

Comprehensive Analysis of Differentially Expressed Profiles of circRNAs in Diabetic Cardiomyopathy

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Abstract

Objective: To assess the circular RNAs (circRNA) expression profile and explore their potential functions in diabetic cardiomyopathy (DCM).

Methods: Using an STZ induced DCM model, microarray analysis was adopted to assess the circRNAs profiles. Then 6 differentially expressed circRNAs were confirmed by quantitative Real-Time PCR. Furthermore, gene ontology (GO) analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and TargetScan were used to reveal the underlying function of the differentially expressed circRNAs. Cytoscape was used to visualize the interaction networks as well.

Results: We revealed a total of 171 dysregulated circRNAs, including 89 upregulated, and 82 downregulated circRNAs in DCM. And confirmed 5 circRNAs distinct expressions (rno_circRNA_000466, rno_circRNA_000964, rno_circRNA_003395, rno_circRNA_000173, rno_circRNA_013989) in DCM by qRT-PCR. GO, and KEGG pathway enrichment revealed the differentially expressed circRNAs might participate in the insulin signaling pathway, autophagy, HIF-1 signaling pathway, inflammatory mediator regulation of TRP channels. rno_circRNA_000466 function through competing endogenous RNA mechanism, and may involve in the TGF- β signaling pathway, regulation of glucose transmembrane transport, endocrine process and response to lipoprotein particle.

Conclusions: This study opens new avenues for a better understanding of the involvement of circRNA leading to DCM. And unveiled the specific role of rno_circRNA_000466 in the pathogenesis of DCM. These results may provide important information and direction for the future development of novel targets for the treatment of DCM.

Background

Diabetes Mellitus is a global disease with high morbidity and mortality, which is one of the most important diseases severely harmful to the health of mankind[1,2]. It can cause myocardial microvascular disease and metabolic disorders[3,4]. On this basis, pathological changes such as myocardial microvascular injury and myocardial interstitial fibrosis can occur, leading to cardiac dysfunction, eventually leading to congestive heart failure, cardiogenic shock or arrhythmia, and sudden death in severe cases[5]. Although the advancement in pharmaceutical treatment and interventional and surgical techniques, it has not yet to find an effective therapeutic method to halt the deterioration of cardiac function due to high levels of blood glucose[6]. There is an urgent need to deepen the understanding of the pathogenesis and molecular mechanism of DCM to assist the development of novel therapeutic approaches⁷.

Circular RNA (circRNA) is a kind of non-coding RNA molecules with a closed circular structure, [without 5' cap-structure and 3' poly\(A\)-tail](#)[7]. CircRNA mainly exists in cytoplasm or exosomes and has the characteristics of tissue specificity, disease specificity, timing specificity and high stability[8]. In recent years, a large number of studies have shown that circRNA is closely related to biological growth and

development, stress response, disease occurrence and development, etc. CircRNA can act as a sponge of microRNA, involving in the regulation of disease-related genes in the transcriptional level and post-transcriptional level through a competing endogenous RNA mechanism[9]. Although increasing evidence has been found to reveal the function of circRNA in biological processes, especially in various kinds of diseased conditions, there's still a long way to go to bridge the knowledge gap. Recently, a series of studies reveal the association between circRNA and cardiovascular disease. For example, Garikipati reported that circular RNA CircFndc3b actively participated in cardiac repair after myocardial infarction through regulation of FUS/VEGF-A Axis[10]. What's more, circRNAs are of prime importance in heart failure. Hsa_circ_0097435 is overexpressed in heart failure patients and can sponge multiple microRNAs to accelerate the progression of heart failure. To develop a new therapeutic method, hsa_circ_0097435 can be a promising target[11]. Additionally, circRNAs play a role in atherosclerosis, coronary artery disease, myocardial infarction, as well as cardiomyopathy[12].

Although mRNA[13], lncRNA[14], and circRNA[15] were studied in DCM, the mechanism of circRNAs in the pathogenesis of DCM has not been systematically studied. Therefore, a comprehensive study of the functions of circRNAs in the heart is an advantageous strategy to understand this metabolic disease.

In this study, we performed circular RNA array analysis; screen differentially expressed circRNAs; and reveal their biological functions and molecular mechanisms, combined with bioinformatics analysis. The circRNAs' expression and enrichment analysis of pathways would greatly facilitate the study of DCM pathogenesis and provide potential novel targets for DCM therapeutics.

Methods

Animals and specimens

20 male Sprague Dawley rats, weighted 210–240 gram, were intraperitoneally injected streptozocin (60mg/Kg, Sigma) to induce type 1 diabetes mellitus (T1DM). After 3 days, if fasting blood glucose was greater than 11.1mmol/L, it is considered to be a successful induction of T1DM model. 8 weeks after modeling, the rats were anesthetized and its left ventricular free wall tissues were cut off for isolation of total RNA. The study was approved by the medical ethics committee of Chinese PLA General Hospital.

Total RNA isolation and quality control

Total tissue RNA was extracted from normal and diabetic Sprague Dawley rats's left ventricular free wall tissues using TRIzol reagent (Invitrogen, NY, USA), according to the manufacturer's instructions. The quality and concentration of RNA samples (Supplementary Table 1) were determined using the NanoDrop ND–1000 (Thermo Fisher Scientific, Wilmington, DE, USA). The RNA Integrity and genomic DNA (gDNA) contamination test were achieved by electrophoresis on a denaturing agarose gel (Supplementary Fig 1). The samples were preserved at –80 °C for further experiments.

circRNA microarray analysis

Sample labeling and array hybridization were performed according to the manufacturer's protocol. Briefly, total RNA was digested with RNase R (Epicentre, Inc.) to remove linear RNAs and enrich the circular RNAs. These circular RNAs were amplified and transcribed into fluorescent cRNA utilizing a random priming method (Arraystar Super RNA Labeling Kit; Arraystar). The labeled cRNAs were hybridized onto the Arraystar Rat circRNA Array V2.0 (8×15 K), with a total of 14,145 circRNA probes on the microarray. After washing the slides, the arrays were scanned using an Agilent G2505C Scanner. Agilent Feature Extraction software (version 11.0.1.1, USA) was used to analyze the acquired array images.

Validation of array results by qPCR

To validate the reliability of microarray analysis and explore the expression trend of circRNAs in DCM, ten circRNAs were randomly selected to verify their differential expression levels by qRT-PCR. Total RNAs isolated from DCM and control samples were reverse transcribed into cDNA for qRT-PCR analysis. Quantitative RT-PCR was conducted in ViiA 7 Real-time PCR System (Applied Biosystems) following the manufacturer's instructions. The expression of circRNAs was defined based on the threshold cycle (Ct), and relative expression levels were calculated via the $2^{-\Delta\Delta Ct}$ method. Outward-facing primers were designed to amplify the fragment across the junction from cDNA using software Primer 5.0 and are listed in Table 1. Both DCM and control groups had three independent samples and all reactions were processed in triplicate.

GO enrichment and KEGG analysis of host genes

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed to predict the biological function of the host gene of differential expressed circRNA using the clusterProfiler (version 3.8.0) <http://bioconductor.org/packages/release/bioc/html/clusterProfiler.html>. We described the enrichment results from three aspects, including biological process (BP), cellular component (CC), and molecular function (MF). GO terms and pathway analyses with P value < 0.05 adjusted by Benjamin and Hochberg method were considered to be significantly enriched functional annotations.

Construction of circRNA-miRNA interaction

Since the physiological and pathological roles of circRNAs potentially act through a competing endogenous RNA mechanism, therefore, it is of prime importance to identify the association between miRNAs and circRNA. What's more the discovery of the molecules involved in the crosstalk among miRNAs and circRNAs now represents an important challenge for scientists in elucidating the complexity of biological processes. Using the Arraystar's home-made miRNA target prediction software based on TargetScan & miRanda, the interaction between circRNA and microRNA was predicted and the

differentially expressed circRNAs were annotated in detail with the circRNA/miRNA interaction information. Then, the crosstalk networks were represented in Cytoscape (www.cytoscape.org/) and the topological properties were analyzed using the Cytoscape 'Network Analysis' plugin. CytoHubba, a plugin of Cytoscape, was used to predict the important nodes and subnetworks in the network using the Degree method.

Results

Successful establishment of the diabetic cardiomyopathy rat model

After the STZ induction, blood sugar level and body weight of rats were measured and documented in a certain time interval. A distinct difference between DCM and normal control rats was observed. In the DCM group, the rat had significantly higher blood sugar levels and lower body weight, which were inconsistent with the typical manifestations and symptoms of diabetes mellitus (*Figure 1A, B*). This experiment reflects left ventricular systolic function in rats through left ventricular ejection fraction (LVEF) and short-axis shortening rate (FS). LVEF values were significantly decreased in the DCM group ($56.33 \pm 1.662\%$) compared with the normal control group ($74.06 \pm 2.631\%$). (*Fig 1C*). In diabetic cardiomyopathy, the increased deposition of collagen and interstitial fibrosis in the myocardium is one of the characteristic structural changes within it. In this study, LV collagen deposition and fibrosis ratio were markedly increased in DCM rats compared to normal controls (5.74 ± 0.3472 vs 0.94 ± 0.07924), using Masson staining method (*Figure 1D*).

Identification of differential expressed circRNAs

To investigate the circRNA expression profile of diabetic cardiomyopathy, we have completed the Arraystar Rat circRNA Array analysis of specimen from 3 DCM samples and 3 normal controls. A total of 14139 circRNAs were detected by Arraystar Rat circRNA Microarray. When comparing two groups of profile differences, the "fold change" between the groups for each circRNA is computed. The statistical significance of the difference is estimated by t-test. CircRNAs having |fold changes| 2.0 and p-values 0.05 are selected as the significantly differentially expressed. Eventually, 171 differentially expressed circRNAs, including 89 upregulated and 82 downregulated circRNAs were identified. A Scatter plot (*Fig 2A*) and a volcano plot (*Fig 2B*) showed the distributions of circRNAs more directly. The top 20 dysregulated circRNAs based on fold changes were summarized in *Table 1*. The result of hierarchical clustering showed a distinguishable circRNA expression profiling among samples. The data suggested that the circRNA in DCM was different in normal control samples (*Fig 2C*).

Real-time qPCR validation of 6 differentially expressed circRNAs

After the comparison and the analysis of the differentially expressed multiple circRNAs in the microarray experiments, we randomly verified 6 circRNAs (rno_circRNA_000466, rno_circRNA_000964,

rno_circRNA_003395, rno_circRNA_000173, rno_circRNA_013989, and rno_circRNA_003643), using GAPDH as the internal control. There were significant differences in all of the selected circRNAs ($P < 0.05$) except for rno_circRNA_003643 ($P = 0.06361$). Among the 6 selected circRNAs, rno_circRNA_000466 was founded to be the highest increase of nearly 28-fold change. rno_circRNA_000964 was down-regulated with a 0.51-fold decrease. The results are shown in *Fig 3*. There was a great consistency between the real-time qPCR results and microarray analysis data, which demonstrated the high reliability of the microarray expression data.

Functional profiles of the parental genes of differentially expressed circRNAs in DCM

circRNA cis-regulates the expression of parental genes. On the one hand, circRNA can combine with RNA binding proteins to affect the expression of the parental gene mRNA. On the other hand, competitive complementary pairing between introns during the formation of circular RNA can achieve a balance between linear RNA and affect mRNA expression, even protein translation. Parental genes from 171 differentially expressed circRNAs were subjected to GO and KEGG pathway enrichment analyses. The top 10 classes of GO enrichment terms and the top 10 classes of KEGG pathway enrichment terms are presented (*Figure 4*). Compared to the normal heart sample, the data demonstrated that the gene expression profile of linear counterparts of differentially expressed circRNAs in DCM involves in the insulin signaling pathway, autophagy, HIF-1 signaling pathway, inflammatory mediator regulation of TRP channels, insulin secretion, regulation of lipolysis in adipocytes and so on. As for the insulin signaling pathway, *CALM3*, *CBLB*, *INSR*, *PIK3R3*, *PRKACA*, *SORBS1* were dysregulated in DCM heart tissue. And *ATP1B1*, *CALM3*, *CAMK2A*, *GRIA2*, *PIK3R3*, *PRKACA* in the cAMP signaling pathway were down in DCM heart tissue. In the autophagy pathway, differentially expressed *IGF1R*, *MTMR3*, *PIK3R3*, *PRKACA*, *SH3GLB1* were found to be altered. To be noted, many genes participated in cell membrane biological activity, including regulation of transmembrane transport, regulation of potassium ion transmembrane transporter activity, etc.

circRNA-microRNA interaction network construction

CircRNA is generally produced by RNA alternative splicing and circRNA has been found to play the role of miRNA sponge. As a competitive endogenous RNA (ceRNA) binding to intracellular miRNA, blocking the inhibition of miRNA on its target gene is its main regulatory mode. The circRNA/microRNA interaction was predicted with Arraystar's home-made miRNA target prediction software based on TargetScan and miRanda, and the differentially expressed circRNAs within all the comparisons were annotated in detail with the circRNA/miRNA interaction information. All the differentially expressed circRNAs prediction was based on the principle of complementary sequence pairing, and targeted miRNAs were ranked according to their mirSVR scores, and five miRNAs with the highest mirSVR score were identified as MREs for each circRNAs. In the topological network (*Fig 5A*), the orange oval nodes represent upregulated circRNAs and the yellow rectangle nodes represent down-regulated circRNAs. It can be founded in the network that one

circRNA interacts with a couple of miRNAs and some miRNA can also interact with multiple circRNAs. Furthermore, we using CytoHubba plugin to screen the top 20 nodes in network ranked by the Degree method, to assess the key modules that significantly regulated the pathogenesis of DCM. The co-expression network reveals that rno-miR-22, rno-miR-27, rno-miR-28, rno-miR-466, rno-miR-320 may play important roles in the development of DCM. And the magnified network was visualized using Cytoscape (v.3.8.0) as well (*Fig 5B*).

The construction of rno_circRNA_000466-associated ceRNA networks

In our previous array and qRT-PCR test, rno_circRNA_000466 was highly expressed in DCM heart tissue with a 28-fold change. And based on the hypothesis that circRNAs may function through the ceRNA mechanism, we used Arraystar's home-made miRNA target prediction software to predict the interaction miRNA of rno_circRNA_000466. Five miRNAs, including rno-miR-1306-3p, rno-miR-665, rno-miR-3068-5p, rno-miR-223-3p, rno-miR-3593-5p, were identified most likely to be interacted with rno_circRNA_000466. And then we use DIANA microT-CDS online tools to predict the target of the five miRNAs. At last, 139 targets were found with the threshold set to 0.9. The topical relationship was visualized through Cytoscape software. The functional annotation was performed using the CluGO and CluPedia plugins. The results show that some of the target genes were enriched in the TGF- β signaling pathway, regulation of glucose transmembrane transport, endocrine process and response to lipoprotein particle, which are related to metabolic remodeling and cardiac fibrogenesis in DCM. These results were presented in *Fig 6*.

Discussion

Circular RNA Microarray is a common method to reveal the differentially expressed circRNAs profiles between the diseased group and the normal control group. It has the characteristics of high sensitivity, high throughput, and parallel detection. The present work was a systematic study to find out the differentially expressed circRNA profile of DCM. And we have studied in depth its regulatory role in the development of DCM and pave the way for further verification and research.

CircRNAs may play a role in the pathogenesis of diseases by regulating the expression of their parental gene. We performed Go analysis and KEGG analysis of the parental genes of circRNAs that were differentially expressed in DCM. Gene ontology analysis is an informatics method for linking differentially expressed genes to the GO classification for functional analysis, including classifying and analyzing differentially expressed genes in terms of participation in molecular functions, biological processes, and cellular components. And the KEGG analysis can identify the altered pathways in the pathogenesis process. The inference from the parental gene analysis of the differentially up-regulated circRNAs in DCM is that the abnormally expressed parental genes of circRNAs participate in various biological processes, specifically, insulin receptor signaling pathway, regulation of potassium ion transmembrane transporter

activity, regulation of glycoprotein metabolic process. These are all consistent with the characteristics of DCM myocardial metabolism abnormality and impaired myocardial cell function. Impaired glucose metabolism, manifesting insulin resistance and metabolic syndrome, promotes the pathogenesis of diabetes and related complications[16]. Abnormal insulin receptor inactivation is often considered an important mechanism for insulin resistance, both in vivo and in cell models[17]. CircRNAs are a widespread RNA species in various species and have important biological functions. They are involved in the regulation of islet cell function and insulin metabolism[18], also mediate vascular damage caused by high glucose as well[19,20].

MAP3K5, host gene of the up-regulated rno_circRNA_000466, has been reported to be elevated in DCM[21,22]. We speculate that rno_circRNA_000466 may also participate in the regulation of cell apoptosis, oxidative stress injury and cardiac fibrogenesis. In terms of rno_circRNA_003643 and its parental gene *Insr*, the phosphorylation of IRSs proteins leads to the activation of the PI3K-AKT/PKB pathway and the Ras-MAPK pathway. Thus, we predicted that rno_circRNA_003643 may play an important role in the metabolic actions of insulin and the control of cell growth and differentiation. Because of cardiac insulin resistance contributes to diabetic cardiomyopathy to a large extent, further studies of rno_circRNA_003643 will advance our understanding of insulin resistance in the pathogenesis of DCM. Hopefully, it can be considered as a novel therapeutic target and used as a biomarker in monitoring disease outcomes.

In our six verified circRNAs, rno_circRNA_000466 was identified to be significantly differentially expressed with a 28-fold in DCM heart tissue. Its ceRNA regulatory network shows that the target genes involved in the TGF- β signaling pathway, which participates in cardiac remodeling and fibrosis in the high glucose damaged myocardium[23,24]. It is noteworthy that myocardial fibrosis is of prime importance in the structural remodeling of diabetic hearts and ultimately affect cardiac function. Thus, finding a therapeutic target of cardiac fibrosis for DCM will be helpful to improve the prognosis of DCM. Focusing on the role of rno_circRNA_000466 in the regulation of cardiac fibrosis will provide us a novel approach to prevent the development of fibrosis and halt the deterioration of cardiac function. Moreover, rno_circRNA_000466 may also involve in the regulation of glucose transmembrane transport. Disorders of glucose metabolism will dramatically affect the functional phenotype of cardiomyocytes and the surrounding stromal cells. Consequentially, alterations in cardiac structure and function will manifest in DCM.

Although this study will contribute to our knowledge of circRNAs in DCM, there is still some limitation. First, the sample size is relatively small, so there will be a possible selection bias in the present study. Larger sample sizes are required to confirm and validate the conclusions. Second, there are differences in type 2 and type 1 diabetes mellitus. In this study, we STZ-induced T1DM model was adopted and thus the difference between these two disease conditions was unable to be clarified. Third, more functional experiments are needed to verify these results for most of our findings were based on bioinformatic analysis.

Conclusion

In conclusion, we have demonstrated, for the first time, a comprehensive circRNAs expression profiles of DCM. Bioinformatics analysis predicted that differentially expressed circRNAs were involved in the regulation of glucose metabolism, insulin resistance and cardiac remodeling. This study opens new avenues for a better understanding of the pivotal role of circRNA in DCM.

List Of Abbreviations

DCM

diabetic cardiomyopathy

T1DM

Type 1 diabetes mellitus

circRNAs

circular RNAs

GO

gene ontology

KEGG

Kyoto Encyclopedia of Genes and Genomes

Declarations

Ethics approval and consent to participate: The experimental protocol was approved by the Ethics Committee for Animal Research from the General Hospital of the PLA. All the rats were cared for and used in accordance with the Guide for the Care and Use of Laboratory Animals.

Consent for publication: Not applicable.

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

Funding: Not applicable

Authors' contributions: YW, NC, RW contributed to the conception of the study; YZ performed the induction of DCM animal model; YW, NC performed the data analyses and wrote the manuscript; LM, LL helped perform the analysis with constructive discussions.

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Tables

Name	Primer sequence	Annealing temperature (°C)	Product length (bp)
rno_circRNA_000964	F:5' CACAAGGACCCCGACATAAAGA 3' R:5' ACGGAAGGCATCCACCCA 3'	60	213
rno_circRNA_003643	F:5' CACAATCAGAGTGAGTATGACGAC 3' R:5' CGTAGAAAGAATAGTTCCTGGG 3'	60	160
rno_circRNA_003395	F:5' ACGTTCAGCCGGCAGGTG 3' R:5' AATGGCTCCGAGGGATCTTCT 3'	60	107
rno_circRNA_000466	F:5' GATGTTACTGTCGTTAGATTTCCA 3' R:5' GCTGCTCAGTTTAACTCCTTGT 3'	60	149
rno_circRNA_013989	F:5' CAAGAGGACTCATGTTCAAATCAAG 3' R:5' TCTCAGGACCTTTGCGGTCAT 3'	60	118
rno_circRNA_000173	F:5' ATCGCTGCCAGAAAATTTG 3' R:5' TGAGCAGGATGTGGAGGAA 3'	60	111
GAPDH(RAT)	F:5' GCTCTCTGCTCCTCCCTGTTCTA3' R:5' TGGTAACCAGGCGTCCGATA3'	60	124

Table 1: Polymerase chain reaction primer design

Table 2. The top 20 up-regulated and down-regulated circRNA ranked by fold changes in microarray data.

circRNA	Regulation	P-value	FC (abs)	circRNA_type	GeneSymbol
rno_circRNA_009571	up	0.0104174	3.1413473	exonic	Fkbp5
rno_circRNA_014008	up	0.0155209	3.1271052	sense overlapping	Nrxn1
rno_circRNA_000466	up	0.0001248	3.0597797	exonic	RGD1306565
rno_circRNA_004268	up	0.039481	3.0011175	exonic	Glul
rno_circRNA_004582	up	0.0358735	2.9601857	exonic	Ehbp1
rno_circRNA_000473	up	0.0001721	2.8824967	exonic	RGD1306565
rno_circRNA_013985	up	0.0209465	2.8074637	exonic	Nrxn1
rno_circRNA_000461	up	0.0001381	2.7596524	exonic	RGD1306565
rno_circRNA_005394	up	0.0020762	2.7409516	sense overlapping	Cadps
rno_circRNA_000472	up	0.0002831	2.6766347	exonic	RGD1306565
rno_circRNA_011134	down	0.0072243	4.9127607	exonic	Gpsm1
rno_circRNA_007259	down	0.0084149	4.0044609	exonic	Dpysl3
rno_circRNA_013379	down	0.0214775	4.003262	exonic	Ptpn3
mmu_circRNA_31698	down	0.009449	3.9550431	exonic	Dpysl3
rno_circRNA_000964	down	0.0010194	3.3290136	exonic	Fads2
rno_circRNA_009978	down	0.026106	3.1780586	sense overlapping	Fmn1
rno_circRNA_006485	down	0.0320876	3.0679901	sense overlapping	Atxn1
mmu_circRNA_23123	down	0.0123396	2.9430241	exonic	Rtn4
rno_circRNA_007790	down	0.0018526	2.9189362	exonic	Nfix
rno_circRNA_004393	down	0.0286942	2.8169464	sense overlapping	Atp1b1

Figures

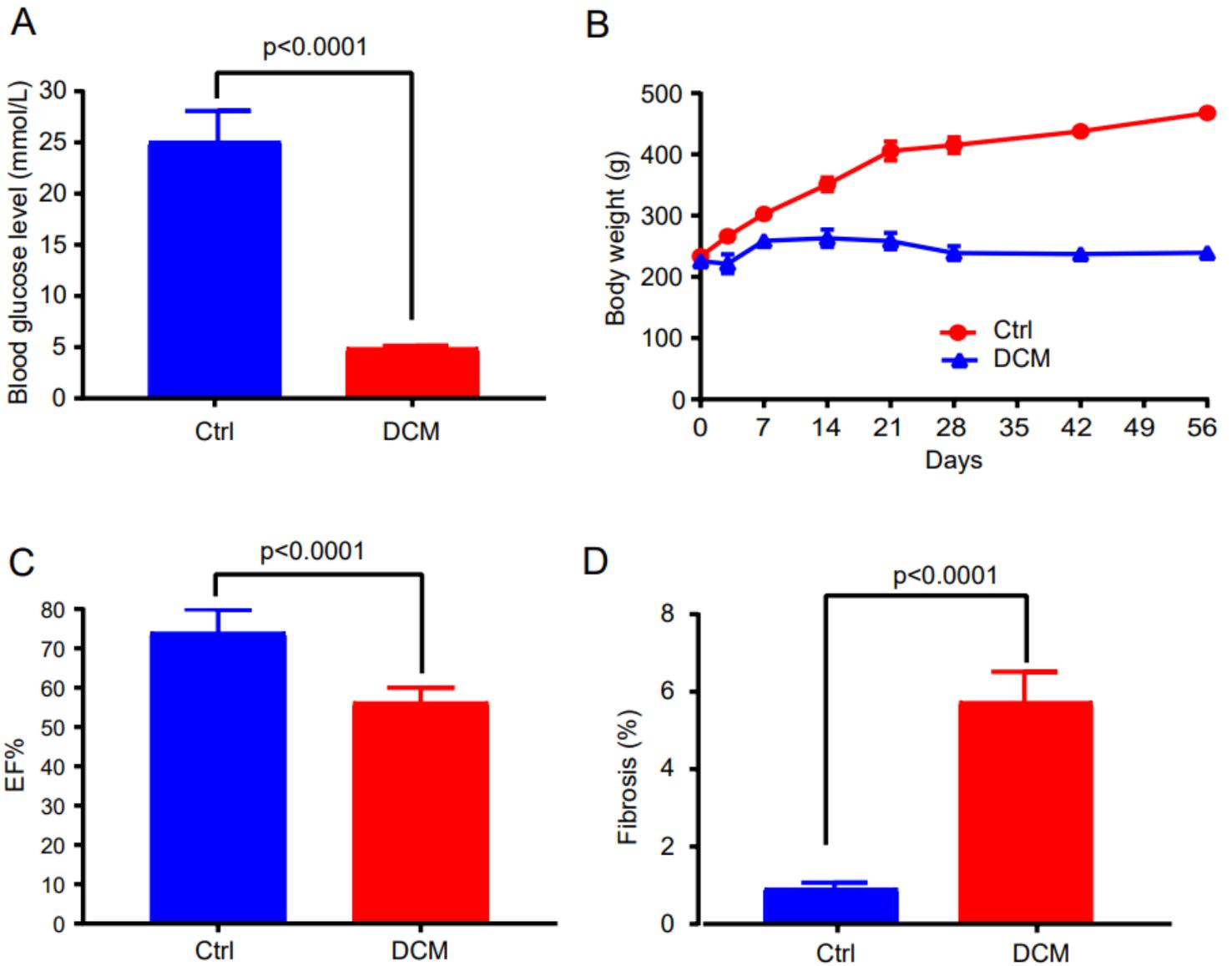


Figure 1

Successful modeling of type 1 diabetic cardiomyopathy. A: Random blood glucose levels were higher in DCM group ($P < 0.0001$); B: Body weight of rats subsequent to establishing the DCM model; C: The DCM group has decreased LVFF values compared with the normal group ($p = 0.0005$); D: The degree of interstitial fibrosis in DCM animals was greater than that in normal control ($P < 0.0001$).

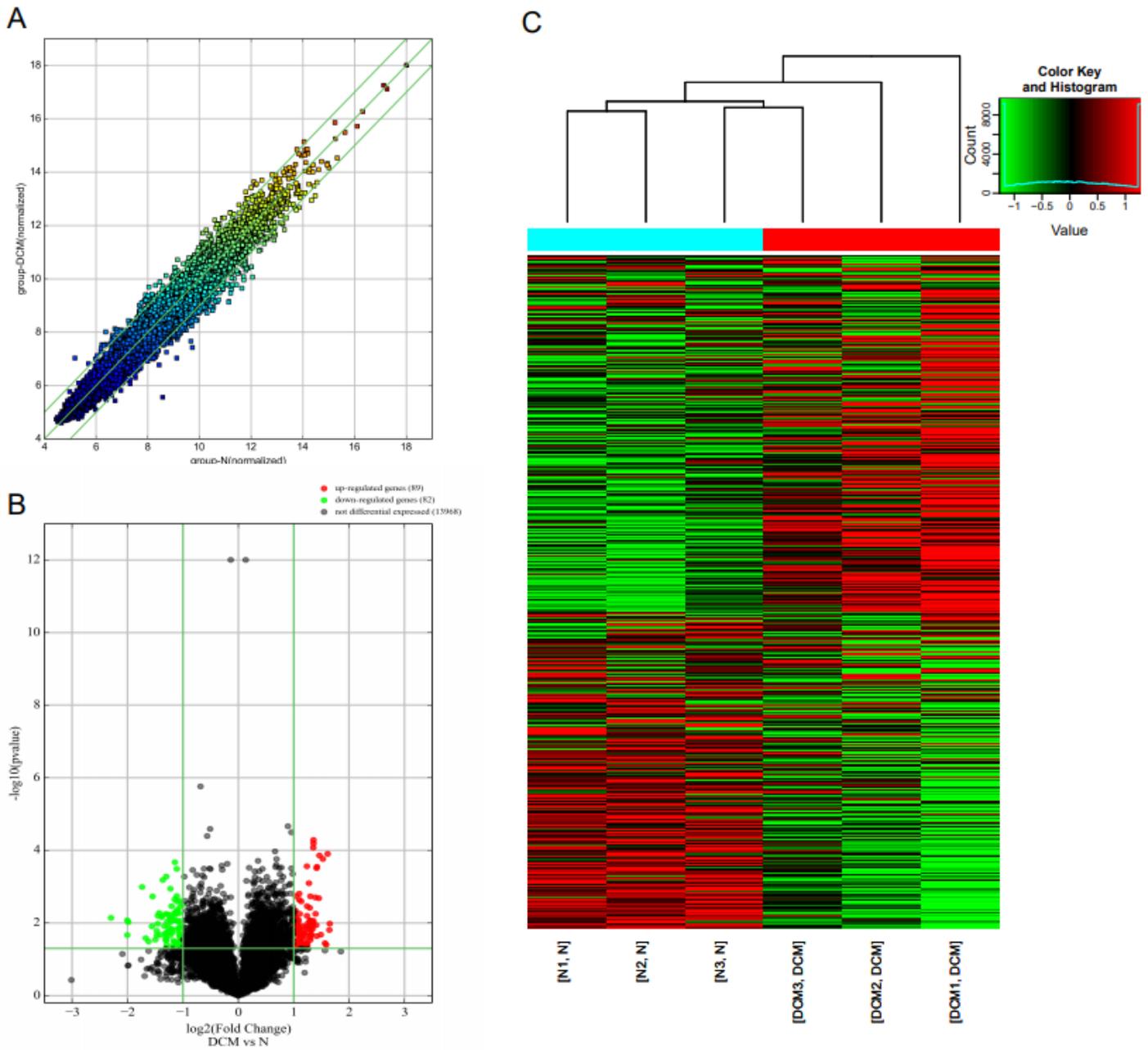


Figure 2

Differential expression of circRNA by (A) scatter plot, (B) volcanic map and (C) cluster analysis in normal group and DCM group.

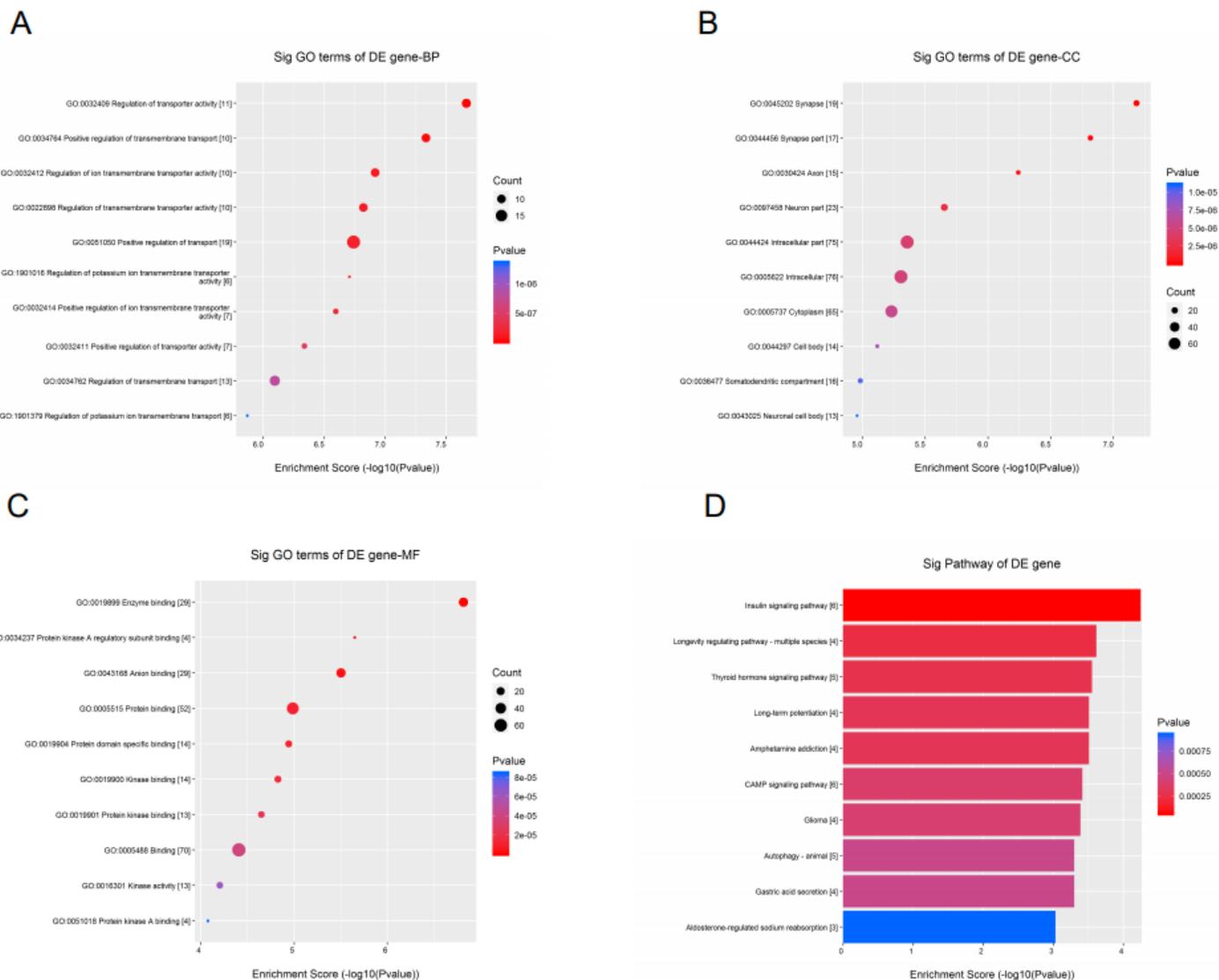


Figure 3

GO enrichment analysis and KEGG analysis of host genes of differential expressed circRNAs showed (A) biological process (BP), (B) cellular component (CC), (C) molecular function (MF) and KEGG pathway (D).

