Expression profiling of circular RNAs and their potential role in early-stage diabetic cardiomyopathy

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Abstract. Diabetic cardiomyopathy (DCM) is a severe cardiovascular complication of diabetes mellitus (DM). Detecting DCM during the early stages of the disease remains a challenge, as the molecular mechanisms underlying early-stage DCM are not clearly understood. Circular RNA (circRNA), a type of non-coding RNA, has been confirmed to be associated with numerous diseases. However, it is still unclear how circRNAs are involved in early-stage DCM. In the present study, heart tissues harvested from BKS-db/db knock-out mice were identified through high-throughput RNA sequencing technology. A total of 58 significantly differentially expressed circRNAs were identified in the db/db sample. Among these, six upregulated circRNAs and seven downregulated circRNAs were detected by reverse transcription-quantitative PCR and analyzed using Gene Ontology and Kyoto Encyclopedia of Genes and Genomes. Furthermore, based on the predicted binding site with microRNAs (miRNAs) involved in DCM, five circRNAs (mmu_circ_0000652, mmu_circ_0000547, mmu_circ_0001058, mmu_circ_0000680 and novel_circ_0004285) were shown to serve as competing endogenous (ce)RNAs. The corresponding miRNAs and mRNAs of the ceRNAs were also verified, and two promising circRNA-miRNA-mRNA regulatory networks were determined. Finally, internal ribosome entry site prediction combined with open reading frame prediction indicated that it was highly possible that mmu_circ_0001160 encoded a protein. In the present study, a comprehensive analysis of the circRNA expression profile during the early phase of DCM was performed, and two promising circRNA-miRNA-mRNA regulatory networks were identified. These results lay the foundation for unravelling the underlying pathogenesis of DCM, and highlight potential biomarkers and therapeutic targets for the treatment of DCM at an early stage.

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Introduction

Diabetes mellitus (DM) is a chronic disease worldwide, and is a major risk factor for cardiovascular disease. The incidence of diabetes is increasing; according to the International Diabetes Federation Diabetes Atlas (8th edition, 2017), >425 million individuals are living with diabetes, 50% of whom are undiagnosed (1). Among the complications of DM, cardiovascular disease has been confirmed to be the leading cause of death (2). DM can result in both structural and functional changes in the myocardium, thus leading to diabetic cardiomyopathy (DCM) (3).

In the past four decades, despite extensive molecular and cellular based research, which have unraveled the mechanisms underlying DM and DCM, the pathogenesis of DCM remains controversial. DCM is a lifelong and progressive disease, the progress of which can be divided into three stages: Early, advanced and late stage (4). During the early stage, the majority of the patients with DCM typically exhibit changes in the structure of the heart, without any noticeable symptoms, such as diastolic and contractile dysfunction. Therefore, these patients remain asymptomatic during the early stages. As the disease advances, it progresses to irreversible heart failure, for which there is no proven effective treatment (5). Hence, unveiling the underlying pathogenesis and identifying early and specific diagnostic indicators of DCM is paramount for preventing heart failure. Recently, numerous imaging techniques and biochemical markers have been used to address this issue. In type 2 diabetes mellitus (T2DM) mice, 2D-Echo-Doppler was used to detect early changes in diastolic function and myocardial hypertrophy (6). Shaver *et al* (7) identified a panel of biomarkers to detect modifications in cardiac structure and function; although none of these were conclusive, and only served as a compensatory index. Thus, there is an urgent need for accurate early detection and

effective therapeutics for the diagnosis and treatment of DCM. Therefore, an improved understanding of the pathogenesis of DCM is required, particularly for early-stage DCM.

Circular RNAs (circRNAs) are a unique type of endogenous non-coding RNAs, which were initially considered to be abnormal splicing byproducts with limited functional potential (8). However, in recent years, increasing evidence has demonstrated that circRNAs play important roles in a range of diverse physiological and pathological processes (9). Although the functions of the majority of circRNAs remains to be fully elucidated, the established functions include acting as a sponge to sequester certain microRNAs (miRNAs), modulating transcription and interfering with splicing, and even translation into polypeptides (10-12). Characterized by their stability and tissue-specificity, circRNAs may not only serve as an ideal biomarker, but may also assist in elucidating the underlying mechanisms in various disease, including DM. For example, Zhao *et al* (13) detected circRNAs in the peripheral blood of patients with T2DM and found that hsa_circ_0054633 may be used as a diagnostic biomarker of pre-diabetes. Fang *et al* (14) showed that circANKRD36 was associated with inflammation in patients with T2DM. Nevertheless, relatively less is known on the expression profile and potential role of circRNAs in early-stage DCM. Therefore, in the present study, secondary sequencing technology was used to examine the expression of circRNAs in early-stage DCM mice. Key circRNAs were screened for further analysis in the pathogenesis of early-stage DCM, as well as their potential as biomarkers, for early diagnosis and treatment of DCM.

Materials and methods

Animals. A total of 5 male C57BL/BKS-Leprdb (db/db) mice (8 weeks old; weight, 42-47 g) were obtained from Gempharmatech Co., Ltd. A total of 5 monogenic LepR mutated mice (db-/db-; 8 weeks old; weight, 20-22 g) from the same company were used as control mice. Mice were maintained under controlled conditions at 20-22˚C with 12-h light/dark cycles and 40-60% humidity, with free access to food and water. Body weight was measured and the fasting blood glucose levels were detected using the glucose oxidase-peroxidase method (15). All experiments were approved by the Institutional Animal Care and Use Committee of Fudan University (China).

Myocardium sample collection and RNAseq. Mice were anesthetized by intraperitoneal injection of 1% pentobarbital sodium solution at 55 mg/kg, and then sacrificed by cervical dislocation and the myocardium samples were harvested for RNAseq. A total of 5 μg RNA in each db/db and control sample was extracted for preparation of RNA samples. Epicentre Ribo-Zero™ rRNA Removal kit (Epicentre; Illumina, Inc.) was used to remove ribosomal RNA. To obtain enriched pure circRNA, linear RNA was digested with 3 U of RNase R (Epicentre; Illumina, Inc.) per μg of RNA. According to the manufacturer's protocol, the sequencing libraries were constructed using a NEBNext® Ultra™ Directional RNA Library Prep kit for Illumina® (New England Biolabs, Inc.). Briefly, circRNAs were interrupted randomly by adding a fragment reagent. Subsequently, the first strand cDNA was synthesized using random hexamer primers and M-MuLV reverse transcriptase (RNaseH), and subsequently the second strand of cDNA was synthesized. After the repair of the end of the DNA strand and adenylation of 3'ends of DNA fragments, AMPure XP beads were used to select cDNA fragments with the appropriate length. Subsequently, 3 μl USER Enzyme (New England Biolabs, Inc.) was used to degrade the second strand containing cDNA. Finally, PCR was performed to amplify the circRNA library using the same materials and methods as below, and products were purified using the AMPure XP system.

Reverse transcription-quantitative (RT-q)PCR. Total RNAs in heart tissue were extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Using a PrimeScript RT-PCR kit (cat. no. RR014; Takara Bio, Inc.) extracted RNA was reverse transcribed to cDNA following the 20 μl system protocol, the reaction condition were as follows: 37˚C for 5 min, 85˚C for 15 sec and 4˚C for preservation. The expression levels of circRNAs and related mRNAs, brain natriuretic peptide (BNP), atrial natriuretic peptide (ANP) and interleukin (IL)-6 were detected using a SYBR Green PCR kit (Qiagen, Inc.) in a 7500 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were: Initial denaturation at 95˚C for 2 min; followed by 40 cycles of 95˚C for 5 sec and annealing at 60˚C for 34 sec; then 95˚C for 15 sec; 60˚C for 1 min and 95˚C for 15 sec. The miRNA levels were detected using miRNA First Strand cDNA Synthesis kit (cat. no. B532451; Sangon Biotech Co., Ltd.), according to manufacturer's protocols. U6 and β-actin were used as internal controls. The $2^{-\Delta\Delta Cq}$ method (16) was used for quantitative analysis of gene expression. The primer sequences are presented in Table I.

TUNEL assay. Cardiomyocyte apoptosis was detected using terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) technique. Mice were sacrificed by cervical dislocation following anesthesia, then heart tissue was isolated and immediately fixed in 4% formalin solution for 24 h at 4˚C and then embedded in paraffin. Next, the specimens were sectioned into 4-μm thick slides and stained with a DeadEnd™ Fluorometric TUNEL system kit at 37˚C for 1 h (cat. no. G3250; Promega Corporation), following the manufacturer's instructions.

Prediction of circRNA-miRNA interactions and identification of key circRNAs. The bioinformatics platform miRanda version 3.3a (microrna.org/) was used to predict putative miRNAs that exhibited a potential association with one of the 13 differential circRNAs identified by RT-qPCR. The major parameters were as follows: i) -sc, 140; ii) -en, -10; iii) -scale, 4; iv) -strict. The circRNA-miRNA interaction network was constructed using Cytoscape version 3.01 (17). Subsequently, Gene Ontology (GO) enrichment analysis (18-20) was used to identify the processes with the highest degree of enrichment, in which the mRNA targets of the miRNAs were involved. Similarly, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed to identify the significant pathways associated with these mRNAs (genome.jp/kegg/) (21). To

Table I. Sequences of the primers used in the present study.

Gene	Primer sequence (5'→3')
BNP	F: GAGGTCACTCCTATCCTCTGG
	R: GCCATTTCCTCCGACTTTTCTC
ANP	F: GTGCGGTGTCCAACACAGAT
	R: TCCAATCCTGTCAATCCTACCC
IL-6	F: CCAAGAGGTGAGTGCTTCCC
	R: CTGTTGTTCAGACTCTCTCCCT
mmu_circ_0001697	F: AGATGGCTTCTGAGCTGCTTT
	R: TAGCTTTCCGCTGGTGGTTG
mmu_circ_0001160	F: TGGTGTAATTGCCTCTGCCATC
	R: CTGCCAATCCGGCCAATATG
novel_circ_0008273	F: CCAGAGATCTGGGAGGAGTAGA
	R: CCTCAGGAACTGAAGGTAAAGT
novel_circ_0009344	F: TGATGCTGGCTTTGTTCCCAA
	R: TTCAAACCCCGACTGGAGCTA
mmu_circ_0001625	F: CATCCTGGCATTGGTTTTGCC
	R: GGGCTCATGATTTTCGTGACTT
mmu_circ_0000431	F: ACTCTGAACGGCGAGATCCT
	R:TGTCATCTCTAACCATCACCAACA
mmu_circ_0000652	F: GCAGGAGACAAGGAGCTACA
	R: AGTTGCTGGTGTAAGAGGCA
mmu_circ_0000058	F: GGTAGACCTGACTGATGCCA
	R: TAGTAAAGTGTTCGCCCTCG
mmu_circ_0001058	F: AGGAGCGTCTGAATGAGGACT
	R: GCGATACTGTGAACACCAGGG
mmu_circ_0000680	F: ATTCAAACTGTGCCTTCCCA
	R: TTCCAGGGAAACAAAGTGACA
novel_circ_0000824	F: GAAGTGCCTCTTCAGGGGTG
	R: AGTCCTTCTCTCTGTGTTGCTC
mmu_circ_0000547	F: GGCGACGGCAGATGAAAACA
	R: GTCAGACAGTGGTCGTGGC
novel_circ_0004285	F: GGTTGAAGAATGGAGGAGGGT
	R: GCAGATACTCGTGAAGGAAGCA
IGF-1	F: AAATCAGCAGCCTTCCAACTC
	R: GCACTTCCTCTACTTGTGTTCTT
FOXO3A	F: GGGGAACCTGTCCTATGCC
	R: TCATTCTGAACGCGCATGAAG
CAB39	F: TGCTGTTGGACAGACACAACT
	R: GGAGGTTCATCATTAGCTTGAGG
BCL2	F: GCTACCGTCGTGACTTCGC
	R: CCCCACCGAACTCAAAGAAGG
SPRY1	F: GGTCATAGGTCAGATCGGGTC
	R: GTCCCGTATTCCACCATGCT
miR-195	F: TAGCAGCACAGAAATATTGGC
	R: Universal PCR Primer R
miR-21	F: TAGCTTATCAGACTGATGTTGA
	R: Universal PCR Primer R
miR-320	F: AAAAGCTGGGTTGAGAGGGCGA
	R: Universal PCR Primer R
miR-451	F: AAACCGTTACCATTACTGAGTT
	R: Universal PCR Primer R
miR-30d	F: TGTAAACATCCCCGACTGGAAG
	R: Universal PCR Primer R

F, forward; R, reverse; BNP, brain natriuretic peptide; ANP, atrial natriuretic peptide; IL-, interleukin; circRNA, circular RNA; IGF-1, insulin-like growth factor 1; FOXO3A, forkhead box protein O3; CAB39, calcium-binding protein 39; SPRY1, sprouty homolog 1; miR, microRNA.

identify the key circRNAs, known DCM-related miRNAs were searched by reviewing the relevant literature in pubmed (pubmed.ncbi.nlm.nih.gov/), and the common miRNAs between the known DCM-related miRNAs and the predicted miRNAs were extrapolated to identify key miRNAs. The circRNAs that were associated with these key miRNAs were used to construct an integral circRNA-miRNA-mRNA regulatory network using Cytoscape version 3.01. Finally, these key miRNAs and their target mRNAs were verified by RT-qPCR, following the aforementioned protocol.

Internal ribosome entry site (IRES) and open reading frame (ORF) prediction of circRNAs. To extensively explore the protein-coding function of the 13 differentially expressed circRNAs, IRES finder (github.com/xiaofengsong/IRESfinder) and circAtlas 2.0 (circatlas.biols.ac.cn) were used to predict the presence of IRES and ORF elements, which are essential elements of circRNAs with protein coding capacity. The IRES score and ORF numbers were plotted using R software version 3.5 (r-project.org/).

Statistical analysis. Data are presented as the mean ± standard deviation of three independent experiments each performed in triplicate. Statistical significance was calculated using a Student's t-test in GraphPad Prism version 5 (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Early changes in the diabetic myocardium. A series of biochemical biomarkers have been determined to crudely indicate functional changes in early-stage DCM (22,23). BNP, ANP and IL-6 expression levels were assessed to detect early modifications in db/db myocardial tissue. The body weight of the five db/db mice were measured and the fasting blood-glucose levels were measured three times on different days. The average weight of db/db mice was 44.34 g, whereas the control mice were 22.8 g (Fig. 1A). The fasting blood-glucose level of db/db mice ranged from 22.2-26.4 mmol/l with a mean of 23.8 mmol/l (Fig. 1B). Furthermore, the qPCR results exhibited a significant increase in the expression levels of BNP, ANP and IL-6 ($P<0.05$). In addition, TUNEL staining also exhibited an increasing trend of cardiomyocyte apoptosis in the db/db model (Fig. 1F), suggesting successful establishment of a model of early-stage DCM.

circRNA expression in the myocardial tissue of db/db mice. To detect circRNA expression in the myocardial tissues of db/db mice, RNA-seq analysis was performed. Then, a volcano plot and heatmap were constructed using R software version 3.5 (r-project.org/). The length of the detected circRNAs was primarily in the 100-500 bp range (Fig. 2A), and a majority of the reads were covered in the exon of the genome (Fig. 2B). Overall, 58 circRNAs were determined to be significantly differentially expressed ($P<0.05$; Table SI), including 29 upregulated circRNAs and 29 downregulated circRNAs (Fig. 2C and D). Of these, three-quarters were newly identified circRNAs. In addition, all candidate circRNAs were found to be distributed among all the chromosomes (Fig. 3).

To validate the RNA-seq results, RT-qPCR was performed to analyze circRNA expression. According to the RT-qPCR results, six upregulated circRNAs (mmu_circ_0001697, mmu_circ_0001160, novel_circ_0008273, novel_circ_0009344, mmu_circ_0001625 and mmu_circ_0000431) and seven downregulated circRNAs (mmu_circ_0000652, mmu_circ_0000058, mmu_circ_0001058, mmu_circ_0000680, novel_circ_0000824, mmu_circ_0000547 and novel_circ_0004285) were verified to be differentially expressed in the tissue samples (Fig. 4).

Establishment of the circRNA-miRNA network. Increasing evidence has suggested that circRNAs may regulate the function of miRNAs by acting as competing endogenous RNAs (ceRNAs) (24). In an attempt to identify the functions of these differentially-expressed circRNAs in DCM, circRNA-miRNA co-expression networks were constructed based on the miRanda algorithm. The results showed there were 610 related miRNAs that exhibited close binding competency to these circRNAs (Fig. 5), the detailed data are listed in Table SII. Total scores of the targeting relationship for all binding sites were predicted and displayed on a color scale, where higher values indicated an increased likelihood of targeting. To further probe the function of these target genes related to these differentially-expressed circRNAs, GO enrichment analysis was performed. The results showed that these target genes participated in various biological processes, such as 'metabolic process', 'binding' and 'negative regulation of cellular process' (Fig. S1). KEGG analysis showed these target genes may have effects on several vital signaling pathways, such as 'glycerophospholipid metabolism', 'glycolysis/gluconeogenesis', 'ether lipid metabolism' and 'fructose and mannose metabolism' (Fig. S2). The results suggested that the detected circRNAs may be related to glucose and lipid metabolism, which is consistent with the fasting blood-glucose result. However, whether these circRNAs function in DCM and the underlying mechanisms need to be further explored.

mmu_circ_0000652 and mmu_circ_0001058 may play an important role in early-stage DCM. To construct a potential ceRNA network in early-stage DCM, the functions of the predicted miRNAs were explored. Based on bioinformatics analysis and literature retrieval, five miRNAs (miR-30d, miR-320, miR-451, miR-195 and miR-21) were determined to be associated with DCM (Table II). Additionally, their possible regulatory mechanisms has been confirmed in previous studies (25-29). The predicted binding sites between these key circRNAs and DCM-related miRNAs are exhibited in Fig. 6A. To further investigate the interactions between these RNAs, the five DCM-related miRNAs and the target mRNAs were also verified by RT-Qpcr (Fig. 6B). The results showed that two regulatory networks, mmu_circ_0000652/miR-195/BCL2 and mmu_circ_0001058/miR-21/sprouty homolog 1 (SPRY1), were representative of a classical ceRNA regulatory mechanism. A schematic plot of the two potential ceRNA networks is presented in Fig. 7. As BCL2 serves as a key regulatory molecule in the process of apoptosis and SPRY1 is associated with interstitial fibrosis (30,31), the results suggested that mmu_circ_0000652 and mmu_circ_0001058 may serve an important role in early-stage DCM.

Table II. The five potential ceRNA regulatory networks and functions in DCM.

circRNA	miRNA	mRNA	Function	Refs.
mmu_circ_0000680	miR-30d	FOXO3A	Cardiomyocyte pyroptosis	(26)
mmu_circ_0000547	miR-320	IGF-1	Impaired angiogenesis	(28)
novel_circ_0004285	miR-451	CAB39	Cardiomyocyte lipotoxicity	(25)
mmu_circ_0000652	miR-195	BCL2	Cardiomyocyte apoptosis	(27)
mmu_circ_0001058	miR-21	SPRY1	Interstitial fibrosis	(29)

miRNA/miR, microRNA; circRNA, circular RNA; ceRNAS, competing endogenous RNAs; IGF-1, insulin-like growth factor 1; FOXO3A, forkhead box protein O3; CAB39, calcium-binding protein 39; SPRY1, sprouty homolog 1.

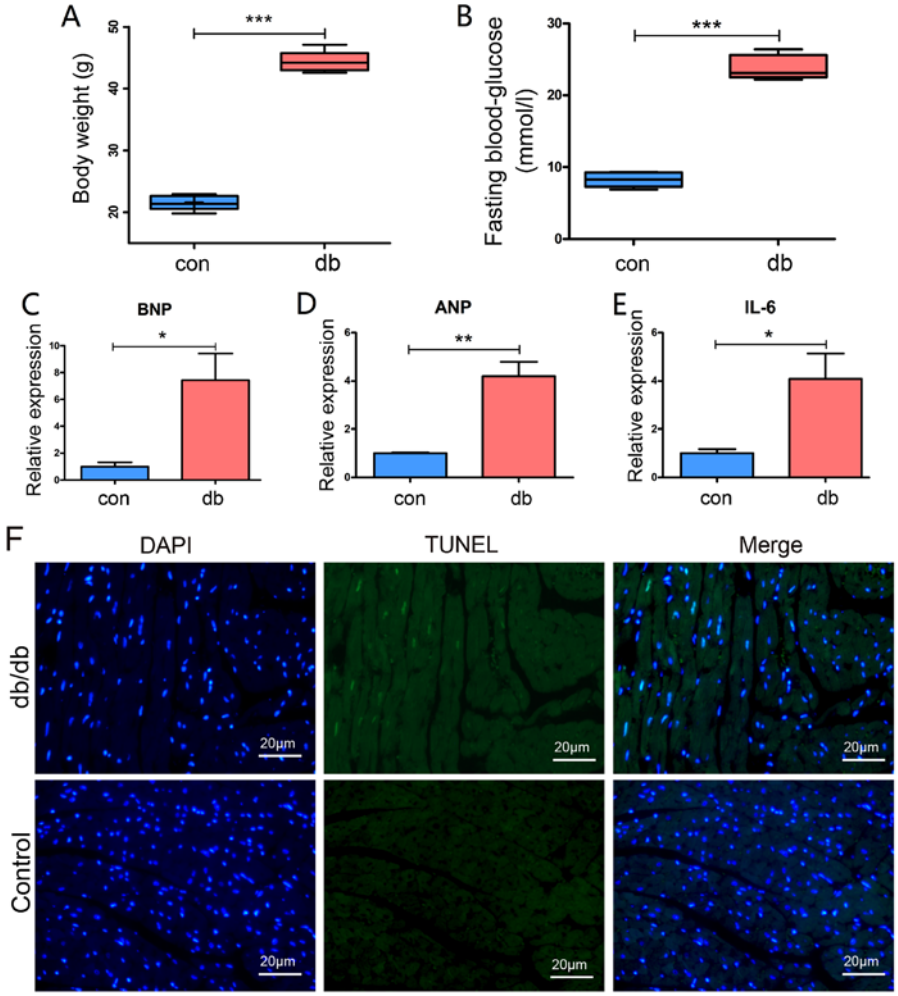

Figure 1. Biochemical analysis of the myocardial tissues of db/db mice. (A) Body weights of the five db/db and control mice. (B) Fasting blood-glucose levels of the db/db and control mice. Relative mRNA expression levels of (C) BNP, (D) ANP and (E) IL-6. (F) TUNEL staining of apoptotic cardiomyocytes. Blue stain shows intact nucleus and the green fluorescence indicates TUNEL-positive nuclei (scale bar, 20 μm). n=3 per group. *P<0.05, **P<0.01, ***P<0.001 vs. control. BNP, brain natriuretic peptide; ANP, atrial natriuretic peptide; IL-6, interleukin-6.

Mmu_circ_0001160 exhibits high protein-coding potential. Recent studies have reported that a fraction of circRNAs exhibit protein-coding potential based on the presence of IRES and ORF elements (32-34). IRES and ORF are both important regulatory elements for circRNA translation without the need for a 5' cap structure. To extensively explore the function of these circRNAs, IRES-finder software and circAtlas 2.0 were used to predict whether a circRNA sequence possessed potential IRES and ORF elements. Among the differentially expressed circRNAs, there were 9 circRNAs that had a potential IRES element. The closer the score value was to 1, the higher the credibility of the IRES in the circRNA was (Fig. 8). Based on the results, mmu_circ_0001160 had a relatively high IRES and ORF score, suggesting that this circRNA may function in a novel way by producing a polypeptide, the potential regulatory mechanisms of which are worthy of future studies.

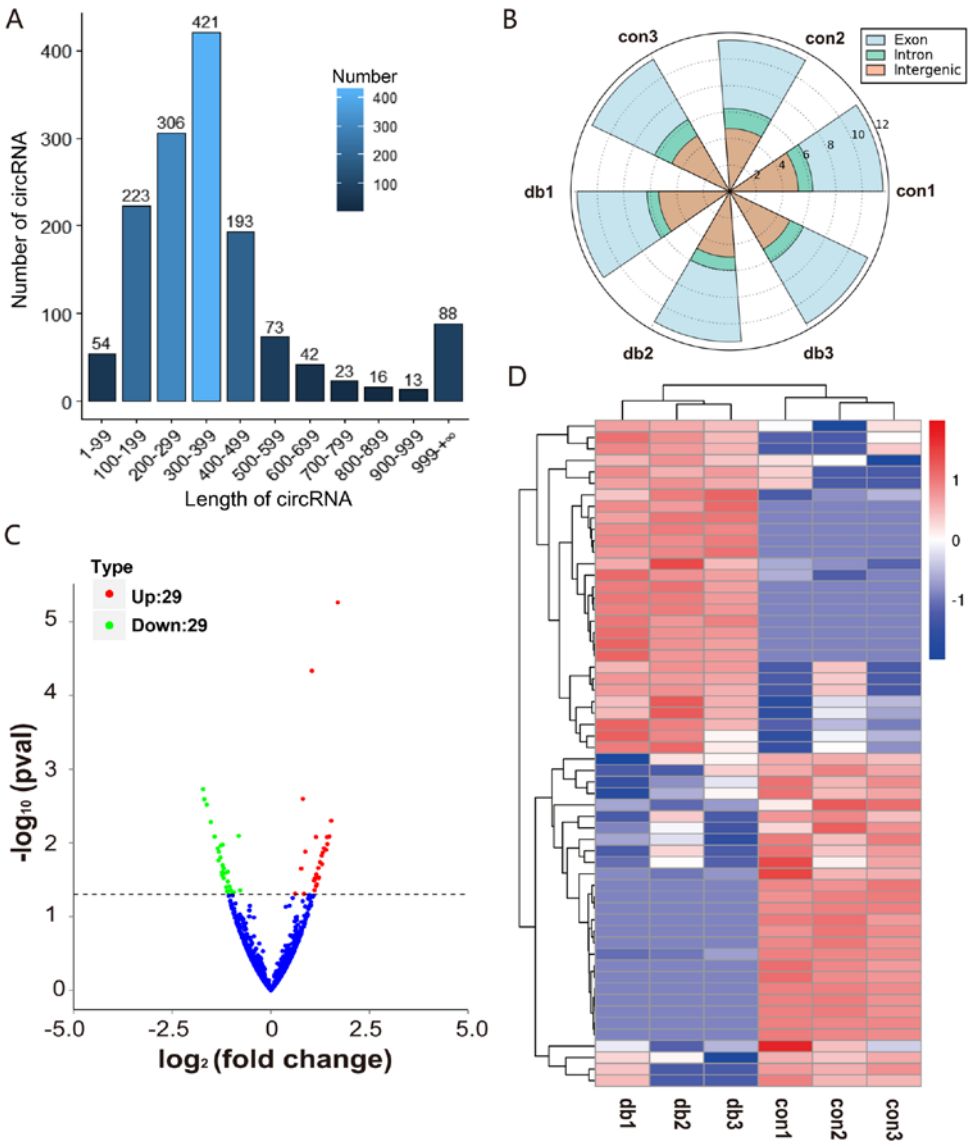

Figure 2. Overview of the detected circRNAs. (A) Length distribution of the detected circRNAs. (B) Polar coordinate sector for circRNAs arising from different genomic locus (exonic, intronic and intergenic). (C) Volcano plots for differentially expressed circRNAs in myocardial tissues from control and db/db groups. (D) Hierarchical cluster analysis (heatmap) for differentially expressed circRNAs between controls and db/db groups. circRNA, circular RNA.

Discussion

circRNAs can regulate the expression of genes at the transcriptional or post-transcriptional levels (24,35). In recent years, expression of circRNAs have been shown to be dysregulated in several types of diseases, including DM (1,11,36-39). However, their function and the underlying mechanisms in DCM remains largely unknown. In the present study, the circRNA expression profile was analyzed in a mouse model of early-stage DCM to improve our understanding of the pathogenesis of early-stage DCM and to find potential circRNA biomarkers. To the best of our knowledge, the present study was the first to explore the significance of circRNAs in early-stage DCM. Two promising circRNAs and their regulatory networks were identified, which may serve an important role in early-stage DCM.

An increasing number of studies have suggested that circRNAs serve a significant role in DM (13,14,36,40-42). For example, Zhao *et al* (13) found that hsa_circ_0054633 may serve as a diagnostic biomarker for pre-diabetes. However, relatively less is known about circRNAs in early-stage DCM. In the present study, 58 circRNAs were shown to be differentially expressed in a mouse model of early-stage DCM. RT-qPCR analysis results were consistent with the results of RNA-seq analysis, confirming the reliability of the RNA-seq data. KEGG analyses indicated that the related mRNAs were primarily involved in glucose metabolism and lipid metabolism pathways, both of which serve a significant role in triggering further alterations of the myocardial tissue. For example, lipid metabolism disorders may contribute to cardiomyocyte lipotoxicity, which is an initial deleterious response in the DM heart through the formation of fatty acid metabolites, as well as the release of mitochondrial and cytosolic ROS (43,44). Although the alterations in the KEGG pathway may be caused by high levels of blood-glucose in our model, the results still preliminarily suggested that these circRNAs may have an association with early stage DCM and the function of these circRNAs needs further investigation.

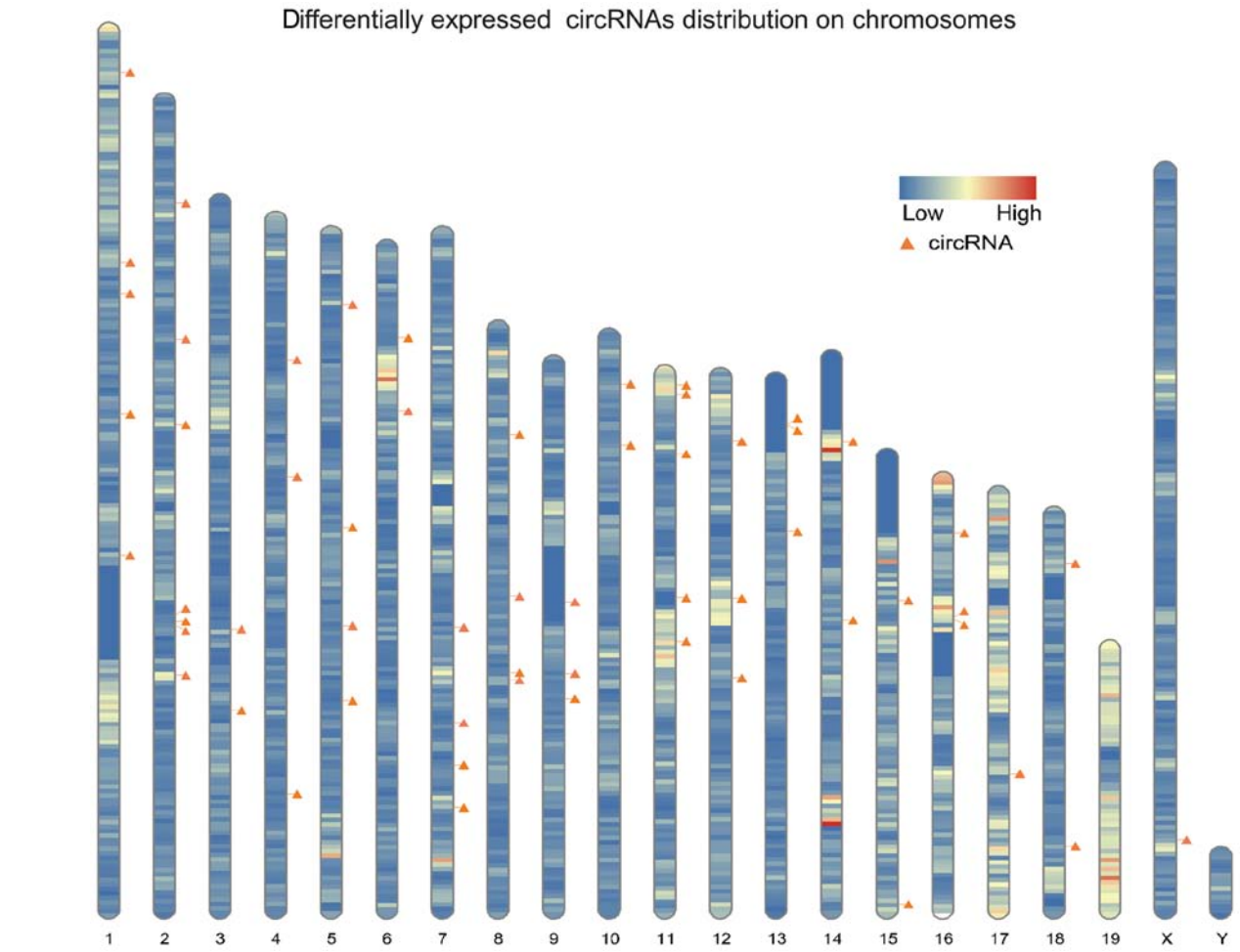

Figure 3. Differentially expressed circRNAs on mouse chromosomes. The yellow triangle represents the upregulated and downregulated circRNAs between the db/db and normal samples. circRNA, circular RNA.

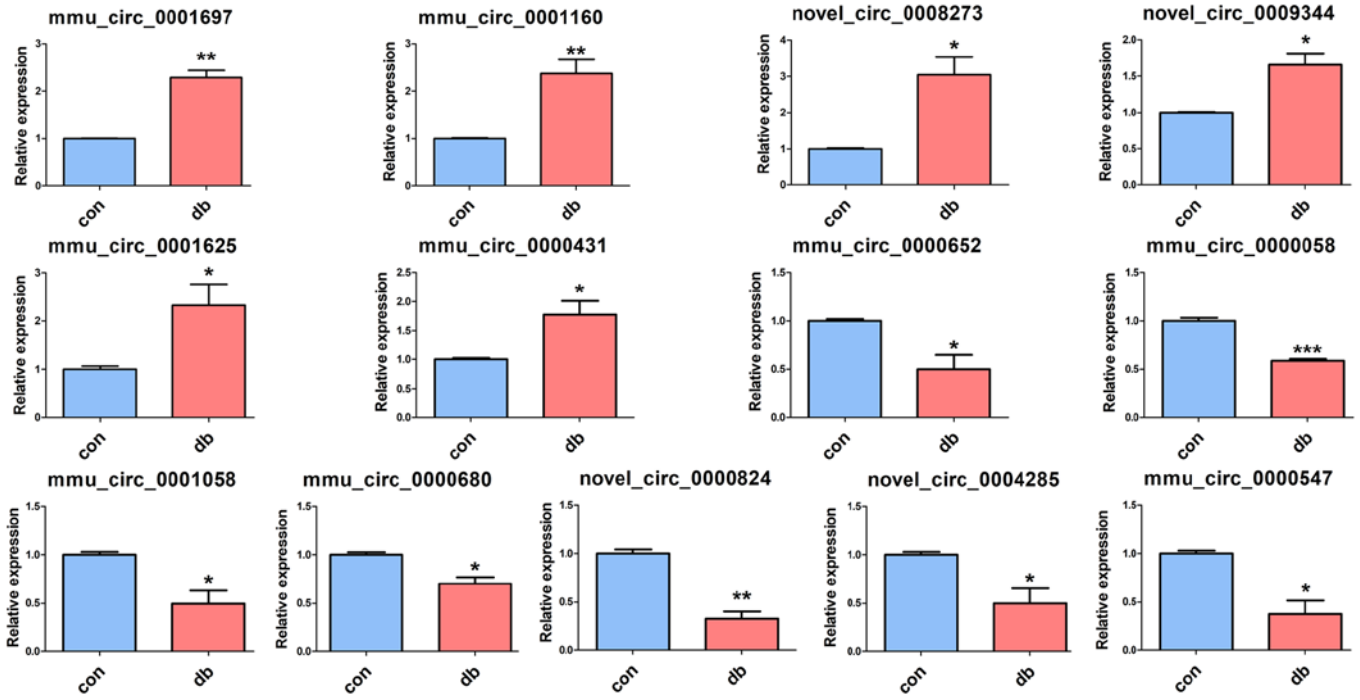

Figure 4. Reverse transcription-quantitative PCR validation of differentially-expressed circRNAs. Relative expression levels of the six upregulated and seven downregulated circRNAs between the control group and db/db group. Data are presented as the mean ± standard deviation. n=3 per group. *P<0.05, **P<0.01, ***P<0.001 vs. control. circRNA, circular RNA.

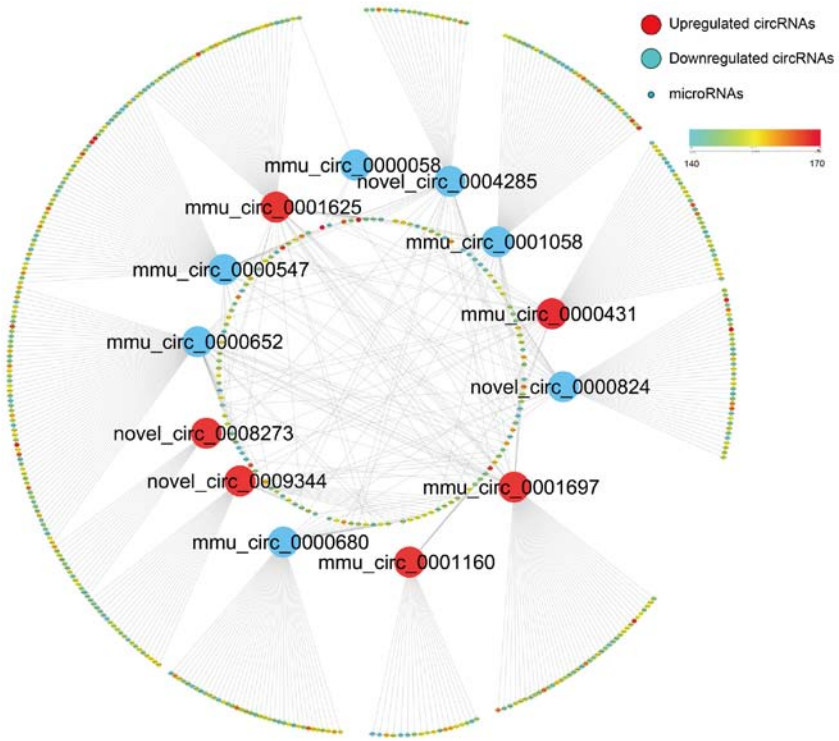

Figure 5. Network map of circRNA-miRNA interactions. The six significantly upregulated circRNAs are represented by red nodes and the seven downregulated circRNAs are represented by blue nodes. Target miRNAs are represented by green and red nodes with the binding score ranging from 140-170. miRNA, microRNA; circRNA, circular RNA.

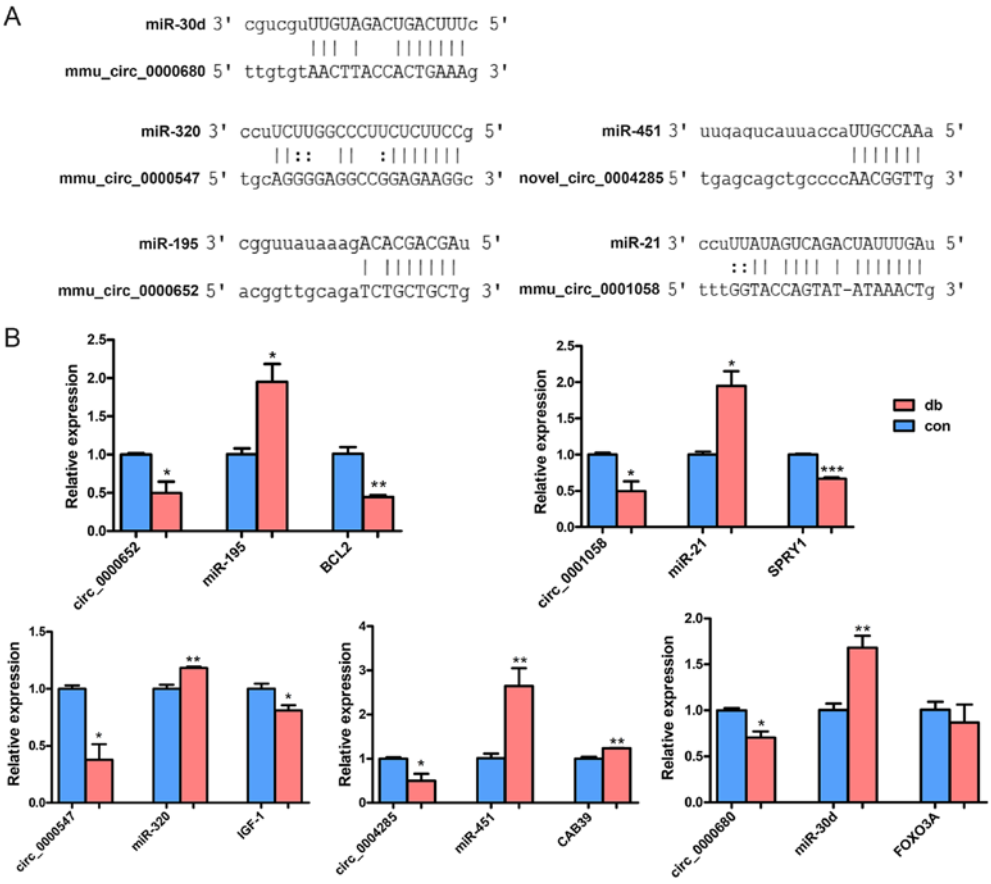

Figure 6. Key circRNA identification. (A) Predicted binding sites of key circRNAs and miRNAs. The dots between the bases represent the wobble base pairing between the purines and pyrimidines. (B) Reverse transcription-quantitative PCR validation of DCM-related miRNAs and the target mRNAs. Data are presented as the mean ± standard deviation. n=3 per group. *P<0.05, **P<0.01, ***P<0.001 vs. control. circRNA, circular RNA; miRNA, microRNA; DCM, diabetic cardiomyopathy; SPRY1, sprouty homolog 1; CAB39, calcium-binding protein 39; FOXO3A, forkhead box protein O3.

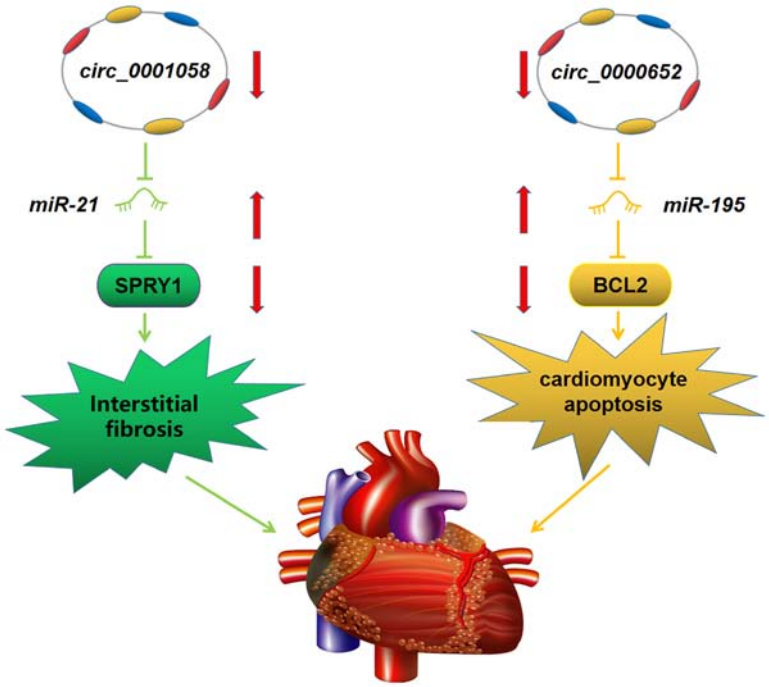

Figure 7. Schematic diagram of the two possible circRNA-miRNA-mRNA regulatory networks in early-stage diabetic cardiomyopathy. miRNA, microRNA; circRNA, circular RNA; SPRY1, sprouty homolog 1.

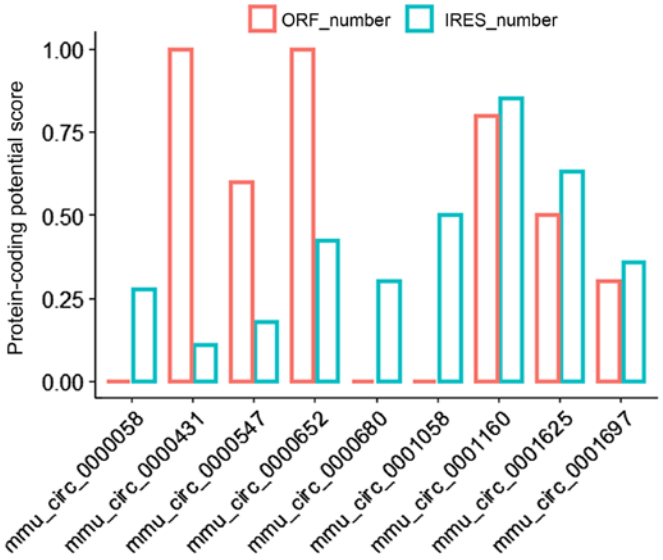

Figure 8. Prediction of protein-coding potential of the selected circRNAs. circRNA, circular RNA; IRES, internal ribosome entry site; ORF, open reading frame.

Numerous studies have shown that circRNAs may negatively regulate the inhibitory effects of miRNAs on their target mRNAs by directly binding to miRNAs, thereby acting as miRNA sponges (24,35,40,45-47). In the present study, mmu_circ_0000652 and mmu_circ_0001058 were found to potentially interact with miR-195 and miR-21, both of which have been shown to serve roles in the metabolism of DCM. miR-195 is associated with a number of pathological myocardial conditions, including hypertrophy, fibrosis, apoptosis, arrhythmia and heart failure (27). Zheng *et al* (48) showed that silencing miR-195 attenuated myocardial hypertrophy, promoted coronary circulation and reduced myocardial dysfunction in streptozotocin-induced diabetic mice. In addition, further experiments showed that these protective effects were associated with decreased apoptosis, generated by the induction of the direct targets of miR-195, BCL2 and sirtuin 1. In the present study, upregulation of miR-195 and downregulation of BCL2 were observed, suggesting that downregulation of mmu_circ_0000652 may promote apoptosis by indirectly inhibiting BCL2 in the db/db model. Apoptosis is a relatively early event in DCM, when apoptotic cardiomyocytes are lost, collagen is deposited to replace dead cardiomyocytes, which contributes to cardiac fibrosis and dysfunction (49). The function of miR-21 differs from miR-195. miR-21 regulates the ERK-MAP kinase signaling pathway by affecting SPRY1, ultimately resulting in fibrosis, hypertrophy and cardiac dysfunction (29). Similarly, mmu_circ_0001058 serves an important role in the progress of DCM by interfering with miR-21. Further studies on mmu_circ_0000652 and mmu_circ_0001058 may highlight the roles of these circRNAs in the underlying pathogenesis of early-stage DCM, thus potentially improving early diagnosis and treatment of early-stage DCM and minimizing its complications.

In addition to acting as an miRNA sponge, studies have reported that circRNAs exhibit protein/peptide coding capacity. To exhibit protein-coding potential, an IRES and ORF are required. The present results showed that mmu_circ_0001160 possessed a relatively high coding possibility amongst the identified circRNAs. Zn^{2+} transporter 7 (ZNT7), the linear parental gene of mmu_circ_0001160, has been shown to be associated with DM by interfering with insulin secretion (50,51). Furthermore, Tuncay *et al* (52) demonstrated that the novel role of ZNT7 in hyperglycemic cardiomyocytes by affecting sarco(endo)plasmic reticulum-mitochondria coupling. Based on the hypothesis that circRNAs may affect its linearly expressed

gene, it was hypothesized that mmu_circ_0001160 may interfere with early-stage DCM by producing a ZNT7-related protein. However, to validate whether this circRNA could encode a protein, in-depth studies are required. As the function of circRNA still remains to be fully elucidated, further functional experiments on mmu_circ_0001160 are required to extensively explore the role of circRNAs in various diseases.

There are several limitations of the present study. First, since the metabolic disturbances could cause a series of cardiac structural and functional alterations in the early stage of DCM (53), some additional structure and function parameters, such as echocardiographic examination and extra cardiac function biomarkers, are also important to confirm the successful establishment of our model (54), it will be applied in our future experiments. Second, although mice exhibit a high degree of homology with humans, the expression profile of its homologous sequences in humans have yet to be determined. Therefore, whether these results can be applied to humans requires further confirmation. In addition, due to the low validation rate of circRNA RNA-seq data compared with the RT-qPCR experiments, it is not possible to exclude the possibility of other dysregulated circRNAs that participate in the development of DCM. Thus, additional studies on these issues are required. As circRNAs are stably expressed in the blood, identifying circulating circRNAs in patients with DCM may provide a promising avenue for research, and should thus be an integral part of future studies.

In conclusion, the present study provided a comprehensive analysis of the circRNA expression profile during early phase DCM, and identified two promising circRNA-miRNA-mRNA axes. Further functional studies are required to determine the underlying pathogenesis of early-stage DCM, and the potential role of these axes in diagnosis and treatment of DCM.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SD was a major contributor in writing the manuscript and performing the experiments. JL and YS designed the study and were in charge of reviewing the manuscript. SD, CT, XY and SC performed the experiments and analyzed the data. LL, MZ, AX, ZZ and BC interpreted the data and edited the important intellectual content of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were approved by the Institutional Animal Care and Use Committee of Fudan University (approval no. 20190221-124).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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