential for lipid metabolism in the heart.

Roles of miRNAs in Fatty Acids Transport in the Heart

Fatty acids (FAs) from albumin or lipoprotein triacylglycerol enter cardiomyocytes through passive diffusion or by protein carrier including fatty acid translocase (FAT)/CD36, fatty acid transport protein (FATP), and plasma membrane isoform of fatty acid-binding protein (FABPpm) (99, 100). Importantly, CD36 could translocate FAs across the membrane of cardiac myocytes. Many studies suggested that 50–60% of FA uptake and oxidation in heart was facilitated by FAT/CD36-mediated transport (101, 102). Unlike FATP or FABPpm, in the regulatory control of FA uptake, CD36 can translocate among the intracellular endosome, the sarcolemmal membrane, and the membrane to promote FA uptake (101, 102).

Many studies have reported that different miRNAs targeted CD36 mRNA and regulated its expression at the posttranscriptional level in a tissue-specific manner (103, 104). For example, in the process of bone marrow cell differentiation to the monocytic-macrophage line, Zhou et al. (105) reported that CD36 was increased and its expression level was associated with seven miRNAs, including miR-134, miR-130a, miR-199a, miR-141, miR-152, miR-363, and miR-342-3p. During the erythropoiesis, miR-26a, miR-22, miR-16, and miR-223 were detected to correlate with the level and appearance of CD36 as an erythroid surface antigen by performing the expression profiling of miRNAs (106). It should be noticed that the role of miRNAs targeting CD36 to regulate FA transport in the normal heart remains to be further investigated (**Figure 2A**).

In the cardiac pathological processes, the FA transport would be changed. During diabetic cardiomyopathy, e.g., energy source will be shifted from glucose to FAs and the FA transport is enhanced to meet the increasing demand of ATP (107). However, lipid accumulation in cardiomyocytes might eventually lead to lipid toxicity that promote contractile abnormalities and cell death (108, 109). Therefore, exploring whether miRNAs are involved in FA transport in diabetic hearts might be helpful in discovering potential therapeutic strategies for diabetes-induced cardiac dysfunction (**Figure 2B**). Our previous research showed that miR-320 could target the CD36 promoter directly resulting in increased transportation of FAs into diabetic cardiomyocytes *via* enhancing CD36 transcription by forming a complex with Ago2 (13). In addition, Xu et al. (110) also found miR-200b-3p expression was significantly reduced in diabetic cardiomyopathy tissues and cells, which could target CD36 directly to reduce cardiomyocytes apoptosis in diabetic cardiomyopathy.

Roles of miRNAs in Fatty Acid Oxidation in the Heart

FAs are the main energy source in adult heart. Acyl-CoA synthetases (ACS) activate cytoplasmic FAs to acyl-CoA esters and then imported into mitochondrion by two acyltransferases, carnitine acyl-carnitine translocase (CACT) and carnitine palmitoyl transferases 1 and 2 (CPT1 and CPT2). Acyl-CoAs are degraded *via* β-oxidation, finally producing acetyl-CoA to fuel the tricarboxylic acid (TCA) cycle inside the mitochondrion (111). Impaired fatty acid oxidation (FAO) led to the decrease of

TABLE 1 | Roles of microRNAs in the glucose and lipid metabolism in the heart.

miRNA(s)	Validated targets	Key observation	References
miR-223	Glucose transporter type 4 (GLUT4)	Regulate glucose uptake in cardiomyocytes	Cardiovasc Res. 2010;86:410
miR-133	Kruppel-like factor 15 (KLF15)	Reduce the level of the downstream target GLUT4	Biochem Biophys Res Commun. 2009;389:315
miR-133a	Glucose transporter type 1/4 (GLUT1/GLUT4)	Increase GLUT1/GLUT4 glucose transporters ratio on the cell membranes	Front Physiol. 2018;9:1475
miR-200a-5p	Stress-related selenoproteins	Lead to glucose metabolism disorder	J Cell Physiol. 2019;234:4095
let-7	Glucose transporter type 4 (GLUT4)	Inhibition of the let-7 family microRNAs improves glucose uptake	Ann Thorac Surg. 2016;102:829
miR-150	Glucose transporter type 4 (GLUT4)	Reduce the glucose utilization	Acta Biochim Biophys Sin. 2020;52:1111
miR-378/miR-378*	Lactate dehydrogenase A (LDHA)	Balance between oxidative phosphorylation and glycolysis in cardiomyocytes	Mol Cell Proteomics. 2014;13:18
miR-27a-3p	TNFR-associated factor 5 (TRAF5)	Promote glycolysis of hypoxia-induced AC16 cells	Life Sci. 2020;262:118511
miR-294	Wee1/CyclinB-CDK1 complex	Enhance oxidative phosphorylation and glycolysis after myocardial infarction	Circ Res. 2019;125:14
miR-21	Period circadian clock 2 (PER2)	Facilitates glycolysis and cardioprotection	PLoS ONE. 2017;12:e0176243
miR-138	Pyruvate dehydrogenase kinase 1 (PDK1)	Inhibit glycolysis but promotes mitochondrial respiration	Biosci Rep. 2017;37
miR-125b	Hexokinase 2 (HK2)	Regulation of lncRNA-XIST in activating glucose metabolism	In vitro Cell Dev Biol Anim. 2020;56:349
miR-34a	Actate dehydrogenase-A (LDHA)	Inhibited the restoration of glycolysis in dysfunctional cardiomyocytes	Biosci Rep. 2017;37
miR-199a	Hexokinase-2 (Hk2); pyruvate kinase-M2 (Pkm2)	Facilitate the upregulation of glycolysis	EMBO J. 2015;34:2671, Circ Res. 2009;104:879
miR-135	Phosphofructokinase-1 (PFK1)	Inhibit aerobic glycolysis in pancreatic cancer cell	Nat Commun. 2019;10:809
miR-195	Pyruvate dehydrogenase complex (PDH)	Increase acetylation of PDH and ATP synthase	Circulation. 2018;137:2052
miR-499, miR-761, miR-140	Mitochondrial fusion/fission proteins	Prevent mitochondrial fission and apoptosis in cardiomyocytes	Free Radic Biol Med. 2013;65:371 PLoS Genet. 2010;6:e1000795
miRNA-181c	Cytochrome *c* oxidase subunit 1 (mt-COX1)	Increase production of ROS in hypoxic conditions of heart	Circ Res. 2012;110:1596
miR-210	Iron-sulfur cluster assembly proteins ISCU1/2	Suppress the iron-sulfur cluster assembly proteins ISCU1/2	Cell Death Dis. 2014;5:e1090
miR-1s	Estrogen-related receptorβ (ERRβ)	Lead to glycogen storage, cardiac dilation, and sudden cardiac death	Cell Res. 2014;24:278
miR-21-3p	Histone deacetylase 8 (HDAC8)	Attenuate cardiac hypertrophy	Cardiovasc Res. 2015;105:340
miR-199a	Glycogen synthase kinase-3β (GSK3β)	Involved in glycogen synthesis	Cell Death Differ. 2017;24:1205
miR-26			J Cardiovasc Pharmacol. 2013;62:312
miR-378			J Biol Chem. 2013;288:11216
miR-29c-3p, miR-144-3p, and miR-195a-3p			J Cell Physiol. 2016;231:1771
miR-126			Cell Mol Life Sci. 2013;70:4631
miR-99b-3p	Glycogen synthase kinase-3β (GSK3β)	Involved in the pathological process of myocardial fibrosis	Acta Pharmacol Sin. 2021;42:715
miR-154			Eur Rev Med Pharmacol Sci. 2018;22:2052
miR-382-3p, miR-3126-5p, and miR-450a-2-3p			J Thorac Dis. 2020;12:5617
miR-122 and miR-34a	Glycogen synthase kinase-3β (GSK3β)	Involved in I/R injury	Biol Trace Elem Res. 2020;196:1
miR-335			J Cell Mol Med. 2019;23:8420
miR-199a-5p			Mol Med Rep. 2019;19:5335-5344 Cell Physiol Biochem. 2016;39:1021
miR-322/503			Am J Physiol Cell Physiol. 2019;317:C253

(Continued)

TABLE 1 | Continued

miRNA(s)	Validated targets	Key observation	References
miR-26a			Eur Rev Med Pharmacol Sci. 2020;24:2659 Yonsei Med J. 2018;59:736 Eur Rev Med Pharmacol Sci. 2019;23:7073
miR-374			Cell Physiol Biochem. 2018;46:1455
miR-378			Cardiovasc Res. 2013;100:241
miR-130a, miR-134, miR-141, miR-199a, miR-363, miR-152, and miR-342-3p	Fatty acid translocase (FAT)/CD36	Involved in fatty acids transport	Oncotarget. 2016;7:28806
miR-16, miR-22, miR-26a, and miR-223	Fatty acid translocase (FAT)/CD36	Regulate fatty acid transport	Exp Hematol. 2007;35:551
miR-320	Fatty acid translocase (FAT)/CD36	Increase transportation of fatty acid into diabetic cardiomyocytes	Circ Res. 2019;125:1106
miR-200b-3p	Fatty acid translocase (FAT)/CD36	Regulate fatty acids transport and activate PPAR-γ signaling pathway	J Cell Biochem. 2019;120:5193
miR-197, miR-146b	Fatty acid binding protein (FABP4)/carnitine palmitoyltransferase 1B (CPT1B)	Suppress genes that drive FAO in primary cardiomyocytes	Sci Transl Med. 2018;10
miR-30c	Peroxisome proliferator–activated receptors (PPARs)	Improved lipid and glucose utilization, reduce excessive ROS production	Cardiovasc Diabetol. 2019;18:7
miR-483-3p	Growth/differentiation factor-3 (GDF-3)	Modulated the capacity of adipocytes to store lipids and differentiate	Cell Death Differ. 2012;19:1003
miR-107	Cyclin-dependent kinase 6 (CDK6)	Attenuate differentiation and lipid accumulation	Mol Cell Endocrinol. 2019;479:110
miR-494-3p	Peroxisome proliferator-activated receptor γ (PPARγ)	Prevented TG synthesis, uptake, hydrolysis, and storage in the heart	Eur Heart J. 2019;40:997
miR-451	Calcium-binding protein 39 (Cab39)	Ameliorate palmitate-induced lipotoxicity in cardiomyocytes	Circ Res. 2015;116:279

the capacity for ATP production and accumulation of toxic lipid intermediates in the heart, while enhanced FAO was associated with increased oxidative stress (2, 112).

Ekaterina et al. found that miR-146b and miR-197 were upregulation in the failing right ventricular of pulmonary arterial hypertension patients and suppressed genes that drive FAO (CPT1b and FABP4) in primary cardiomyocytes (113). Peroxisome proliferator-activated receptors (PPARs), a class of ligand-activated nuclear receptors, control FAO enzymes expression, while PPARγ coactivator-1β (PGC-1β) is an important coactivator of PPARs (114–116). Our previous work showed that exogenous miR-30c delivery improved lipid and glucose utilization, reduced excessive ROS production and thereby attenuated cardiac dysfunction *via* PGC-1β/PPARα signals in a mouse model of diabetic cardiomyopathy (117) (**Figure 2C**).

Roles of miRNAs in Lipid Storage in the Heart

Cardiomyocytes could reserve multiple energy substrates, among which accumulation of non-polar and polar lipids could activate intracellular signaling pathways (98). The FAs are stored as triacylglycerol (TAG) in lipid droplets. The accumulation of excess lipids is prevented by the physiological balance of lipid uptake and oxidation (98). However, various processes that affect this balance might lead to hypoxia, obesity, diabetes mellitus, sepsis, cardiac dysfunction, and even heart failure.

Many studies suggested that miRNAs play an important role in the lipid storage (**Figure 2D**). For example, Pegoraro et al. (118) suggested that miR-133a, miR-133b, miR-1, and miR-206, might be useful biomarkers for neutral lipid storage disease with myopathy. Ferland-McCollough et al. (119) demonstrated that miR-483-3p modulated the capacity of adipocytes to store lipids and differentiation by manipulating growth/differentiation factor-3 expression. Moreover, overexpression of miR-107 attenuated differentiation and lipid accumulation in pre- and mature human adipocytes of Simpson-Golabi-Behmel syndrome *via* regulating CDK6 and Notch signaling (120).

Considering the heart, metabolic cardiomyopathy is the main cause of heart failure in obese patients characterized by lipotoxic damage and intramyocardial triglyceride (TG) accumulation (121). JunD could enable transcription of genes involved in TG synthesis, uptake, hydrolysis, and storage by directly binding to PPARγ promoter. Costantino et al. found that miR-494-3p prevented TG synthesis, uptake, hydrolysis, and storage in the heart from diet-induced obese mice by suppressing JunD/PPARγ signaling, which was also associated with myocardial left ventricular (LV) dysfunction and TG

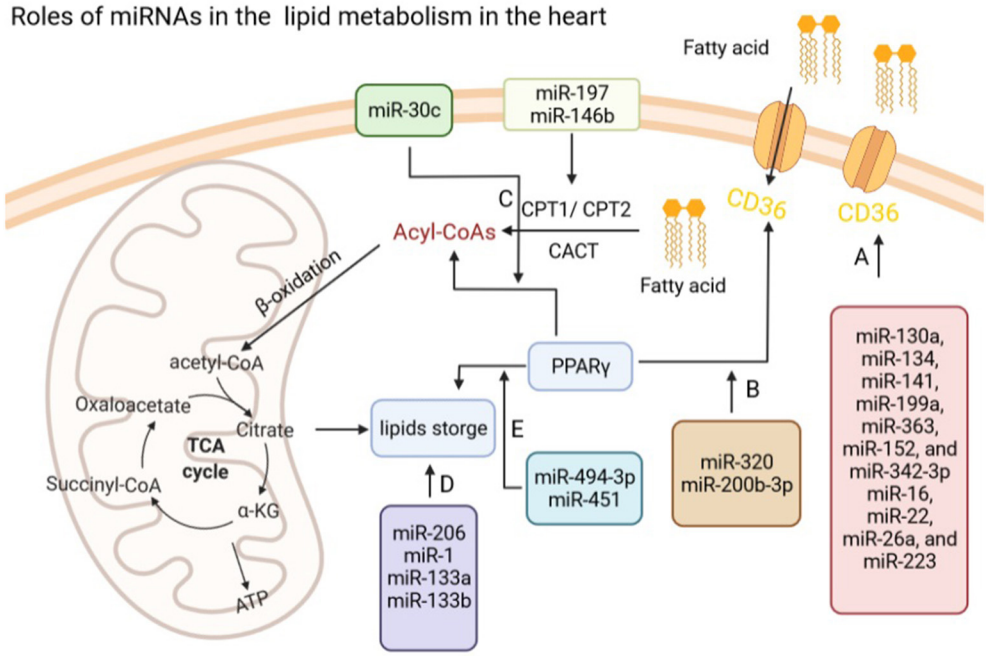

FIGURE 2 | Roles of miRNAs in lipid metabolism in the heart. **(A)** miRNAs regulate fatty acids transport *via* modulating the expression of CD36 in the heart. **(B)** miR-320 and miR-200b-3p regulate fatty acid transport *via* PPARγ. **(C)** miR-197 and miR-146b modulate Acyl-CoAs by CPT1/2, while miR-30c regulates the production of Acyl-CoAs through targeting PPARγ. **(D)** miR-206, miR-1, and miR-133a/b influence lipids storage in the heart. **(E)** miR-494-3p and miR-451 regulate lipids storage *via* modulating the expression of PPARγ in the heart. CD36, fatty acid translocase (FAT)/CD36; CPT1/2, carnitine palmitoyltransferase 1/2; PPARγ, peroxisome proliferator-activated receptors (PPARγ).

accumulation (122). Besides, increased miR-451 were reported in the mouse heart with high-fat diet (HFD), while loss of miR-451 alleviated palmitate-induced lipotoxicity in cardiomyocytes *via* inhibiting calcium-binding protein 39 (Cab39), which is an AMP-activated protein kinase (AMPK) upstream kinase (123) (**Figure 2E**).

POTENTIAL miRNA-BASED THERAPY IN CVDs

Therapeutic strategies targeting miRNAs for CVDs have been highlighted in many studies (124). For instance, miR-15 family was found to be consistently upregulated during postnatal development of the heart and CVDs, knockdown of the miR-15 family by anti-miRNAs could increase the number of mitotic CMs and reduce the infarct size after ischemia-reperfusion injury in neonatal mice (125–127). As miRNAs can affect different genes simultaneously to alter glucose and lipid metabolism in the pathological processes of diseased heart, they attracted increasing attentions for potential therapeutic targets and treatments (128, 129).

Various strategies were developed for the delivery miRNAs into cardiomyocytes. A novel technique called ultrasound-mediated sonoporation, which carry genetic material to target sites, using albumin-shelled microbubbles, has been considered for miRNA delivery in the myocardium (130). Importantly, Su et al. (131) has used this approach to prevent coronary microembolization-induced cardiac dysfunction by delivering hsa-miR-21-5p in pig myocardium by ultrasound-targeted microbubble. In addition, local injection is a nicely method to overcome the systemic effects on other organs and obtain better transfection efficiency. Many trials have attempted to inject miRNAs *via* intramyocardial or intracoronary directly during heart surgeries (132). Moreover, with the development of new techniques such as positron emission tomography and electromechanical mapping, clinicians can achieve high efficiency around the site of injection by better targeting site of myocardial ischemia (7, 133).

However, there are limitations of miRNA-based therapy, which should be solved before clinical use. Compared with the physiological miRNA expression levels, gain- and loss-of function assays using synthesized oligonucleotides often induce very high abundance of miRNA into the cells, which may lead to irreproducible and misguided interpretation of the results. Most of miRNA studies have been focused on site-specific phenotypic effects *in vivo*, which might ignore the signaling pathways responsible for their effects on other organs and the whole genome targets. Moreover, the off-target effects cannot be ignored. Thus, studies are needed to use both site-specific deliveries and systemic approach to focus on the *in vivo* miRNA effects.

CONCLUSION

An increasing number of studies have provided important clues of miRNAs and their potential roles in the glucose and lipid metabolism in CVDs. Current studies revealed the biological and pathological process that miRNAs involved, which might broaden the treatment strategies for CVD patients with or without metabolism disorders. In this review, we systematically described the effects of miRNAs on the glucose and lipid metabolism in cardiomyocytes and compared the advantages and limitations in miRNA-based therapy in CVDs. In addition, we provided a summary table to better illustrate the various miRNAs that participate in glucose and lipid metabolism in the heart. However, considering the multiple targets of one certain miRNA, there are still uncertainties that remain regarding the systemic effects of miRNAs on other organs and biological processes. In summary, miRNAs play critical roles in the regulation of glucose and lipid metabolism in CVDs. MiRNAs and miRNA-based therapies are one of the most promising innovative applications in CVD treatment in the future.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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LncRNA PVT1 Knockdown Ameliorates Myocardial Ischemia Reperfusion Damage via Suppressing Gasdermin D-Mediated Pyroptosis in Cardiomyocytes

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Objective: Myocardial ischemia reperfusion (I/R) damage is a life-threatening vascular emergency after myocardial infarction. Here, we observed the cardioprotective effect of long non-coding RNA (lncRNA) PVT1 knockdown against myocardial I/R damage.

Methods: This study constructed a myocardial I/R-induced mouse model and a hypoxia/reoxygenation (H/R)-treated H9C2 cells. PVT1 expression was examined via RT-qPCR. After silencing PVT1 via shRNA against PVT1, H&E, and Masson staining was performed to observe myocardial I/R damage. Indicators of myocardial injury including cTnI, LDH, BNP, and CK-MB were examined by ELISA. Inflammatory factors (TNF-α, IL-1β, and IL-6), Gasdermin D (GSDMD), and Caspase1 were detected via RT-qPCR, western blot, immunohistochemistry, or immunofluorescence. Furthermore, CCK-8 and flow cytometry were presented for detecting cell viability and apoptosis.

Results: LncRNA PVT1 was markedly up-regulated in myocardial I/R tissue specimens as well as H/R-induced H9C2 cells. Silencing PVT1 significantly lowered serum levels of cTnI, LDH, BNP, and CK-MB in myocardial I/R mice. H&E and Masson staining showed that silencing PVT1 alleviated myocardial I/R injury. PVT1 knockdown significantly lowered the production and release of inflammatory factors as well as inhibited the expression of GSDMD-N and Caspase1 in myocardial I/R tissue specimens as well as H/R-induced H9C2 cells. Moreover, silencing PVT1 facilitated cell viability and induced apoptosis of H/R-treated H9C2 cells.

Conclusion: Our findings demonstrated that silencing PVT1 could alleviate myocardial I/R damage through suppressing GSDMD-mediated pyroptosis *in vivo* and *in vitro*. Thus, PVT1 knockdown may offer an alternative therapeutic strategy against myocardial I/R damage.

Keywords: lncRNA, PVT1, myocardial ischemia reperfusion, pyroptosis, gasdermin D

INTRODUCTION

Myocardial ischemia reperfusion (I/R) may trigger acute myocardial infarction, with increasing morbidity and morbidity in modern society and trending to be younger (1–3). Myocardial I/R represents an intricate pathological process in which the blood supply is restored following myocardial ischemia, leading to metabolic dysfunction as well as structural injury (4–6). The potential mechanism of myocardial I/R is of complexity, involving systematic networks (7). Thus, mitigating the injury mediated by myocardial I/R has attracted scholars' attention of globally.

Pyroptosis, a type of inflammatory cell deaths, has the characteristics of gasdermin D (GSDMD) or gasdermin E (GSDME)-mediated necrosis, activation of proinflammatory caspase-1 and excessive release of inflammatory factors [such as interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α)] (8). GSDMD-mediated pyroptosis of cardiomyocytes represents a critical event for myocardial I/R damage and caspase-11/GSDMD signaling is essential for this process (9). Suppressing GSDMD distinctly alleviates I/R-mediated myocardial damage via reducing pyroptosis of cardiomyocytes (10). Long non-coding RNA (LncRNA) is a class of non-coding RNA with >200 nt length (11). Growing evidence demonstrates that lncRNAs exert key roles in myocardial I/R via mediating different biological processes including pyroptosis (12). For instance, lncRNA H19 may initiate microglial pyroptosis as well as neuronal deaths in retinal I/R damage (13). LncRNA KLF3-AS1 may ameliorate cardiomyocyte pyroptosis and myocardial infarction (14). LncRNA GAS5 induces pyroptosis of cardiac fibroblasts through mediating NOD-like receptor protein 3 (NLRP3) (15). LncRNA MEG3 facilitates cerebral I/R injury via enhancing pyroptosis (16). Nevertheless, the influence of lncRNAs in regulating pyroptosis in cardiovascular diseases especially myocardial I/R damage is mostly unclear. Previous research has reported that lncRNA plasmacytoma variant translocation 1 (PVT1) up-regulation is involved in pathogenesis of cardiovascular diseases (17). Knockdown of PVT1 suppresses apoptosis of vascular smooth muscle cells as well as extracellular matrix destruction for abdominal aortic aneurysms (18). Inhibiting PVT1 alleviates atrial fibrosis and atrial fibrillation (19). Serum PVT1 levels are distinctly up-regulated in coronary artery disease and distinguish mild and severe patients (20). Nevertheless, it remains unknown about the influence and mechanisms of PVT1 on myocardial I/R. Here, our study found that lncRNA PVT1 was markedly up-regulated in myocardial I/R mouse model and hypoxia/reoxygenation (H/R)-induced cellular model. Knockdown of PVT1 alleviated myocardial I/R damage via suppressing GSDMD-mediated pyroptosis in cardiomyocytes.

Abbreviations: I/R, ischemia reperfusion; GSDMD, gasdermin D; GSDME, gasdermin E; IL-1β, interleukin 1β; IL-6, interleukin 6; TNF-α, tumor necrosis factor alpha; lncRNA, long noncoding RNA; NLRP3, NOD-like receptor protein 3; PVT1, plasmacytoma variant translocation 1; H/R, hypoxia/reoxygenation; shRNA, short hairpin RNA; NC, negative control; LAD, left anterior descending; RT-qPCR, Real-time quantitative reverse transcription PCR; ELISA, Enzyme-linked immunosorbent assay; LDH, lactate dehydrogenase; CK-MB, creative kinase isoenzyme MB; α-MHC, α-myosin heavy chain; β-MHC, β-myosin heavy chain; CCK-8, cell counting kit-8.

MATERIALS AND METHODS

Animals and Grouping

Totally, 50 C57BL/6 mice (Nanjing Junke Biological Engineering Co., Ltd.) were fed in a specific pathogen-free environment under a 12 h light/12 h dark cycle. Each animal experiment strictly followed the Guide for the Care and Use of Laboratory Animal by International Committees. The mice were randomly separated into the following five groups before surgery (10 mice/group): control group; sham operation group; I/R group; I/R + short hairpin RNA (shRNA) against negative control (sh-NC) group; I/R + shRNA against PVT1 (sh-PVT1) group. This study gained the approval of the Institutional Animal Care of Guangdong Provincial People's Hospital (GDREC2016255H).

Preparation of I/R Animal Model

After the mice were adaptively reared for 1 week, they were anesthetized by intraperitoneal injection of sodium pentobarbital (45 mg/kg). After anesthesia, there was no response in the clip toe test and the trachea was intubated. The ventilator parameters were set to synchronize with the respiratory rate of the anesthetized mice. Then, the endotracheal tube was connected to the ventilator. Before the operation, a physiological signal collection and processing system was used to record the rat's electrocardiogram. The chest was opened through the third intercostal space on the left edge of the sternum, and the left anterior descending (LAD) coronary artery was ligated with 7-0 silk thread. After 45 min, the ligature was loosened for reperfusion. No treatment was done for mice in the control group. This study only opened the chest of mice in the sham operation group, but threaded the corresponding part without ligating the LAD. After the operation, mice were continuously monitored by ECG for 15 min and placed on a heating pad at 30°C until they were awakened. 48 h before modeling, mice in the I/R + sh-NC and I/R + sh-PVT1 groups were injected by 10 μL sh-NC or sh-PVT1 (GenePharma, Shanghai, China) that was premixed with Lipofectamine 2000 reagent (Thermo Fisher Scientific, USA). The sequence of sh-PVT1 was as follows: 5′-CCUGCAUAACUAUCUGCUUTT-3′.

Sample Collection and Preparation

Under anesthesia, abdominal aortic blood was firstly collected. Then, the limbs were fixed, the chest was opened and the mouse's heart was obtained. After removing the auricle and vascular tissue, the heart was transected into two parts. Half of the tissue at the bottom of the heart was quick-frozen by liquid nitrogen, and then transferred to a −80°C refrigerator for subsequent experiments. Apical 1/2 tissue was fixed in 40 g/L paraformaldehyde and transferred to 70% ethanol solution on the second day. Then, through the Excelsior™ AS tissue processing system, the tissue was dehydrated by gradient ethanol, transparent by xylene and waxed in liquid paraffin. Subsequently,

the HistoCore Arcadia paraffin embedding system was used for pathological examination after embedding and retention.

Real-Time Quantitative Reverse Transcription PCR (RT-qPCR)

Total RNA was extracted from myocardial tissues and cells utilizing TRIzol (Beyotime, Shanghai, China) and cDNA was reverse transcribed with PrimeScript RT Master Mix (Servicebio, Wuhan, China). These primer sequences were as follows: PVT1: 5′-TGAGAACTGTCCTTACGTGACC-3′ (F), 5′-AGAGCACCAAGACTGGCTCT-3′ (R); IL-1β: 5′-CACCTCTCAAGCAGAGCACAG-3′ (F), 5′-GGGTT CCATGGTGAAGTCAAC-3′ (R); IL-6: 5′-GCTACAGCAC AAAGCACCTG-3′ (F), 5′-GACTTCAGATTGGCGAGGAG-3′ (R); TNF-α: 5′-GGCAGCCTTGTCCCTTGAAGAG-3′ (F), 5′-GTAGCCCACGTCGTAGCAAACC-3′ (R); GSDMD: 5′-CCAACATCTCAGGGCCCCAT-3′ (F), 5′-TGGCAAGTTTC TGCCCTGGA-3′ (R); GAPDH: 5′-CTGGGCTACACTGAGC ACC-3′ (F), 5′-AAGTGGTCGTTGAGGGCAATG-3′ (R). The expression of above targets was quantified with the $2^{-\Delta\Delta Ct}$ method, while GAPDH served as an internal control.

Enzyme-Linked Immunosorbent Assay (ELISA)

Abdominal aortic blood samples were centrifuged at 3,000 g. Then, the serum samples were harvested. Following the instructions of ELISA kits, brain natriuretic peptide (BNP; Biocompare, USA), cardiac troponin I (cTnI; Biocompare, USA) and IL-1β (Biocompare, USA) levels were tested in serum or cell supernatant. Through automatic biochemical analyzer, lactate dehydrogenase (LDH) and creative kinase isoenzyme MB (CK-MB) levels were examined in serum or cell supernatant.

Histological Analysis

The paraffin-embedded mouse myocardial tissue was sectioned to 5 μm. H&E staining and Masson staining were carried out following the kit instructions (Servicebio, Wuhan, China). The paraffin sections were deparaffinized to water. Then, H&E staining was successively performed. The sections were stained with hematoxylin for 5 min and rinsed by tap water, followed by being differentiated by differentiation solution for 3 s. After being rinsed by tap water, the sections returned to blue for about 3 s, rinsed by tap water and dehydrated by 85 and 95% ethanol in turn for 4 min. Afterwards, the sections were stained by eosin dye solution for 5 min and dehydrated with anhydrous ethanol for 3 times (5 min each time) and transparent by xylene twice for 2 min. Then, the sections were mounted with neutral gum. Masson staining was carried out according to the following procedures. The sections were stained through Masson A solution lasting 15 h as well as heated in a 65°C oven lasting 30 min. After being washed using tap water, the sections were stained by Masson B solution and C solution mixed dyeing for 1 min and differentiated with 1% hydrochloric acid alcohol lasting 1 min. Then, the sections were washed by tap water as well as stained utilizing Masson D solution lasting 6 min and immersed by Masson E solution for 1 min and Masson F solution for 15 s. Afterwards, the sections were differentiated by 1% glacial acetic acid for 3 times (8 s each time) and dehydrated with absolute ethanol for 3 times (5 min each time). After being transparent, the slides were mounted with neutral gum. All slides were scanned at 200× by TissueFAXS PLUS scanning system (Taborstrasse, Austria).

Western Blot

According to the instructions of the reagents, protein specimens were extracted from myocardial tissues, serum samples and cells through RIPA lysate. After centrifugation at 14,000 g, the supernatant was collected and mixed with the loading buffer. The protein was denatured by boiling at 100°C for 10 min. About 50 μg protein was electrophoresed in 10% SDS-PAGE (Beyotime, Shanghai, China), then transferred to 0.22 μm PVDF membrane (Merck KGaA, Germany). Afterwards, the membrane was incubated with primary antibodies against α-myosin heavy chain (α-MHC; 1/1,000; ab174640; Abcam, USA), β-myosin heavy chain (β-MHC; 1/1,000; ab170867; Abcam, USA), TNF-α (1/1,000; ab215188; Abcam, USA), IL-1β (1/1,000; ab254360; Abcam, USA), IL-6 (1/1,000; ab259341; Abcam, USA), GSDMD (1/200; ab219800; Abcam, USA), TLR4 (1/50; ab1355; Abcam, USA), MyD88 (1/1,000; ab219413; Abcam, USA), NLRP3 (1/1,000; ab263899; Abcam, USA), NF-κB phosphorylation p-p65 (1/1,000; ab76302, USA), NF-κB p65 (#8242, 1:1,000; Cell Signaling Technology, USA), Cleaved caspase-3 (1/5,000; ab21443; Abcam, USA), Bax (1/1,000; ab23247; Abcam, USA), Bcl-2 (1/1,000; ab259833; Abcam, USA), Caspase1 (1/1,000; ab207802; Abcam, USA), and GAPDH (1/1,000; ab9484; Abcam, USA) overnight at 4°C. The next day, the membrane was washed utilizing TBST as well as incubated with HRP-conjugated secondary antibodies (1/10,000; ab7090; Abcam, USA) lasting 1 h at room temperature. Then, the ECL substrate (Thermo Scientific, USA) was dropped on the membrane, followed by being exposed in the FluorChem™E chemiluminescence imaging system. The integrated optical density of the protein band was measured using ImageJ software (version 1.8.0).

Immunohistochemistry

Paraffin-embedded sections of myocardial tissue were baked in a 60°C incubator for 2 h. The sections were deparaffinized by xylene and hydrated with ethanol gradient. 3% hydrogen peroxide was added to eliminate endogenous peroxidase activity. After washing, antigen retrieval was carried out in PBS buffer. Then, the sections were blocked by goat serum for 15 min. The primary antibody against GSDMD (1/200; sc-393581; Santa Cruz Biotechnology, USA) and Caspase1 (1/1,000; ab207802; Abcam, USA) was added dropwise to the sections and incubated overnight at 4°C. After washing with PBS, the sections were incubated with secondary antibody (1/10,000; ab7090; Abcam, USA) lasting 2 h. Then, the color was developed with DAB color developing solution. The sections were counterstained by hematoxylin, dehydrated, transparent, and mounted. The results were investigated under a microscope, which were analyzed by Image Plus image analysis software.

Cell Culture, Treatment, and Transfection

H9C2 cells (ATCC, USA) were grown in DMEM cell culture medium containing 1.5 g/L $NaHCO_3$, 10% FBS, 1% glutamine, and 1% penicillin in a constant temperature incubator of 5% CO_2 at 37°C. For H/R, the cells were cultured in an environment of 95% N_2 and 5% CO_2 at 37°C lasting 4 h. After changing the medium, the cells were reoxygenated with 5% CO_2 at 37°C lasting 3 h. PVT1 sequence was designed and synthesized by GenePharma company (Shanghai, China), which was subcloned into pcDNA3.1 (Invitrogen, USA). The pcDNA3.1 vector served as a control. Lipofectamine 2000 (Invitrogen, USA) was utilized for transfection of plasmids (500 ng pcDNA3.1 vector (empty vector), 500 ng PVT1) into H9C2 cells for 48 h. The sense and antisense oligonucleotides of the sh-PVT1 (5′-CCUGCAUAACUAUCUGCUUTT-3′) were synthesized and cloned into the pENTR^{TM}/U6 vector (Invitrogen, USA). For silencing PVT1, H9C2 cells (5×10^4) were transfected by 1 μg sh-NC or 1 μg sh-PVT1 for 48 h via Lipofectamine 2000 reagent before H/R. After transfection for 48 h, above transfected cells were used for the subsequent experiments. Furthermore, the cells were treated with 25 nM Necrosulfonamide (NSA; abs814352; Absin, Shanghai, China), followed by PVT1 overexpression vector transfection and H/R treatment.

Cell Counting Kit-8 (CCK-8)

H9C2 cells in each group were planted onto a 96-well plate (1×10^4/well). Each group set eight multiple holes. A blank group was set, which was only added by culture medium without cells. Each well was incubated with 10 μL of CCK-8 solution (Dojindo, Japan) lasting 1 h at 37°C. After incubation, a microplate reader was utilized for measuring the absorbance at 450 nm wavelength.

Flow Cytometry for Apoptosis Detection

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) cell apoptosis kit (Beyotime, Shanghai, China) was utilized for detecting cellular apoptosis. H9C2 cells were collected and washed utilizing PBS. The cells were resuspended by 250 μL prepared binding buffer. The density of the cells was adjusted to 1×10^9/L. Then, 100 μL cell suspension was inoculated into a 5 mL flow tube. The cells were incubated with 5 μL Annexin V/FITC and 10 μL PI in the dark at room temperature. After 15 min, the apoptosis rate was detected by flow cytometry.

Immunofluorescence

H9C2 cells were seeded and climbed on the 6-well plate. When the cell fusion was about 80%, the cells were treated with H/R, transfection and drug treatment. Then, the cells were fixed by paraformaldehyde, permeabilized by PBS containing 0.1% TritonX-100, and blocked by BSA. Then, the sections were incubated with primary antibody against GSDMD (1/200; ab219800; Abcam, USA), followed by FITC-labeled fluorescent secondary antibody (1/200; ab7064; Abcam, USA). After the nucleus was stained with 1 μg/ml DAPI for 10 min, images were investigated under a confocal laser microscope.

Statistical Analysis

All analyses were presented utilizing Graphpad Prism software (version 7.0). Statistical differences between multiple groups were compared through one-way analysis of variance (ANOVA) followed by Tukey's test. The results were displayed as the mean ± standard deviation. Each experiment was carried out in triplicate. $P < 0.05$ was indicative of statistical significance.

RESULTS

LncRNA PVT1 Is Up-Regulated in Myocardial I/R Tissues and Its Knockdown Alleviates Myocardial I/R Damage

Here, this study constructed a myocardial I/R mouse model. Our results showed that lncRNA PVT1 displayed significant up-regulation in myocardial I/R tissues compared with sham myocardial tissues (**Figure 1A**). This indicated that PVT1 might participate in myocardial I/R progression. For investigating this influence of PVT1 on myocardial I/R damage, PVT1 was silenced by its shRNA. In **Figure 1A**, the expression of PVT1 was markedly inhibited by sh-PVT1 in myocardial I/R tissues compared with sh-NC. Indicators of myocardial injury including cTnI, LDH, BNP and CK-MB were examined by ELISA. As demonstrated by our data, serum levels of cTnI (**Figure 1B**), LDH (**Figure 1C**), BNP (**Figure 1D**), and CK-MB (**Figure 1E**) were markedly elevated in myocardial I/R mice compared with sham mice. But PVT1 knockdown significantly decreased serum cTnI, LDH, BNP and CK-MB levels in myocardial I/R models. To evaluate the effect of PVT1 knockdown on the morphology of I/R heart tissue, H&E and Masson staining of heart tissue was performed at the end of the intervention. The results of H&E staining showed that the myocardial fibers of the control group and the sham group were arranged regularly; there was no breakage or necrotic gap; and the myocardial cell nucleus was fusiform or oval (**Figure 1F**). For I/R and I/R + sh-NC groups, the myocardial fiber structure was damaged; the myocardial fiber was broken and dissolved; the intermuscular space was enlarged; there were inflammatory cellular infiltrations in the infarct. The destruction of myocardial fiber structure and infiltrations of inflammatory cells were improved in I/R + sh-BCRT1 group. As shown in Masson staining, the myocardial tissue fibers were neatly arranged, evenly stained, and there was no collagen in the control group and the sham group (**Figure 1G**). For I/R and I/R + sh-NC groups, the degree of myocardial fibrosis was obvious; myocardial cells were significantly reduced; and there was increased collagen. For I/R + sh-BCRT1 mice, myocardial fibrosis was markedly ameliorated; the reduction of myocardial cells was improved; and the collagen was decreased. These findings demonstrated that PVT1 knockdown could alleviate myocardial I/R injury.

LncRNA PVT1 Knockdown Alleviates Cytokine Release in Myocardial I/R Mice

Western blot was applied for testing the expression of cardiac function markers including α-MHC and β-MHC in

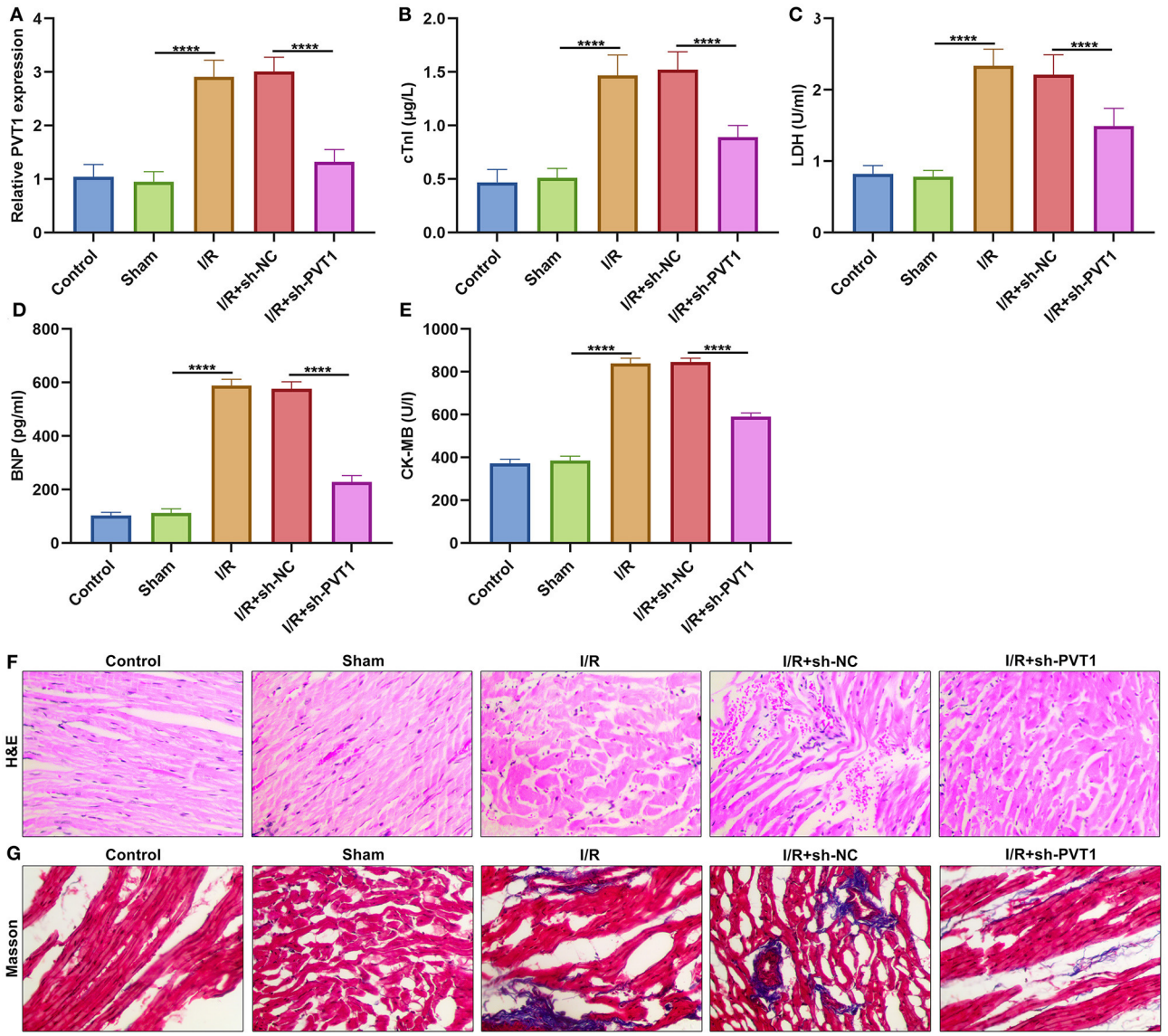

FIGURE 1 | LncRNA PVT1 is up-regulated in myocardial I/R tissues and silencing PVT1 alleviates myocardial I/R damage. **(A)** RT-qPCR of the expression of lncRNA PVT1 in myocardial tissues in five groups: control, sham, I/R, I/R + sh-NC, and I/R + sh-PVT1 groups. Mice in the I/R + sh-NC and I/R + sh-PVT1 groups were injected by 10 μL sh-NC or sh-PVT1 via Lipofectamine 2000 reagent. After 48 h, myocardial I/R model was constructed. No treatment was done for mice in the control group. The chest of mice in the sham operation group was only opened, but was threaded the corresponding part without ligating the LAD. There were 10 mice in each group. **(B–E)** ELISA for examining the serum levels of **(B)** cTnI, **(C)** LDH, **(D)** BNP, and **(E)** CK-MB in above five groups. **(F)** HandE staining of myocardial tissues in five groups. Bar = 100 μm. **(G)** Masson staining of myocardial tissues in five groups. Bar = 100 μm. ****$p < 0.0001$.

myocardial tissues. Our data showed that α-MHC expression was significantly decreased while β-MHC expression was significantly increased in I/R myocardial tissues compared with sham tissues (**Figures 2A–C**). However, silencing PVT1 markedly alleviated I/R-induced the decrease in α-MHC and the increase in β-MHC. This indicated that PVT1 knockdown could improve cardiac function in myocardial I/R mice. Serum TNF-α, IL-1β, and IL-6 levels were examined by RT-qPCR. In comparison to sham mice, there were increased serum levels of TNF-α (**Figure 2D**), IL-1β (**Figure 2E**), and IL-6 (**Figure 2F**) mRNAs in myocardial I/R mice. Nevertheless, silencing PVT1 distinctly decreased their serum levels in myocardial I/R mice. Western blot was also carried out to test the expression of TNF-α, IL-1β, and IL-6 in serum samples (**Figure 2G**). Consistently, the expression TNF-α (**Figure 2H**), IL-1β (**Figure 2I**), and IL-6 (**Figure 2J**) proteins was markedly up-regulated in serum of myocardial I/R mice compared with sham mice. Their expression was significantly ameliorated by PVT1 knockdown. Therefore, silencing PVT1 could alleviate I/R-induced cytokine release.

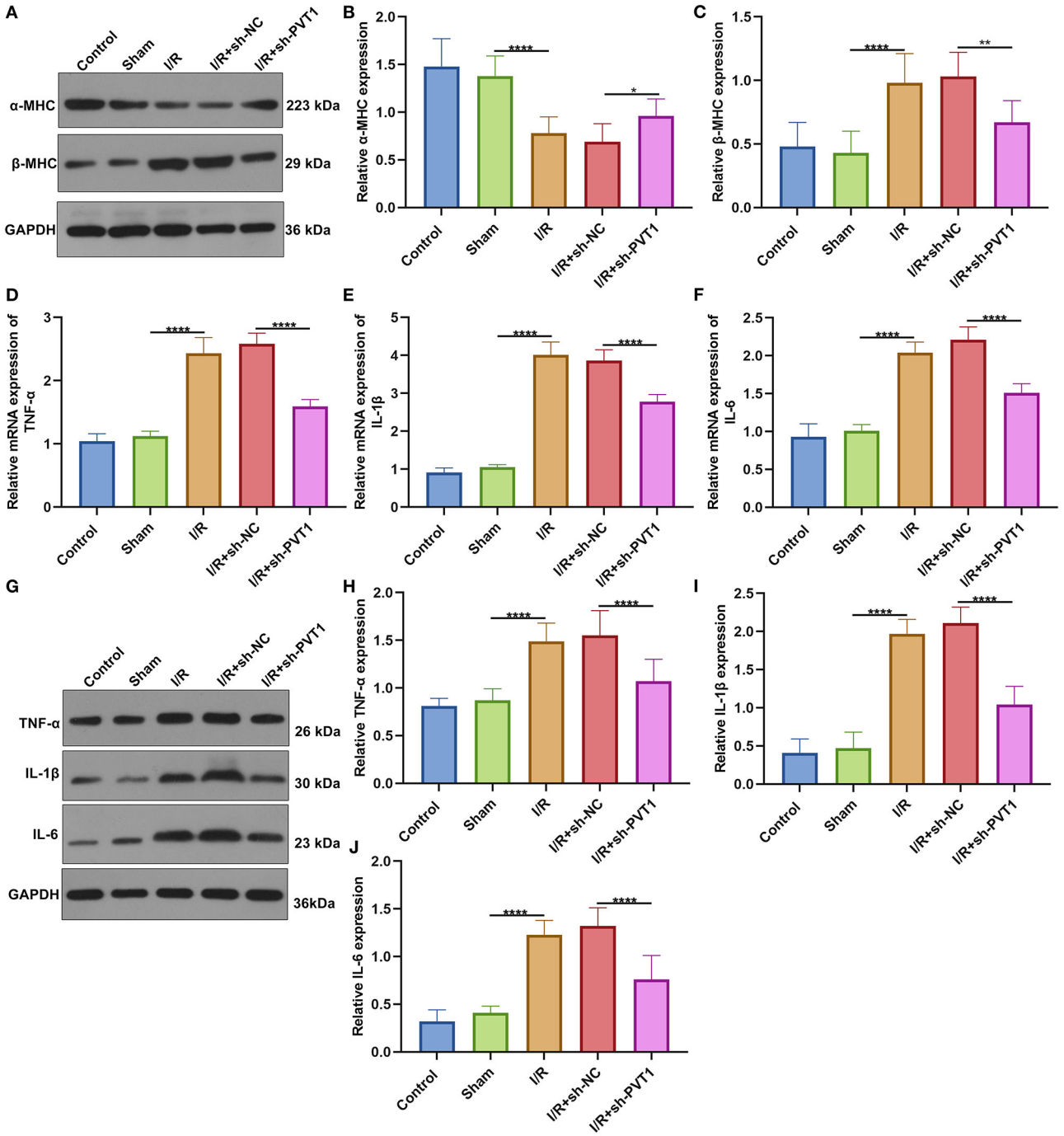

FIGURE 2 | LncRNA PVT1 knockdown ameliorates cardiac function and cytokine release in myocardial I/R mice. **(A–C)** Western blot of the expression of **(B)** α-MHC and **(C)** β-MHC in myocardial tissues of control, sham, I/R, I/R + sh-NC, and I/R + sh-PVT1 groups. Mice in the I/R + sh-NC and I/R + sh-PVT1 groups were injected by 10 μL sh-NC or sh-PVT1 via Lipofectamine 2000 reagent. After 48 h, myocardial I/R model was constructed. No treatment was done for mice in the control group. The chest of mice in the sham operation group was only opened, but was threaded the corresponding part without ligating the LAD. There were 10 mice in each group. **(D–F)** RT-qPCR of the expression of **(D)** TNF-α, **(E)** IL-1β, and **(F)** IL-6 mRNAs in serum samples from above groups. **(G–J)** Western blot of the expression of **(H)** TNF-α, **(I)** IL-1β, and **(J)** IL-6 proteins in serum samples of each group. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$.

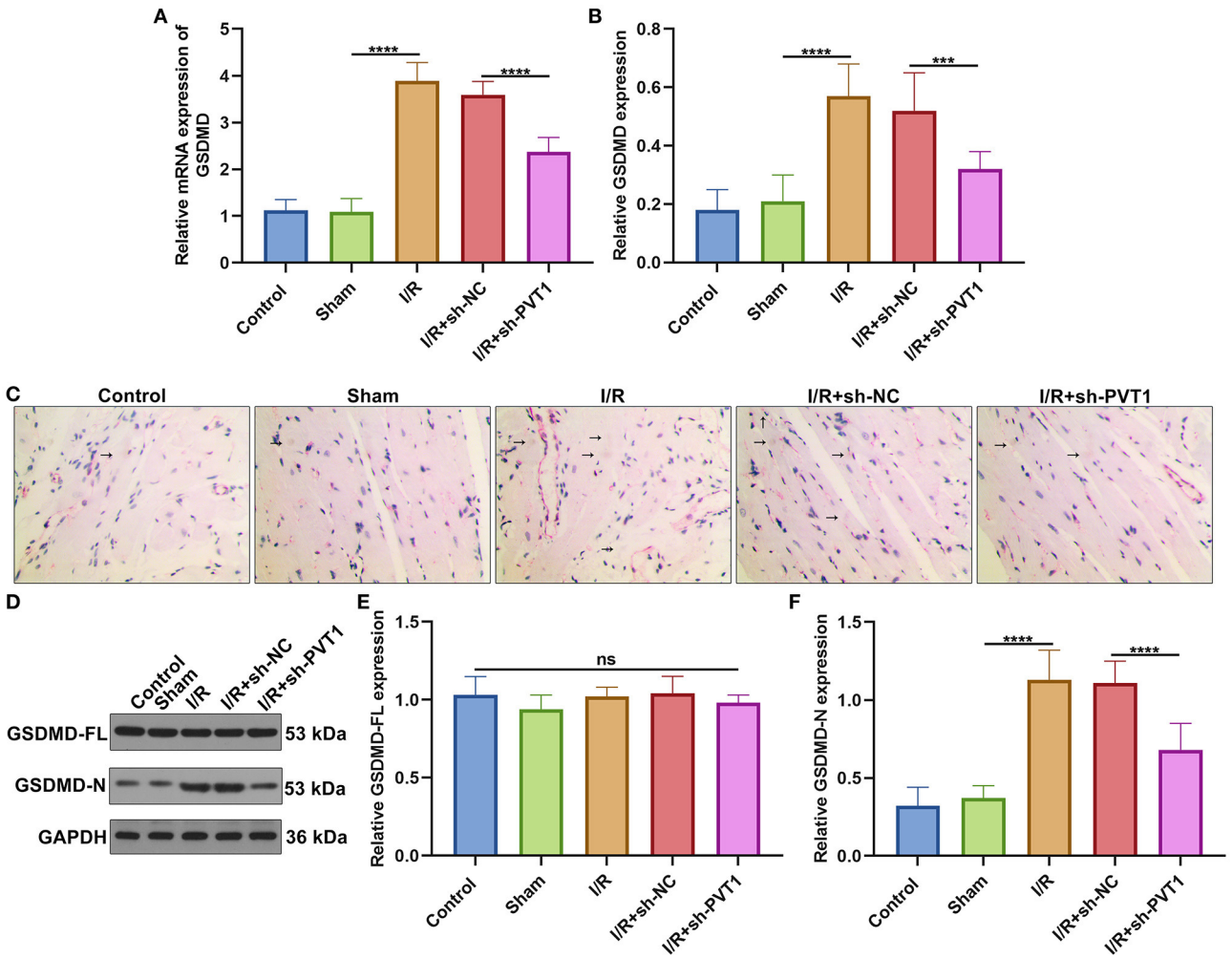

FIGURE 3 | LncRNA PVT1 knockdown alleviates GSDMD-mediated pyroptosis in myocardial I/R mice. **(A)** RT-qPCR of the expression of GSDMD mRNA in myocardial tissues of five groups: control, sham, I/R, I/R + sh-NC, and I/R + sh-PVT1 groups. Mice in the I/R + sh-NC and I/R + sh-PVT1 groups were injected by 10 μL sh-NC or sh-PVT1 via Lipofectamine 2000 reagent. After 48 h, myocardial I/R model was constructed. No treatment was done for mice in the control group. The chest of mice in the sham operation group was only opened, but was threaded the corresponding part without ligating the LAD. There were 10 mice in each group. **(B,C)** Immunohistochemistry of the expression of GSDMD protein in myocardial tissues of above groups. Arrow highlights the positive staining of GSDMD. **(D–F)** Western blot of **(E)** GSDMD-FL and **(F)** GSDMD-N expressions in myocardial tissues of each group. Ns: not significant; ***$p < 0.001$; ****$p < 0.0001$.

LncRNA PVT1 Knockdown Ameliorates GSDMD-Mediated Pyroptosis in Myocardial I/R Mice

Here, we detected the expression of GSDMD mRNA in myocardial tissues by RT-qPCR. In **Figure 3A**, GSDMD exhibited the up-regulated mRNA expression in myocardial I/R tissues than sham specimens. But silencing PVT1 markedly decreased the expression of GSDMD mRNA in myocardial I/R tissues. Immunohistochemistry was carried out to examine the expression of GSDMD protein. In comparison to sham mice, increased GSDMD expression was found in myocardial I/R tissues, which was distinctly lowered by PVT1 knockdown (**Figures 3B,C**). Western blot showed that I/R or PVT1 knockdown did not affect GSDMD-FL expression but affected GSDMD-N expression (**Figures 3D–F**). GSDMD-N expression was distinctly up-regulated in myocardial I/R tissues, which was lowered by PVT1 knockdown. Above data suggested that GSDMD was required for myocardial I/R-induced pyroptosis and silencing PVT1 alleviated GSDMD-induced pyroptosis in myocardial I/R mice.

Suppression of Pyroptosis by lncRNA PVT1 Knockdown Involves TLR4/MyD88/NF-κB/NLRP3 Inflammasome Pathway

Increasing evidence suggests that inhibition of the TLR4/MyD88/NF-κB/NLRP3 inflammasome pathway may

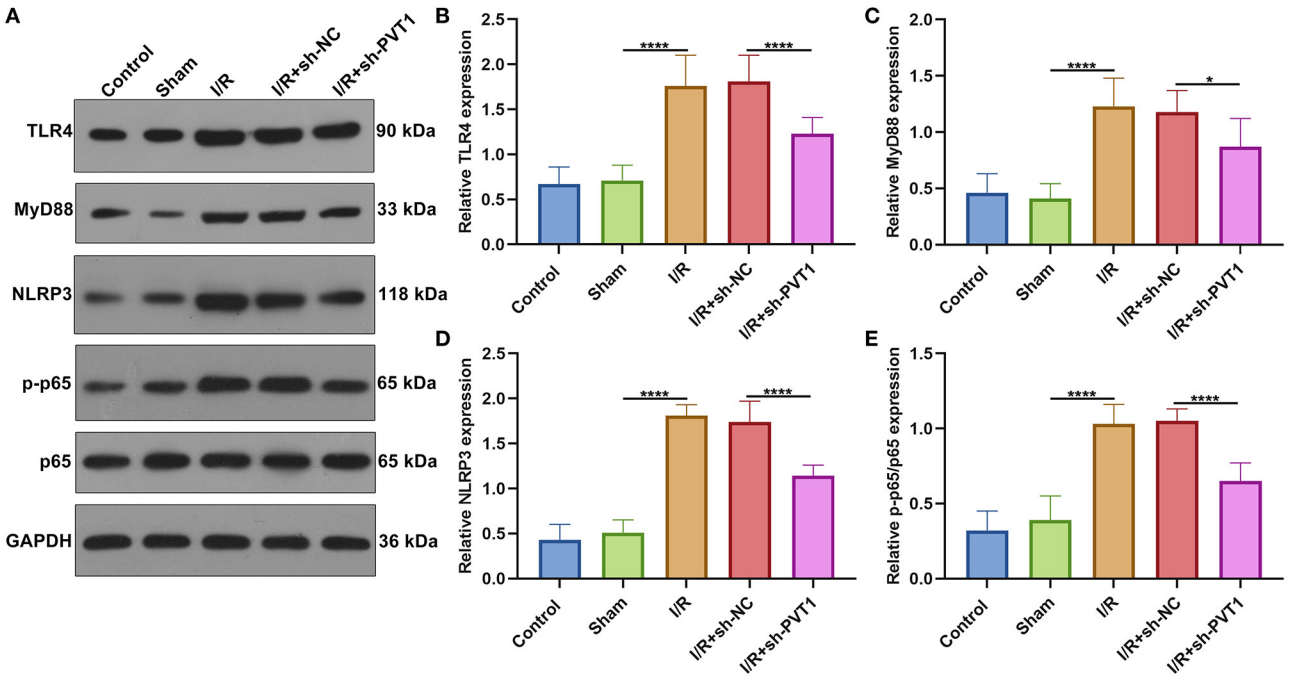

FIGURE 4 | Suppression of pyroptosis by lncRNA PVT1 knockdown involves TLR4/MyD88/NF-κB/NLRP3 inflammasome pathway in myocardial I/R tissues. **(A–E)** Western blot of **(B)** TLR4, **(C)** MyD88, **(D)** NLRP3, and **(E)** p-p65/p65 expressions in myocardial tissues of control, sham, I/R, I/R + sh-NC, and I/R + sh-PVT1 groups. Mice in the I/R + sh-NC and I/R + sh-PVT1 groups were injected by 10 μL sh-NC or sh-PVT1 via Lipofectamine 2000 reagent. After 48 h, myocardial I/R model was constructed. No treatment was done for mice in the control group. The chest of mice in the sham operation group was only opened, but was threaded the corresponding part without ligating the LAD. There were 10 mice in each group. $^{*}p < 0.05$; $^{****}p < 0.0001$.

reduce myocardial infarction-induced pyroptosis (9, 21). The activation of TLR4/MyD88/NF-κB/NLRP3 inflammasome pathway was examined in myocardial tissues by western blot (**Figure 4A**) (22). Our western blot showed that the expression of TLR4 (**Figure 4B**), MyD88 (**Figure 4C**), NLRP3 (**Figure 4D**), and p-p65/p65 (**Figure 4E**) was distinctly elevated in myocardial I/R tissues than sham specimens. However, their expression was markedly suppressed by PVT1 knockdown. These data suggested that suppression of pyroptosis through PVT1 knockdown involved TLR4/MyD88/NF-κB/NLRP3 inflammasome pathway in myocardial I/R tissues.

LncRNA PVT1 Knockdown Ameliorates I/R-Induced Apoptosis and Caspase1 Involving Pyroptosis in Myocardial Tissues

We also investigated whether PVT1 knockdown affected cardiomyocyte apoptosis. Western blot was presented for examining Cleaved caspase-3, Bax, and Bcl-2 expression in myocardial tissues (**Figure 5A**). We found that the expression of Cleaved caspase-3 (**Figure 5B**) and Bax proteins (**Figure 5C**) was distinctly increased as well as Bcl-2 expression (**Figure 5D**) was markedly decreased in myocardial I/R tissues than sham tissues. Moreover, silencing PVT1 markedly alleviated I/R-induced the increase in Cleaved caspase-3 and Bax proteins as well as the decrease in Bcl-2 protein in myocardial tissues. Pyrolysis is dependent on Caspase1. Here, Caspase1 expression was examined by immunohistochemistry. The up-regulation of Caspase1 was found in myocardial I/R tissues than sham tissues, which was suppressed when silencing PVT1 expression (**Figures 5E,F**).

LncRNA PVT1 Knockdown Alleviates H/R-Induced Apoptosis in Cardiomyocytes

This study established H/R-induced cellular models. The up-regulation of PVT1 was detected in H/R-induced H9C2 cells than controls (**Figure 6A**). After transfection with sh-PVT1, PVT1 expression was markedly suppressed. CCK-8 results showed that H/R markedly lowered cellular viability of H9C2 cells (**Figure 6B**). But PVT1 knockdown ameliorated the decrease in cell viability induced by H/R. In **Figures 6C,D**, H/R treatment markedly elevated apoptotic levels of H/R-treated H9C2 cells. Nevertheless, silencing PVT1 markedly lowered the apoptotic levels of H/R-induced H9C2 cells.

LncRNA PVT1 Knockdown Ameliorates H/R-Induced Pyroptosis in Cardiomyocytes

ELISA was presented for testing the levels of CK-MB and LDH in the supernatant of H9C2 cells. Our data showed that H/R markedly elevated CK-MB (**Figure 6E**) and LDH (**Figure 6F**) production in the supernatant, which was alleviated by PVT1 knockdown. Furthermore, we tested IL-1β production in the cell supernatant via ELISA. IL-1β was markedly up-regulated in

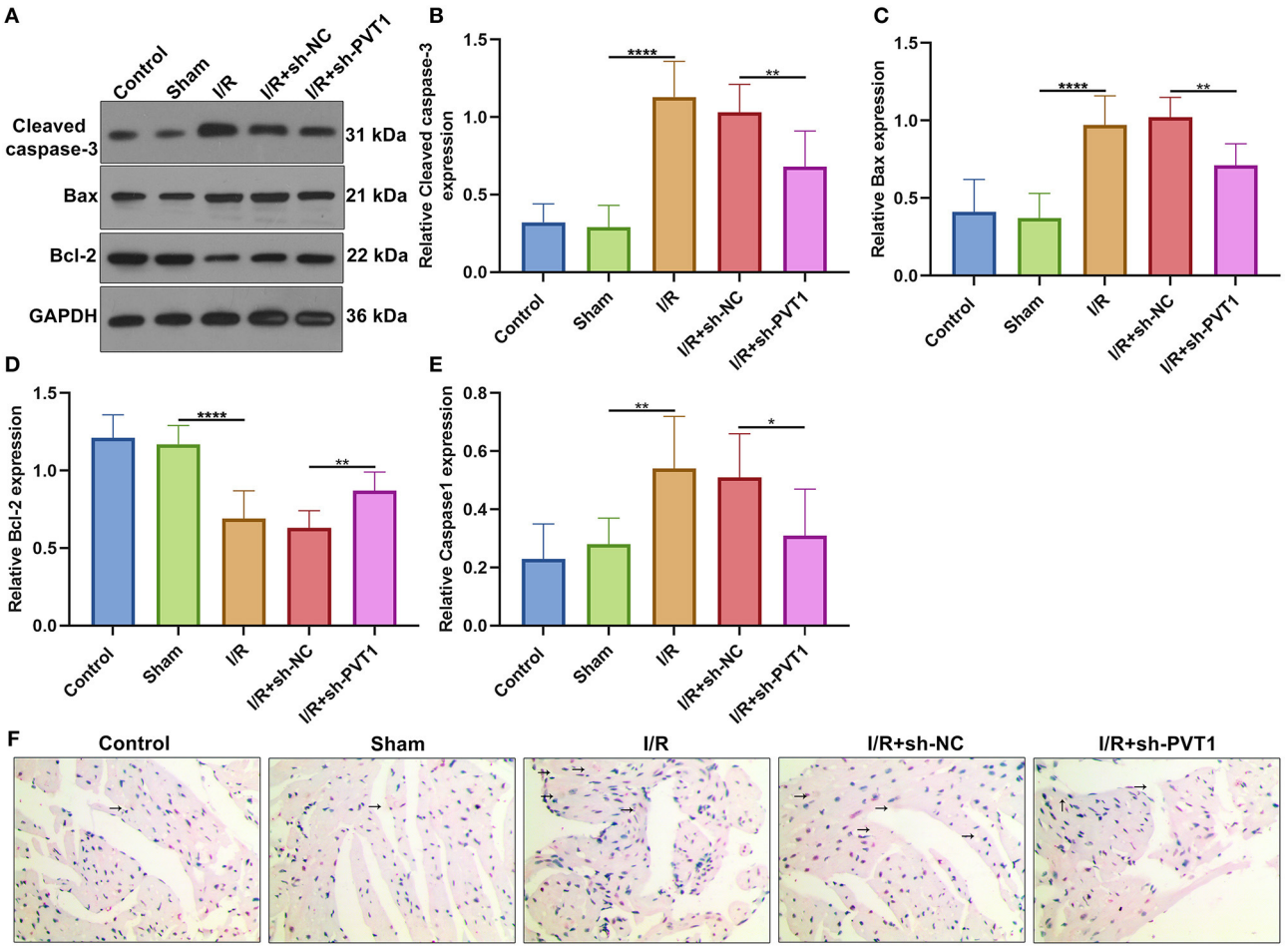

FIGURE 5 | LncRNA PVT1 knockdown suppresses I/R-induced apoptosis and Caspase1 involving pyroptosis in myocardial tissues. **(A–D)** Western blot of the expression of **(B)** Cleaved caspase-3, **(C)** Bax, and **(D)** Bcl-2 proteins in myocardial tissues of five groups: control, sham, I/R, I/R + sh-NC, and I/R + sh-PVT1 groups. Mice in the I/R + sh-NC and I/R + sh-PVT1 groups were injected by 10 μL sh-NC or sh-PVT1 via Lipofectamine 2000 reagent. After 48 h, myocardial I/R model was constructed. No treatment was done for mice in the control group. The chest of mice in the sham operation group was only opened, but was threaded the corresponding part without ligating the LAD. There were 10 mice in each group. **(E,F)** Immunohistochemistry of the expression of Caspase1 protein in myocardial tissues of above groups. Bar = 100 μm. Arrow highlights the positive staining of Caspase1. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$.

H/R-induced H9C2 cells than controls (**Figure 7A**). Meanwhile, its production was significantly inhibited under silencing PVT1 in H9C2 cells. As shown in western blot, H/R or PVT1 knockdown did not alter GSDMD-FL expression in H9C2 cells (**Figures 7B,C**). But H/R treatment distinctly elevated the expression of GSDMD-N, which was markedly alleviated by PVT1 knockdown (**Figure 7D**). Moreover, Caspase1 expression was significantly up-regulated in H/R-induced H9C2 cells than controls (**Figure 7E**). Nevertheless, sh-PVT1 transfection significantly lowered its level H/R-treated cardiomyocytes. Immunofluorescence assays were also carried out to verify GSDMD expression in H9C2 cells. Consistently, the up-regulation of GSDMD was mediated by H/R treatment, which was lowered through PVT1 knockdown in H9C2 cells (**Figures 7F,G**).

Overexpression of lncRNA PVT1 Promotes H/R-Mediated Pyroptosis in Cardiomyocytes

For further investigating the influence of PVT1 on H/R-induced pyroptosis, H9C2 cells were transfected by PVT1 overexpression vector. In **Figure 8A**, RT-qPCR demonstrated that H/R-induced PVT1 up-regulation was enhanced by PVT1 overexpression vector transfection in H9C2 cells. CCK-8 showed that PVT1 overexpression promoted the decrease in cell viability induced by H/R (**Figure 8B**). Furthermore, its overexpression strengthened the increase in IL-1β production induced by H/R treatment in H9C2 cells (**Figure 8C**). Our western blot showed that pyroptosis inhibitor NSA did not affect GSDMD-FL expression but significantly suppressed the expression of GSDMD-N in H/R-induced H9C2 cells (**Figures 8D–F**). Nevertheless, PVT1

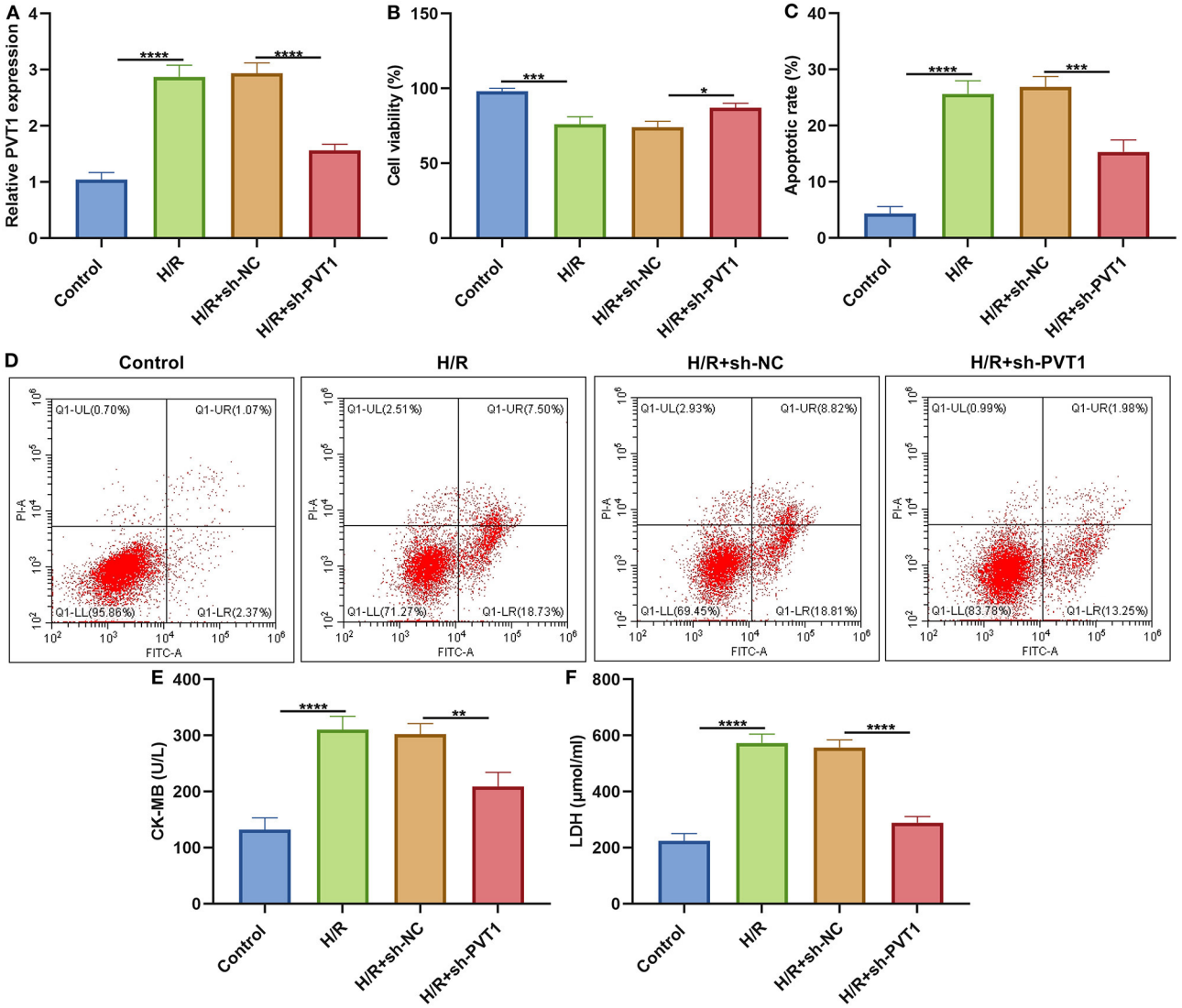

FIGURE 6 | LncRNA PVT1 knockdown alleviates H/R-induced apoptosis in H9C2 cells. **(A)** RT-qPCR of PVT1 expression in H9C2 cells in four groups: control, H/R, H/R + sh-NC, and H/R + sh-PVT1 groups. H9C2 cells in the H/R, H/R + sh-NC, and H/R + sh-PVT1 groups were treated by H/R for 4 h. For the H/R + sh-NC or H/R + sh-PVT1 groups, H9C2 cells (5×10^4) were transfected by 1 μg sh-NC or 1 μg sh-PVT1 for 48 h. No treatment was done for H9C2 cells in the control group. **(B)** CCK-8 for the cell viability of H9C2 cells in above groups. **(C,D)** Flow cytometry of apoptotic levels of H9C2 cells in each group. **(E,F)** ELISA of the levels of CK-MB and LDH in the supernatant of H9C2 cells in above groups. Each experiment was repeated three times. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

overexpression significantly alleviated the inhibitory effect of NSA on GSDMD-N expression in H9C2 cells.

DISCUSSION

In our study, lncRNA PVT1 expression displayed distinct up-regulation in I/R-induced myocardial tissues as well as H/R-induced H9C2 cells. Consistently, Mao et al. reported the up-regulation of PVT1 in H/R-induced AC16 cells (23). In previous studies, lncRNA MALAT1 knockdown decreased cTnI, LDH and CK-MB levels for oxygen-glucose deprivation and reoxygenation (OGD/R)-mediated cardiomyocytes (24). Silencing HIF1A-AS1 could lower serum BNP, cTnI, LDH and CK-MB levels in myocardial I/R rat models (25). Furthermore, targeting FOXD3-AS1 markedly decreased the levels of cTnI and CK-MB in OGD/R-induced H9C2 cells (26). Here, in I/R-induced mouse models, we found that PVT1 knockdown significantly alleviated the expression of myocardial damage markers including cTnI, LDH, BNP as well as CK-MB in serum samples from I/R-induced mouse models. H&E and Masson staining confirmed that silencing PVT1 could alleviate I/R-induced myocardial damage.

The α-MHC and β-MHC are major contractile proteins of cardiomyocytes (27). The β-MHC is mainly expressed in the embryonic stage of mice and is almost replaced by α-MHC after birth (28). However, the expression will increase under pathological conditions such as heart failure

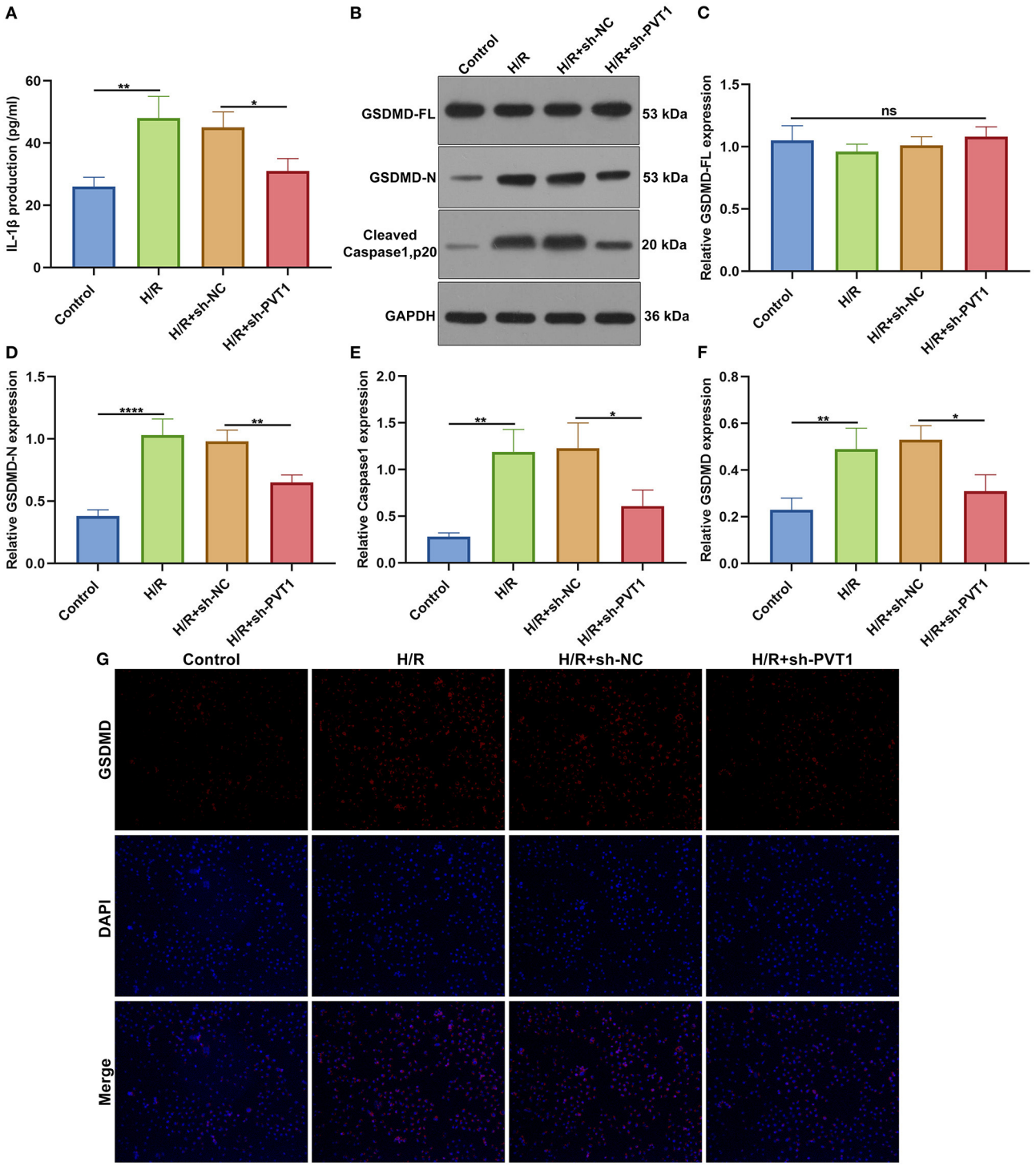

FIGURE 7 | LncRNA PVT1 knockdown ameliorates H/R-induced pyroptosis in cardiomyocytes. **(A)** ELISA for the levels of IL-1β production in H9C2 cells in four groups: control, H/R, H/R + sh-NC, and H/R + sh-PVT1 groups. H9C2 cells in the H/R, H/R + sh-NC, and H/R + sh-PVT1 groups were treated by H/R for 4 h. For the H/R + sh-NC or H/R + sh-PVT1 groups, H9C2 cells (5×10^4) were transfected by 1 μg sh-NC or 1 μg sh-PVT1 for 48 h. No treatment was done for H9C2 cells in the control group. **(B–E)** Western blot for **(C)** GSDMD-FL, **(D)** GSDMD-N, and **(E)** cleaved Caspase1 (p20) expressions in H9C2 cells in above groups. **(F,G)** Immunofluorescence for the expression of GSDMD in H9C2 cells in above groups. Each experiment was repeated three times. Ns: not significant; $^*p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$.

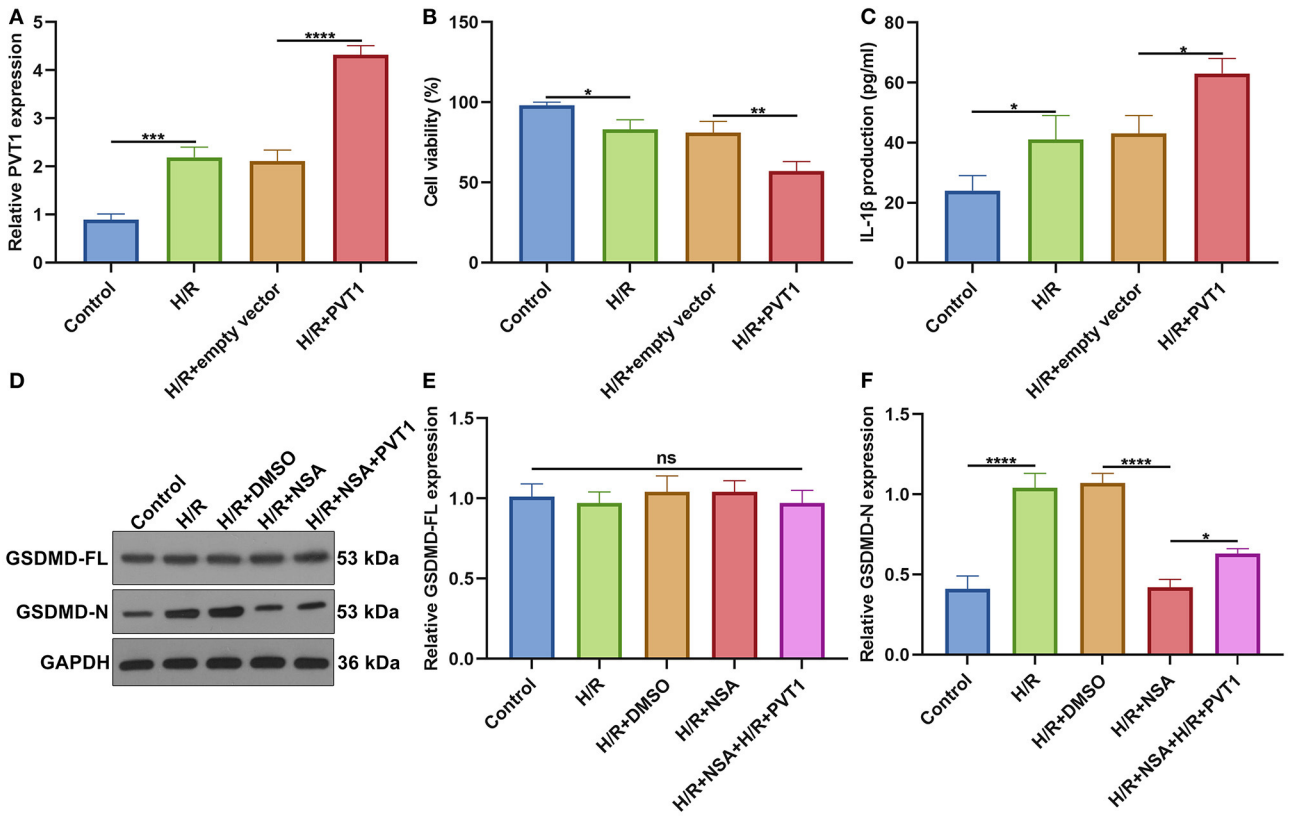

FIGURE 8 | Overexpression of lncRNA PVT1 promotes H/R-mediated pyroptosis in H9C2 cells. **(A)** RT-qPCR for the expression of PVT1 in H9C2 cells in four groups: control, H/R, H/R + empty vector, and H/R + PVT1 groups. H9C2 cells in the H/R, H/R + empty vector, and H/R + PVT1 groups were treated by H/R for 4 h. For the H/R + empty vector and H/R + PVT1 groups, H9C2 cells were transfected by plasmids of 500 ng pcDNA3.1 vector (empty vector) or 500 ng PVT1 for 48 h. No treatment was done for H9C2 cells in the control group. **(B)** CCK-8 for the cell viability of H9C2 cells in above groups. **(C)** ELISA for IL-1β levels in the cell supernatant in above groups. **(D–F)** Western blot of **(E)** GSDMD-FL and **(F)** GSDMD-N expressions in H9C2 cells in five groups: control, H/R group, H/R + DMSO, H/R + NSA, and H/R + NSA + PVT1 groups. H9C2 cells were treated with 25 nM NSA, followed by plasmids of 500 ng PVT1 and H/R treatment. Each experiment was repeated three times. Ns: not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

and increased angiotensin II. Therefore, conversion of α-MHC to β- MHC is also regarded as an important sign of heart failure (29). PVT1 knockdown significantly increased α-MHC and decreased β-MHC expressions in I/R-induced myocardial tissues, indicating that targeting PVT1 could improve cardiac function of mice following I/R damage. As previously reported, silencing PVT1 lowered β-MHC expression in cardiac hypertrophic mouse models (30). PVT1 knockdown markedly reduced the production and secretion of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in I/R-induced myocardial tissue specimens. TNF-α antagonism may alleviate myocardial I/R damage through elevating adiponectin expression (31). Our data indicated the anti-inflammatory effect of PVT1 knockdown on myocardial I/R damage. GSDMD-induced pyroptosis of cardiomyocytes accelerates myocardial I/R damage (10). A recent study demonstrated that PVT1 may modulate NLRP3-induced pyroptosis in septic acute kidney damage (32). Here, we found that silencing PVT1 alleviated GSDMD-N not GSDMD-FL expression in I/R-induced myocardial tissue specimens as well as H/R-treated cardiomyocytes. Thus, PVT1 knockdown could ameliorate GSDMD-mediated pyroptosis in cardiomyocytes.

It has been confirmed that TLR4/MyD88/NF-κB/NLRP3 inflammasome pathway is responsible for pyroptosis (9, 21). For instance, Nicorandil suppresses TLR4/MyD88/NF-κB/NLRP3 axis to alleviate pyroptosis in rats with myocardial infarction (21). Emodin reduces myocardial I/R injury-induced pyroptosis through inhibiting the TLR4/MyD88/NF-κB/NLRP3 inflammasome pathway (9). Furthermore, octreotide and melatonin reduce inflammasome-induced pyroptosis through suppressing TLR4-NF-κB-NLRP3 pathway in hepatic I/R damage (33). In this study, TLR4/MyD88/NF-κB/NLRP3 inflammasome pathway was activated in myocardial I/R tissues, consistent with a previous study (34). Silencing PVT1 decreased the activation of TLR4/MyD88/NF-κB/NLRP3 pathway. This indicated that suppression of pyroptosis through PVT1 knockdown involved TLR4/MyD88/NF-κB/NLRP3 inflammasome pathway in myocardial I/R. Except for GSDMD-induced pyroptosis, our data showed that PVT1 knockdown alleviated I/R-induced the increase in cleaved caspase3 and Bax and the decrease in Bcl-2 in myocardial tissues. For H/R-treated cardiomyocytes, silencing PVT1 elevated cellular viability as well as suppressed apoptosis. As reported,

PVT1 could protect human AC16 cardiomyocytes from H/R-induced apoptosis (23). Thus, targeting PVT1 could ameliorate myocardial I/R damage via suppressing cardiomyocyte apoptosis.

Our findings proposed for the first time that inhibition of PVT1 could alleviate myocardial I/R damage through reducing GSDMD-mediated pyroptosis *in vivo* and *in vitro*, which provided a novel direction for prevention and treatment of myocardial I/R damage.

CONCLUSION

Collectively, our study established myocardial I/R mouse models and H/R-induced cellular models and confirmed the up-regulation of lncRNA PVT1 following myocardial I/R damage. Silencing PVT1 ameliorated myocardial I/R damage through inhibiting GSDMD-mediated pyroptosis. Hence, PVT1 knockdown might be an alternative therapeutic strategy against myocardial I/R damage.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The study was approved by the Ethics Committee of Guangdong Provincial People's Hospital (GDREC2016255H).

AUTHOR CONTRIBUTIONS

YL, YW, and HD conceived and designed the study. CL, HS, and CC conducted most of the experiments and data analysis and wrote the manuscript. SC, QZ, DL, and JL participated in collecting data and helped to draft the manuscript. All authors reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Multidimensional Mechanistic Spectrum of Long Non-coding RNAs in Heart Development and Disease

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With the large-scale genome-wide sequencing, long non-coding RNAs (lncRNAs) have been found to compose of a large portion of the human transcriptome. Recent studies demonstrated the multidimensional functions of lncRNAs in heart development and disease. The subcellular localization of lncRNA is considered as a key factor that determines lncRNA function. Cytosolic lncRNAs mainly regulate mRNA stability, mRNA translation, miRNA processing and function, whereas nuclear lncRNAs epigenetically regulate chromatin remodeling, structure, and gene transcription. In this review, we summarize the molecular mechanisms of cytosolic and nuclear lncRNAs in heart development and disease separately, and emphasize the recent progress to dictate the crosstalk of cytosolic and nuclear lncRNAs in orchestrating the same biological process. Given the low evolutionary conservation of most lncRNAs, deeper understanding of human lncRNA will uncover a new layer of human regulatory mechanism underlying heart development and disease, and benefit the future clinical treatment for human heart disease.

Keywords: long non-coding RNA, microRNA, heart development, heart disease, ceRNA, PRC2 complex

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INTRODUCTION

The heart is a central organ of the circulatory system, which pumps blood and drives oxygen and nutrients throughout the whole body. According to American Heart Association, heart disease is one of the leading causes of death in the United States. Approximately 655,000 Americans die of heart disease each year (1). Although heart disease could be caused by various factors, the most direct and common reason has been recognized as genetic variations in coding genes. During the past decade, accumulated evidence demonstrates that non-coding RNAs (ncRNAs) are also highly relevant to cardiovascular diseases (2). Non-coding RNAs are transcripts without prominent protein coding potential, which include two major groups, short non-coding RNAs (sncRNAs) and long non-coding RNAs (lncRNAs) (3). SncRNAs include transfer RNAs (t-RNAs), ribosomal RNAs (r-RNAs), small nuclear RNAs (snRNAs), microRNAs (miRNAs), small interfering RNAs (siRNAs) and P-element-induced wimpy testis (PIWI) interacting RNAs (piRNAs). SncRNAs are broadly involved in transcriptional and translational regulations (3). LncRNAs are over 200 bp transcripts and lncRNA genes compose a large portion of the human genome. LncRNAs display multidimensional functions at various regulatory levels, such as histone modification, DNA

methylation, gene transcription, post-transcription, translation, RNA and protein stability (4). Many lncRNAs have been reported to be involved in cardiovascular development and disease, although their underlying molecular mechanisms in pathological process remain elusive (5). Therefore, understanding the roles of lncRNAs in heart development and disease will reveal the molecular basis of cardiogenesis, and the molecular etiology of human cardiovascular diseases. For example, a conserved lncRNA *H19* represses cardiac hypertrophy by preventing and reversing experimental pressure-overload-induced heart failure, and Duchenne and Becker muscular dystrophy associated cardiomyopathy (6, 7). Hence, we summarize the current knowledge of characterized lncRNA mechanisms in heart development and disease (**Table 1**), and further discuss the clinical potential of lncRNA in heart disease therapy.

LONG NON-CODING RNA FUNCTIONS IN HEART DEVELOPMENT AND DISEASE

The establishment of *in vitro* cardiomyocyte (CM) differentiation from mouse and human pluripotent stem cells (hPSCs) allows modeling early events of cardiogenesis in dish. Furthermore, whole transcriptomic profiling and CRISPR/Cas-9 mediated approaches have paved the way toward discovering and functional assessment of crucial lncRNAs in early human cardiac development by using hPSCs (45). Currently, hundreds of lncRNAs have been identified in the human cardiac precursor cells (CPCs), such as cardiac mesoderm enhancer-associated non-coding RNA (*Carmen*), which promotes cardiac specification and differentiation of CPCs (46). A human-specific lncRNA, Heart Brake LncRNA 1 (*HBL1*), represses CM differentiation from human hPSCs via counteracting *miR-1* function (8). A mouse-specific lncRNA, *Braveheart* (*Bvht*), is required for the commitment of nascent mesoderm toward a cardiac fate (33). A heart field related lncRNA, *Linc1405*, controls cardiac mesoderm specification and cardiogenesis in mESC and *in vivo* (42). A lateral mesoderm-specific lncRNA *Fendrr* (FOXF1 Adjacent Non-Coding Developmental Regulatory RNA) plays an essential role in heart and body wall development *in vivo* (35). In addition to control early cardiac lineage specification, lncRNAs also play important roles in CM maturation and proliferation via various mechanisms, such as regulating the expression ratio of Myh6/Myh7 (47), sarcomere organization (43), cardiac myogenesis (36), metabolic maturation (44, 48) and cardiac conduction (27, 31, 49). LncRNA *Mhrt* (myosin heavy-chain-associated RNA transcripts) is required for maintaining the ratio of Myh6/Myh7 during mouse heart development and maturation, which is important for CM maturation (47). LncRNA *CPR* (cardiomyocyte proliferation regulator) induces hypertrophic responses of mature CMs, including increased sarcomere organization and CM surface area (43).

Evidence of the association between deregulation of lncRNAs and heart diseases has been reports for various cardiovascular disease models, such as cardiac hypotrophy (6), muscular dystrophy (7), coronary artery disease (CAD) (50–52), myocardial infarction (32, 53), diabetic cardiomyopathy (54), non-Ischemic cardiomyopathy (NICM) and heart failure (55). Murine and human lncRNA *H19* display an anti-hypotrophy function, and CM-restricted *H19* gene delivery can suppress the development of cardiac hypertrophy and later on heart failure (6). Recently, Zhang et al. found that *H19* inhibits dystrophin degradation, preserves skeletal and cardiac muscle histology, and improves cardiomyocyte strength and heart function in muscular dystrophy cells and murine model (7). *H19* also suppresses apoptosis and autophagy of CMs under diabetic condition (12, 56). In myocardial infarction, lncRNA *Meg3* is upregulated in infarcted mouse heart and promotes CM death (32). Although a large number of lncRNAs have been found to be associated with heart development and disease (**Table 1**), the mechanisms of most lncRNAs remain elusive. Particularly, the deeper understating of lncRNA mechanisms will shed light on the clinical potential of lncRNAs, with the findings of novel therapeutic targets or druggable lncRNAs. Interestingly, many lncRNAs show restricted expression patterns in the cytoplasm or nucleus although some lncRNAs express in both, suggesting the differential functions executed by lncRNAs in different subcellular localizations, which are summarized in the following sections.

MECHANISMS OF LONG NON-CODING RNAs IN CYTOPLASM

The subcellular localization is considered as a key factor determining lncRNA function (57, 58). Although the nucleus is the location for RNA biogenesis and processing, many mature lncRNAs are transported into cytoplasm, showing high cytosolic expressing levels (59). In the cytoplasm, lncRNA-mediated mechanisms have been found to mainly regulate mRNA stability, translation of mRNA, and microRNA (miRNA) related functions (60).

Long Non-coding RNA Counteracts microRNA

Since the first discovery of competing endogenous RNA (ceRNA), hundreds of lncRNAs have been found to function as miRNA sponge to counteract endogenous miRNAs. The ceRNAs can modulate miRNA activity through sequestration, thereby increasing the expression of miRNA target genes (61). During heart development, several lncRNAs have been identified to counteract miRNAs and regulate expressions of genes essential for stem cells pluripotency or lineage specification. Using hPSCs, *HBL1* was identified as a modulator to fine-tune human CM development via sponging *miR-1* (8). *HBL1* is a human-specific lncRNA highly expressed in hPSCs and gradually diminishes during CM differentiation.

Abbreviations: lncRNA, long non-coding RNA; ceRNA, competing endogenous RNA; miRNAs microRNAs; iPSCs, induced pluripotent stem cells; CPCs, cardiac precursor cells; CM, cardiomyocyte; CAD, coronary artery disease; NICM, non-Ischemic cardiomyopathy; PRC2, polycomb-repressive complex 2; AS, alternative splicing; JARID2, jumonji and AT-rich interaction domain containing 2; EED, embryonic ectoderm development; EZH2, enhancer of zeste homolog 2.

TABLE 1 | Roles of lncRNAs in heart development and diseases.

	Cellular location	Validated target(s)	Cardiac functions	References
HBL1	Cytoplasm	*miR-1*	Cardiac development	(8, 9)
	Nucleus	JARID2 and EED		
HOTAIR	Cytoplasm	*miR-1*	Acute myocardial infarction	(10)
LINCMD1	Cytoplasm	*miR-133*; *miR-135*; *pre-miR-133b*	Myogenesis	(11)
H19	Cytoplasm	*let-7*; *miR-877-3p*; *miR-22-3p*; *miR-19a*; *miR-675-3p*; *miR-675-5p*; KSRP; Dystrophin.	Muscle differentiation and regeneration; MI-induced myocardial injury; Senescence; Diabetic cardiomyocyte; Muscular dystrophy	(6, 7, 12–20, 56)
	Nucleus	EED; EZH2; SUZ12		
UCA1	Cytoplasm	*miR-184*	Cardiac hypertrophy	(22)
MIAT	Cytoplasm	*miR-150*	Cardiac hypertrophy	(23)
CHRF	Cytoplasm	*miR-489*	Cardiac hypertrophy	(24)
ROR	Cytoplasm	*miR-133*	Cardiac hypertrophy	(25)
Plscr4	Cytoplasm	*miR-214*	Cardiac hypertrophy	(26)
MALAT1	Cytoplasm	*miR-220C*	Cardiomyocyte electrophysiology; cardiac remodeling and failure	(27–29)
	Nucleus	BRG1; HDAC9		
CARL	Cytoplasm	*miR-539*; *miR-296*	Cardiac apoptosis, replication, and regeneration	(30)
CCRR	Cytoplasm	CIP85	Cardiac conduction	(31)
Meg3	Cytoplasm	FUS	Cardiac apoptosis	(32)
Bvht	Nucleus	SUZ12	Cardiovascular lineage commitment	(33, 34)
Fendrr	Nucleus	PRC2; TrxG/MLL	Lateral plate or cardiac mesoderm differentiation	(35)
PPP1R1B	Nucleus	Ezh2	Myogenic differentiation	(36)
Ahit	Nucleus	SUZ12	Cardiac hypertrophy	(37)
Chaer	Nucleus	EZH2	Cardiac hypertrophy	(38)
Uc.323	Nucleus	EZH2	Cardiac hypertrophy	(39)
Mhrt	Nucleus	Brg1	Cardiac hypertrophy and failure	(41, 47)
Linc1405	Nucleus	Eomes	Cardiac differentiation	(42)
CPR	Nucleus	DNMT3A	Cardiac proliferation	(43)
MDRL	Cytoplasm	*miR-361*; *miR-484*	Cardiac apoptosis	(48)
	Nucleus	*Pre-miR-484*		

Loss of *HBL1* increases CM differentiation from hPSCs. *HBL1* expresses in both nucleus and cytoplasm of undifferentiated hPSCs. In the cytoplasm, *HBL1* binds with *miR-1* to fine-tune its activity and further regulate cardiogenic gene expressions (**Figure 1**). Additionally, lncRNA *HOTAIR* (HOX antisense intergenic RNA), which was initially described as a regulator of cancer progression, also displays a cardioprotective role in acute myocardial infarction, which is partially through the interaction and negative regulation of *miR-1* (10).

LINCMD1 (Long Intergenic Non-protein Coding RNA, Muscle Differentiation 1) is a muscle-specific ceRNA, which is required for muscle differentiation and plays an important role in myogenesis. *LINCMD1* acts as ceRNAs for two muscle-specific microRNAs, *miR-133* and *miR-135*, which target the MAML1 (expression of mastermind-like-1) and MEF2C (myocyte-specific

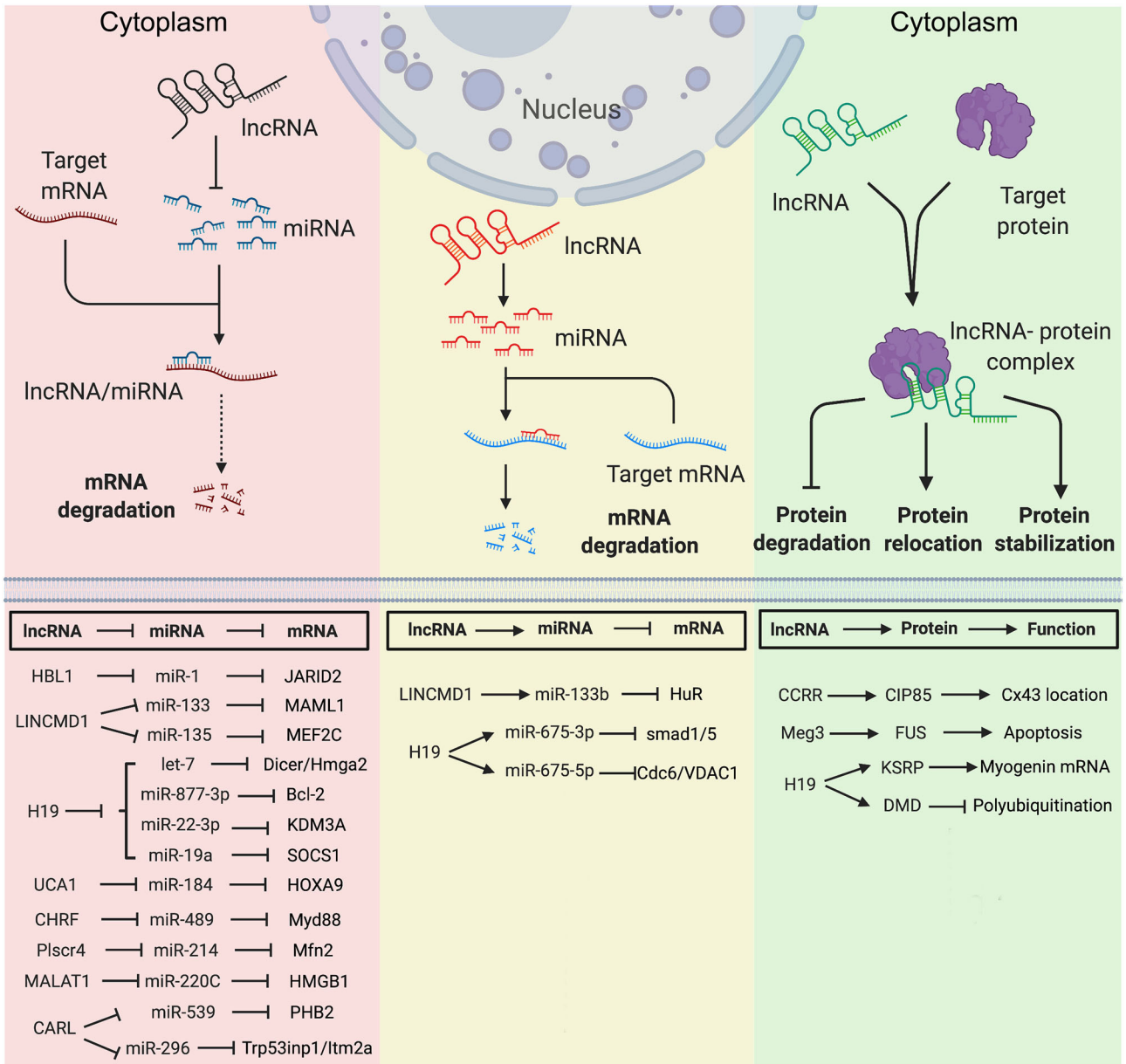

FIGURE 1 | Mechanisms of Long non-coding RNAs in cytoplasm in heart development and diseases. **(Left)** lncRNA functions as miRNA sponge. **(Middle)** lncRNA functions as miRNA precursor. **(Right)** lncRNA functions as protein scaffold. Created with BioRender.com.

enhancer factor 2C) genes, respectively (**Figure 1**). MAML1 and MEF2C are transcriptional coactivators which positively regulate muscle-specific gene expression. Depletion of *LINCMD1* represses the expression of MAML1 and MEF2C, whereas overexpression of *LINCMD1* increases MAML1 and MEF2C expression levels and promotes muscle differentiation (11).

It was reported that approximately 378,295 ceRNA interactions appeared in the cardiovascular disease-related ceRNA interactions (62). *H19* is a lncRNA with high expression level in embryos (63, 64). *H19* is conserved in both human and mouse and has no coding potential. *H19* is required for muscle differentiation and regeneration via acting as a natural molecular sponge for the *let-7* family of miRNAs (13). Depletion of *H19* causes precocious muscle differentiation, which can be repressed by *let-7* overexpression (**Figure 1**) (14). In H_2O_2-treated CMs and mouse ischemia-reperfusion (I/R) hearts, *H19* functions as a ceRNA for *miR-877-3p*, which targets Bcl-2 to further regulate mitochondria-mediated apoptosis in myocardial I/RI (**Figure 1**) (15). Additionally, Zhang et al. reported that *H19* functions as a ceRNA of *miR-22-3p*, which directly targets KDM3A gene to ameliorate MI-induced myocardial injury (**Figure 1**) (16). *H19* is also a pro-senescence lncRNA in CMs

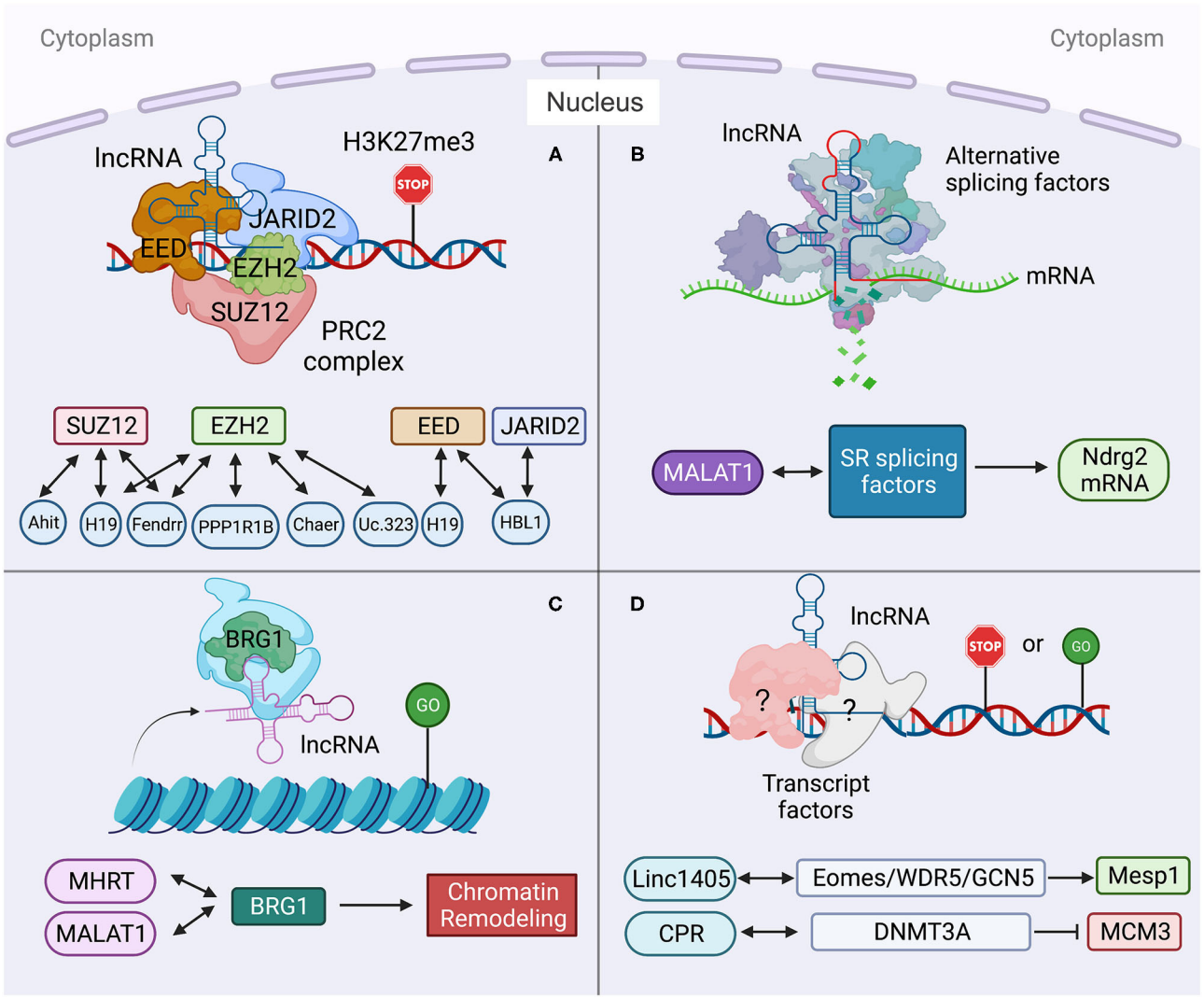

FIGURE 2 | Long non-coding RNA mechanisms in nucleus in heart development and diseases. **(A)** lncRNA targets to PCR2 complex and regulates its downstream gene transcription. **(B)** lncRNA regulates pre-mRNA alternative splicing. **(C)** lncRNA regulates chromatin remodeling. **(D)** lncRNA interacts with transcription factors to regulate gene transcription. Created with BioRender.com.

by counteracting *miR-19a* to upregulate SOCS1 expression and further activate the p53/p21 pathway to promote CM senescence (**Figure 1**) (17).

Many lncRNAs have been reported to play a ceRNA role in hypertrophic cardiomyopathy. LncRNA *UCA1* regulates cardiac hypertrophy via the *UCA1*/*miR-184*/HOXA9 axis (**Figure 1**) (22). *MIAT* promotes cardiac hypertrophy through targeting *miR-150* (23). LncRNA *CHRF* (cardiac hypertrophy related factor) regulates cardiac hypertrophy via the *CHRF*/*miR-489*/Myd88 axis (24). LncRNA *ROR* mediates cardiac remodeling and promotes cardiac hypertrophy via interacting with *miR-133* (25). *Plscr4* negatively regulates cardiac hypertrophy *in vivo* and *in vitro* via the *miR-214*/Mfn2 axis (26). *MALAT1* (metastasis-associated lung adenocarcinoma transcript 1) reduces transient outward potassium current of CMs by targeting *miR-220C* and its downstream target gene HMGB1 (**Figure 1**) (27). *CARL* (cardiac apoptosis-related lncRNA) significantly increases in CMs since the neonatal stage of mouse (44). *CARL* can negatively regulate mitochondrial fission and apoptosis through the *miR-539*/PHB2 axis (44). It can also directly target *miR-296* and its downstream genes Trp53inp1 and Itm2a, further regulating CM replication and cardiac regeneration after injury (**Figure 1**) (30).

To date, hundreds of publications have reported the ceRNA role of lncRNAs under normal and diseased conditions. Given the cascading effects exerted by the gene networks comprising ceRNA-miRNA-coding genes, lncRNA and its downstream gene networks are potential new targets for cardiovascular disease therapy.

Long Non-coding RNA Forms miRNA Precursor

LncRNAs can be transcribed as miRNA precursors, which produce mature miRNAs via further processing. Therefore, lncRNAs could indirectly regulate the expression of miRNA downstream target genes. For example, transcript of *LINCMD1* hosts a *pre-miR-133b* transcript. The RNA-binding protein HuR is a component of *LINCMD* regulatory circuitry to regulate muscle differentiation (65). During the early stage of muscle differentiation, HuR binds to *LINCMD1* and promotes *miR-133* biogenesis from the *LINCMD1* transcript. HuR/*LINCMD1* complex is then targeted by *miR-133* in the cytoplasm (**Figure 1**). Thus, the ceRNA function of *LINCMD1* reinforces HuR expression via counteracting *miR-133* in a positive feedforward loop (65). In this case, *LINCMD1* plays dual roles in fine-tuning the dynamic of muscle differentiation and regeneration.

Interestingly, the exon 1 of *H19* hosts transcripts of *miR-675-3p* and *miR-675-5p*. *MiR-675-3p* regulates the bone morphogenetic protein (BMP) signaling pathway by directly targeting Smad1 and Smad5 mRNAs (**Figure 1**) (18). *MiR-675-5p* could target DNA replication initiation factor Cdc6 mRNA (18). Therefore, *H19* exhibits a pro-differentiation function in primary myoblasts and regenerating skeletal muscles (19). In the rat model of diabetic cardiomyopathy, overexpression of *H19* can attenuate apoptosis of diabetic CMs and improve left ventricular function, whereas knockdown of *H19* shows opposite functions. Mechanistically, *H19* expression is significant downregulated in the hearts of rats with diabetic cardiomyopathy, which leads to a reduced level of *miR-675* and an increased level of *miR-675* target-gene VDAC1. Enhanced VDAC1 can induce apoptosis of CMs when exposed to high glucose (12).

Long Non-coding RNA Functions as Protein Scaffold

In cytoplasm, lncRNA can regulate protein location and stability by directly binding with target protein(s). As an anti-arrhythmic lncRNA, *CCRR* (cardiac conduction regulatory RNA) is downregulated in both mouse and human heart failure (31). *CCRR* knockdown induces arrhythmias, and its overexpression improves cardiac conduction. *CCRR* is also required for maintaining the proper distribution of connexin43 (CX43) in the intercalated discs (**Figure 1**). Mechanically, *CCRR* directly binds with CX43-interacting protein CIP85 and prevents CX43 from backward trafficking and subsequent degradation in the cytoplasm of CMs (31).

Meg3 is upregulated in infarcted mouse hearts and human failing hearts. *Meg3* expression is directly regulated by p53 under hypoxic condition. It has been reported that *Meg3* has a pro-apoptotic function in rodent CMs (32). *Meg3* shRNA delivered by the adeno-associated virus serotype 9 (AAV9) can significantly improve cardiac function. *Meg3* functions as protein scaffold to direct bind with RNA-binding protein FUS and regulates apoptotic signaling pathway (**Figure 1**) (32).

Except for the functions mentioned above, *H19* also interacts with proteins in the cytoplasm. In the undifferentiated multipotent mesenchymal C2C12 cells, *H19* interacts with a multifunctional RNA binding protein KSRP (K homology-type splicing regulatory protein) (20). To maintain the undifferentiated state of C2C12 cells, cytoplasmic *H19* post-transcriptionally modulates gene expression via acting as a protein scaffold of KSRP and promotes its interaction with RNA exosome, which further enhances the KSRP-promoted mRNA decay of myogenic genes (20). Recently, in muscular dystrophy (MD) patients, *H19* was found to directly interact with dystrophin and inhibit E3-ligase-dependent polyubiquitination at Lys3584 for protein degradation. Non-silent mutation (C3340Y) of dystrophin results in defective interaction between dystrophin and *H19*, which causes ubiquitination and degradation of dystrophin (**Figure 1**) (7). In both *Dmd* mouse model and human iPSC-derived skeletal muscle cells from patients with Becker MD, simultaneous administration of *H19* RNA mimic and nifenazone, an analgesic for rheumatic conditions, could effectively inhibit dystrophin degradation, preserve skeletal and cardiac muscle histology, and improve cardiac strength and heart function. This suggests a protective role of *H19* in both Becker and Duchenne muscular dystrophy, providing a potential RNA therapy for MD patients (7).

LONG NON-CODING RNA FUNCTIONS IN NUCLEUS

Compared to cytoplasm, RNAs are processed in nucleus where many lncRNAs reside and execute functions. Nuclear lncRNAs play a variety of crucial roles with complex molecular mechanisms, including regulating chromatin organization, transcription, and different nuclear condensates (66).

Long Non-coding RNA Interacts With the Polycomb-Repressive Complex 2 (PRC2)

Multiple nuclear lncRNAs have been found to regulate lineage differentiation by interacting with PRC2. Histone-modifying complex PRC2 plays a pivotal role in determining the epigenetic state of genes controlling pluripotency, lineage commitment, and cell differentiation (67). A heart-associated lncRNA, *Bvht* is required for the commitment of nascent mesoderm to a cardiac fate from mouse ESCs (33). In the nucleus, *Bvht* can activate the core cardiovascular gene network by interacting with SUZ12, a component of PRC2, during CM differentiation (**Figure 2**). In *Bvht*-depleted cells, SUZ12 and PRC2 associated chromatin modification H3K27me3 are deposited at promoters of cardiogenic genes, such as Mesp1, which is a master regulator of cardiovascular fate commitment (33). Additionally, deletion of a 5′ asymmetric G-rich internal loop (AGIL) in *Bvht* can dramatically impair CM differentiation (34). Through AGIL, *Bvht* can interact with a cellular nucleic acid binding protein CNBP (ZNF9), which is known as a zinc-finger protein to bind with single-stranded G-rich sequences. Together, in the nucleus, *Bvht* controls cardiovascular lineage commitment by interacting with SUZ12/PRC2 and CNBP through defined RNA motifs (33, 34).

In mouse, a lateral mesoderm-specific lncRNA *Fendrr* is essential for heart development (35). During mouse embryo

development, *Fendrr* binds with both PRC2 via the EZH2 subunit and TrxG/MLL complexes and acts as modulators of PRC2 or TrxG/MLL activity (**Figure 2**) (35). *Fendrr* deficient embryos show upregulation of several transcription factors controlling lateral plate or cardiac mesoderm differentiation, accompanied with a drastic reduction of PRC2 occupancy and decreased H3K27 trimethylation and/or increased H3K4 trimethylation at those gene promoters. So, similar to *Bvht*, *Fendrr* plays an essential role in controlling cardiac lineage fate commitment via PRC2 (35).

The interaction between lncRNA and PRC2 complex is conserved in human and mouse. LncRNA *PPP1R1B* was found to bind with EZH2, a key PRC2 subunit (**Figure 2**) (36, 68). Silencing of *PPP1R1B* compromises myotube development in both mouse C2C12 and human skeletal myoblasts (36). In hiPSCs-CMs, *PPP1R1B* deficient also impairs myogenic differentiation (36). *PPP1R1B* regulates the expression of myogenic transcription factors, such as MyoD, Myogenin, and Tbx5, by interacting with PRC2 at the chromatin interface. *PPP1R1B* interacts with PRC2 to suppress H3K27me3 histone modification on the MyoD1 and Myogenin promoters. In the nucleus, *PPP1R1B* modulates PRC2 occupancy on promoters of essential myogenic genes to regulate myogenic differentiation during heart and skeletal muscle development (36).

Our recent study found that nuclear *HBL1* interacts with two PRC2 subunits, JARID2 and EED in human pluripotent stem cells (**Figure 2**) (9). During human cardiogenesis, loss of *HBL1* disrupts genome-wide PRC2 occupancy, reduces H3K27me3 chromatin modification on essential cardiogenic genes, and therefore enhances cardiogenic gene transcription in undifferentiated hPSCs and later-on differentiation. At the pluripotency stage, deletions of *HBL1* and JARID2 both reduce PRC2 occupancy on 62 overlapped cardiogenic genes. Therefore, *HBL1* precisely controls cardiogenic gene transcription via modulating PRC2 occupancy.

H19 plays important functions in both cytoplasm and nucleus. In diabetic cardiomyopathy, cytosolic *H19* forms *miR-675-3p* and *miR-675-5p* and attenuates apoptosis of CMs (12). Under the same pathological condition, Zhuo et al. reported that *H19* directly binds with EZH2, a subunit of PRC2, in CM nucleus to affect the anti-autophagy function (56). Loss of H19 was found to reduce EZH2 and H3K27me3 occupancy on the promoter of DIRAS3, which regulates the formation of autophagosome initiation complex (**Figure 2**) (21), and causes DIRAS3 downregulation. Consistent with its cytosolic function (12), overexpression of *H19* can inhibit cell death of CMs caused by high glucose via this nuclear mechanism. Recently, Viereck et al. reported the interaction between *H19* and PRC2 complex subunits EED, EZH2 and SUZ12 in the nuclear lysate of HL-1 CMs (**Figure 2**) (6). In pressure overload-induced left ventricular hypertrophy mice, *H19* ablation aggravates cardiac hypertrophy compared to wild-type mice. Taken together, *H19* physically interacts with PRC2 to suppress H3K27me3 modification at the Tescalcin locus, which is an anti-hypertrophic gene, to promote Tescalcin expression and in turn repress the NFAT signaling pathway (6).

Many other lncRNAs also have been found to interact with PRC2 complex under heart disease conditions. *Ahit* suppresses cardiac hypertrophy through binding with SUZ12 to regulate PRC2 occupancy on the MEF2A (myocyte enhancer factor 2A) promoter (**Figure 2**) (37). *Chaer* is required for the development of cardiac hypertrophy through direct binding with PRC2 subunit EZH2 to further regulate expressions of Anf, Myh7 and Acta1 genes (**Figure 2**) (38). *Uc.323* protects CMs against cardiac hypertrophy by binding with EZH2 to regulate CPT1b gene expression (**Figure 2**) (39).

Taken together, lncRNAs play important roles in cardiac development and diseases by interacting with PRC2 complex to affect PRC2-related epigenetic modifications.

Long Non-coding RNA Regulates Alternative Splicing of Pre-mRNA

Alternative splicing (AS) of pre-mRNA enhances diversities of transcriptome and proteomic of the genome in higher eukaryotes (69). During tissue- or cell-type specification, the serine/arginine (SR) splicing factors regulate AS in a concentration or phosphorylation dependent manner (70, 71). During human cardiovascular differentiation, stage-specific RNA alternative splicing and lineage-enriched lncRNAs were identified by whole RNA-seq (45). As a long nuclear-retained regulatory RNA (nrRNA), *MALAT1* interacts with SR splicing factors in the nuclear speckle domains (**Figure 2**) (28). *MALAT1* regulates mRNA alternative splicing by modulating the levels of phosphorylated SR proteins (28). During pressure overload-induced cardiac remodeling and failure, *Malat1* was found to be an alternative splicing regulator of Ndrg2, which shows skipped exon 3 in hypertrophic mouse hearts (72, 73).

Long Non-coding RNA and Chromatin Remodeling

In addition to interactions with splicing factors and epigenetic factors, lncRNAs have also been shown to interact with chromatin remodeling complexes (74). *Mhrt* (myosin heavy-chain-associated RNA transcripts) is a cardiac-specific lncRNA located in the murine myosin heavy chain 7 locus and is suppressed by the BRG1-HDAC-PARP chromatin repressor complex in cardiomyopathy (75). Overexpression of *Mhrt* protects mouse heart from hypertrophy and failure (47). *Mhrt* directly binds with BRG1, which is a chromatin-remodeling factor and the ATPase subunit of the SWI/SNF complex (**Figure 2**) (40), to remove SWI/SNF from its occupied genomic regions on target genes, thus regulating chromatin remodeling and gene transcription. *Mhrt* binds with the helicase domain of BRG1, which is crucial for tethering BRG1to its targets. In turn, BRG1 represses *Mhrt* in stress-induced cardiac hypertrophy and failure (41). This *MHRT*-BRG1 feedback circuit is also conserved in the human heart (47). *MALAT1* can also form RNA-protein complex with chromatin-remodeling enzyme BRG1 and histone deacetylase HDAC9 in vascular smooth muscle cells (**Figure 2**). This HDAC9-*MALAT1*-BRG1 complex represses expression of contractile protein genes in association with gain of H3K27me3 histone modification (29).

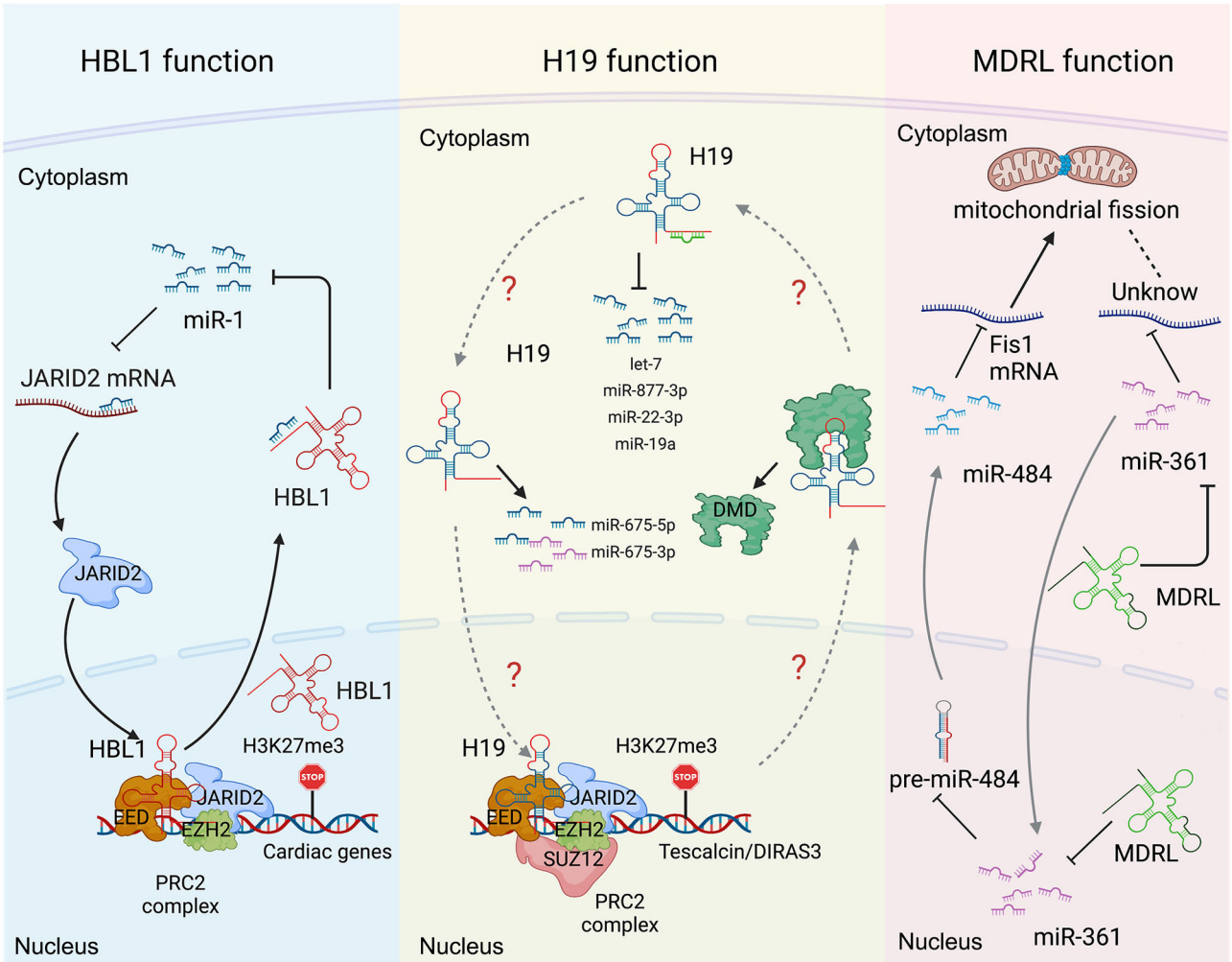

FIGURE 3 | Long non-coding RNA mechanisms cohere with cytoplasm and nucleus in heart development and diseases. **(Left)** the functions of *HBL1* in both cytoplasm and nucleus. **(Middle)** *H19* has multiple functions in both cytoplasm and nucleus. **(Right)** the functions of *MDRL* in both cytoplasm and nucleus. Created with BioRender.com.

Long Non-coding RNA Interacts With Transcription Factors

Besides chromatin-remodeling factors and epigenetic factors, transcription factors have also been found to interact with lncRNAs in heart development and disease. *Linc1405* is highly expressed in heart during mouse embryo development and critical for proper cardiac differentiation (42). *Linc1405* interdependently interacts with Eomes, which physically mediates Eomes/WDR5/GCN5 complex binding at the enhancer region of Mesp1 gene to activate its expression (**Figure 2**) (42). Mesp1 is one of the earliest key regulators of cardiac lineage specification (76). Disruption of Mesp1 in mice results in embryonic lethality due to a cardiac mesoderm deficiency (77). Therefore, *linc1405* guides Eomes/WDR5/GCN5 complex to directly target Mesp1 and affect expression of Mesp1 downstream genes to control cardiac differentiation (42).

Recently, lncRNA *CPR* (cardiomyocyte proliferation regulator) was found to play an important role in the regulation of CM proliferation (43). Deletion of *CPR* in CMs increases CM proliferation, reduces scar formation, and improves heart function after myocardial injury. Mechanically, *CPR* represses CM proliferation by suppressing the transcription of MCM3, which regulates initiation of eukaryotic genome replication and cell cycle (78) by direct binding with DNMT3A. Further, DNMT3A promotes CpG methylation of MCM3 promoter and represses transcription of MCM3 (**Figure 2**) (43).

CROSSTALK OF CYTOSOLIC AND NUCLEAR PORTIONS OF THE SAME LONG NON-CODING RNA

Many lncRNAs, such as *HBL1* (8, 9), *H19* (6, 7, 13, 14, 19, 20), *MDRL* (48) and *LncMyoD* (79), express in both cytoplasm and

nucleus to display different functional mechanisms. However, how the cytosolic and nuclear mechanisms mediated by the same lncRNA could crosstalk with each other has been rarely studied. Recently, we reported the function of nuclear *HBL1* in human cardiogenesis (9), following our previous characterization of cytosolic *HBL1* role during human CM differentiating (8). We also defined the mechanism by which cytosolic and nuclear *HBL1* crosstalk to control cardiogenic gene transcription (9). *HBL1* functions as a *miR-1* sponge in cytoplasm and governs *PRC2* occupancy on cardiogenic genes in nucleus (**Figure 3**). In the meanwhile, *miR-1* was found to bind with 3'UTR of *JARID2* mRNA to repress its expression, and JARID2 deficiency reduces PRC2 occupancy on cardiogenic genes. This conserved *miR-1*-JARID2 axis thus allows precise regulation of nuclear PRC2 occupancy on cardiogenic genes through *miR-1* activity in cytosol (**Figure 3**). In the cytoplasm, *HBL1* counteracts *miR-1*, which further determines mRNA and protein level of JARID2. After JARID2 protein entering nucleus, nuclear *HBL1* binds with JARID2 and EED to determine PRC2 occupancy on cardiogenic genes (**Figure 3**). Together, this *HBL1*/*miR-1-HBL1*/JARID2/PRC2 mechanism coordinates to fine-tune the chromatin state of essential cardiogenic genes in human cardiogenesis (8, 9).

H19 has been well-studied in heart development and various heart diseases, including diabetic cardiomyopathy (12, 56), myocardial infarction (15), cardiac hypertrophy (6), muscular dystrophies (7) and heart failure (17). *H19* displays multiple functions in both cytoplasm and nucleus. Under cardiac hypertrophy, *H19* acts as a *miR-675* precursor to regulate the expression of miR-675 downstream gene VDAC1 and CM apoptosis in cytoplasm (**Figure 3**) (12); In nucleus, *H19* regulates PRC2 occupancy on the promoters of DIRAS3 and Tescalcin to repress cardiac hypotrophy (**Figure 3**) (6, 56). Consistently, all those studies reported that overexpression of *H19* in CMs can mitigate cardiac hypertrophy (6, 12, 56). These observations raise a question whether the cytosolic and nuclear functions of *H19* could coordinate to regulate cardiac hypertrophy, which remains to be further investigated.

MDRL (mitochondrial dynamic related lncRNA) is another well-studied lncRNA with defined mechanisms in both nucleus and cytoplasm. *MDRL* functions as a ceRNA of *miR-361*, which directly affects *miR-484* expression in mouse CMs (**Figure 3**) (48). *MDRL* inhibits mitochondrial fission and apoptosis through two miRNAs, *miR-361* and *miR-484*. In nucleus, *MDRL* affects the processing of *pre-miR-484* by targeting miR-361. In cytoplasm, *MDRL* regulates the mitochondrial network through both *miR-361* and *miR-484* (**Figure 3**). This work defined the complex functions of *MDRL* in both miRNA processing and downstream gene expression (48). All these findings suggest that clinical application of lncRNA should rely on deeper mechanistic studies, especially the differential roles of the same lncRNA in nucleus and cytoplasm.

CONCLUSIONS

We summarized the biological functions and molecular mechanisms of lncRNAs in heart development and disease. In heart development, lncRNAs *Carmen, HBL1, Bvht, Fendrr, Bvht* and *CRP* regulate cardiac fate commitment, lineage differentiation, CM maturation/proliferation, and sarcomere organization etc. via both nuclear and cytoplasmic mechanisms. In heart diseases, lncRNAs are involved in the pathogenesis of cardiac hypotrophy, muscular dystrophy, myocardial infarction, diabetic cardiomyopathy, non-Ischemic cardiomyopathy (NICM) and heart failure and so on. With current progresses of genome-wide sequencing and functional screening studies, more functional lncRNAs have been identified in organogenesis and diseases, although the detailed molecular mechanisms of most lncRNAs have not been clearly defined. For example, lncRNAs *ALIEN* is expressed in undifferentiated pluripotent stem cells and impairs cardiovascular differentiation from pluripotent stem cells with molecular mechanism to be further studied (80). LncRNA *GASL1* is downregulated in chronic heart failure and can inhibit CM apoptosis through TGF-β1 signaling pathway, but how it regulates TGF-β1 is unclear (81). A group of lncRNAs are enriched in peripheral blood under different heart disease conditions (82, 83). For example, lncRNA *Heat2* expression is increased in the blood of heart failure patients (84); lncRNA *MT-LIPCAR*, transcribed from mitochondrial DNA, is positively associated with left ventricular diastolic dysfunction (54, 85). Although these lncRNAs might be utilized as disease markers or possess therapeutic penitential, their molecular mechanisms still require further characterizations.

The subcellular location of lncRNA is critical for its function, particularly for those lncRNAs highly expressed in both nucleus and cytoplasm (86). Cytosolic lncRNAs mainly function as regulators of mRNA stability, mRNA translation, miRNA processing and function, whereas nuclear lncRNAs can epigenetically regulate chromatin remodeling, structure, and gene transcription. Therefore, the balanced doses and transportation of lncRNA between cytoplasm and nucleus are expected to be a new research topic in the lncRNA field. During the last two decades, the translational potential of non-coding RNAs in heart disease therapy has gradually emerged. Nowadays, accumulated evidence indicates that lncRNAs provide a new layer of regulatory mechanism on top of coding genes. Since many lncRNAs have low evolutionary conservation (87), studies of lncRNAs might also reveal unique molecular mechanisms of heart development and disease in the human. Given the complex mechanisms, it is expected lncRNAs could offer new preventive and treatment approaches for human diseases including cardiovascular disease. Although, currently, there is no lncRNA therapeutic approach has progressed into preclinical or clinical trial, *H19* has been tested as a potential clinical therapeutic target in the Yucatan mini-pig (88). The expression changes of lncRNAs under different setting of heart diseases make it difficult for clinical applications. For example, in cardiac hypertrophy, *Mhrt* is downregulated (47), while *Chaer* and *Chrf* are upregulated (24, 38). *MALAT1* and *Whispr* expressions are upregulated in cardiac fibrosis, whereas *Meg3* and *GAS5* expressions are downregulated (89–92). Nevertheless, upregulated lncRNAs can

be repressed by using shRNA, locked nucleic acids (LNAs) or GapmeR, and downregulated lncRNAs can be enhanced by using virus such as adenovirus, adeno-associated virus (AAV), and lentivirus (93). Although no clinical trial exists for lncRNA therapy in heart disease, the success of non-coding RNA *miR-132* based clinical trial paved the way. Recently, phase 1b clinical study to assess safety, pharmacokinetics and pharmacodynamics parameters of CDR132L, a *miR-132* inhibitor, has been completed (94). CDR132L is safe and well tolerated. Importantly, it improves cardiac function of heart failure patients. Therefore, the clinical applications of lncRNAs have a bright future, with fully and clearly characterized molecular mechanisms.

AUTHOR CONTRIBUTIONS

LH and LY summarized the references and prepared the manuscript. LH drafted the illustrations. LY supervised the project. All authors contributed to the article and approved the submitted version.

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REVIEW
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miR-208a in Cardiac Hypertrophy and Remodeling

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Various stresses, including pressure overload and myocardial stretch, can trigger cardiac remodeling and result in heart diseases. The disorders are associated with high risk of morbidity and mortality and are among the major health problems in the world. MicroRNAs, a class of ∼22nt-long small non-coding RNAs, have been found to participate in regulating heart development and function. One of them, miR-208a, a cardiac-specific microRNA, plays key role(s) in modulating gene expression in the heart, and is involved in a broad array of processes in cardiac pathogenesis. Genetic deletion or pharmacological inhibition of miR-208a in rodents attenuated stress-induced cardiac hypertrophy and remodeling. Transgenic expression of miR-208a in the heart was sufficient to cause hypertrophic growth of cardiomyocytes. miR-208a is also a key regulator of cardiac conduction system, either deletion or transgenic expression of miR-208a disturbed heart electrophysiology and could induce arrhythmias. In addition, miR-208a appeared to assist in regulating the expression of fast- and slow-twitch myofiber genes in the heart. Notably, this heart-specific miRNA could also modulate the "endocrine" function of cardiac muscle and govern the systemic energy homeostasis in the whole body. Despite of the critical roles, the underlying regulatory networks involving miR-208a are still elusive. Here, we summarize the progress made in understanding the function and mechanisms of this important miRNA in the heart, and propose several topics to be resolved as well as the hypothetical answers. We speculate that miR-208a may play diverse and even opposite roles by being involved in distinct molecular networks depending on the contexts. A deeper understanding of the precise mechanisms of its action under the conditions of cardiac homeostasis and diseases is needed. The clinical implications of miR-208a are also discussed.

Keywords: miR-208a, cardiomyopathy, miRNA, hypertrophy, Trbp, Sox6

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INTRODUCTION

The heart acts like a pump. It incessantly contracts to deliver oxygen and nutrient-rich blood throughout the body. In response to cardiac damage, pressure overload and a variety of other pathologic insults, the heart often undergoes complex molecular, cellular and interstitial changes, termed cardiac remodeling. The progression of cardiac remodeling eventually leads to cardiac dysfunction, which is the major threat to human health and has become one of the leading causes of death in world (1).

Hypertrophy is a common type of cardiac remodeling. It is the primary response of cardiac muscle to elevated workload or myocardial infarction (2). Cardiac hypertrophy is believed to be adaptive and have a "compensatory" role in the premise that it can diminish oxygen consumption, normalize the systolic wall stress, and improve ejection performance. However, long-term and chronic stress (ex. hypertension or valvular disease) can result in pathological remodeling, characterized by the increase in the size of cardiomyocytes, the abnormal enlargement and thickening of the heart muscle, cardiac dysfunction and fibrosis (3, 4). Multiple biological processes participate in modulating cardiac hypertrophy and remodeling. At the cellular level, hypertrophic growth of cardiomyocytes can be induced by numerous signal cues, including biomechanical stress, neurohumoral and endocrine hormones, involves MAPK, PI3K-AKT, Calcineurin-NFATc and other signal pathways (5), and is accompanied by enhanced protein synthesis, reorganization of the cytoskeleton, metabolic shift from oxidative phosphorylation to glycolysis and adult-to-fetal switch on expression program of myosin isoforms (3, 6–8).

A class of small noncoding RNAs, termed microRNAs (miRNAs), was discovered as key regulators of gene expression more than 2 decades ago (9–11). These small transcripts are composed of approximately 21–25 nucleotides, and exert their functions primarily through translational repression or messenger RNA degradation by base-pairing with the mRNA targets. MiRNAs are widely presented in many kinds of organisms (11, 12), and most of them, are well conserved during evolution (13, 14). Presently, more than 2000 miRNAs have been discovered in humans, and it is believed that these miRNAs can modulate approximately 1/3 of genes in the genome (15, 16). Because miRNAs frequently have only modest effects on the expression of individual genes, they are often conceptualized as "fine-tuners". However, a single miRNA can target numerous mRNA transcripts, thus, the accumulative effects of coordinated modulation of multiple downstream mRNA transcripts can substantially influence the functional outcomes (17).

Extraordinary effort has been devoted to study cardiac hypertrophy and remodeling, yet, the underlying mechanisms remain elusive. Many clues to the regulatory events were derived from the identification and characterization of new factors involved in the processes. Numerous miRNAs, including miR-1, miR-133, miR-208a, miR-499 and miR-22, have been implicated in cardiac remodeling and pathogenesis, adding a new dimension to the regulatory networks of cardiomyopathy (18–22). Studies on miRNAs have uncovered previously unrecognized mechanisms and provided novel insights into cardiac remodeling. In addition, due to the relative ease of pharmacological manipulation, the identified cardiomyopathy-related miRNAs also hold great potential as promising targets for therapeutic intervention. Here, we focus on a cardiac muscle-specific miRNA, miR-208a (miR-208), which plays key roles in regulating heart function and appears to be master organizer of cardiac remodeling to pathogenic stress. We summarize the findings on its regulatory effects and mechanisms, and propose several intriguing topics, which need to be resolved in the future. The clinical potential of miR-208a as a diagnostic biomarker and a therapeutic target is also discussed.

EXPRESSION OF miR-208a AND ITS REGULATION

MiR-208a belongs to the miRNA family, which also includes miR-208b and miR-499 (21, 23). Members of this family contain nearly identical seed sequences, thus can target certain common downstream mRNAs and may be functionally redundant (21). These three miRNA are encoded by the intron regions of Myh6, Myh7, and Myh7b genes, respectively (**Figure 1**) (21, 23). These three genes encode myosin proteins involved in multiple muscle pathophysiological processes. In mouse, the Myh7 gene encodes the β-isoform of myosin heavy chain (β-MHC) and is highly expressed in embryonic or neonatal cardiomyocytes, while Myh6 encodes the α-isoform of myosin heavy chain (α-MHC), the predominant myosin heavy chain (MHC) isoform in adult heart (24, 25). Cardiac remodeling is usually accompanied by myofibrillar remodeling, a shift in MHC isoform content from α(adult) to β(fetal) in cardiac muscle. Such α-MHC to β-MHC switch may be a maladaptive response and can accelerate the pathogenic remodeling (26–28).

The intronic miR-208a, miR-208b and miR-499 are co-transcribed with their host genes. Spatially, miR-208b and miR499 are expressed in both skeletal and cardiac muscle tissues, while miR-208a is specifically presented in the heart (20, 21, 23). In parallel with the expression of α-MHC and β-MHC in the heart which is developmentally regulated, cardiac miR-208b is mainly expressed in the embryonic or neonatal stages, while miR-208a is enriched in the adult cardiac muscle (21, 23). Notably, humans display an entirely distinct myosin expression pattern. MHY7 is the major left ventricular MHC in the adults, whereas *MYH6* eocodes the myosin enriched in developing human ventricle and adult atrium (29). Owing to the expression pattern of their host genes, expression of miR-208 family members in adult human hearts showed prominent chamber specificity. MiR-208a is abundant in atrial myocardium, while miR-208b is preferentially expressed in left ventricles (30).

Expression of miR-208a in the heart is also regulated at the posttranscription level. A double-strand RNA binding protein TRBP is required for the normal posttranscriptional processing of miR-208a. TRBP functions as co-factor of DICER and may confer the dicing specificity or preference for the cleavage of pre-miRNAs (31–33). Genetic abrogation of TRBP in the heart led to the dysregulation of a small subset of miRNAs, among which, miR-208a, miR-208b and miR-499 were the significantly and substantially downregulated ones. The previous study indicates that these three myomiRs appear to be the primary targets of TRBP (34). It is not clear how precursors of these miRNAs are recognized by TRBP machinery or how TRBP "selectively" regulates processing of pre-miR-208a, pre-miR-208b and pre-miR-499 (and several other pre-miRNAs) in the heart. Since TRBP is a double-strand RNA binding protein,

FIGURE 1 | Gene structure and expression pattern of miR-208a, miR-208b and miR-499 and their host genes in mouse. The intronic miR-208a, miR-208b and miR-499 are co-transcribed with their host genes, Myh6, Myh7 and Myh7b respectively.

the stem regions of pre-miR-208a, pre-miR-208b and pre-miR-499, which have certain sequence similarity, may act as the *cis*-elements mediating the recognition. Intriguingly, abrogation of TRBP in the skeletal muscle did not alter the level of miR-499 or its target Sox6 (34, 35). This observation indicates that regulation of miR-499 (and likely also miR-208a) processing by TRBP is context-dependent. One possibility is that additional cardiac-specific cofactors may exist, act in *trans* and participate in reshaping such specificity or preference. It is unclear whether the posttranscriptional regulatory events are conserved in humans. Further investigation on the underlying mechanisms will offer important clues for understanding of the specificity of miRNA post-transcriptional processing.

Intriguingly, expression of miR-208 exhibits certain gender differences. The level of miR-208 in female is 16 and 21-fold higher than that in male at 15 and 21 weeks of age, displaying a female-biased pattern (36). In addition, in both Zucker Lean and in Zucker diabetic fatty rats, which exhibit cardiac hypertrophy, the expression of cardiac miR-208a is much higher in female compared to male, further demonstrating the gender differences (37). The mechanisms need to be addressed in the future.

THE FUNCTIONAL ROLES OF miR-208a

Abnormal expression of miR-208a has been observed in the onset of diseases such as cardiac hypertrophy and heart failure, suggesting that this cardiac specific miRNA may participate in modulating heart function (38–41). Indeed, miR-208a has been found to be involved in a broad array of cellular processes in cardiac pathogenesis by targeting a wide range of downstream mRNAs (**Figures 2**, **3**). Yet, its functional roles and effects in the heart are more complicated than expected, and many questions remain regarding the underlying mechanisms.

miR-208a and Cardiac Hypertrophy

Although it is one of the most abundant miRNAs in adult cardiomyocytes, when miR-208a was deleted in mouse (*miR-208a*$^{-/-}$ or miR-208a KO), no obvious cardiac phenotype, but only a slight reduction in fractional shortening, was seen (20). Abrogation of miR-208a only resulted in very mild phenotypes in the heart at the basal level, whereas it had more profound functional influences in stress models (20, 23). In response to pressure overload or activation of calcineurin signal, miR-208a knockout mouse exhibited resistance to cardiac remodeling, showing virtually no hypertrophic growth of cardiomyocytes or fibrosis (20). Thus, miR-208a is required for stress-induced cardiac pathogenesis. The pro-hypertrophic effect of miR-208a was further demonstrated in the gain-of-function studies. Overexpression of miR-208a in mouse heart is sufficient to induce cardiac hypertrophy, which is evidenced by increased ventricle wall thickness and cross-sectional cell area of cardiomyocytes, and enlarged ventricle chambers (23). At the molecular level, inactivation of miR-208a broadly altered gene expression in mouse hearts. In particular, the transcripts of genes encoding early response factors, heat shock proteins, and skeletal muscle fast-twitch myofiber proteins were substantially upregulated in miR-208a mutant hearts (20). However, the pathophysiological relevance of the altered gene expression pattern is not clear, and the mechanisms by which miR-208a regulates cardiac hypertrophy are still elusive.

There are several topics with regard to miR-208a and cardiac hypertrophy yet to be resolved. Expression of β-MHC in adult cardiac muscle not only is one of the hallmarks of hypertrophy, but appears to be a maladaptive response in the heart, since it can accelerate the pathogenic remodeling (26–28). In miR-208a mutants, cardiac stress failed to upregulate β-MHC, while miR-208a transgene in mouse heart can potently induce the expression of β-MHC and lead to hypertrophy (20, 23). Intriguingly, elevated expression of miR-208a has been implicated in diabetic cardiomyopathy in human patients. The functional consequences were linked to the up-regulation of β-MHC and the α/β-MHC switch, too (42). These findings raise the question of whether the pro-hypertrophic/pro-remodeling function of miR-208a is attributed to its regulatory effects on β-MHC. If yes, how does miR-208a regulate β-MHC? Previous studies have suggested that it could be mediated by numerous transcription regulators, including Med13 (or thyroid hormone–associated protein 1, Thrap1) and Sox6, the transcripts of which

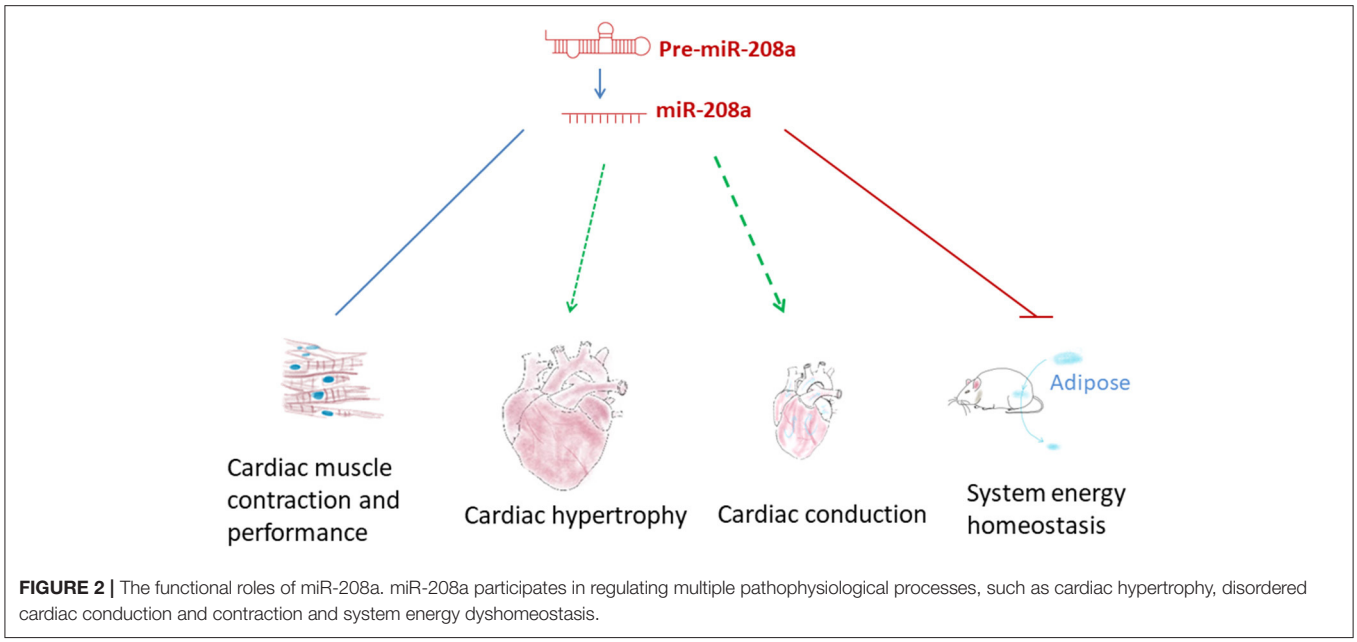

FIGURE 2 | The functional roles of miR-208a. miR-208a participates in regulating multiple pathophysiological processes, such as cardiac hypertrophy, disordered cardiac conduction and contraction and system energy dyshomeostasis.

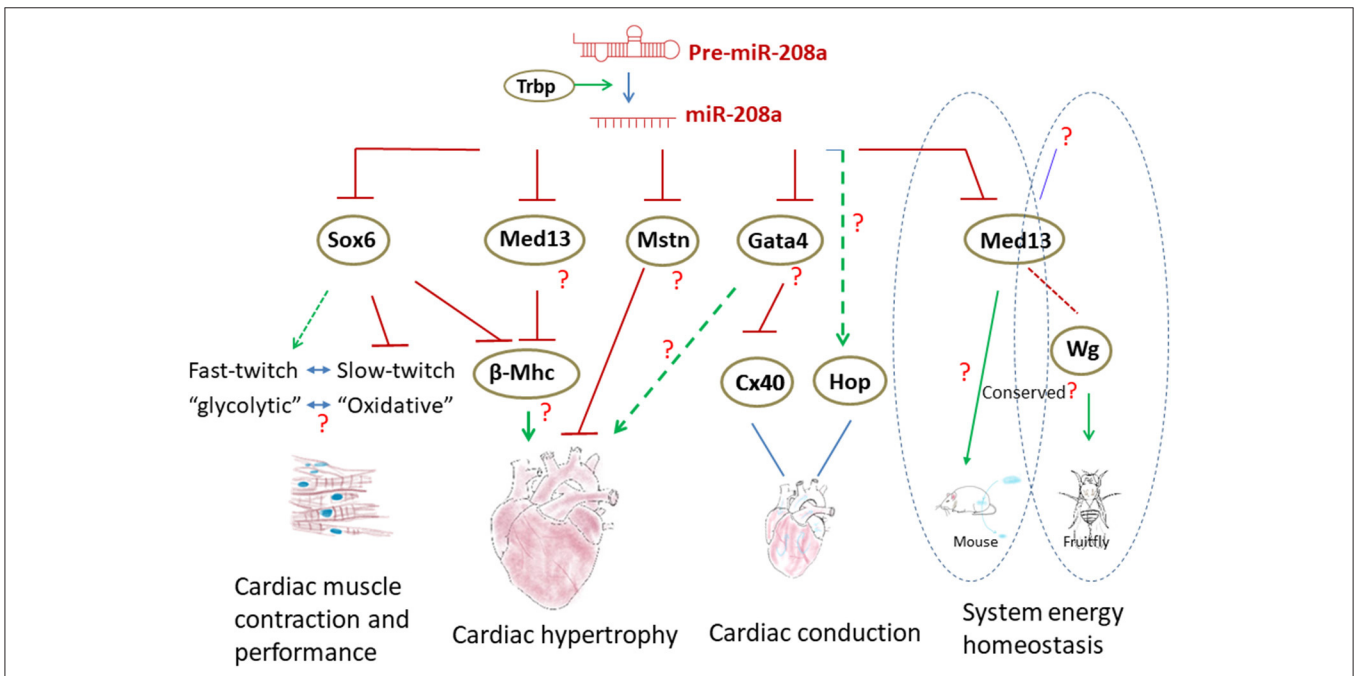

FIGURE 3 | The regulatory networks involving miR-208a in the heart. Normal biogenesis of miR-208a in the heart requires TRBP. miR-208a regulates cardiac function by modulating the expression of downstream genes. In addition, miR-208a in the heart can also control the system energy homeostasis through MED13. Wg was found to be a downstream factor of MED13 in *Drosophila* to regulate metabolism, yet it is not clear if the processes are conserved in mammals.

contain the binding sites and may be the direct targets of miR-208a (20, 23, 34). The amount of Sox6 transcripts was increased in miR-208 null mice, thus,miR-208a can inhibit Sox6 at the mRNA level in the heart (21, 34). Overexpression of Sox6 in heart did result in the downregulation of β-MHC, which was also observed in *miR-208a*$^{-/-}$ mice (21, 43, 44). However, in miR-208a transgenic mice exhibiting upregulation of β-MHC (23), the mRNA level of Sox6 was unaltered (34), indicating additional unrecognized molecular events may be involved. Med13 (Thrap1) is another target and has been thought to function downstream of miR-208a to regulate of myosin expression. Nevertheless, as shown in a study by Grueter et al. (45), transgenic expression of Med13 resulted in upregulation of β-MHC, which appears contradictory to the observation in

miR-208a$^{-/-}$ mice. In addition, no hypertrophy was detected when Med13 gene was deleted in cardiac muscle (45). Thus, the roles of miR-208a/Sox6 and miR-208a/Med13 axes in modulating β-MHC expression and hypertrophy in the heart appear obscure.

Notably, the induced expression of β-MHC *in vivo* by miR-208a transgene was heterogenous in the myocardium (23). It may be caused by the "mosaicism" of transgene expression. It is also likely due to the heterogeneity of the cardiomyocytes in the heart, that individual ones may differentially respond to miR-208a overexpression (23). In addition, β-MHC upregulation in the heart may not be a primary effect, but secondary to the cardiac abnormalities induced by miR-208a transgene (46). The intra- and inter-cellular molecular events underlying these observations need to be further investigated in the future.

Although can accelerate the pathogenesis in the heart, elevated expression of β-MHC alone is not sufficient to induce cardiac remodeling. Thus, it is still unclear how and how much the upregulated β-MHC expression is relevant to cardiac hypertrophy in miR-208a transgenic (miR-208TG) mouse. Is it a correlation or the causality? As shown in the study by Callis, there was no association between the state of β-MHC induction and hypertrophic growth of individual cardiomyocytes, suggesting that upregulation of β-MHC may be not an obligate component of miR-208a-induced hypertrophy (23). Then, what else can participate in mediating the pro-hypertrophic effects of miR-208a? Cardiac hormone atrial natriuretic factor (ANF) is another molecular hallmark of cardiac remodeling (4). However, no significant change of ANF mRNA abundance was detected in miR-208TG hearts (23). Levels of miRNA markers, including miR-1, miR-133 and miR-29a (downregulated in hypertrophy), miR-125b (upregulated in hypertrophy) were not altered either (23). These observations indicate that overexpression miR-208a may induce hypertrophic growth of cardiomyocytes without affecting the aspects of these known pathways.

MiR-208a has been shown to target *Myostatin(Mstn)* (23), which encodes a negative regulator of muscle growth (47). Genetic inactivation of Mstn signaling in the adult murine heart caused cardiac hypertrophy, which phenotypically resembled the consequences of miR-208a overexpression (23, 48). This finding indicates that *Mstn* may be *one* of the downstream targets mediating the pro-hypertrophic function of miR-208a. Yet, it is necessary to experimentally validate the role of Mstn particularly in miR-208a-induced hypertrophy and further confirm the regulatory effects of miR-208a/*Mstn* axis on cardiac remodeling.

One thing, which makes it challenging to decipher the mechanisms of miR-208a, is that this versatile miRNA can target not only anti-hypertrophic genes, but also those encoding pro-hypertrophic factors. For instance, expression of Gata4 in the heart, a transcription factor required for cardiac hypertrophy, was found to be inhibited by miR-208a (23). It is unclear what the role(s) of Gata4 in miR-208a-induced cardiac hypertrophy is. How does miR-208a exert its pro-hypertrophic effects when the downstream targets with opposing functions (anti-hypertrophic Mstn vs. pro-hypertrophic Gata4) co-present in the heart? It may be dependent on miR-208a abundance, the availability of the different targets and the physiological contexts. Comprehensive and systemic characterization of the "miR-208a network" and a deeper understanding of miR-208a activity in the different relevant physiological and pathological contexts are needed, in order to ultimately define the functions of this important miRNA in cardiac hypertrophy. The in-depth further investigation on miR-208a will also offer important insights into the regulatory mechanisms of cardiac remodeling (**Figure 3**).

miR-208a and Cardiac Conduction

miR-208a is also a key regulator of cardiac conduction. Loss of miR-208a resulted in cardiac conduction abnormalities (23). Expression of transcription factor homeodomain-only protein (Hop) and gap junction protein connexin 40 (Cx40) was dramatically down-regulated in the hearts of miR-208a null mice, suggesting that miR-208a is required for the normal expression of these cardiac conduction-related genes (23). MiR-208a may indirectly regulate Hop and Cx40 by targeting GATA4 (23). However, loss of miR-208a only slightly increased the level of GATA4 (23), which appears unlikely to be sufficient to cause such dramatic alteration of Hop and Cx40 expression. Thus, additional unidentified miR-208a downstream targets and other factors may participate in and contribute to the process. The miR-208a transgenic mice exhibited cardiac conduction defects, too (23). Yet, apparently, the abnormalities were not concurrent with the dysregulation of Hop and Cx40. As shown in the study by Callis, overexpression of miR-208a did not alter the expression of Cx40 (23). Growing evidences have suggested that the targeting specificity and capacity of miRNAs are dose-dependent and are often sensitive to the biological contexts (49). Although it is unknown whether the expression of Gata4 was downregulated in miR-208aTG hearts, the cardiac conduction abnormalities observed in miR-208a gain- and loss-of function backgrounds may be attributed to different regulatory pathways and mediated by different downstream targets (**Figure 3**).

miR-208a and Slow-/Fact- Twitch Contractile Gene Expression

The primary function of the heart is to circulate blood by beating and contracting. Defects in contraction often result in severe cardiac disorders. The contractile apparatus in myocytes consists of sarcomeric proteins, which can be broadly classified into 2 major types: fast-twitch and slow-twitch (24, 50). The major type of contractile proteins in cardiomyocytes is slow-twitch, and fast-twitch genes are expressed at much lower levels in the heart (24, 25, 50, 51). However, it has not been well documented how the pattern of fast-/ slow-twitch gene expression is established in cardiomyocytes, and the pathophysiological relevance is not fully understood. As shown in the study by Ding et al., heart-specific inactivation of Trbp (*Trbp*^{cKO}) in mice resulted in progressive cardiac remodeling, concurrent with a "slow-to-fast" shift in myofiber gene expression in the heart, downregulation of normal cardiac slow-twitch myofiber genes and increased expression of genes encoding fast-twitch contractile proteins (34). Dramatic downregulation of miR-208a was observed in *Trbp*^{cKO} hearts and appeared to be responsible for the cardiac defects. Indeed, reintroduction of miR-208a into Trbp mutant hearts substantially corrected the fast- and slow- twitch myofiber

gene expression pattern and rescued the cardiac abnormalities (34). These findings not only implied that the unbalanced fast-/ slow-twitch gene expression and the possible desynchronized myofilament activation could be the cause of the cardiac defects, but also demonstrated the crucial role of miR-208a in establishing or maintaining the proper expression pattern of slow-/ fast-twitch myofiber genes in the heart.

The regulatory effects of miR-208a on slow-/fast- twitch contractile gene expression in the heart appears to be mediated mainly by its downstream target Sox6 (34). Sox6 has been shown to modulate the expression of slow-/fast- twitch myofiber genes in skeletal muscle (43, 44, 52, 53). The similar regulatory events also occur in cardiac muscle. Overexpression of Sox6 in cardiac muscle resulted in the "slow-to-fast" shift in myofiber gene expression, recapitulating the effects of Trbp inactivation (34). Intriguingly, the regulatory effect of Trbp on Sox6 and contractile gene expression appeared to be quantitively correlated with the postnatal ages. It was subtle in neonatal cardiac muscle in which miR-208b is more abundant, but more substantial at adult stage when miR-208a is predominant in the heart (34). Similar to miR-208a, miR-208b is regulated by Trbp, too. Sox6 can also be targeted by miR-208b (21), which has an identical seed sequence to miR-208a, only differing at 3 nucleotides in the 3' region. What is the observed "age-dependence" accounted for, then? It is likely that the targeting capacity of miR-208b on Sox6 is not as strong as that of miR-208a, due to the dissimilarity in sequence outside of the seed region. Or, it is possible that the effects are context-/stage-dependent, and the regulatory axis is effective preferentially in adult hearts.

Skeletal muscle consists of two major types of myofibers, that type I myofibers mainly expressing slow-twitch contractile genes produce relatively less force but can sustain long-lasting contractions, and type II fibers which express fast-twitch contractile genes can support high-intensity and short-duration contractions (51). The molecular motors in myocytes require energy to sustain the contraction. These two types of myofibers have different energy demands and thus preferentially utilize different metabolic pathways to generate ATP. The fast -twitch (type II) myofibers are usually "glycolytic" (anaerobic), whereas the slow ones (type I) are "oxidative" (aerobic) (50, 51, 54). The energetic states are correlated with the myofiber types. Although cardiomyocytes, unlike skeletal muscle cells, appear not to undergo fast- and slow-twitch fiber-type speciation, the findings from the study by Ding et al. (34), raised a question of whether the "slow-to-fast" shift in myofiber gene expression in Trbp mutant hearts is accompanied with a change(s) in metabolic pathways (fatty acid oxidation to glycolysis) (55). It will be interesting to study the link or coupling between these two events in cardiac muscle and the involvement of miR-208a in the processes in the future (**Figure 3**).

miR-208a and Systemic Energy Homeostasis

miR-208a not only regulates heart function, but also participates in modulating systemic energy homeostasis. As shown in the study by Grueter et al. (45), mice administered with miR-208a inhibitor were resistant to high-fat diet-induced obesity, and exhibited improved insulin sensitivity as well as glucose tolerance. Transgenic expression of Med13, a target of miR-208a, in cardiac muscle attenuated the metabolic defects in mouse models of obesity, phenocopying the effects of miR-208a inhibition. The study demonstrated that miR-208a/Med13 axis in cardiac muscle was involved in regulating the energy homeostasis in distant organs, including fat tissue and liver, and indicated that miR-208a could be a promising therapeutic target for metabolic disorders such as type 2 diabetes and obesity (**Figures 2**, **3**). In addition, rapamycin and nebivolol were reported to inhibit weight gain in rodent model of obesity and in human patients (56–59). The effects are similar to the effect of miR-208a inhibition. A further study indicates that both rapamycin and nebivolol may suppresses the up-regulation of miR-208a via inhibiting mTORC1 activation, thus increasing the level of MED13 and conferring the resistance of obesity (60).

The heart is more than a "pump" and may act as an endocrine organ to regulate the energy storage or dissipation of the whole body (45, 61). A tantalizing question is, what mediates endocrine function of the heart. Is there a "slimming" factor(s) produced in cardiomyocytes, regulated by the miR-208a/Med13 axis, and secreted into the bloodstream, so that it modulates the whole-body metabolism? This hypothetical answer can be tested with the parabiosis model, which allows exchange of whole blood between 2 animals (62). It is also possible that the signal output downstream of miR-208a/Med13 axis in cardiac muscle may be initially delivered to the brain as a relay system to other tissue and organs (61).

Intriguingly, Med13 has been found to inhibit lipid accumulation in *Drosophila* (63), thus the anti-obesity function appears to be conserved in invertebrates. Taking advantages of *Drosophila*, which is a good *in vivo* model for genetic analysis of obesity and an ideal system for studying inter-organ crosstalk, Lee et al. (63). Identified the Wingless(Wg) peptide as a circulating "slimming" factor downstream of Med13 and released by cardiac and skeletal muscle. Although autonomous activation of Wnt signaling in adipose can reduce fat mass in mice (64), it is not clear whether the Wnt ligand(s), in particular Wnt1, the mammalian ortholog of Wg, is regulated by miR-208a/Med13 axis in the heart and mediates the cross-organ communications, as well (**Figure 3**).

miR-208a AS A BIOMARKER

Though exclusively expressed in cardiac muscle, miR-208a can be secreted by cardiomyocytes into serum and plasma, in response to cardiac stresses. The abundance of circulating miR-208a was found to be altered concurrently with cardiac pathogenesis in numerous studies (65–67). For instance, in mouse models treated with isoproterenol, plasma level of miR-208a was closely correlated to circulating cardiac troponin I, which is a widely used biomarker for myocardial injury (65, 68). Raised level miR-208a in plasma was also detected in human patients with myocardial injury (66). Although it is unclear whether the upregulation of circulating miR-208a is adaptive or maladaptive and whether it has any functional consequence(s) in cardiac remodeling, growing evidences suggested that it could be a promising, non-invasive diagnostic biomarker for cardiac defects.

miR-208a AS A THERAPEUTIC TARGET

Animals with genetic deletion of miR-208a, at the basal level, appeared to be phenotypically normal or only have very mild phenotypes, while exhibited resistance to cardiac remodeling in response to stresses (20). Inhibition of miR-208a by subcutaneous delivery of the locked nucleic acid-modified antisense oligonucleotides (69–71) (antimiR-208a) could dose-dependently blunts stress-induced cardiac pathogenesis in a rat model of diastolic heart failure (Dahl salt-sensitive rats) (72). The antimiR-208a significantly improved the cardiac function, overall health and survival of the animals, and no adverse side effects were detected on the treatment (72). These findings indicated that targeting miR-208a could be an efficient and a safe way to prevent cardiac remodeling in the heart with minimal side effects on normal tissues.

Altered expression of miR-208 family has been implicated in the onset of cardiac hypertrophy and heart failure in human patients (38–41), indicating that this miRNA and its family members (miR-208b, miR-499) hold great potential as the therapeutic targets. Although their expression patterns in humans are different from those in rodents, the studies using mouse and rat models have been offering important insights and clinical implications. miRNAs exert their functions by regulating their downstream targets, most of which are protein-coding genes. For many identified miRNA:mRNA pairs, the sequence complementation is well conserved in both primates and rodents. Yet, owing to the difference of α/β MHC (their host genes) expression between mice and humans, investigations using non-human primate animal models may be needed to further validate the therapeutic effects prior to full clinical application.

PERSPECTIVE

MiR-208a is one of the cardiac miRNAs which relatively have been well investigated. Yet, as discussed above, our understanding of this miRNA appears still rudimentary. It may be not just for miR-208a. According to Dr. Eric Olson, "there has been a tendency to oversimplify the mechanistic basis of miRNA functions…" (73). Numerous topics need to be addressed. For miR-208, the biological functions and the mechanisms of this miRNA, its multiplicity, the broad range of downstream targets and the complex regulatory network(s), remain very elusive. This miRNA holds great potential as a therapeutic target, however, extensive studies and analyses are required prior to full clinical application.

Safety is always an important issue for therapeutics. Despite the promise of miR-208a-based therapeutics, it is necessary to evaluate the long-term effects of antimiR-208a or other miR-208a inhibitors in various settings. There is a long way to go, but further investigations on this unique miRNA will contribute to the development of new therapeutic approaches to treat heart diseases.

AUTHOR CONTRIBUTIONS

X-HH, J-LL, and JD conceived the presented idea and prepared the manuscript. X-HH, J-LL, X-YL, S-XW, Z-HJ, and S-QL summarized the literature and produced the figures. X-YL, S-XW, Z-HJ, S-QL, and JL reviewed and edited the manuscript. X-HH, J-LL, JL, and JD drafted the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Role of N6-methyladenosine Modification in Cardiac Remodeling

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Cardiac remodeling is the critical process in heart failure due to many cardiovascular diseases including myocardial infarction, hypertension, cardiovascular disease and cardiomyopathy. However, treatments for heart failure focusing on cardiac remodeling show relatively limited effectiveness. In recent decades, epitranscriptomic modifications were found abundantly present throughout the progression of cardiac remodeling, and numerous types of biochemical modifications were identified. m6A modification is the methylation of the adenosine base at the nitrogen-6 position, and dysregulation of m6A modification has been implicated in a wide range of diseases. However, function of m6A modifications still remain largely unknown in cardiac diseases, especially cardiac remodeling. LncRNAs are also shown to play a vital role in the pathophysiology of cardiac remodeling and heart failure. The crosstalk between lncRNAs and m6A modification provides a novel prospective for exploring possible regulatory mechanism and therapeutic targets of cardiac remodeling. This review summarizes the role of m6A modification in cardiac remodeling in the current researches.

Keywords: cardiac remodeling, m6A modification, lncRNAs, heart failure, epigenetic modifications

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INTRODUCTION

Cardiac remodeling is a physiologic and pathologic condition that may occur after various cardiovascular diseases including myocardial infarction, hypertension, cardiovascular disease and cardiomyopathy (1). Cardiac remodeling is a dynamic process in which the heart changes in size, mass, geometry and function in response to mechanical stress such as pressure or volume overload, neurohormones or cytokines in order to maintain hemodynamic homeostasis. When cardiac remodeling progresses, the transition from possible adaptive to maladaptive remodeling occurs, which may likely to establish heart failure (HF) eventually. Treatment for cardiac remodeling have been focused on neurohormonal inhibition such as renin-angiotensin-aldosterone system (RAAS) or sympathetic nervous systems (SNS) inhibition, but the proportion of poor prognosis of HF remained high. As a result, understanding the underlying pathophysiological processes involved in cardiac remodeling is critical for developing novel therapeutic strategies.

Epigenetic modifications are abundantly present throughout the physiological processes of life. It is characterized by the modification in temporal and spatial expression patterns of chromatins and genes driven by particular enzymes, without changing the nucleotide sequence of DNA and subsequent functional alterations of heritable gene (2, 3). RNA modification, also term epitranscriptomic modification, is one of these epigenetic modifications. So far, at least 170 types of RNA modifications have been discovered (4). N6-methyladenosine (m6A) is one of the abundant

mRNA modifications in almost all eukaryotes. It refers to methylation of the adenosine base at the nitrogen-6 position (5). It is written by a complex of m6A methyltransferase, erased by m6A demethylase, and read by m6A binding proteins. m6A modifications govern RNA processing, including splicing, nuclear exports, RNA stability and translation, by recognizing m6A binding proteins (6). m6A has been proved to be involved in the regulation of a wide variety of pathological processes including obesity, metabolic disease and carcinogenesis. For example, one of the m6A component, the RNA methyltransferase METTL3, was discovered to promote proliferation and invasion of human lung cancer cells (7). As for cardiovascular disorders, continuous dynamic control of m6A have been shown to play a critical role as well. METTL14, another methyltransferase of RNA, enhanced the m6A modification of pri-miR-19a and promoted mature miR-19a processing to increasing atherosclerosis vascular endothelial cells proliferation and invasion (8). At the moment, researches of m6A modification in the field of heart failure and cardiac remodeling are still in their initial phases. Zhang et al. revealed the different expression profiles of m6A regulators in heart failure with preserved ejection fraction (HFpEF) patients in combination with a clinical case-control study and animal experiment, and highlighted the relationship between m6A modification and the risks of HFpEF (9). Given the comprehensive regulation of m6A modification in diseases, much remains to be explored in cardiac remodeling and heart failure.

Long non-coding RNAs (lncRNAs) are non-protein coding transcripts with a length more than 200 nucleotides. LncRNAs could govern the cellular processes, such as cell cycles regulation, differentiation through diverse mechanisms like transcription, translation, splicing, etc, in various disease states (10). Emerging data have shown that lncRNAs play a key role in the pathogenesis of cardiac remodeling and heart failure (11). Many mature lncRNAs are modified after transcription, and numerous types of biochemical modifications were found in lncRNAs (12). The relationship between lncRNAs and m6A modifications still remain largely unknown, provides a fresh viewpoint for exploring the possible regulatory mechanism and suggests that m6A modification and lncRNAs interplay might be significant treatment targets for various diseases (13). This review provides an overview of recent advances in m6A modifications and gives an updated outline of the association between m6A modification and cardiac remodeling, and provide an insight into potential molecular biomarkers associated with the m6A modification of lncRNAs and therapeutic targets in cardiac remodeling.

Abbreviations: m6A, N6-methyladenosine; LncRNAs, long non-coding RNAs; HF, heart failure; RAAS, renin-angiotensin-aldosterone system; SNS, sympathetic nervous systems; HFpEF, heart failure with preserved ejection fraction; METTL3, Methyltransferase like-3; METTL14, Methyltransferase like-14; WTAP, Wilms' tumor 1–associated protein; RBM15, RNA Binding Motif Protein 15; ZC3H13, Zinc Finger CCCH-Type Containing 13; RBM14, RNA Binding Motif Protein 14; FTO, fat mass and obesity-associated gene; ALKBH5, AlkB Homolog 5; YTHDF/YTHDC, YT521-B homology (YTH) domain family of proteins; IGF2BPs, insulin-like growth factor 2 mRNA-binding protein; HNRNPA2B1, heterogeneous nuclear ribonucleoproteins A2/B1; eIF3, eukaryotic initiation factor 3; ECM, extracellular matrix; ECs, endothelial cells; VSMCs, vascular smooth muscle cells; ADSCs, adipose-derived stem cells; H/R, hypoxia/reoxygenation; I/R, ischemia/reperfusion; Mhrt, myosin heavy chain associated RNA transcript; XIST, X-inactive specific transcript; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; MAPKs, mitogen-activated protein kinases.

MACHINERY AND BIOLOGICAL ROLES OF M6A MODIFICATION

m6A modification is found throughout species in evolutionary patterns, having a consensus sequence of the m6A center site. Their critical roles in epigenetics and physiological connections to numerous human diseases have given them a huge scientific and medical attention. m6A modification is a dynamic and reversible posttranscriptional modification process which was implemented in three distinct kinds of protein complexes ("writer" and "reader" and "eraser" proteins) and can alter important biological processes by adding, removing, or preferring identify the m6A sites (**Figure 1**).

Writer

Methyltransferase (m6A "writers") are protein components that catalyze m6A methylation of RNAs. m6A modification is achieved by a large methyltransferase complex (MTC). The m6A MTC mainly consists of the methyltransferase-like proteins, such as METTL3 and METTL14, and various protein factors indispensable for their proper catalysis, including WTAP, RBM15, ZC3H13, and RBM14. METTL3 is responsible for the installation of m6A on RNA. According to a phylogenetic study, METTL14 is a homolog of METTL3, with 43% similarity. The METTL3/METTL14 form a stable heterodimer core complex exhibits far more m6A methyltransferase activity than either component alone. The METTL3/METTL14 complex preferentially methylates RNA on sites of RRACH (R = A/G, H = A/U/C) *in vitro*, in accordance with the RRACH motif distribution of m6A transcriptome-wide (14). WTAP, as the scaffold protein of m6A-MTC, exhibits an influence on the METTL3/METTL14 compound structure and substrate composition (15). Additionally, several proteins have been shown to involved in substrate binding, catalytic efficiency, stability, and localization in addition to the core of m6A complex. RBM15, ZC3H13, HAKAI, and VIRMA, for example, have been identified as WTAP components, and this association offers a scaffold for METTL3/METTL14 recruitment for methylation (16).

Eraser

Demethylases (m6A "erasers") are enzymes that remove the methyl group from RNA. m6A is one of the few intrinsic RNA modifications that may be reversed. The identification of RNA demethylase FTO, was the crucial breakthrough that revived the m6A function. FTO catalyzes oxidative demethylation of m6A in an Fe(II)- and α-KG-dependent manner which preferentially bind to pre-mRNA strands in intronic regions, in the proximity of alternatively spliced exons and poly(A) sites (17). FTO is a member of the ALKB family. Another family member, ALKBH5, has also been discovered as a demethylase. Both ALKBH5 and FTO are confined to nucleus and located by nuclear speckles, which show the splicing effects of methylation (18). Instead of

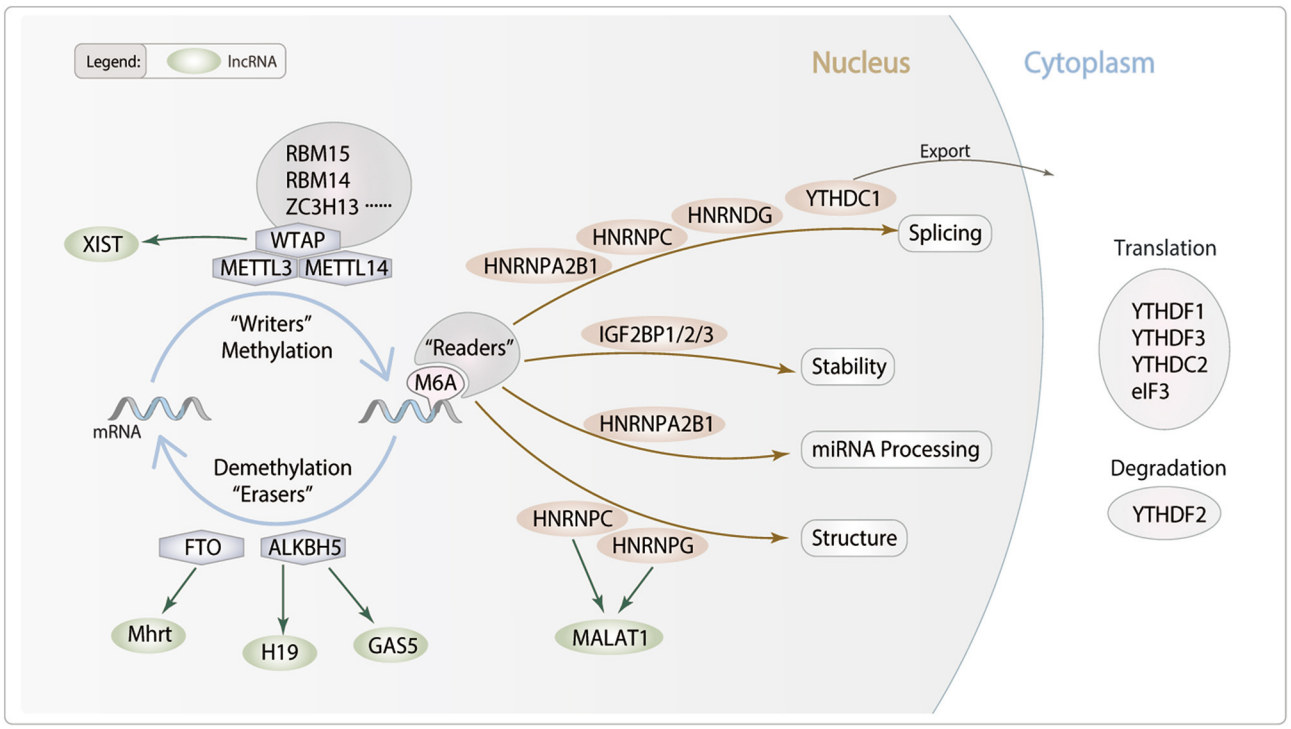

FIGURE 1 | Potential role of lncRNA m6A modification in cardiac remodeling. m6A is deposited by "Writers" (METTL3/14, WTAP, RBM14/15 and ZC3H13), removed by "Erasers" (FTO and ALKBH5), and recognized by "Readers" (YTHDC1/2, YTHDF1/2/3, IGF2BP1/2/3, HNRNPA2B1, HNRNPC, HNRNPG and eIF3). m6A modifications can regulate RNA processing, including splicing, nuclear exports, stability and translation. We hypothesis lncRNA m6A modification which may modulate particular pathophysiological process of cardiac remodeling.

the FTO oxidative demethylation, ALKBH5 catalytic reaction directly removes the methyl group from the m6A methylated adenosine (19). ALKBH5 found as m6A mRNA demethylase employing Fe^{2+} and 2OG cofactor, which work together to remove the methyl group in m6A containing substrates.

Reader

The biological function of m6A modification is regulated by m6A-binding proteins, also known as the "readers," including YTHDF1/2/3, YTHDC1/2, IGF2BP1/2/3, HNRNPA2B1 and eIF3. Reader proteins that act as functional mediators, selectively identity target m6A-modified mRNA and regulate varies of RNA metabolism processes such as RNA splicing, transport, translocation, translation and degradation (20). YTHDC1 is a nuclear protein involved in gene splicing. However, YTHDF1-3 are cytoplasmic m6A readers. YTHDF1 and YTHDF3 collaborate to influence the translation of m6A-containing mRNAs, whereas YTHDF2 speeds up mRNA decay and YTHDC1 influences the nuclear processing of its targets (21). Moreover, a number of recent papers claim to have discovered the presence of additional sorts of readers. IGF2BPs (IGF2BP1-3), HNRNPA2B1 and eIF3 can affect the splicing, translation, stability and degradation of mRNA (21). IGF2BPs belong to a conserved family of RNA-binding, and associate with target mRNAs in cytoplasmic ribonucleoprotein complexes, and enhance the stability and storage of their target mRNAs, therefore regulating the output of gene expression (22). HNRNPA2B1 can directly binds nuclear transcripts to induces alternative splicing effects and boosting microRNA processing (23). eIF3 plays an important role in recruitment of the pre-initiation complex PIC to mRNA. Moreover, YTHDF1 relocates from the cytoplasm to the nucleus and regulates translation may depends on interaction with eIF3 (24).

m6A METHYLATION IN CARDIAC REMODELING

Cardiac remodeling involves almost all the cell types in the heart including cardiomyocytes, cardiac fibroblasts, vascular smooth muscle cells and vascular endothelial cells. Remodeling encompasses cellular changes including cardiomyocytes hypertrophy, necrosis, apoptosis, vascular differentiation and fibroblast proliferation. Recently, it was discovered that m6A methylation plays an important role in mediating significant structural alterations in the failing heart. Researchers observed that the overall level of m6A modification of the transcripts in the healthy mouse and human heart is increased by using next-generation sequencing. Moreover, the changes in m6A methylation exceeded changes in gene expression with the course of heart failure in both mice and humans (25).

TABLE 1 | Role of m6A modification in cardiac remodeling.

Cardiac remodeling	m6A regulators	m6A levels	Cell (tissue) types	Main functions and mechanisms	References
Cardiomyocytes hypertrophy	METTL3 upregulated	Increased	Cardiomyocytes under hypertrophic conditions.	METTL3 promotes the expression of MAPKs family in cardiomyocytes.	(27)
	METTL3 upregulated	Increased	Human failing myocardium.	m6A regulates translational efficiency by affecting transcript stability.	(28)
	FTO upregulated	Decreased	Cardiomyocytes by leptin stimulation.	FTO upregulation *via* JAK2/STAT3-dependent CUX1 upregulation.	(29)
	FTO downregulated	Increased	Cardiomyocytes.	FTO regulates Intracellular $Ca2^{+}$ and sarcomere dynamics in cardiomyocytes.	(34)
Cardiomyocytes death	METTL3 upregulated ALKBH5 downregulated	Increased	H/R-treated cardiomyocytes and I/R-treated mice heart.	METTL3 enhances the binding of TFEB to HNRNPD, which decreases TFEB expression, thereby impairing autophagic flux and enhancing apoptosis. ALKBH5 exerts the opposite effects.	(32)
	FTO downregulated	Increased	Failing human (both ischemic and non-ischemic), post-MI pig and mouse hearts.	FTO regulated SERCA2A, MYH6/7 and RYR2 expression and prevented cardiac contractile transcription deterioration.	(34)
Extracellular matrix remodeling	METTL3 upregulated	Increased	Cardiac fibroblasts treated with TGF-β1 and in the chronic MI murine hearts.	METTL3 promotes cardiac fibrosis through Smad-mediated pathway.	(35)
	METTL3 upregulated	Increased	Mice hearts.	METTL3 overexpression following TAC operation decreases fibrosis and collagen transcription.	(28)
	FTO upregulated	Decreased	Murine MI hearts.	Reduces scar size.	(34)
Vascular remodeling	METTL3 upregulated	Increased	ADSCs undergoing VSMCs differentiation induction.	Stimulates the differentiation of ADSCs into vascular VSMCs and regulates the secretion of VEGF, HGF, TGF-β, GM-CSF, bFGF, and SDF-1.	(38)
	FTO upregulated	Decreased	Murine MI hearts.	Reduces cardiac fibrosis (decreases of scar size%) and increases angiogenesis (higher number of CD31− positive cells).	(34)
	METTL14 upregulated	Increased	TNF-α stimulated HUVECs.	METTL14 modifies FOXO1 mRNA to promote TNF-α-induced endothelial monocyte adhesion.	(39)
	METTL14 upregulated	Increased	ASVEC.	METTL14 regulates the maturation of pri-miR-19a, to promotes invasion and proliferation of cardiovascular ECs.	(8)

m6A, N^{6}-methyladenosine; METTL3, methyltransferase-like 3; FTO, fat mass- and obesity-related protein; MAPKs, mitogen-activated protein kinases; JAK2, Janus kinase 2; STAT3, signal transduction and activator of transcription 3; CUX1, Cut Like Homeobox 1; MI, myocardial infarction; TFEB, Transcription Factor EB; H/R, hypoxia/reoxygenation; I/R, ischemia/reperfusion; ALKBH5, AlkB Homolog 5; HNRNPD, Heterogeneous Nuclear Ribonucleoprotein D; SERCA2a, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 2a; MYH6/7, β-myosin heavy chain 6/7; Ryr2, ryanodine receptor 2; TAC, transverse aortic constriction; ADSCs, adipose-derived stem cells; VSMCs, vascular smooth muscle cells; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; TGF-β, transforming growth factor β; GM-CSF, granulocyte-macrophage colony-stimulating factor; bFGF, basic fibroblast growth factor; SDF-1, stromal cell-derived factor-1; METTL14, Methyltransferase like-14; HUVECs, Human umbilical vein endothelial cells; ASVEC, atherosclerotic vascular endothelial cells; FOXO1, forkhead box O1; ECs, endothelial cells.

Cardiomyocytes Hypertrophy

Cardiac hypertrophy is one of the predominant components of cardiac remodeling. During the early stages of stress, cardiac hypertrophy serves as a compensatory mechanism occurred in cardiomyocytes, manifested as myocytes hypertrophy, and progressive thickening of the ventricular wall. Sustained pathological hypertrophy, on the other hand, is a major cause of cardiomyocytes remodeling and HF. In addition to the transcriptional control of gene expression during hypertrophy, posttranscriptional regulation of protein expression is increasingly recognized as a vital mechanism for hypertrophic control (26) and m6A methyltransferase METTL3 is one of them (**Table 1**). Investigation showed that the level of m6A modification was significantly increased in isolated neonatal rat ventricular cardiomyocytes responded to hypertrophic stimulation by using m6A immunoprecipitation followed by RNA sequencing (27). In order to further investigate the possibility that m6A is involved in the regulation of cardiomyocyte homeostasis and hypertrophy *in vivo*, METTL3-overexpressing mice were studied and showed significant cardiac hypertrophy when exposed to pressure overload stress. However, results did not show accelerated cardiac dysfunction. METTL3 knockout mice, on the other hand, revealed indications of failure on both morphological and functional levels (27). These evidences implied that the METTL3-m6A pathway could be a novel critical regulator of cardiac homeostasis. Interestingly,

opposite findings were revealed by Kmietczyk et al. (28). In compared with healthy myocardium, their investigation indicated a higher METTL3 activity and a predominance of RNA transcripts enriched in m6A in human failing heart and dilated cardiomyopathy. Additionally, overexpression and knockdown of METTL3 expression affected the cellular size and cardiomyocytes remodeling both *in vitro* and *in vivo*. Mechanically, according to Dorn et al. (27), m6A peaks were selectively abundant in mRNAs encoding protein kinases and modifiers, such as mitogen-activated protein kinases (MAPKs) family, resulting in the significant increase of cardiomyocyte size. On the other side, Kmietczyk et al. (28) suggested the mRNA methylation is highly dynamic when exposed to stress conditions, which in turn to regulate translational efficiency by modifying transcript stability. These two findings appear to be contradictory, and previous researches have revealed that the genetic background of mice may influence the phenotypic outcome of cardiac disorders post-intervention. It is possible that variances in methodologies and mice strain backgrounds which may explain some of the disparities in the data. However, this also highlights that the underlying mechanisms are highly uncertain, and further investigations would be necessary.

As for FTO, a well-known m6A demethylase protein, has been linked to cardiac hypertrophy and muscular contraction in cardiac remodeling (**Table 1**). Gan et al. revealed that nuclear FTO expression in cardiomyocytes was considerably increased during an adipokine induced cardiomyocyte hypertrophic response in newborn rat cardiomyocytes, leptin-induced FTO upregulation in cardiomyocytes *via* JAK2/STAT3-dependent CUX1 upregulation (29). The researchers also discovered that FTO knockdown reduced the hypertrophic response *in vitro*, indicating that FTO plays an essential regulatory function in cardiac hypertrophy.

Cardiomyocytes Death

Recently, the role of apoptosis in the heart during ischemic and non-ischemic cardiomyopathies has been explored to involved in cardiac remodeling and heart failure. Structural or functional alterations in the myocardium may be intimately associated with myocardial ischemia. Alterations in blood supply that occur over time may result in changes in cardiac tissue which may have impacts on both short- and long-term cardiovascular function and prognosis. Myocardial infarction causes widespread cardiac damage *via* ischemic and ischemia/reperfusion injury, resulting in a deleterious effect on cardiomyocytes and cardiac functions. Cardiomyocyte apoptosis and necrosis, is a hallmark characteristic features of myocardial infarction and cardiac remodeling. Autophagy is triggered during ischemic stress to protect cardiomyocytes from ischemic or ischemia/reperfusion injury. However, excessive autophagy activation may have a deleterious effect on the heart in reperfusion and other stress circumstances, revealing the controversial nature of autophagy in myocardial ischemia. In numerous animal models, blocking apoptosis or regulating autophagy process could effectively reduce cardiac remodeling and heart failure (30, 31).

Song et al. (32) identified that in hypoxia/reoxygenation (H/R)-treated cardiomyocytes as well as the ischemia/reperfusion (I/R)-treated mice heart, the m6A methylations were increased owing to the upregulation of METTL3. Silencing METTL3 enhanced autophagic flux and inhibited apoptosis in H/R-treated cardiomyocytes. Whereas the RNA demethylase ALKBH5 acted in a opposite manner during myocardial I/R (32). Mechanistically, METTL3 methylates the transcription factor EB (TFEB), a critical regulator of lysosomal biogenesis and also stimulated the expression of autophagy genes (33). Silencing of METTL3 promoted TFEB expression and its nuclear translocation. Furthermore, TFEB can reduce METTL3 expression by decreasing the stability of the METTL3 mRNA. TFEB knockdown eliminated the enhanced autophagy driven by METTL3 downregulation, demonstrating that METTL3 mediates autophagic flux in a TFEB-dependent manner (32). Although authors did not evaluate changes to the overall m6A levels following ALKBH5 dysregulated, they demonstrated a vital role for m6A in autophagy regulation in H/R cardiomyocytes (**Table 1**).

Besides, Mathiyalagan et al. (34) demonstrated the m6A modifications were increased in failing human (both ischemic and non-ischemic), post-MI pig and mouse hearts and hypoxic cardiomyocytes, resulting in a decrease in contractile performance. To identify the m6A regulators in the ischemic myocardium, the expressions of the major methylases, demethylases, and reader proteins were measured. They found FTO expression was significantly decreased in failing mammalian hearts and hypoxic cardiomyocytes. It is remarkable that loss of FTO exhibited a significantly increased number of arrhythmic events which are mediated primarily by cardiac contractile genes regulation, such as SERCA2A, MYH6/7 and RYR2, resulting in contractile dysfunction in cardiomyocytes. *In vivo*, FTO overexpression improved cardiac function remarkably during the chronic stage of post-myocardial infarction (34). FTO prevented cardiac contractile transcription deterioration and enhanced protein expression by selective demethylation under ischemic circumstances, thereby safeguarded cardiomyocyte contractile function by regulating intracellular Ca^{2+} dynamics (34).

Extracellular Matrix Remodeling

Cardiac fibrosis, defined by pathological activation of cardiac fibroblasts and excessive buildup of extracellular matrix (ECM) in the afflicted tissue. Cardiac fibroblasts are activated and transform into myofibroblasts which produce a large amount of matrix metalloproteinases (MMPs), leading to accumulation and composition alteration of ECM. In the cardiac remodeling, cardiac fibrosis contributes to the dysfunction of failing hearts by making the heart stiffer and reducing its function due to its inability to contract or conduct electric impulses required for the transmission of the contraction. The activation and differentiation of cardiac fibroblasts are also influenced by m6A methylation. Increased METTL3 expression was shown in cardiac fibroblasts treated with TGF-β1 and in the murine hearts suffered from chronic myocardial infarction (35). METTL3 overexpression promoted cardiac fibrosis, fibroblast-to-myofibroblast transition, and enhanced extracellular matrix production and accumulation, whereas METTL3 silencing had

the opposite effect. *In vivo*, silencing METLL3 significantly suppressed cardiac fibrosis progression. Smad2/3 are key regulators of the fibrogenesis process in cardiac fibroblasts, whereas silencing METTL3 reduced the upregulation of Smad2/3 induced by TGF-β1, suggesting that METTL3 regulated cardiac fibrosis at least partially through Smad-mediated pathway (35). Consistent with these results, Kmietczyk et al. (28) carefully analyzed and revealed that AAV9-mediated METTL3 overexpression following TAC operation significantly decreased fibrosis and collagen transcription in mice hearts. In addition to modifying cardiomyocyte hypertrophy, AAV9-mediated METTL3 overexpression may partially modulate the cardiac fibroblast phenotype. These evidences indicated METTL3-mediated m6A methylation played a critical role in cardiac fibrosis regulation (**Table 1**).

In addition, along with the positively regulating contractile protein expression, FTO-dependent demethylation was also indicated to affected critical non-contractile processes involving tissue morphogenesis, angiogenesis, extracellular matrix organization, fibrosis, and cell proliferation and differentiation in murine MI hearts (34). In FTO-overexpressing mice post-MI models, fibrosis in mice overexpressing FTO was significantly decreased compared with their respective controls, as measured by scar size (%) (34).

Vascular Remodeling

Vascular remodeling refers to alterations in the structure of resistance vessels contributing to elevated systemic vascular resistance. It is a dynamic structural change that involves alterations to numerous cellular including endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and fibroblasts and non-cellular components, which may lead to vascular stiffness. During vascular remodeling and heart failure, abnormalities in ECs and VSMCs proliferation and migration, endothelial dysfunction, inflammatory processes, and the production or breakdown of extracellular matrix components are present and predominant (36, 37). METTL3 and FTO have been shown to be involved in the regulation of vascular or cardiac dysfunction under stress conditions (**Table 1**). Under hypoxia stimulations, METTL3 can stimulate the differentiation of adipose-derived stem cells into vascular VSMCs (38). Also, evidences showed that overexpression of FTO in the ischemic myocardium could reduce cardiac fibrosis and increase angiogenesis (34). For angiogenesis, anti-CD31 immunostaining was performed by Mathiyalagan et al. (34). They measured the angiogenic response by detecting CD31-positive endothelial cells in the infarct border zone of murine hearts 4 weeks after MI, and discovered FTO-overexpressing hearts had a much higher proportion of CD31-positive cells than their respective control group (34). METTL14, as a methylase, was discovered to stimulate inflammatory response in ECs and promote atherosclerotic plaque formation by interacting with forkhead box O1 (FOXO1). Through YTHDF1 recognition, METTL14 enhanced the translation of FOXO1 mRNA, therefore increased the expression of the endothelial adhesion molecules VCAM-1 and ICAM-1, which promoting endothelial adhesion (39). In addition, researchers revealed that METTL14 mediated the m6A modification of pri-miR-19a, promoted its processing and maturation, which promoting the invasion and proliferation of cardiovascular endothelial cells (8).

LncRNAs in Cardiac Remodeling

Numerous investigations have revealed that long non-coding RNAs played critical regulatory roles under stressful situation: lncRNAs Mhrt, XIST, MIAT, Chast, CHRF, ROR, H19, and Plscr4 are involved in myocardial hypertrophy, whereas XIST, MALAT1, GAS5, Neat1, AK139328, APF, CAIF, AK088388, CARL, HOTAIR, and NRF are involved in cardiomyocytes apoptosis and necrosis. Moreover, MIAT, MALAT1, Wisper, MEG3, and H19 are involved in extracellular matrix reconstruction. In addition, MALAT1, GAS5, H19, TUG1, AK098656, TRPV1, Giver, and Lnc-Ang362 have been implicated in vascular remodeling (11, 40). m6A modification has been identified in long non-coding RNAs by methylated RNA immunoprecipitation sequencing (MeRIP-seq) m6A on lncRNAs accounts for ∼12% of the total m6A peaks (41). The m6A methylation of lncRNAs has been demonstrated to modify their structure (42), stability (43), transport and destruction (44), hence altering the biological processes or the interaction with other RNA molecules (45–47). However, only a few of m6A modification on lncRNA have been reported in cardiac diseases. In this section, we will focus on the potential function of m6A of lncRNAs in cardiac remodeling (**Table 2**).

LncRNA myosin heavy chain associated RNA transcript (Mhrt) have been proven in regulating the cardiac remodeling. Mhrt is related to heart failure due to its ability to modulate cardiac hypertrophy. Mhrt increased the expression of KLF4 through direct binding to miR-145a-5p or inhibiting phosphorylation of KLF4, to prevent ERK and KLF4 interaction, hence inhibiting myocardin expression and the development of cardiac hypertrophy (48). Besides, Mhrt could protect cardiomyocytes from apoptosis against the H_2O_2 or H/R exposure (49). Shen et al. (50) observed that the expression of FTO and Mhrt were downregulated in heart failure mouse model. FTO overexpression inhibited the m6A modification of Mhrt, hence increased the expression of Mhrt, which inhibited the apoptosis of cardiomyocytes induced by the H/R. These results indicated that m6A modification of Mhrt participates in the development of cardiac disease (50).

LncRNA X-inactive specific transcript (XIST), plays a critical role in the regulation throughout the whole spectrum of human diseases and can be utilized as a novel diagnostic and prognostic biomarker for human disease (51, 52). XIST was investigated positively regulates S100B expression and enhance TLR2 expression, thereby modulates the progression of cardiomyocyte hypertrophy by miR-330-3p/S100B pathway and miR-101/TLR2 axis (53, 54). XIST was up-regulated in cardiomyocytes after infarction. Overexpression of XIST might promote cardiomyocyte apoptosis and inhibit proliferation by mediating PDE4D expression *via* targeting miR-130a-3p (55). In acute myocardial infarction, XIST protected hypoxia-induced cardiomyocyte injury and repressed myocardial apoptosis by interacting directly with various miRNAs and positively regulated expression of anti-apoptotic biomarkers such as Bax,

TABLE 2 | Potential role of lncRNA m6A modification in cardiac remodeling.

LncRNAs	LncRNAs in cardiac remodeling	m6A regulators	LncRNAs modified with m6A
Mhrt	Mhrt regulated the expression of KLF4 to prevent ERK and KLF4 interaction, hence inhibiting the development of cardiac hypertrophy (48).	FTO	FTO downregulated in heart failure mouse model. FTO overexpression increased the expression of Mhrt, which inhibited the apoptosis of cardiomyocytes induced by the H/R (50).
	Mhrt protects cardiomyocytes from apoptosis against the H_2O_2 or H/R exposure (49).		
XIST	XIST could modulate the progression of cardiomyocyte hypertrophy by regulating miR-330-3p/S100B pathway and miR-101/TLR2 axis (53, 54).	RBM15/ RBM15B, WTAP, METTL3	XIST is highly methylated with at least 78 m6A residues (45). m6A methylation of XIST creates a multiprotein complex and recruits the silencing complex, thereby promotes XIST-mediated transcriptional repression (45).
	Overexpression of XIST promoted cardiomyocyte apoptosis and inhibit proliferation by mediating PDE4D expression *via* targeting miR-130a-3p (55).		
	In acute myocardial infarction, XIST regulated expression of anti-apoptotic biomarkers Bax, hexokianse 2 and Notch1 (56, 57).		
MALAT1	MALAT1 could increase cardiomyocytes proliferation or apoptosis in myocardial I/R rats through activating PI3K/Akt and β-catenin signaling pathways (59, 60).	/	SCARLET verified MALAT1 was also highly modified by m6A and contained several m6A motifs (A2515, A2577, A2611, and A2720) (66).
	MALAT1 promoted cardiomyocyte apoptosis of HL-1 or H9c2 cells under H/R conditions *via* interacting with microRNAs (61, 62).	HNRNPC and HNRNPG	The binding of MALAT1 and the m6A "reader" HNRNPC and HNRNPG would increase if A2577 and A2515 of MALAT1 modified by m6A, thereby alter the expression of MALAT1(67).
	MALAT1 promoted human endothelial cells pyroptosis by affecting NLRP3 expression through competitively binding miR-22 (63). MALAT1 mediated cardiac fibrosis in MI mice model (64).		
GAS5	GAS5 hasten myocardial I/R injury by sponging miR-532-5p in myocardial cells (68).	ALKBH5, YTHDF2	ALKBH5 inhibited m6A modification of GAS5 to its stability. Also, m6A promoted the degradation of GAS5 in a YTHDF2-dependent manner (70).
	GAS5 knockdown would aggravate microvascular dysfunction by altering β-catenin signaling activity (69).	YTHDF3	Silencing m6A "reader" YTHDF3 enhances the degradation of GAS5 (44).
H19	H19 promoted myocardial apoptosis (71). H19 overexpression promoted VSMCs proliferation and inhibited its apoptosis (72).	ALKBH5	ALKBH5 regulated the expression of H19 by mediating its m6A modification levels in H9c2 cells with H_2O_2-induced senescence (73).

hexokianse 2, and Notch1 (56, 57). Researchers have shown XIST is highly methylated with at least 78 m6A residues. m6A methylation of XIST creates a multiprotein complex with RBM15/RBM15B, WTAP, and METTL3, which in turn recruits the silencing complex, thereby promotes XIST-mediated transcriptional repression (45).

LncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is widely expressed and highly conserved in human tissues (58). MALAT1 has been reported to increase cardiomyocytes proliferation or apoptosis in rats with myocardial I/R induced injury through activating canonical signaling pathways, such as PI3K/Akt and β-catenin signaling pathways (59, 60). Also, MALAT1 was discovered to promote cardiomyocyte apoptosis of HL-1 or H9C2 cells under H/R conditions *via* interacting with microRNAs (61, 62). LncRNA MALAT1 profoundly induced by stimuli such as hypoxia, cytokine and oxidative stress in ECs, and it could regulate various pathophysiological processes of cardiac remodeling regarding to vascular remodeling (63, 64). For example, ox-LDL is thought to be a critical factor in the initiation and progression of endothelial dysfunction. MALAT1 induced by ox-LDL was also found to protect against endothelial injury by sponging the miR-22-3p *via* activation of AKT pathway (65). Recently, several m6A-deposition sites of MALAT1 have been identified, by the method which can accurately determines m6A status at any site in mRNA/lncRNA, named SCARLET. It has been verified that lncRNA MALAT1 was also highly modified by m6A and contained several m6A motifs (A2515, A2577, A2611, and A2720) (66). m6A modifications on site A2577 and A2515 of MALAT1 destabilize the RNA hairpin of MALAT1, releasing the poly-U tract and increasing the binding with HNRNPC and HNRNPG (42, 67), indicating MALAT1 could be m6A modified to regulate the development of various diseases including cardiac remodeling.

LncRNA GAS5 was found to promote myocardial I/R injury by sponging miR-532-5p in myocardial cells (68). LncRNA GAS5 was also confirmed to participate in the vascular remodeling. Wang et al. showed that lncRNA GAS5 regulated ECs and VSMCs function, GAS5 knockdown would aggravate microvascular dysfunction by altering β-catenin signaling activity, thus increased neovascularization and capillary leakage (69). The m6A modifications of GAS5 have been found. ALKBH5 inhibited m6A modification of GAS5 to its stability. Besides this, m6A promoted the degradation of GAS5 in a YTHDF2-dependent manner (70). Additionally, it was discovered that silencing m6A "reader" YTHDF3 enhances the degradation of GAS5 (44). These findings suggests that m6A-modified GAS5 may play a role in the development of cardiac remodeling.

In recent decades, lncRNA H19, a potential serum marker for coronary heart disease, is involved in the regulation of vascular remodeling. For example, in I/R condition, lncRNA H19 is reported to largely participated and promoted myocardial apoptosis (71). Researchers have investigated that H19 overexpression promoted VSMCs proliferation and inhibited its apoptosis (72). The m6A modified-H19 has been revealed involving in the development of cardiac diseases. In H_2O_2-induced senescence, lncRNA H19 expression decreased and m6A modification increased following H/R, ALKBH5 regulated the expression of H19 by mediating its m6A modification levels (73).

CONCLUSIONS AND FUTURE PROSPECTS

In cardiac remodeling which is the fundamental step for the progression of heart failure, m6A modification shows great potential in mechanistic studies and therapeutic target explorations. However, m6A has complicated impacts on gene expression and is hard to be isolated at a cellular level. It is hard to totally remove the complex of m6A methyltransferase, as knockout typically results in cell death. Studies were significantly impeded by this situation. It will require more studies to completely illustrate how m6A impacts the *in vivo* behavior of target RNAs and its direct downstream effects on gene expression in the setting of cardiac remodeling.

It is worth noting that, the interplay of m6A and lncRNAs is prevalent and inspiring, which significantly broadening the scope of epitranscriptomic regulation. m6A modifying lncRNAs still needs to interact with other components, such as other proteins, miRNAs and mRNAs. m6A modification on lncRNAs are emerging regulators in diverse process but remained in the level of phenotype researches rather than further mechanism manifestations. In addition, it remains unknown how m6A modifications on lncRNAs differs from the those on mRNAs in cardiac remodeling. As a whole, improvements in RNA modification sequencing and mapping technologies will definitely lead to a deeper understanding of RNA epigenetics mechanisms, and encourages the development of novel molecular treatments for cardiac remodeling in the future.

AUTHOR CONTRIBUTIONS

MC and RX wrote the manuscript. YW drew the figure. WF, YD, and CL edited the manuscript. All authors contributed to the article and approved the submitted version.

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Downregulation of Uncoupling Protein 2(UCP2) Mediated by MicroRNA-762 Confers Cardioprotection and Participates in the Regulation of Dynamic Mitochondrial Homeostasis of Dynamin Related Protein1 (DRP1) After Myocardial Infarction in Mice

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Acute myocardial infarction (MI) is one of the leading causes of death in the world, and its pathophysiological mechanisms have not been fully elucidated. The purpose of this study was to investigate the role and mechanism of uncoupling protein 2 (UCP2) after MI in mouse heart. Here, we examined the expression and role of UCP2 in mouse heart 4 weeks after MI. The expression of UCP2 was detected by RT-PCR and western blotting. Cardiac function, myocardial fibrosis, and cardiomyocyte apoptosis were assessed by echocardiography and immunohistochemistry. Phosphatase dynamin-related protein1 (*P*-DRP1) and myocardial fibrosis-related proteins were measured. Cardiomyocytes were exposed to hypoxia for 6 h to mimic the model of MI. Mdivi, an inhibitor of *P*-DRP1, was used to inhibit DRP1-dependent mitochondrial fission. Mitochondrial superoxide, membrane potential, oxygen consumption rate, and cardiomyocyte apoptosis were detected after hypoxia. It is shown mitochondrial superoxide, membrane potential, oxygen consumption rate, and cardiomyocyte apoptosis were dependent on the level of *P*-DRP1. UCP2 overexpression reduced cardiomyocyte apoptosis (fibrosis), improved cardiac function and inhibit the phosphorylation of DRP1 and the ratio of *P*-DRP1/DRP1. However, inhibition of DRP1 by mdivi did not further reduce cell apoptosis rate and cardiac function in UCP2 overexpression group. In addition, bioinformatics analysis, luciferase activity, and western blot assay proved UCP2 was a direct target gene of microRNA-762, a up-regulated microRNA after MI. In conclusion, UCP2 plays a protective role after MI and the mechanism is involved in microRNA-762 upstream and DRP1-dependent mitochondrial fission downstream.

Keywords: myocardial infarction, UCP2, DRP1, mitochondrial fission, microRNA

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Liu D, Zou S, Li G, Zhang Q, Chen C, Li C, Song H, Chen S, Wang J, Wu Y and Liu Y (2022) Downregulation of Uncoupling Protein 2(UCP2) Mediated by MicroRNA-762 Confers Cardioprotection and Participates in the Regulation of Dynamic Mitochondrial Homeostasis of Dynamin Related Protein1 (DRP1) After Myocardial Infarction in Mice. Front. Cardiovasc. Med. 8:764064. doi: 10.3389/fcvm.2021.764064

INTRODUCTION

Acute myocardial infarction (MI) caused by coronary heart disease (CHD) is very common in clinical practice and is associated with high morbidity and mortality (1). MI leads to cardiac dysfunction, arrhythmias and abnormal remodeling of the ventricles. The pathophysiological process of this disease is complex and its mechanisms have not been entirely elucidated (2). A deeper understanding of the pathophysiological mechanisms is necessary to better treat this disease.

Uncoupling protein 2 (UCP2) is a mitochondrial transport protein, which is increasingly recognized as an important molecule in the defense of various cardiovascular diseases such as atherosclerosis, coronary heart disease, heart failure and hypertension (3–7). The protective effect of UCP2 on atherosclerosis has been studied, and its mechanism may be related to macrophage metabolism and thermogenesis (8). It has been reported that transgenic mice with UCP2 are protected against salt-induced hypertension (9). There are also some pieces of evidence show UCP2 has a significant protective effect on ischemia/reperfusion injury (10). However, the roles of UCP2 in the heart have not yet been fully elucidated. Even in the same disease, UCP2 may play different roles. For example, concerning CHD, some studies demonstrated that diabetic patients after MI cohort with the UCP2-866A allele have poorer survival (11, 12). However, another study showed the UCP2-866A allele is associated with reduced risk of CHD in type 2 diabetic men in a 6-year prospective study (13). Therefore, there is an urgent need to establish the roles of UCP2 in CHD, and this study aims to explore the role of UCP2 in mouse heart after MI.

In this study, we found the expression of UCP2 is down-regulated by miR-762 and UCP2 has a protective role in cardiomyocyte apoptosis and fibrosis, and improve cardiac function after MI. The protective mechanism of UCP2 on the process is involved in DRP1-dependent mitochondrial fission. UCP2 may be a new therapeutic target for patients after MI.

MATERIALS AND METHODS

Materials

The chemical substances, antibodies, and reagents used herein were obtained from various sources. Dulbecco's modified Eagle medium (DMEM), phosphate buffer saline (PBS), Collagenase, fetal bovine serum (FBS), and Lipofectamine 3,000 were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany); Mitochondrial division inhibitor 1 and MitoSOX Red Mitochondrial Superoxide Indicator were purchased from MedChemExpress(Shanghai); TRIzol reagent and mRNA qRT-PCR Sybr Green Detection Kit were purchased from Invitrogen (USA). An Annexin V-FITC, Propidium Iodide (PI) Detection Kit,m and A terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) kit were purchased from BD Biosciences (New Jersey, USA); The following primary antibodies were used in this experiment: anti-GAPDH (1:100, Cell Signaling Technology, USA), anti-UCP2 (1:5,000, R&D Systems, Inc, USA), anti-MMP9 (1:1,000, R&D Systems, Inc. USA), anti-TGF-beta (1:1,000, Abcam, Cambridge, Britain), and anti-p-DRP1 (1:1,000, Cell Signaling). MTT assay (Beyotime); LDH assay (Beyotime).

Establishment of the Mouse MI Model

The left anterior descending (LAD) coronary artery of male C57BL/6 (20–25 g) was subjected to ligation to induce MI induce MI as described previously (14). In short, pentobarbital sodium (50 mg/kg) was used to anesthetize mice intraperitoneally and mice were ventilated artificially with a rodent ventilator. After sedation, thoracotomy, and pericardiotomy were performed, followed by ligation of the beginning of the LAD coronary artery with 6-0 proline suture. The ST-segment elevation of ECG indicates the successful induction of myocardial ischemia. Mice in the sham-operated group received the same treatment but did not receive LAD coronary artery ligation. After 4 weeks, all mice were examined by echocardiography to confirm that the models of heart failure were successfully induced, and then they were euthanized by cervical dislocation so that their tissues could be used for subsequent experiments. All animal experiments were approved by the Animal Care and Use Committee of the Institute of Medicine, Guangdong Provincial People's Hospital. All animal experiments were conducted following the guidelines for the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978, UK).

Masson's Trichrome Staining

Masson's trichrome staining was performed to detect collagen fibers in tissue that were fixed in paraformaldehyde (4%) and embedded in paraffin. After staining, the collagen fibers were blue, the nuclei were black, and the cytoplasm was red.

Cell Culture and Hypoxia Treatment

Established procedures were used to isolate primary neonatal mouse cardiomyocytes (NMCMs) (15). Neonatal C57BL/6 mice (1–2 d old) were euthanized by decapitation and hearts were immediately extracted. The ventricles were quickly and finely minced, followed by digestion with 0.1% collagenase. Cell suspensions were collected, centrifuged, and then resuspended in DMEM with 100 U/ml penicillin, 10% FBS, and 100 μg/ml streptomycin. Cells were expanded under standard culture conditions (37°C in 5% CO2 and 95% O2) for 1.5 h to obtain fibroblast attachment to the culture plates. Then, the cell suspension primarily containing NMCMs was collected and plated onto culture dishes. After 24 h, NMCMs were attached and cultured in the complete medium containing 0.1 mM 5-BrdU.

Abbreviations: CHD, Coronary heart disease; DRP1, Dynamin-related protein1; DMEM, Dulbecco's modified eagle medium; EF, Ejection fraction; FS, Fractional shortening; FBS, Fetal bovine serum; IVSs, Interventricular septal systolic diameter; IVSd, Interventricular septal diastolic diameter; LAD, Left anterior descending; LVIDd, Left ventricular end diastolic diameter; LVIDs, Left ventricular end systolic diameter; LVPWd, Left ventricular posterior wall diastolic diameter; LVPWs, Left ventricular posterior wall systolic diameter; MMP9, Matrix metallo proteinase 9; MI, Myocardial infarction; NMCMs, Neonatal mouse cardiomyocytes; PBS, Phosphate buffer saline; ROS, Reactive oxygen species; RT-PCR, Reverse transcription-quantitative polymerase chain reaction; TGF-β, Transforming growth factor-beta; TUNEL, Transferase-mediated dUTP nick end-labeling; UCP2, Uncoupling protein 2.

Cell purity was confirmed by cellular morphology (beating cells) and immunostaining. Monoclonal antibodies against GAPDH were used to identify cardiomyocytes.

To establish the hypoxia model, the culture medium was replaced with serum-free DMEM and the cells were placed in an anaerobic chamber containing 94% N2, 5% CO2, and 1% O2. In the normoxia group, cells were cultured in serum-free DMEM and placed in a normoxic incubator. The cells were subjected to hypoxia for 24 h unless indicated otherwise.

Transfection of miR-762 Mimic or Inhibitor Into NMCMs

The cells were transfected for 24 h with 50 nM miR-762 mimic (Product Number: miR1171220024715-1-5, Ribobio, Guangzhou, China) or 100 nM miR-762 inhibitor (Product Number: MIR2171220024749-1-5, Ribobio) using lipofactamin RNAiMAX (Invitrogen, US) according to the manufacturer's protocol. Cells transfected with negative control of mimic (NCm) (Product Number: miR1N0000001-1-5, Ribobio) or negative control of inhibitor (NCi) (Product Number: miR2N0000001-1-5, Ribobio) served as controls.

Luciferase Reporter Gene Assay

In order to construct reporter vector with miRNA-762 target site, wild-type or mutant UCP2 mRNA 3′UTR sequence was amplified by polymerase chain reaction (PCR) and cloned into pGL3-promoter construct (Promega). The wild-type or mutant UCP2 3′UTR firefly luciferase reporter gene was obtained. Myocytes were co-transfected with 80 ng wild-type or mutant UCP2 3′UTR firefly luciferase reporter gene, 40 ng Renilla luciferase reference plasmid PRL-TK, and miR-762 (final concentration, 20 nM) in each control. Luciferase activity was measured 48 h after transfection using the dual luciferase reporter assay system (Promega, USA). The final data is the ratio of firefly fluorescent value to Renilla fluorescence value.

Quantitative Real-Time RT-PCR

To determine the expression of UCP2 mRNA, total RNA was separated from cardiac tissues and cultured cells using TRIzol reagent. NanoDrop −2,000 spectrophotometer (Thermo Fischer Scientific) was used to assess RNA quality and purity. To quantify the mRNA expression of UCP2, SYBR RT-PCR was used to synthesize cDNA and make a quantitative RT-PCR analysis. Ct (threshold cycle) values were used to quantify mRNA expression levels. GAPDH served as the internal control for mRNA. The mRNA expression levels were expressed as fold changes relative to the levels of the appropriate control samples and were determined using the 2-ΔΔCt method. The following primers were used for the experiment:

mouse UCP2:
Forward Primer
5-ATGGTTGGTTTCAAGGCCACA-3
Reverse Primer
5-TTGGCGGTATCCAGAGGGAA-3
mouse GAPDH:
Forward Primer
5-AGGTCGGTGTGAACGGATTTG-3
Reverse Primer
5-TGTAGACCATGTAGTTGAGGTCA-3

Western Blot Analysis

The procedure was followed as in a previous study (16). Protein samples of cells or tissues were washed with PBS and lysed on ice in lysis buffer supplemented with protease inhibitors and phosphatase inhibitors. Then the samples were centrifuged at a speed of 12,000 × g for 15 min at 4°C. Protein was separated by 10% SD polyacrylamide gel electrophoresis and then transferred to PVDF membranes. These membranes were immersed in 5% non-fat dry milk in TBST with 0.1% Tween-20 for 1.5 h at room temperature, rinsed, and incubated overnight at 4°C with specific antibodies against UCP2, P-DRP1, MMP9, and TGF-ß in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T). Primary antibodies were cleaned by washing the membranes three times in TBS-T and then incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody (1: 1,000–2,000 dilution). Membranes were was cleaned three times with TBS-T. Then, immunopositive bands were visualized by enhanced chemiluminescence and exposed to X-ray film. The levels of protein expression were presented as fold changes relative to expression levels in the control sample.

Flow Cytometry Analysis

To analyze the effects of treatments on cell survival, Annexin V-FITC and PI Detection Kit were used to stain the cells before flow cytometry analysis. The steps are as follows in detail: NMCM cells were collected at the logarithmic phase, digested with 0. 25% trypsin, and then washed with pre-cooled PBS 2 × 5 min. Annexin V-FITC (5 μl) and PI (5 μl) were added

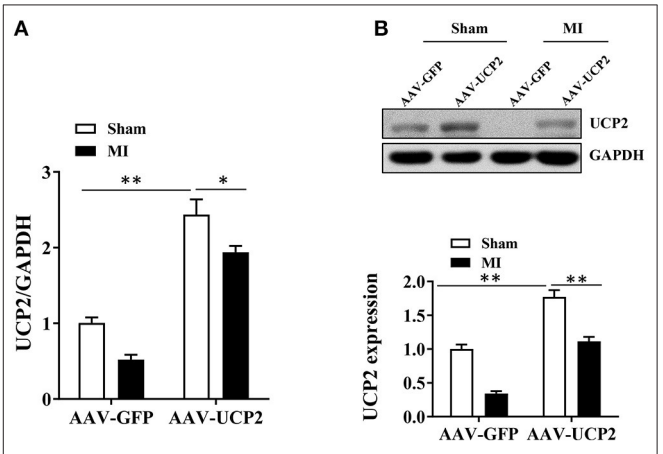

FIGURE 1 | The expression of UCP2 in the infarcted marginal zone was significantly down-regulated 4 weeks after MI in mice. **(A)** qRT-PCR was performed to detect the expression of UCP2 in myocardial tissue under sham and over-expressed UCP2 condition 4 weeks after MI. ($n = 5$); **(B)** Western blotting was performed to detect the expression levels of UCP2 in myocardial tissue under sham and over-expressed UCP2 condition 4 weeks after MI. ($n = 5$); MI, myocardial infarction; *$p < 0.05$; **$P < 0.01$ compared with each other.

to a buffer with 500 μl suspended cells. The apoptosis rate was determined by flow cytometry. Total apoptosis rate (%) = early apoptosis rate + late apoptosis rate. Repeat three times for each sample.

Cell Viability and Cell Injury Evaluation

As previously reported, the MTT assay (Beyotime) was used to determine cell (NRCMs) viability. According to the manufacturer's instructions, cell damage is assessed by determining the release of LDH from the cell using the LDH detection kit (Beyotime).

TUNEL Assay

After MI, mice were sacrificed and the left ventricle was removed. The left ventricular myocardium was placed in a 10% formaldehyde solution and fixed for 24 h. The tissue was embedded with paraffin. Twelve slices were taken from each group, and 20- 400-fold fields were randomly selected from each section to count the number of cells in the field that were positive for staining. AI (%) = the number of positive cells in the field/the total number of cells in the field × 100%.

OCR Assay

To assess the Oxygen Consumption Rate (OCR), NMCMs cells were obtained from the mice after hypoxia or Ad-UCP2/LacZ treatment and rinsed briefly in Seahorse XF medium supplemented with 2 mM L-glutamine, 10 mM glucose, and 1 mM sodium pyruvate. The OCR was assessed at 37°C in a Seahorse XF24 extracellular flux analyzer (Agilent) to the value effects of Ad-UCP2 or AdLacZ on OCR. Once the XF experiment was completed, cells were homogenized to determine the protein concentration for normalization. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (HyClone Pierce). The production of ATP and basal respiration rate were detected by observing

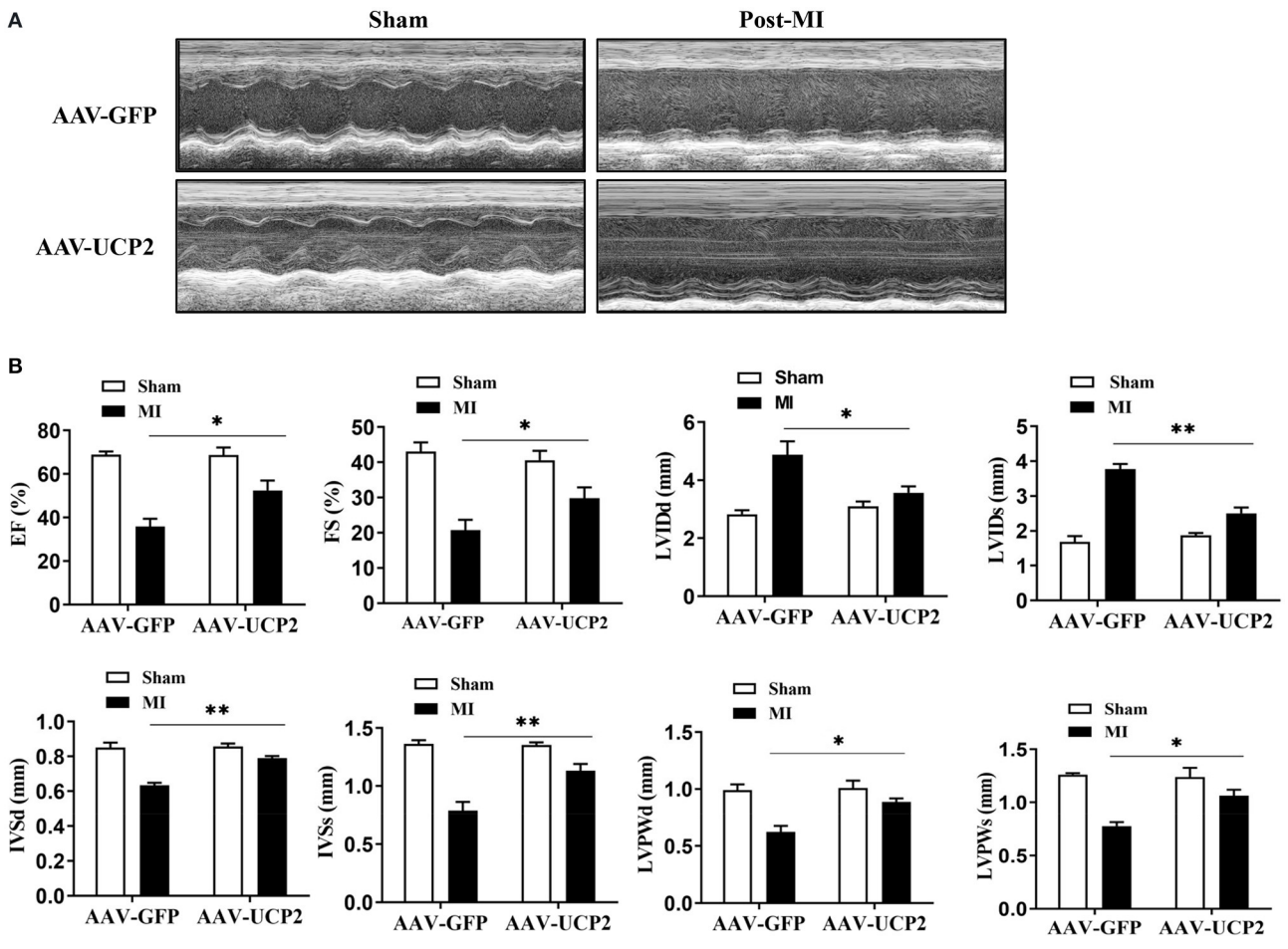

FIGURE 2 | Left ventricular functional and quantitative echocardiographic analysis in sham operation and over-expressed UCP2 group 4 weeks after MI. **(A)** Two-dimensional ultrasound diagram in sham-operated group and over-expressed UCP2 group 4 weeks after MI. **(B)** Functional and quantitative echocardiography in sham-operated group and over-expressed UCP2 group 4 weeks after MI. EF, Ejection fraction; FS, Fractional shortening; LVIDd, Left ventricular end diastolic diameter; LVIDs, Left ventricular end systolic diameter; LVPWd, Left ventricular posterior wall diastolic diameter; LVPWs, Left ventricular posterior wall systolic diameter; IVSs, Interventricular septal systolic diameter; IVSd, Interventricular septal diastolic diameter; MI, Myocardial infarction; $^{*}p < 0.05$, $^{**}P < 0.01$ compared with each other.

how the OCR changes in response to treatments that modulate mitochondrial activity.

Measurement of Mitochondria Reactive Oxygen Species

Cells were collected in different treatment groups, washed with PBS 3 times, and then resuspended in PBS. Then, the cells and MitoSOX with appropriate concentration were placed in the cell suspension for 30 min. After 30 min, they were washed with PBS. After centrifugation, a flow cytometer (Thermo Fischer Scientific) was used to detect the fluorescence intensity of MitoSOX in 510–580 waves.

Determination of Mitochondrial Membrane Potential

After a 24 h hypoxia treatment, NMCMs were perfused with Tyrode's solution containing 50 nM blebbistatin and 50 nM tetramethylrhodamine methyl ester (Guyana Biotech Co., Ltd. (Shanghai, China) for 30 min. Confocal images were taken at 20x magnification from multiple randomly selected regions before and after hypoxia with a Nikon A1 confocal microscope(Nikon AmericaInc., Melville, NY).

Statistical Analysis

All data are presented as the mean ± S.D of at least three independent experiments. Differences between two groups were analyzed by Student's test and multiple comparisons were analyzed by one-way ANOVA using GraphPad Prism version 8.0 (GraphPad). $P < 0.05$ was considered statistically significant.

RESULTS

The Expression of UCP2 After MI

Previous studies have shown UCP2 is expressed in myocardial tissue (17, 18). Increasing evidence indicates the expression of UCP2 is upregulated in the ischemic myocardium (19). To explore the expression of UCP2 in the post-infarction myocardium, we measured UCP2 by PCR and western blotting in infarct border zone. It was found the expression of UCP2 decreased significantly 4 weeks after MI which is shown in the AAV-GFP group in **Figure 1**. To further explore the role of UCP2, we constructed the AAV-UCP2 vector. Mice were injected with this vector intravenously, and the gene and protein levels of UCP2 in the myocardial tissue were detected 4 weeks after MI. It is shown the expression of UCP2 was significantly increased in AAV-UCP2 group, which indicating a successful vector construct.

UCP2 Improves Cardiac Function, Reduces Cardiomyocyte Apoptosis and Fibrosis After MI

To explore the role of UCP2 after MI, we investigated the effect of UCP2 on heart function after AAV-UCP2 transfection. The results showed that UCP2 significantly improved cardiac function. EF value increased from 40 to 55%, and other echocardiographic parameters (FS, LVIDd, LVIDs, IVSd, IVSs, LVPWd, and LVPWs) in the UCP2 group were also improved significantly (**Figure 2**). At the same time, we detected the level of fibrosis and found UCP2 reduced the intensity of collagen fiber (**Figure 3A**). In addition, we also detected apoptosis in

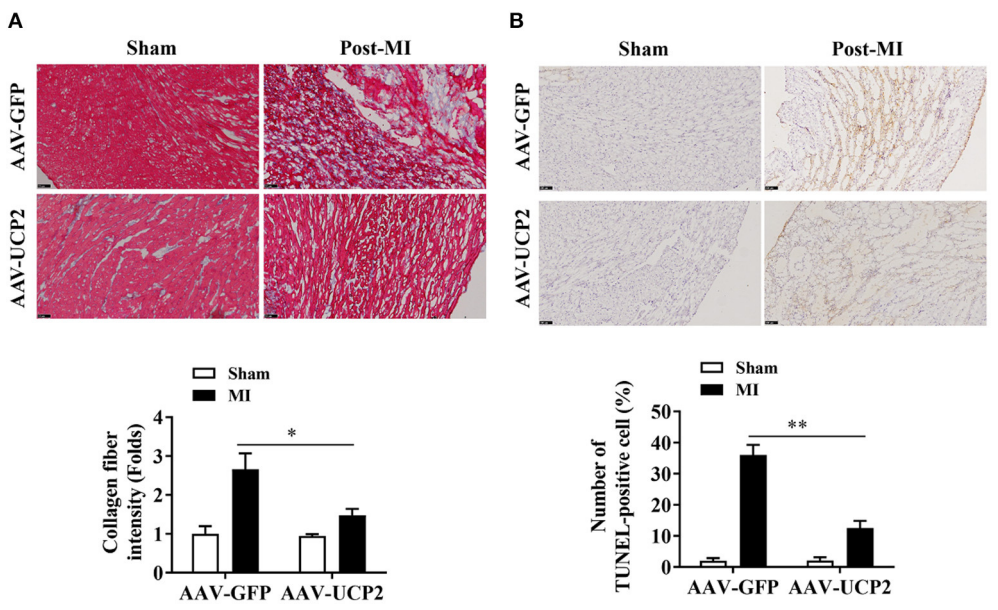

FIGURE 3 | UCP2 overexpression in heart reduce myocardial fibrosis and cardiomyocyte apoptosis 4 weeks after MI. **(A)** Masson's trichrome staining of heart tissue sections showing the presence of myocardial fibrosis in the border zones of the infarcted regions 4weeks after MI ($n = 3$). **(B)** TUNEL assay showing the effects of overexpression UCP2 on cardiomyocyte apoptosis 4 weeks after MI ($n = 3$). MI, myocardial infarction; $^{*}p < 0.05$, $^{**}P < 0.01$ compared with each other.

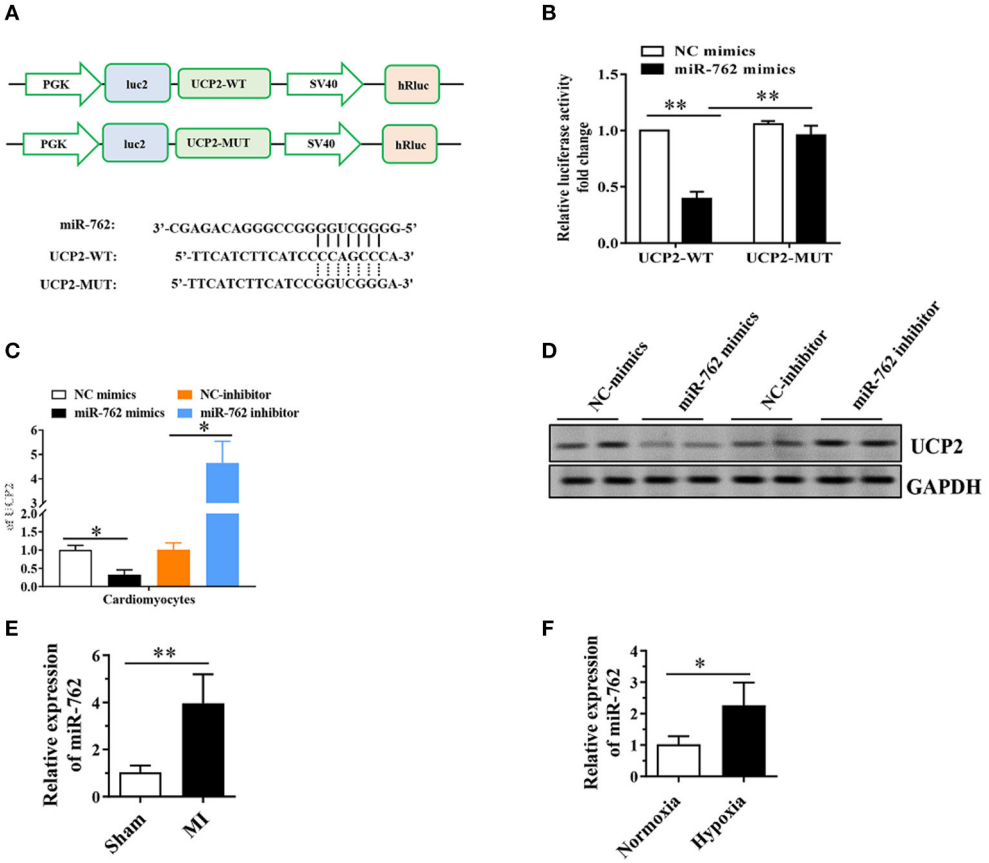

FIGURE 4 | MiR-762 directly targets UCP2. **(A)** The putative miR-762 binding sequence and the mutant sequence in the 3′UTR of UCP2. **(B)** Luciferase reporter activities of vectors carrying luciferase gene and a fragment of UCP2 3′UTR containing the wild-type and mutant binding sites of miR-762. **(C)** Quantitative real-time PCR was performed to detect the expression of UCP2 mRNA. **(D)** Western blot was used to detect the level of UCP2 protein. **(E,F)** MiR-762 was significantly up-regulated in mouse myocardial tissue after MI and NMCMs cells subjected to hypoxia. The results were presented as the mean ± SD, $n = 6$, $^{*}P < 0.05$. $^{**}P<0.01$. MI, myocardial infarction.

different group. The TUNEL positive rate was 13% in the UCP2 group, while it was as high as 37% in the control group (**Figure 3B**).

UCP2 Was a Direct Target Gene of miRNA-762

To clarify the molecular mechanisms and underlying the role of UCP2 in cardiomyocytes, we used the TargetScan online tool to predict potential microRNA targets of UCP2 and discovered a conserved binding site for miR-762 in the 3′UTR region of the UCP2 gene. To confirm whether miR-762 targets UCP2 through its 3′ untranslated region (3′UTR), the wild-type or mutant 3′UTR fragment of UCP2 was cloned into a firefly luciferase reporter plasmid (**Figure 4A**). The luciferase activity assay showed that miR-762 attenuated the luciferase activity of the reporter containing the wild-type UCP2-3′UTR, but did not alter the activity of the reporter with the UCP2-3′UTR 3′UTR binding site mutation (**Figure 4B**). In addition, to verify that miR-762 targets UCP2 under physiological conditions, we have assessed the expression of UCP2 mRNA and protein in cells transfected with miR-762 mimics, mimic controls, miR-762 inhibitors, or inhibitor controls. Our results indicated miR-762 overexpression significantly lowered UCP2 mRNA and protein expression, while miR-762 inhibitor had little effect on UCP2 mRNA and protein expression (**Figures 4C,D**). These findings suggest that miR-762 can directly target UCP2 in NMCMs cells. In addition, quantitative real-time PCR was used to test the expression level of miR-762 in mouse myocardial tissues after MI and NMCMs cells subjected to hypoxia. As noted in **Figures 4E,F**, the level of miR-762 was notably increased in mouse myocardial tissues after MI and NMCMs cells subjected to hypoxia compared with control groups, respectively. Furthermore, we assessed whether manipulation of miR-762 levels could affect injury of NMCMs after hypoxia. These results indicate miR-762 reduce cell viability and increase cell damage after hypoxia; However,UCP2 overexpression could eliminate this effect of miR-762 on NMCMs (**Supplementary Figure 1**). These data suggest that UCP2 was a direct target gene of miRNA-762 and they have an important role in cell injury after hypoxia.

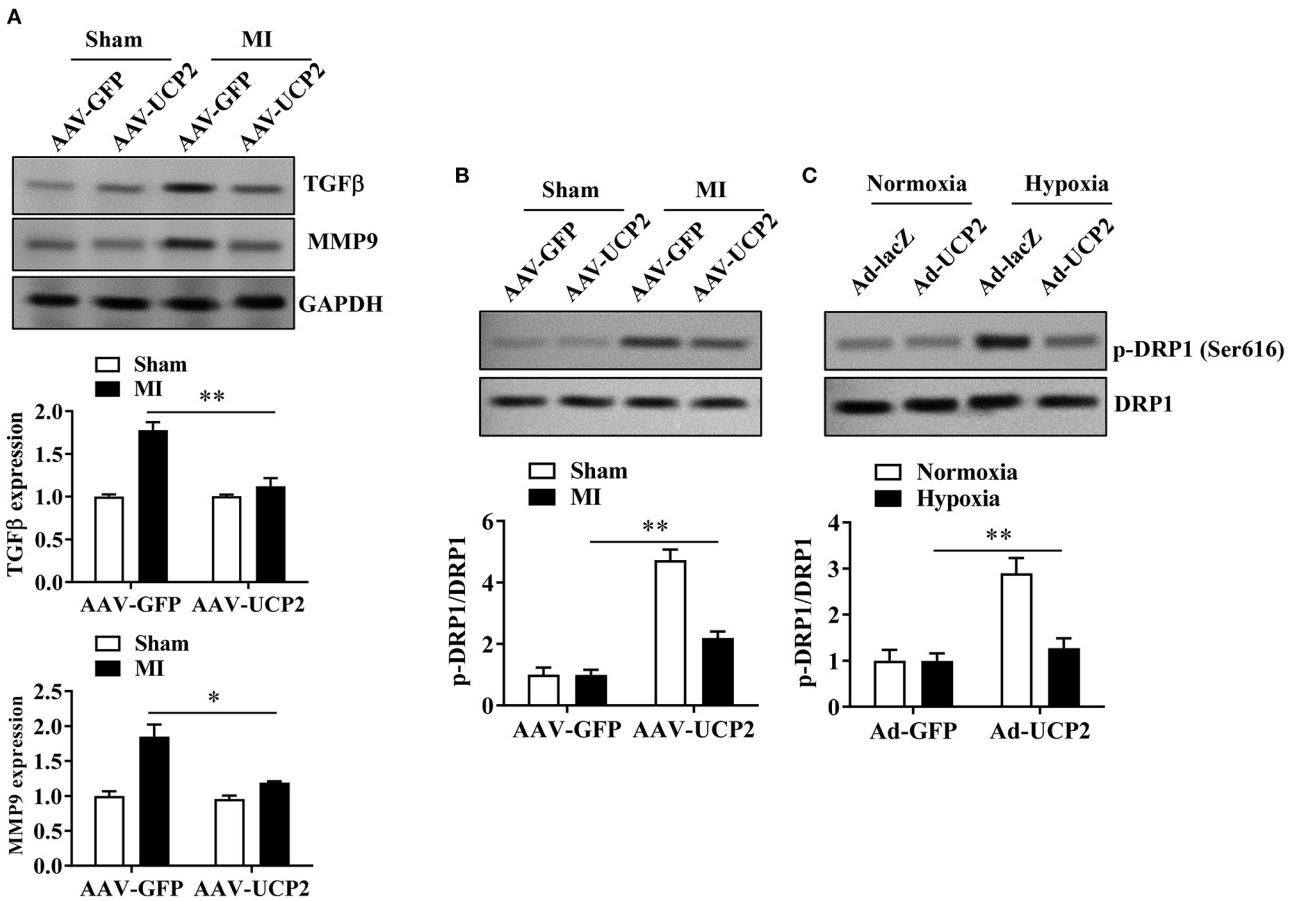

FIGURE 5 | The effect of UCP2 overexpression on fibrosis-related proteins and DRP1 phosphorylation 4 weeks after MI. **(A)** UCP2 overexpression in heart reduces the expression of fibrosis-related protein MMP9 and TGF-β. **(B,C)** UCP2 overexpression in heart or cell increases the expression of *p*-DRP1/DRP1 4 weeks after MI. MI, myocardial infarction;*$p < 0.05$, **$P < 0.01$ compared with each other.

The Effect of UCP2 on Fibrosis-Related Proteins and DRP1 Phosphorylation After MI

To explore the potential mechanism of UCP2 in alleviating myocardial fibrosis, we detected the expression of fibrosis-related proteins (MMP9 and TGF-β) by WB. We found that UCP2 reduced the expression of MMP9 and TGF-β (**Figure 5A**). A recent study showed that UCP2 is responsible for DRP1-dependent mitochondrial fission upon glucose load in SF1 neurons of ventromedial nucleus. Therefore, we infer UCP2 and DRP1 may have a similar interaction mechanism in cardiomyocytes. We have measured the level of DRP1 phosphorylation by WB *in vitro* and *in vivo*. It is found the phosphorylation of DRP1 and the ratio of p-DRP1/DRP1 decreased dramatically in the over-expressed UCP2 group (**Figures 5B,C**).

UCP2 Confers Protection by Regulating DRP1 After Hypoxia

It has been reported UCP2 affects mitochondrion fission and fusion to participate in regulating peripheral glucose homeostasis (20). Based on this, we infer that UCP2 may be involved in the process of cardiac remodeling after MI by affecting mitochondrial dynamic balance. Mdivi is widely used as a mitochondrial division inhibitor of DRP1. In this study, we used mdivi to inhibit DRP1 *in vitro* and *vivo*. As shown in **Figure 6**, the relative fluorescence of mitosox in mitochondria decreased significantly and the mitochondrial membrane potential was well-improved by mdivi. Furthermore, we detected OCR at different time points and found UCP2 significantly increased maximum OCR (**Figure 7A**). The effect of UCP2 on NMCMs' apoptosis and mouse cardiac function were detected by flow cytometry *in vitro* and M-mode echocardiography *in vivo,* respectively. It was shown UCP2 overexpression has a role in reducing apoptosis rate and improving cardiac function. However, compared with the mdivi group, inhibition of DRP1 by mdivi in over-expressed UCP2 group did not further reduce cell apoptosis rate and improve cardiac function, which indirectly indicates DRP1 is a key molecule for UCP2 to play its protective role after hypoxia and MI (**Figures 7B,C**).

DISCUSSION

Data presented in this study demonstrate: (1) The expression of UCP2 is downregulated in mouse heart 4 weeks after

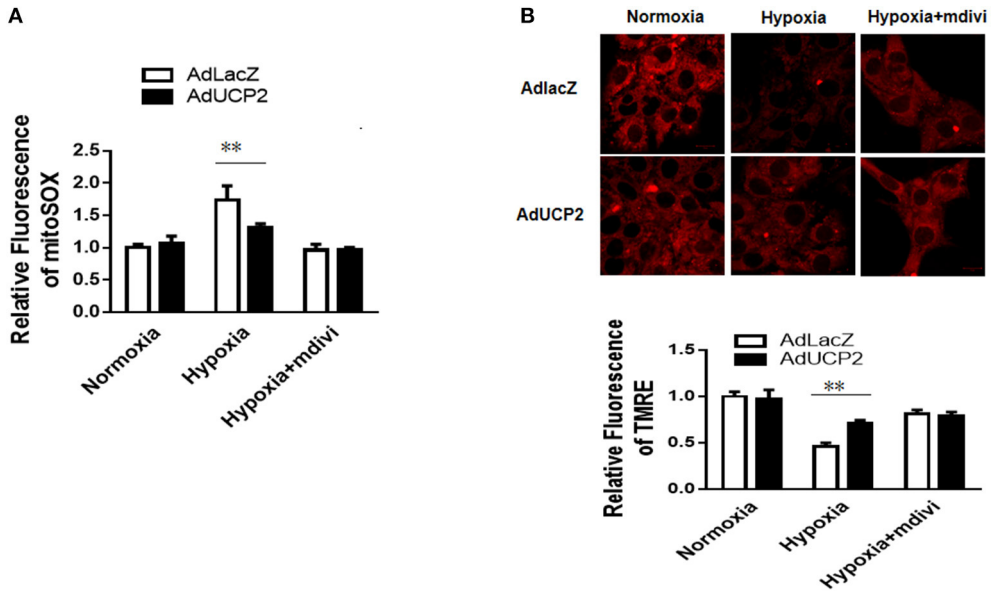

FIGURE 6 | UCP2 affects the production of reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) through DRP1. **(A)** The production of ROS was detected in different groups. **(B)** The MMP was measured in different groups. Mdivi, an inhibitor of DRP1; $^{**}P < 0.01$ compared with each other.

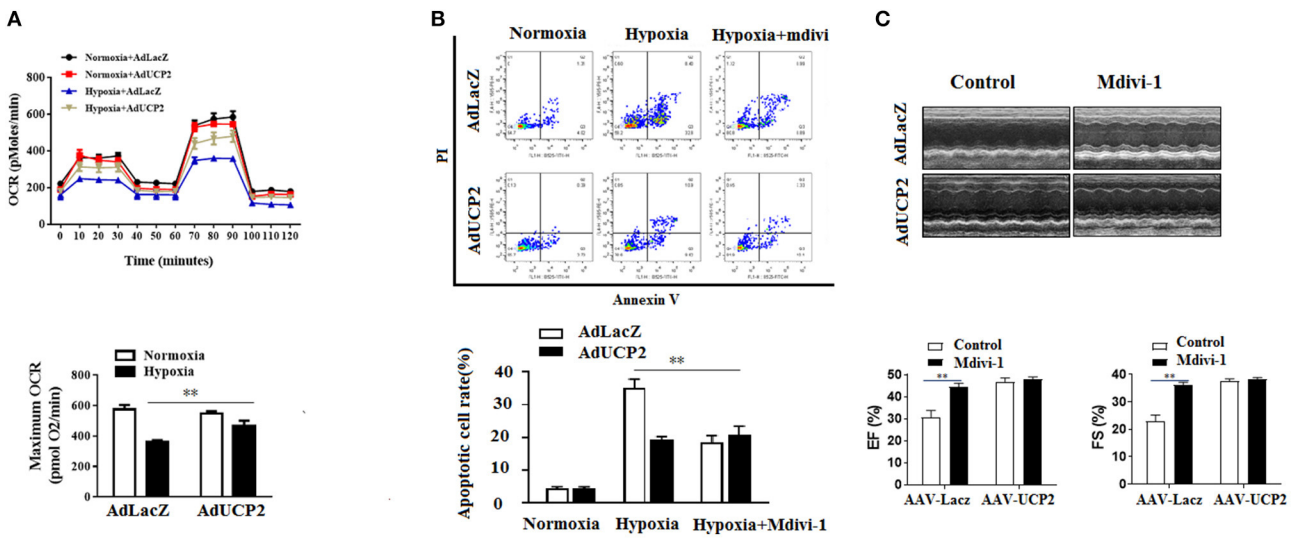

FIGURE 7 | UCP2 affects cardiomyocyte oxygen consumption rate (OCR) and apoptosis through DRP1. **(A)** Cardiomyocyte OCR was measured in different groups. **(B)** Cardiomyocyte apoptosis was detected using flow cytometry in different groups. **(C)** M-mode images of the left ventricle (LV) in different groups were measured after MI. Mdivi, an inhibitor of DRP1; $^{**}P < 0.01$ compared with each other.

MI; (2) UCP2 overexpression in the heart has protective roles on myocardial fibrosis and apoptosis after MI; (3) The potential mechanism of UCP2 is involved in the microRNA-762 in upstream and DRP1-dependent mitochondrial fission downstream.

CHD remains the leading cause of death, accounting for 7.4 million deaths globally every year (21). Acute MI is a subtype of CHD, with high mortality and complicated pathophysiological process. To reduce mortality, it is necessary to explore the pathogenesis of the disease more deeply. UCP2 is one member of the family of mitochondrial anion carriers. In recent years, UCP2 was found to play an important role in heart disease. More and more evidence shows that the upregulation of UCP2 in the ischemic myocardium may be due to increased oxidative stress (17, 18). In this study, we have detected the expression of UCP2 4 weeks after MI with PCR and west blotting. Our results show that the expression of UCP2 decreased significantly,whichis in line with the previous study (22).

It was reported that UCP2 expression alters differently in the settings of coronary artery disease and myocardial ischemia. There is some evidence showing UCP2 reduces the CAD risk (12, 23). However, another study demonstrated the opposite result (13). The reason for this difference is not clear, but it may be due to race and population differences. In this study, we found UCP2 plays a positive role in failing hearts after MI. Overexpression of UCP2 in the heart after MI ameliorates heart failure and reduces myocardial fibrosis and apoptosis. Although UCP2 has an antioxidant effect on the myocardium, it also induces a low energy state of the myocardium through the uncoupling of oxidative phosphorylation. As a result, UCP2 has a potentially harmful effect on failing hearts, with mismatches in energy production and utilization (22). However, in this study, it is found proper external supply of UCP2 protected against heart failure after MI. We speculate the possible reason is that UCP2 keeps the heart after MI in a good state of energy balance.

In the present study, we explored the potential protective mechanism of UCP2 after MI. Although the exact mechanism is not completely clear, some authors believe that UCP2 affects many aspects of mitochondrial function. It is reported that UCP2 prevents cell death caused by mitochondria (18). In addition, recent research has demonstrated UCP2 takes responsibility for DRP1-dependent mitochondrial fission upon glucose load in the ventromedial nucleus' SF1 neurons. Therefore, we focused on exploring the relationship between UCP2 and DRP1 after MI. Not surprisingly, we found UCP2 plays its protective role by regulating DRP1-dependent mitochondrial function and cardiomyocyte apoptosis. DRP1 is the most studied fission protein in heart disease, whose sub-pool colocalizes with mitochondria at sites of future fission. mitochondrial fission induced by DRP1 is an important mediator of myocardial cell death in MI (14, 24). The inhibition of DRP1 also protects the heart from MI by reducing mitochondrial metabolism and fragmentation (25). It was indicated in our study that UCP2 affected the production of mitochondrial superoxide, mitochondrial membrane potential, mitochondrial oxygen consumption rate, and cardiomyocyte apoptosis and improve cardiac function by DRP1. DRP1 is essential for UCP2 to play its protective role after MI.

MicroRNAs (miRNAs) are a big family of small non-coding RNAs that regulate gene expression by binding to their target mRNAs, subsequently leading to translation inhibition or degradation. Numerous studies have shown that miRNAs are involved in various heart diseases such as hypertension, hypertrophy, and remodeling. This study explored the relationship between miRNA and UCP2 after MI. we used bioinformatics analysis, luciferase activity and western blot assay proved UCP2 was a direct target gene of miRNA-762. Previous study show miR-762 participates in the regulation of cardiomyocyte apoptosis and mitochondrial function by NADH dehydrogenase subunit 2 (ND2) (26). In our study, we found miR-762 reduce cell viability and increase cell damage after hypoxia and it plays the role by the target of UCP2. MiR-762 and UCP2 may be new therapeutic targets for patients after MI.

Limitations

However, it should be noted that there are still some shortcomings in this study. Firstly, the study did not use transgenic mice to explore the role and mechanism of UCP2. Secondly, miRNA microarray assay isn't employed to explore the relationship between UCP2 and miRNAs.we just use targetscan software to predict the target of UCP2 and verify the result by luciferase activity and western blot.

In summary, our study revealed the physiological role of UCP2 in mouse heart after MI. The potential protective mechanism of UCP2 is involved in miRNA-762 upstream and DRP1-dependent mitochondrial fission downstream. UCP2 may offer new therapeutic potential for patients after MI.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Guangdong Provincial People's Hospital.

AUTHOR CONTRIBUTIONS

YL: conceptualization, design, and writing. DL: data curation, writing-original draft preparation, and software. SC: visualization. CC: investigation. SZ: supervision. HS and JW: methodology. QZ and CL: validation. YW: writing—reviewing and editing. GL: design and supervision. All authors contributed to the article and approved the submitted version.

FUNDING

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ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.764064/full#supplementary-material

Supplementary Figure 1 | The effect of MiR-762 and UCP2 on cell viability and damage. **(A)** Cell viability was measured by MTT assay. **(B)** LDH was measured using LDH assay kit $*P < 0.05$ compared with each other.

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Predicted Value of MicroRNAs, Vascular Endothelial Growth Factor, and Intermediate Monocytes in the Left Adverse Ventricular Remodeling in Revascularized ST-Segment Elevation Myocardial Infarction Patients

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Background: Primary percutaneous coronary intervention (PPCI) in patients with ST-segment elevation myocardial infarction (STEMI) improves the survival of patients; nevertheless, some patients develop left ventricular adverse remodeling (LVAR) a few months after the intervention. The main objective of this study was to characterize the role of pro-inflammatory cell populations, related cytokines, and microRNAs (miRNAs) released after PPCI as reliable prognostic biomarkers for LVAR in patients with STEMI.

Methods: We evaluated the level of pro-inflammatory subsets, before and after revascularization, 1 and 6 months after PPCI, using flow cytometry. We also performed a miRNA microarray in isolated peripheral blood mononuclear cells (PBMCs) and examined the levels of 27 cytokines in patients' serum of patients by multiplex ELISA.

Results: We observed that the levels of classical and intermediate monocytes increased 6 h after PPCI in patients who developed LVAR later. Multivariate regression analysis and ROC curves indicated that intermediate monocytes, after PPCI, were the best monocyte subset that correlated with LVAR. Within the 27 evaluated cytokines evaluated, we found that the increase in the level of vascular endothelial growth factor (VEGF) correlated with LVAR. Furthermore, the microarray analysis of PBMCs determined that up to 1,209 miRNAs were differentially expressed 6 h after PPCI in LVAR patients, compared with those who did not develop LVAR. Using RT-qPCR we confirmed a significant increase in miR-16, miR-21-5p, and miR-29a-3p, suggested to modulate the expression of different cytokines, 6 h post-PPCI in LVAR patients. Interestingly, we determined that

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the combined analysis of the levels of the intermediate monocyte subpopulation, VEGF, and miRNAs gave a better association with LVAR appearance. Similarly, combined ROC analysis provided high accurate specificity and sensibility to identify STEMI patients who will develop LVAR.

Conclusion: Our data suggest that the combined analysis of intermediate monocytes, VEGF, and miRNAs predicts LVAR in STEMI patients.

Keywords: STEMI, PPCI, inflammatory cells, monocytes, left ventricular adverse remodeling

INTRODUCTION

During a myocardial infarction with ST-segment elevation (STEMI), the time of coronary occlusion is of vital importance until blood flow is reestablished. Primary percutaneous coronary intervention (PPCI) considerably mitigates cardiac cell death and adverse cardiovascular events (1). The occlusion of the coronary artery initiates multiple structural, functional, and metabolic lesions that negatively affect the function of the heart. Several studies have suggested the beneficial effects of early PPCI on left ventricle remodeling, which demonstrated that significant left ventricular dilation occurred in a relevant proportion of patients with STEMI treated with PPCI (2, 3). Pathological remodeling appears because of progressive change in the left ventricle size, shape, and function, causing left ventricular adverse remodeling (LVAR), which can lead to heart failure (HF) (3). There is a general consensus that acute inflammation and especially the innate immune system influences the clinical outcome in HF patients (1). Immune cells play an important role in protecting the myocardium from ischemic damages and promote wound healing in the ischemia-affected tissues (2). Therefore, the control and expansion of pro-inflammatory populations are considered hallmarks of inflammation resolution. Conversely, excessive production and infiltration of innate cells promote LVAR and increase the probability of suffering adverse cardiac events (3, 4). Hence, given its complexity, it is still unclear how the innate immune system influences the development and progression of LVAR toward HF.

Leukocytes proliferate in the bone marrow (BM) and in response to damage-associated molecular patterns (DAMPs) secreted by tissue, they egress from the BM in order to infiltrate the ischemic myocardium (5). These inflammatory cell populations synthesize a broad range of pro-inflammatory mediators as chemokines, cytokines, microRNAs (miRNAs), and resolution factors, which are key regulators of the inflammation in the myocardium after a STEMI (6, 7). Within leukocyte cells, neutrophils have been recognized as the first innate inflammatory cell population to infiltrate the heart following an acute myocardial infarction (AMI) (8), but 3 days after AMI monocytes became the most important inflammatory cells involved in the salvage of the myocardium (4, 9). Monocytes are a heterogeneous cell population divided in subsets with specific functions and phenotypes during the process of inflammation. Three subsets of human monocytes have been immunodefined (10). The differential expression of CD14 (Lipopolysaccharide LPS receptor) and CD16 (the low affinity receptor for IgG, FCγIIIR) distinguishes classical (CD14++ CD16+), intermediate (CD14++ CD16+), and non-classical (CD14+ CD16++) monocytes.

On the other hand, several studies have demonstrated that miRNAs, the most studied small non-coding RNA, are dysregulated in patients with cardiovascular diseases, as compared with healthy patients, some of them predict the LVAR (11). Recently, the expression patterns of circulating miRNAs have been described in patients suffering a myocardial infarction (11) and after a PPCI (12). miRNAs are known to regulate the expression of key proteins involved in the response to AMI (13). However, little is known about their association with changes in the expression of cytokines and the levels of inflammatory cells population, key effectors during AMI responses.

In this study, we analyzed changes in the levels of inflammatory cell subsets during STEMI and after PPCI, and we further correlated their changes with the clinical appearance of LVAR. We also determined miRNAs levels in peripheral blood mononuclear cells (PBMCs) and of pro-inflammatory cytokines in the serum of these patients. Our combined analysis establishes three components that predict the appearance of LVAR in patients with revascularized STEMI.

MATERIALS AND METHODS

This study was conducted following the principles published by the declaration of Helsinki and its modification or similar ethical standards. The study was authorized by the local Ethics Committee on Human Research at the University Hospital "Virgen del Rocio" of Seville (Approval no. 2013PI/096). To report our findings, we followed the "strengthening the reporting of observational studies in epidemiology" (STROBE) guidelines (**Supplementary Figure 1**).

A detailed section of material and methods is provided in **Supplementary Information**. We recruited a cohort of patients with revascularized STEMI and controls with rigorous inclusion criteria. We evaluated LVAR using echocardiography and extracted patients' blood samples before revascularization (0 h), and at different time points after PPCI (6 h, 1, and 6 months). We analyzed inflammatory cell populations using flow cytometry; and serum cytokines levels using Multiplex and ELISA assays. miRNAs expression was examined by miRNA array assay and RT-qPCR.

RESULTS

Study of the Cohort Clinical Data

Demographical and clinical information were obtained from 28 healthy controls and 44 patients with STEMI who underwent PPCI. Healthy controls (46 ± 11 years; 57.14% male sex) were patients who did not suffer from arterial hypertension, dyslipidemia, or type II diabetes mellitus and did not smoke, suggesting a cohort without apparent cardiovascular risk. Within the STEMI patient group (58 ± 10 years; 92.10% male sex), 36.84% have arterial hypertension, 47.37% dyslipidemia, 18.42% diabetes mellitus type II, and 47.37% were smokers.

Analysis of the Level of Inflammatory Cells in ST-Segment Elevation Myocardial Infarction Patients Undergoing Primary Percutaneous Coronary Intervention

Since inflammatory innate cells are key players in the inflammatory response to AMI, we examined by flow cytometry (**Figure 1A**) the level of CD11b + granulocyte populations (neutrophils and eosinophils) at different time points, before PPCI (0 h), and 6 h, 1, and 6 months after PPCI. **Figure 1B** shows a high increase in neutrophil levels (neutrophilia) in STEMI patients, as compared with healthy controls. Neutrophilia was observed at the onset and 6 h after PPCI. This increase was mild even if it was still statistically different 1 and 6 months after the intervention. By contrast, the level of eosinophils decreased significantly before and 6 h after PPCI in patients with STEMI, as compared with the control (**Figure 1C**); while they recovered their basal levels 1 and 6 months after PPCI.

Since monocytes and neutrophils are the main populations egressing from the BM after acute infections or in response to internal injuries as a cardiovascular ischemic event (8), we analyzed the level of monocytes by flow cytometry (**Figure 1D**) and quantified monocytes subsets populations (**Figures 1E–G**). As illustrated in **Figure 1E**, the level of classical monocytes (CD16- CD14++) increased significantly 1 and 6 months after PPCI, but not at the onset neither 6 h after PPCI. Meanwhile, the level of intermediate monocytes (CD16+ CD14++) was significantly increased in STEMI patients before and 6 h after PPCI. This increase lasted up to 6 months, as compared with the healthy controls (**Figure 1F**). In the case of non-classical monocytes (CD16++ CD14-), we observed only a significant increase 1 and 6 months after PPCI (**Figure 1G**). Altogether, these data indicate that neutrophils and intermediate monocytes could be involved in the acute inflammatory responses after PPCI in STEMI patients.

Levels of Inflammatory Cells in ST-Segment Elevation Myocardial Infarction Patients With Left Ventricular Adverse Remodeling

In order to analyze the number of STEMI patients who developed LVAR after PPCI, we performed an echocardiography of all the patients at the hospital discharge and 6 months after the ischemic event. **Table 1** shows that 12 of 38 patients (31.58%) developed LVAR 6 months after PPCI, given that their index value of left ventricular end-diastolic volume (LVEDV) increased 37.91 ± 14.31%, as compared with their values at the hospital discharge. We also observed a significant difference in the level of troponin-T (TnT) and creatine kinase (CK), but not in the elevation of the ST segment, or in the timing of infarction pain during patient admission. In addition, there were no significant differences between LVAR and non-LVAR groups in terms of gender, age, or cardiovascular risk factors.

To characterize the relevance of the observed changes in the level of inflammatory cells, we examined whether they were different in patients who developed LVAR compared with those who did not present cardiovascular adverse events after PPCI. **Figure 2** shows that there were no significant differences in the level of neutrophils (**Figure 2A**) and eosinophils (**Figure 2B**) between the two groups of patients. However, there was a significant difference in total monocyte counts 6 h after PPCI (**Figure 2C**). We further analyzed the level of all monocyte subset populations. **Figure 2D** shows that there was no difference in the level of non-classical monocytes at any time points analyzed. By contrast, levels of pro-inflammatory intermediate monocytes were increased significantly in patients who developed LVAR before and 6 h after PPCI (**Figure 2E**). Meanwhile, classical monocytes increased significantly 6 h after PPCI in patients with LVAR compared with those without LVAR (**Figure 2F**). Moreover, classical monocytes were still increased 6 months after the intervention. These data indicate that these two monocyte subset populations are likely associated with the appearance of the LVAR.

Correlation of Pro-Inflammatory Intermediate Monocyte Levels Increase With Left Ventricular Adverse Remodeling

To distinguish which monocyte subset population could be a fair predictive marker for the appearance of LVAR in STEMI patients after PPCI we performed a multivariate logistic regression analysis. We calculated the odd ratio (OR) of different variables as age, sex, CK, and TnT levels, compared to the level of intermediate (**Figures 3A,B**) and classical (**Supplementary Figures 2A,B**) monocytes before and after revascularization. Multivariate analysis identified the level of intermediate monocytes as an independent predictor of LVAR, especially 6 h ($p = 0.03$) after revascularization (**Figure 3B**). However, multivariate analysis of classical monocytes suggests that they were not independent predictor of LVAR, since the OR was not significant ($p > 0.05$) at 0 or 6 h after the PPCI (**Supplementary Figures 2A,B**).

Linear regression analysis further confirmed that changes in the level of intermediate monocytes were associated with changes of the percentage of LVEDV, indicative of LVAR development. As illustrated in **Figures 3C,D** the levels of intermediate monocytes at 0 h ($r = 0.616$, $p < 0.001$), and 6 h after the PPCI ($r = 0.498$, $p = 0.002$) significantly correlated with changes in the percentage

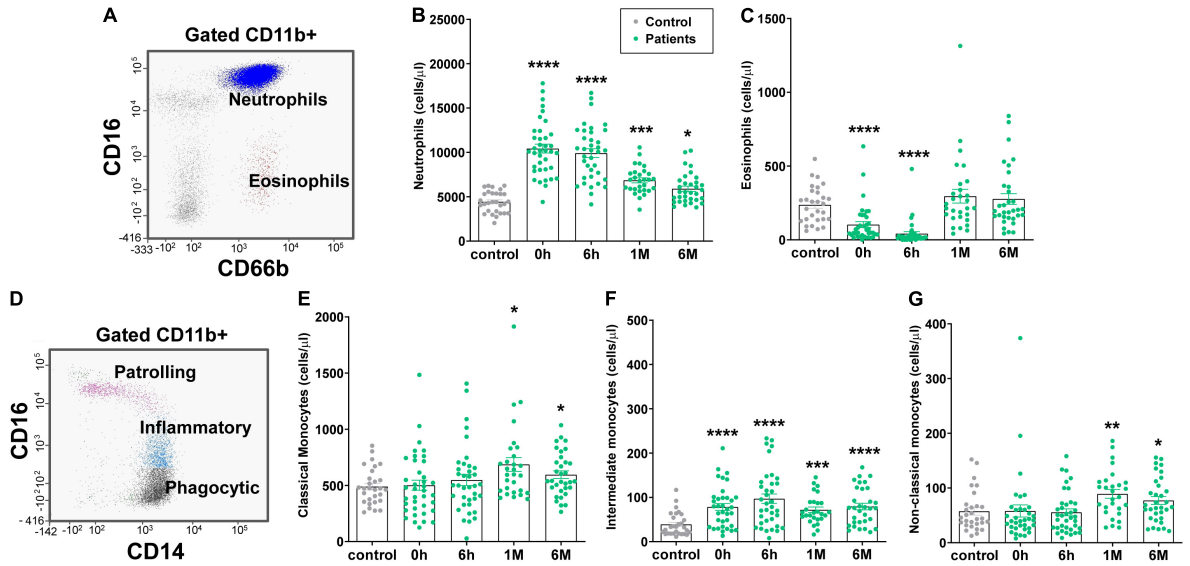

FIGURE 1 | Granulocytes, monocytes and monocyte subsets levels in STEMI patients measured by flow cytometry. **(A)** Flow cytometry strategy with a dot plot gated in CD11b+ cells is used to identify neutrophils (CD11b+, CD16+, CD66b+, blue) and eosinophils (CD11b+, CD16-, CD66b+, red). **(B,C)** Bar graphs show levels of neutrophils **(B,C)** eosinophils in healthy controls (gray dots, $n = 28$) compared with the levels in STEMI patients (green dots, $n = 38$) measured in peripheral blood (PB) at 0 h (before revascularization), 6 h (after revascularization), 1 month and 6 months. **(D)** Representative flow cytometry diagrams of a patient's PB sample depicting monocyte subsets: non-classical (patrolling, CD11b+ CD14+ CD16++, pink), intermediate (inflammatory, CD11b+ CD14+ CD16+, blue), and classical (phagocytic, CD11b+ CD14+ CD16-, black) monocytes. **(E–G)** Quantification of the level of classical **(E)**, intermediate **(F)** and non-classical **(G)** monocytes in controls and STEMI patients at 0 h, 6 h, 1 month and 6 months after the ischemic event. Values are presented as the means ± SEM. Kruskal–Wallis test with multiple comparisons corrected by Dunn's test was performed. (*), (**), (***) and (****) indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively. PB, peripheral blood; STEMI, ST-elevation myocardial infarction.

TABLE 1 | Demographic and clinical characteristics of STEMI patients ($n = 38$).

	STEMI patients ($n = 38$)	Non-LVAR ($n = 26$)	LVAR ($n = 12$)	p-value
Age (years)	58 ± 10	57 ± 10	60 ± 9	0.420
Male sex	35 (92.10%)	24 (92.30%)	11 (91.66%)	0.949
Arterial hypertension	14 (36.84%)	7 (26.92%)	7 (58.33%)	0.085
Smoking	18 (47.37%)	12 (46.15%)	6 (50.00%)	0.834
Dyslipidemia	18 (47.37%)	13 (50.00%)	5 (41.67%)	0.647
Type 2 diabetes mellitus	7 (18.42%)	6 (23.08%)	1 (8.33%)	0.288
LVEDV (ml/m^2)$^{\$\$}$	68.19 ± 20.63	65.99 ± 16.36	72.60 ± 27.62	0.453
LVEDV (ml/m^2)$^{\$\$\$}$	75.84 ± 28.13	62.58 ± 14.85	102.38 ± 30.08	**< 0.001***
Creatine kinase (mg/dL)$^{\$}$	2187 (1044–4611)	1590 (723.5–3335)	4056 (2165–5090)	**0.028***
Troponin-T (ng/mL)$^{\$}$	5835 (3642–11493)	4785 (3503–6802)	11215 (4921–14129)	**0.033***
ST elevation	13 (8.25–20.50)	11 (8.00–16.75)	18 (10.75–21.75)	0.1854
Timing of infarction pain	160 (120–230)	140 (120–235)	180 (150–250)	0.3810

Patients were classified into two groups: no remodeling (n = 26) and remodeling (n = 12). Data are shown as the means ± SEM or median (IQR) and as n (%). Student's t-tests or Mann-Whitney test were performed. (*)Indicates significance at $p < 0.05$. ($^{\$}$)Indicates that the variable was examined at admission, $^{\$\$}$ at hospital discharge and $^{\$\$\$}$ at 6 months after PPCI.

IQR, interquartile range; LVAR, left ventricular adverse remodeling; LVEDV, left ventricular end-diastolic volume; PPCI, primary percutaneous coronary intervention; SEM, standard error of the mean; STEMI, myocardial infarction with ST-segment elevation. Significant p-values are provided in bold.

of the LVEDV. In addition, in **Figures 3E,F**, ROC curves analysis showed that the area under the curve (AUC) of these monocytes at 0 and 6 h, were higher than 0.7 with significant p-values, indicating an accurate level of adjustment. In contrast, the linear regression curves of classical monocytes presented a worse association with the changes in the percentage of the LVEDV with an "r" below 0.30 (**Supplementary Figures 2C,D**). Similarly, ROC curves with classical monocytes at 0 h showed poor sensitivity and specificity to predict LVAR (**Supplementary Figure 2E**). However, the AUC was higher than 0.7 at 6 h with $p = 0.02$ (**Supplementary Figure 2F**).

We also performed a ROC analysis with other classical parameters before and after revascularization, such as CK and troponin-T levels, time until revascularization, ST elevation in

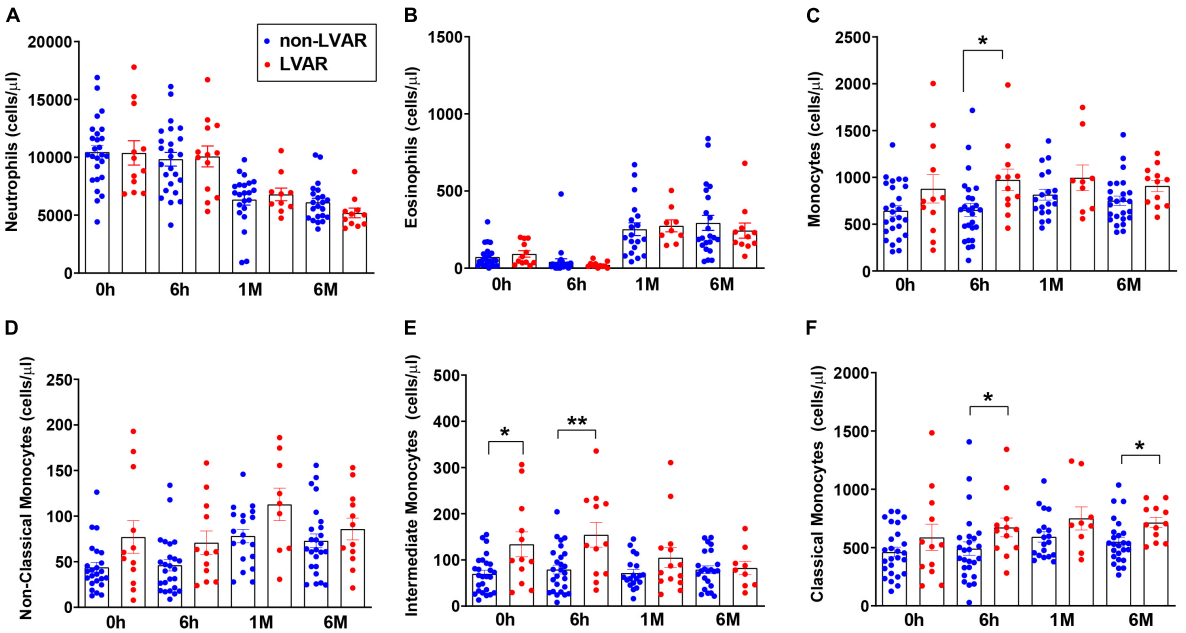

FIGURE 2 | Analysis of the level of granulocytes, monocytes and monocytes subsets in patients with or without LVAR. **(A–F)** Bar graphs show the level of neutrophils **(A)**, eosinophils **(B)**, total monocytes **(C)** and the monocyte subsets: non-classical **(D)**, intermediate **(E)** and classical monocytes **(F)** in PB samples of non-LVAR STEMI patients (blue dots, $n = 26$) and STEMI patients who developed LVAR (red dots, $n = 12$) at 0, 6 h, 1 and 6 months post PPCI. Data are shown as the means ± SEM. Kruskal–Wallis test with multiple comparisons corrected by Dunn's test was performed. (*) and (**) indicate significance at $p < 0.05$ and $p < 0.01$, respectively. LVAR: left ventricle adverse remodeling; PB, peripheral blood; PPCI, primary percutaneous coronary intervention; STEMI, ST-elevation myocardial infarction.

the emergency room and age. The AUC of CK and TnT levels at 0 h were higher than 0.70 with a significant *p*-value, although the multivariate analysis showed that these biomarkers were not independent of age and sex in predicting the LVAR (**Figures 3A,B** and **Supplementary Figures 2A,B**). The rest of the parameters provided weak AUC in the ROC analysis with insignificant *p*-values.

Taken together, these results confirmed that intermediate monocytes, rather than classical monocytes, are the best subset cell population to predict LVAR in STEMI patients after a PPCI.

Analysis of Pro-inflammatory Cytokines in the Serum of ST-Segment Elevation Myocardial Infarction Patients

The main effectors of inflammation are cytokines, chemokines, and inflammatory mediators expressed and secreted from inflammatory cells in the tissues at the sites of infiltration. Therefore, we measured pro-inflammatory cytokines whose level could correlate with the appearance of LVAR. We performed a Bioplex analysis of 27 pro-inflammatory cytokines in the serum of a 13 patients' subgroup (10 non-LVAR and 3 LVAR patients). We observed a significant increase in the secretion of Granulocyte-macrophage colony stimulating factor (GM-CSF) before PPCI (0 h), and interleukin 1β (IL-1β), interferon γ (IFNγ), interleukin 17 (IL-17), and vascular endothelial growth factor (VEGF) 6 h after PPCI in patients with LVAR (**Supplementary Table 1**). By contrast, there were no significant differences in other well-known pro-inflammatory 12 cytokines, as interleukin 1 receptor antagonist (IL-1Ra), tumor necrosis factor α (TNF-α), interleukin 4 (IL-4), interferon γ –inducible protein (IP-10), interleukin 9 (IL-9), interleukin 8 (IL-8), granulocyte colony stimulating factor (G-CSF), fibroblast growth factor basic (FGF basic), macrophage inflammatory protein 1 α (MIP-1α), macrophage inflammatory protein 1 β (MIP-1β), or platelet-derived growth factor BB, and Eotaxin. Next, we performed ELISA to measure the level of these differentially expressed cytokines at the time point of their maximum level of secretion (6 h). As illustrated in **Supplementary Figures 4A–D**, only VEGF increased differentially in LVAR patients, compared with non-LVAR patients. We also observed an incremental trend in the level of the IL-1β. Likewise, ROC curves showed that only the AUC of the levels of VEGF was higher than 0.7 to predict LVAR (**Supplementary Figures 4E–H**).

Study of miRNAs Expressed by Inflammatory Cells in Patients With ST-Segment Elevation Myocardial Infarction

Next, we analyzed the expression of miRNAs in PBMCs isolated from the control healthy group ($n = 3$), a group of non-LVAR ($n = 5$), and LVAR STEMI patients ($n = 5$), before (0 h) and after revascularization (6 h). We found only 256 miRNAs differentially expressed in control compared with the non-LVAR patients (fold change ± 2.5 and $p < 0.05$); meanwhile, 925 miRNAs were differentially expressed between control and LVAR patients (data not shown). Interestingly, we found 577

A

Factor	OR	95% CI	*p*-value
Intermediate monocytes (0 h)	**1.018**	**0.999-1.037**	**0.061**
Age	1.064	0.952-1.190	0.275
Sex	1.467	0.011-190.317	0.877
Creatin Kinase	1.000	1.000-1.001	0.367
Troponin-T	1.000	1.000-1.000	0.482

B

Factor	OR	95% CI	*p*-value
Intermediate monocytes (6 h)	**1.018**	**1.002-1.035**	**0.030***
Age	1.073	0.955-1.204	0.235
Sex	0.151	0.007-3.158	0.223
Creatin Kinase	1.000	1.000-1.001	0.213
Troponin-T	1.000	1.000-1.000	0.681

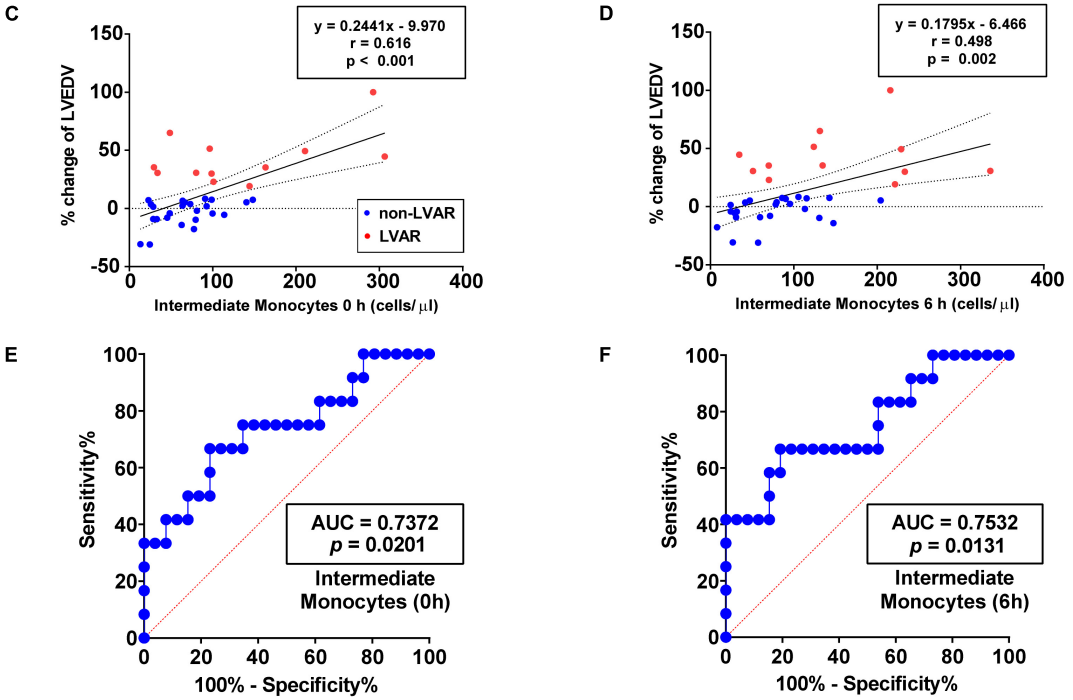

FIGURE 3 | Correlation analysis of the level of intermediate monocytes with LVAR. **(A,B)** Multivariate logistic regressions analysis of intermediate monocytes measured in PB at 0 h **(A)** and 6 h **(B)**, using age, sex, creatine kinase and troponin-T as cofactors. **(C,D)** Linear regression curves analysis using the percentage of change in LVEDV of non-LVAR (blue dots, $n = 26$) and LVAR (red dots, $n = 12$) patients as dependent variable and intermediate monocytes level at 0 h **(C)** or 6 h **(D)** as independent factor. **(E,F)** ROC analysis with the AUC (values given on the graphs) indicating sensitivity and specificity of level of intermediate monocytes at 0 h **(E)** or 6 h **(F)** to predict LVAR. Multivariate logistic regression, linear regression and ROC analysis were performed. (*) Indicates significance at $p < 0.05$. AUC, area under the curve; CI, confidence interval; LVAR, left ventricle adverse remodeling; LVEDV, left ventricular end-diastolic volume; OR, odds ratio; PB, peripheral blood; ROC, Receiver-operating characteristics.

and 1,209 miRNAs differentially expressed in PBMCs from LVAR, as compared with the non-LVAR patients before and after revascularization, respectively (**Figures 4A,B**). Volcano plots show that 154 miRNAs were downregulated and 423 were upregulated (**Figure 4A**) before PPCI, and 85 miRNAs were downregulated and 1,124 were upregulated (**Figure 4B**) 6 h after revascularization, in LVAR patients compared with the non-LVAR patients (fold change ± 2.5 and $p < 0.05$). The hierarchical clustering analysis confirmed that miRNAs were differentially expressed in the two groups of patients with STEMI and they were well clustered (**Figures 4C,D**).

Based on these findings and using *in silico* databases, we selected miRNAs suggested to target cytokine production (**Supplementary Table 2**), focusing on those that might regulate the oversecreted cytokines in LVAR patients, 6 h after PPCI detected in Bioplex analysis. Therefore, we chose 3 miRNAs, miR-16-5p, miR-21-5p, and miR-29a-3p that are predicted to target IL-1β, IFNγ, and VEGF genes. We did not find any miRNA, from the microarray list, targeting IL-17 gene, possibly because of that this cytokine is expressed and secreted mainly by lymphocytes (14). Using qRT-PCR we confirmed significant increase in the level of miR-16-5p, miR-21-5p, and miR-29a-3p in LVAR vs. non-LVAR patients, especially 6 h after revascularization (**Figures 5A–C**). ROC curves analysis indicated that the AUC of miR-16-5p, miR-21-5p, and miR-29a-3p were statistically significant 6 h after PPCI (**Supplementary Figure 5**). Using the online platform Panther to analyze potential target genes of these miRNAs, we found a set of genes involved in immune response and inflammation mediated by cytokine, IFNγ, and IL signaling pathways (detail in **Supplementary Table 3**), which support our hypothesis.

Analysis of Combined Biomarkers to Improve Prediction of Left Ventricular Adverse Remodeling

Looking for better markers to more accurately identify patients who will develop LVAR, we performed combined ROC analysis using classical markers, such as CK and TnT levels at 0 h,

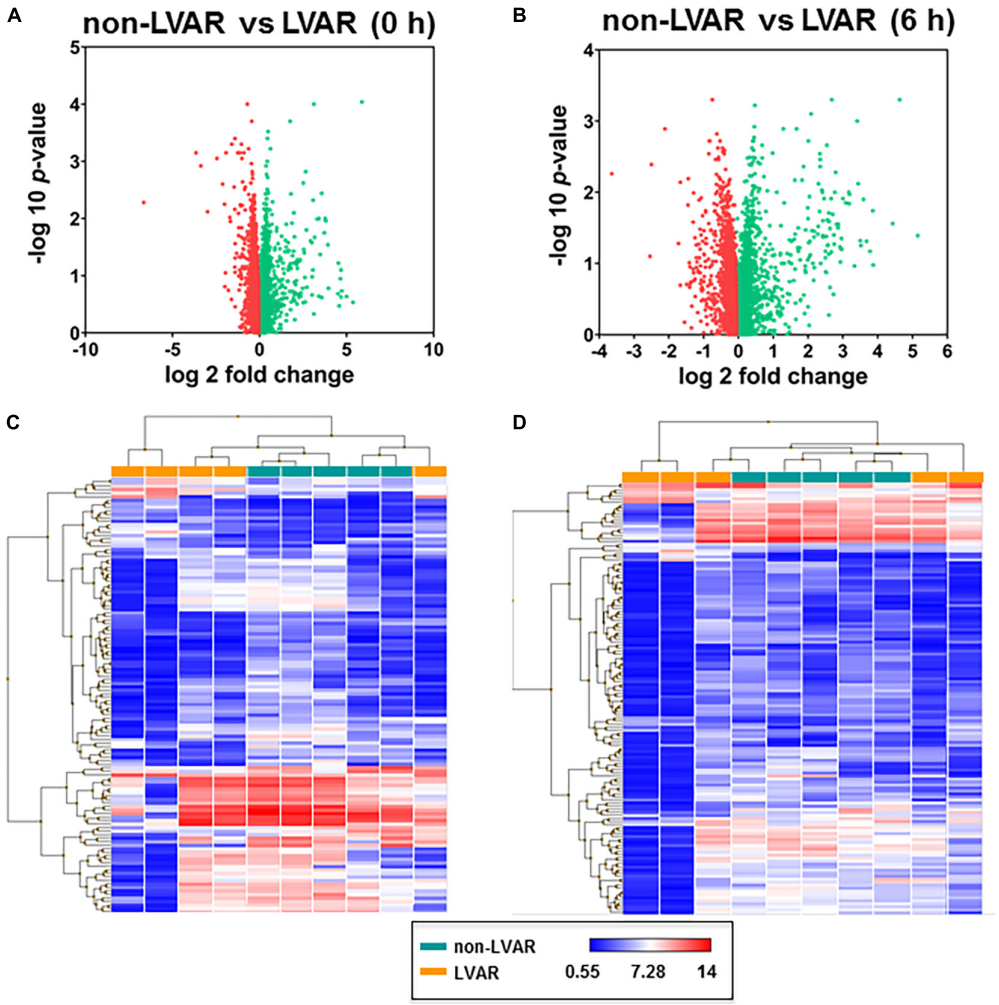

FIGURE 4 | miRNAs differentially expressed between non-LVAR and LVAR patients in PBMCs. **(A,B)** Volcano plots showing miRNAs differentially expressed in LVAR patients compared to non-LVAR patients at 0 h **(A)** and 6 h **(B)** after PPCI (Green dots: up-regulated miRNAs, red dots: down-regulated miRNAs). **(C,D)** Hierarchical clustered sample-centric heat-map plots of –2.5 > fold change > 2.5 value of miRNAs in PBMCs samples from non-LVAR (blue rectangle, $n = 5$) and LVAR patients (orange rectangle, $n = 5$) at 0 h **(C)** and 6h **(D)**. Scale bar, downregulated (blue) and upregulated (red). LVAR, left ventricle adverse remodeling; miRNAs, microRNAs; PBMCs, peripheral blood mononuclear cells; PPCI, primary percutaneous coronary intervention.

and newly identified markers, intermediate monocytes, VEGF, and miRNAs levels at 6 h after PPCI. As described earlier, the analysis of each factor independently showed an AUC greater than 0.70 with significant p values. To improve the power of LVAR prediction, we established a worsening diagnosis score based on the secretion level of all biomarkers in each patient as described in **Supplementary Table 4** and Supplementary Materials and Methods. First, we calculated the score by combining the analysis of classical parameters CK and TnT. As depicted in **Figure 6A**, LVAR patients obtained a significantly higher score compared with the non-LVAR patients. **Figure 6B** indicates that the analysis of the ROC curve of this score was more accurate in predicting LVAR, than those of CK and TnT analyzed separately (**Supplementary Figures 3A,C**). Next, we compared the predictor score combining the analysis of the new markers, pro-inflammatory intermediate monocytes, VEGF, miR-16-5p, miR-21-5p, and miR-29a-3p, 6 h after revascularization. **Figures 6C,D** shows that significance was even higher for LVAR patients compared with non-LVAR patients, and ROC curve analysis presented an AUC of 0.85 with a p-value of 0.003. Finally, as shown in **Figures 6E,F**, the combined analysis of the score of all the examined markers, CK, TnT, pro-inflammatory intermediate monocytes, VEGF, miR-16-5p, miR-21-5p, and miR-29a-3p provided a significantly higher score and the ROC curve achieved a great AUC of 0.9111 with a $p = 0.0005$ (**Figure 6F**), to predict LVAR appearance in STEMI patients after an PPCI. Therefore, this data analysis suggests that a patient with a score higher than 14.50 (being 25 the maximum score) will positively develop LVAR, with 75.00% of sensitivity and 88.89% of specificity, as indicated in **Figure 6F**. These findings revealed that the combined analysis of classical and new biomarkers (CK, TnT, VEGF, intermediate monocytes, and miRNA levels) provides

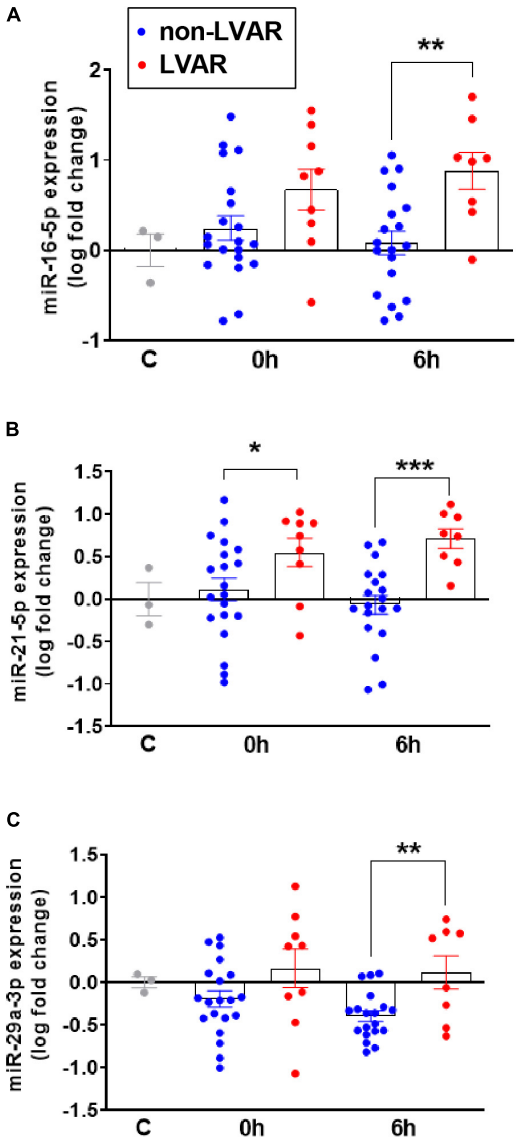

FIGURE 5 | Analysis of miR-16, miR-21 and miR-29a expression in PBMCs from non-LVAR and LVAR patients. **(A–C)** Bar graphs show the levels of expression of miR-16 **(A)**, miR-21 **(B)** and miR-29a **(C)** in controls and non-LVAR (blue dots, $n = 20$) and LVAR patients (red dots, $n = 9$), pre-PPCI (0 h) and post-PPCI (6 h). Values are presented as the means ± SEM. Ordinary one-way ANOVA with multiples comparisons using *t*-test without correction (Fisher's LSD test) was performed. (*) indicates $p < 0.05$, (**) indicates $p < 0.01$ and (***) indicates $p < 0.001$. LVAR, left ventricle adverse remodeling; PBMCs, peripheral blood mononuclear cells; PPCI, primary percutaneous coronary intervention.

highly specific and sensitive value to predict LVAR in patients undergoing PPCI.

DISCUSSION

Acute myocardial infarction and the consequent HF are major causes of mortality and morbidity worldwide. There is a general consensus that early and prompt coronary intervention has significantly improved outcomes in the acute phase of AMI. However, there is still a great incidence of LVAR in STEMI patients who underwent successful PPCI. Patients enrolled in this study were admitted at the hospital less than 12 h after the onset of chest pain and without having previous history of ischemic heart disease, showing a TIMI (Thrombolysis in Myocardial Infarction) flow of 0 in the left anterior descendent coronary artery. The inclusion criteria were rigorous to guarantee consistent results related to the impact of revascularization on inflammatory cells measurements, and the release of cytokines and miRNAs. Almost 32% of patients with STEMI developed LVAR as soon as 6 months after PPCI in our cohort, which agree with other finding (15). In this study, we found that the classical cardiac markers, CK and TnT, were significantly higher in patients who developed LVAR, compared with those without LVAR, which is concordant with a large infarct size of the heart. However, multivariate statistical analysis using these classical markers indicated that they are weak predictors of LVAR after PPCI.

Compelling evidences highlighted the role of the inflammatory response to AMI and its critical role in determining the infarct size and subsequent LVAR (2). However, the use of anti-inflammatory drugs has not achieved any substantial benefits in the clinical trials (16). Here, we focused on the role of monocytes as central regulators of inflammatory process after revascularization (5). Our data revealed that the levels of classical and intermediate monocytes were significantly increased in STEMI patients who later developed LVAR after an PPCI. Previous studies demonstrated significant increase in monocyte subsets levels in non-revascularized STEMI patients (4, 9) and in non-STEMI patients (17, 18). They also suggested that the level of classical monocytes correlated with a reduced left ventricle ejection fraction (LVEF) and a larger infarct size (17, 18). Interestingly, according to our linear regression analysis, the increase in the level of intermediate monocytes was associated significantly with changes in the LVEDV, and their levels are well-clustered in non-LVAR and LVAR patients. Moreover, multivariate regression and ROC analysis indicated that changes in the level of these monocytes were associated with LVAR appearance and provided very accurate sensitivity and specificity. These data also showed that this monocyte subset, but not CK, neither TnT nor sex, nor age, could reliably predict the appearance of LVAR. At the same time, our data demonstrated that classical monocytes could not be considered as an independent predictor of LVAR. Recently, some studies used the level ratio between monocytes and lymphocytes as a prognostic marker for non-STEMI patients (19), or with eosinophils for STEMI patients (20), and with platelets (21) in AMI patients. However, none of these studies analyzed the correlation between monocyte subsets with LVAR. Actually, the importance of all those inflammatory cells populations and their roles after an ischemic event constitute an interesting research field, even if they are still under debate (22).

The inflammatory response is characterized by cytokines and chemokines secretion which can determine the final recovery of ischemic tissue and the occurrence of the LVAR (7). Very few studies analyzed the level of multiple cytokines in STEMI patients after PPCI. In our study, within 27 pro-inflammatory cytokines, we found a significant increase in the level of VEGF in patients

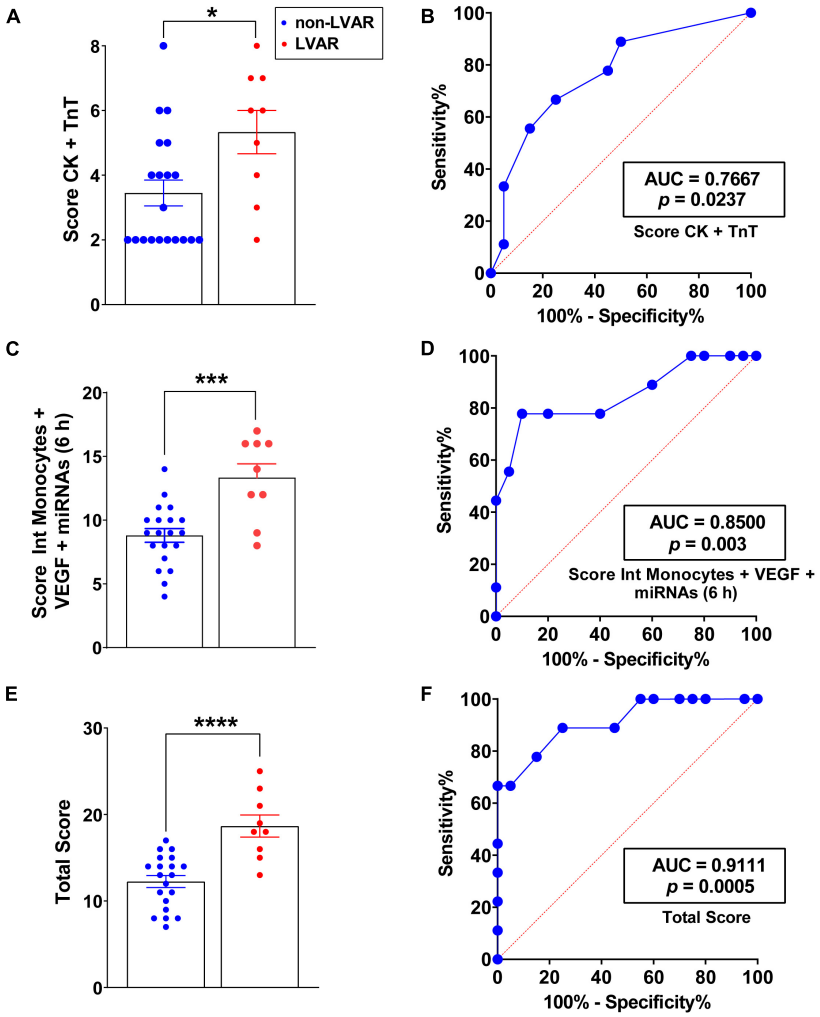

FIGURE 6 | Analysis of the level of the remodeling prediction score in patients with or without LVAR. **(A)** Bar graphs show the CK and TnT, which is the sum of the score, from 1 to 4, assigned to each level of CK and TnT at 0 h, in non-LVAR (blue dots, $n = 20$) and LVAR (red dots, $n = 9$) patients (see section Materials and Methods" and **Supplementary Table 3** for more information about formula of the scores and the ranges). **(B)** ROC analysis with the AUC (values given on the graphs), indicating sensitivity and specificity of the CK and TnT score **(A)** at 0 h to predict LVAR. **(C)** Bar graphs show intermediate monocytes, VEGF and miRNAs score at 6 h in non-LVAR and LVAR patients. **(D)** ROC analysis of intermediate monocytes, VEGF and miRNAs score at 6 h **(C)** to predict LVAR. **(E)** Bar graph shows the total score (summary of assigned score to CK and TnT at 0 h, and intermediate monocytes, VEGF and miRNAs at 6 h post-PPCI) in non-LVAR and LVAR patients. **(F)** ROC analysis with the AUC (values given on the graphs), indicating sensitivity and specificity of the total score to predict LVAR. Values are presented as the means ± SEM. Mann-Whitney (non-parametric) and *t*-test (parametric) and ROC analysis were performed. (*), (***), and (****) indicate significance at $p < 0.05$, $p < 0.001$ and $p < 0.0001$, respectively. AUC, area under the curve; CK, creatine kinase; LVAR, left ventricle adverse remodeling; miRNAs, microRNAs; PPCI, primary percutaneous coronary intervention; ROC, Receiver-operating characteristics; TnT, troponin-T; VEGF, vascular endothelial growth factor.

who developed LVAR 6 h after PPCI. Consistently, other studies highlighted the role of VEGF isoforms to predict MACEs in the clinical practice (23); in addition, the plasma increased level of VEGF was associated with microvascular obstructions and poor prognosis in STEMI patients as described recently in the PREGICA study (ClinicalTrials.gov identifier: NCT01113268) (24). Therefore, a substantial increase in the level of VEGF after PPCI might be detrimental for heart recovery from the ischemic events. In addition to inflammatory cells subsets and cytokines, we also studied the behavior of miRNAs in STEMI patients. Recently, we provided evidence indicating that PBMCs release miRNAs into the blood stream of patients with LVAR (12). In this study, we observed significant dysregulation of miRNAs levels in PBMCs owing to patients who later developed LVAR compared with those without LVAR. Based on miRNAs array and *in silico* analysis we found that three miRNAs, miR-16-5p, miR-21-5p, and miR-29a-3p, are predicted to target genes involved in the inflammatory signaling pathway. miR-21 is a well-known miRNA involved in cardiovascular diseases, as reviewed recently (25). Several reports described changes in the expression of miR-21 in cardiomyocytes, fibroblast, and endothelial cells during ischemic heart disease, but little is known

regarding its possible secretion by inflammatory cells after an AMI. Interestingly and in agreement with our finding, exosomal miR-21 is secreted from macrophages, influencing the migration and proliferation of smooth vascular cells in the atheroma plaque (26). On the other hand, miR-16-5p and miR-29a-3p seem to participate in inflammation process in atherosclerosis (27, 28). Although, the role of miR16-5p and the inflammatory process in heart has been barley addressed. Meanwhile, miR-29 family was associated with LVAR after myocardial infarction in the mouse model (29). Another study determined that changes in the expression of miR-29a-3p was associated with sudden death in patients with coronary heart disease, apparently originated from M1 polarized pro-inflammatory monocytes, correlating with the increase in the level of pro-inflammatory cytokine IL-6 (30). Recently, we demonstrated that the overexpression of miR-29a-3p prevents changes in the expression of apoptotic and fibrotic genes induced by ischemia and reperfusion in rats (31).

Interestingly, our data demonstrated that those three miRNAs and their predicted target cytokines are upregulated in PBMCs and serum, respectively, in patients with LVAR, compared with non-LVAR patients, at the same time point, 6 h after patients' intervention. Based on these data we cannot assure whether miRNAs are up or downstream cytokines production. However, we do not discard that under these conditions, inflammatory cells may increase miRNAs expression in a way to control their own exacerbated expression of cytokines. This intriguing bidirectional miRNA-cytokine regulation was already described in other inflammatory cells, such as primary helper T cells, which were suggested to increase miR-29 expression to correct their aberrant expression of IFNγ (32). In agreement with this analysis, independent studies also showed bidirectional regulation between cytokines and miRNAs (33, 34).

Since it is difficult to predict the outcome of a such complex and multifactorial disease as AMI, we have combined the analysis of intermediate monocytes, VEGF, and miRNAs using a score to stratify STEMI patient and to assess their value as powerful prognostic biomarkers for LVAR development. In fact, the combined analysis measuring the levels of intermediate monocytes, the release of VEGF, and miRNAs provided greater statistical significance compared to the analysis of only classical markers. We demonstrate that combined analysis of classical markers CK and TnT generated an AUC of 0.7667 ($p = 0.0237$), while the combined analysis of intermediate monocytes, VEGF, miR-16, miR-21, and miR-29a generated an AUC of 0.8500 ($p = 0.003$). Interestingly, the joint analysis of classical markers together with the new identified markers analyzed in this study achieved an AUC of 0.911 with a $p = 0.0005$. These findings ensure a great precision to predict LVAR in revascularized patients using this combined analysis. In accordance with our results, a recent study demonstrated that combined analysis of 8 miRNAs and NT-proBNP provided a potent diagnosis results for HF detection (35).

In conclusion, the analysis of combined parameters such as CK, TnT, intermediate monocytes, VEGF, and miRNAs levels, could be useful for an early identification of patients who will develop LVAR. Herein, we demonstrate that the increase in the level of intermediate monocytes, VEGF, and miRNAs in the bloodstream, at the moment of the ischemic event, can serve as more sensitive predictive markers, rather than simple blood indexes, since their combined analysis accurately predicted whose patients have high probability to develop an LVAR and HF in future. Therefore, prophylactic therapy in these patients could prevent HF. Further studies are eagerly needed to establish the therapeutic effect of targeting inflammatory cells subset, miRNAs, and cytokines in STEMI patients undergoing PPCI.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: ArrayExpress Browser, E-MTAB-11002.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Local Ethics Committee on Human Research at the University Hospital "Virgen del Rocio" of Seville (Approval no. 2013PI/096). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IG-O, RDT, EB, FG-M, DF, AG-R, LD-L, GB-E, AO-F, and TS: conceptualization, data acquisition, analysis, and interpretation. IG-O, RDT, EB, FG-M, GB-E, and TS: investigation and methodology. RDT, AO-F, and TS: writing original draft. AG-R, FG-M, LD-L, GB-E, and AO-F: revising the draft and clinical concepts. AO-F and TS: funding acquisition project administration. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.777717/full#supplementary-material

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