Potential role of circular RNA in cyclosporin A-induced cardiotoxicity in rats

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Abstract
Cyclosporin A (CsA) is a well-known and effective drug that is commonly used in autoimmune diseases and allotransplantation. However, kidney toxicity and cardiotoxicity limit its use. Circular RNAs (circRNAs) play a crucial role in disease, especially cardiovascular disease. We aimed to explore the circRNA expression profiles and potential mechanisms during CsA-induced cardiotoxicity. Sixty male adult Wistar rats were randomly divided into two groups. The CsA group was injected with CsA (15 mg/kg/day body weight) intraperitoneally (ip) for 2 weeks, whereas the control group was injected ip with the same volume of olive oil. We assessed CsA-induced cardiotoxicity by light microscopy, transferase-mediated dUTP nick-end labeling (TUNEL) staining, and electron microscopy. Microarray analysis was used to detect the expression profiles of circRNAs deregulated in the heart during CsA-induced cardiotoxicity. Sixty male adult Wistar rats were randomly divided into two groups. The CsA group was injected with CsA (15 mg/kg/day body weight) intraperitoneally (ip) for 2 weeks, whereas the control group was injected ip with the same volume of olive oil. We assessed CsA-induced cardiotoxicity by light microscopy, transferase-mediated dUTP nick-end labeling (TUNEL) staining, and electron microscopy. Microarray analysis was used to detect the expression profiles of circRNAs deregulated in the heart during CsA-induced cardiotoxicity. We confirmed the changes in circRNAs by quantitative PCR. Moreover, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of the microarray data were performed. A conventional dose of CsA induced cardiotoxicity in rats. We identified 67 upregulated and 37 downregulated circRNAs compared with those in the control group. Six of 12 circRNAs were successfully verified by quantitative real-time polymerase chain reaction (qRT-PCR). GO analyses of the differentially expressed circRNAs indicated that these molecules might play important roles in CsA-induced cardiotoxicity. KEGG pathway analyses showed that the differentially expressed circRNAs in CsA-induced cardiotoxicity may be related to autophagy or the Hippo signaling pathway. We identified differential circRNA expression patterns and provided more insight into the mechanism of CsA-induced cardiotoxicity. CircRNAs may serve as potential biomarkers or therapeutic targets of CsA-mediated cardiotoxicity in the future.

KEYWORDS
apoptosis, cardiotoxicity, circular RNAs, cyclosporin A, microarray
1 | INTRODUCTION

Cyclosporin A (CsA) is an important and effective immunosuppressant drug that is widely used to manage immunological organ rejection and autoimmune diseases (Rezza, 2006) because it specifically inhibits the signal transduction of T-cell receptors (Buehler et al., 2000). However, CsA produces side effects in several organs, particularly the kidney (Krejci et al., 2010), liver (Klintmalm et al., 1981), and heart (Florio et al., 2003). CsA-induced cardiotoxicity is controversial. Some studies have indicated that CsA can ameliorate heart failure (Sharov et al., 2007) and cardiac hypertrophy (Schreiner et al., 2004) and protect the heart from ischemia–reperfusion injury (Bies et al., 2005). However, our previous report and other studies have found that CsA results in myocardial structural damage and increased cardiomyocyte apoptosis (Tang et al., 2011; Zhao et al., 2011) and myocardial fibrosis in rats (Rezza et al., 2003). Therefore, CsA-induced cardiotoxicity cannot be ignored. Another study (Tang et al., 2011) showed that CsA induced myocardial injury in a time-dependent manner through intracellular calcium overload. Our previous report indicated that CsA can induce cardiomyocyte apoptosis through the downstream cytochrome c-caspase-3 pathways (Zhao et al., 2011). Zhu et al. (2016) suggested that CsA could induce cardiomyocyte apoptosis through the miR-377-XIAP/NRP2 axis. To date, the underlying mechanism of CsA-mediated cardiotoxicity remains unclear and requires further investigation.

Circular RNA (circRNA) is a new type of noncoding RNA that can form closed continuous loop structures with covalently joined 3'- and 5'-ends, which is in contrast to traditional linear RNAs (including 5' and 3' ends) (Sanger et al., 1976). CircRNAs have been detected in various tissues and organisms (Danan et al., 2012). More than 20 years ago, circRNAs were initially described as scrambled exons (Nigro et al., 1991). Currently, studies have demonstrated that these molecules play a pivotal role in the regulation of gene expression (Zeng et al., 2017). CircRNAs have been shown to have three biological functions: (1) microRNA (miRNA) sponges (Hansen, Jensen, et al., 2013), (2) protein-binding molecules (Jeck & Sharpless, 2014), and (3) transcriptional regulators (Li et al., 2015). Increasing evidence suggests that circRNAs are potential diagnostic and treatment markers in various diseases, playing a key role in tumors (Qin et al., 2016), diabetes mellitus (Wang et al., 2019), cardiovascular diseases (Wang et al., 2016), and neurodegenerative diseases (Lu et al., 2019). CircRNAs have become a research hotspot in recent years. However, to the best of our knowledge, the expression profile and function of circRNAs in CsA-induced cardiotoxicity remain unknown.

In this study, we explored the circRNA expression profiles and mechanisms in the pathogenesis of CsA-induced cardiotoxicity through microarray analysis, quantitative real-time polymerase chain reaction (qRT-PCR) verification, and bioinformatics, aiming to show that circRNAs play potential roles in CsA-induced cardiotoxicity and may represent novel markers for its diagnosis and treatment.

2 | MATERIALS AND METHODS

2.1 | Reagents

CsA was purchased from Novartis Pharma AG (Basel, Switzerland). The transferase-mediated dUTP nick-end labeling (TUNEL) kit was purchased from Roche (Mannheim, Germany). TRIzol reagent was purchased from Invitrogen (NY, USA). Olive oil was purchased from Betis (Sevilla, Spain). The food and pure water of rats were supplied by the Animal Research Center of Harbin Medical University.

2.2 | Animal groups and animal model establishment

Sixty specific pathogen-free male adult Wistar (210–230 g) rats used in this study were provided by the Animal Research Center of Harbin Medical University. All animal experimental procedures were approved by the Animal Management Rules of the National Institutes of Health. Sixty rats were randomly divided into two groups. CsA (250 mg:5 ml) was dissolved and diluted in olive oil. The CsA group (CsA, n = 30) was injected intraperitoneally (ip) daily with CsA at 15 mg/kg body weight (Hagar, 2004; Lexis et al., 2005; Wassef et al., 1985; Zhu et al., 2016), whereas the control group (control, n = 30) was injected ip with 3 ml/kg body weight of olive oil. The rats were maintained in a barrier environment for 2 weeks of quarantine/acclimatization at a temperature of 22 ± 2 °C, relative humidity of 55% ± 5%, on a 12-h light–dark cycle, and food and pure water were supplied ad libitum. Two weeks later, the rats were anesthetized, and the heart tissues were removed for analyses.

2.3 | Hematoxylin and eosin staining

The ventricular tissues of rats were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin; sectioned into 5-μm-thick slices; dewaxed; and stained with hematoxylin and eosin (H&E). The morphology of each specimen was observed by light microscopy.

2.4 | TUNEL assay

The ventricular tissues were fixed in 4% paraformaldehyde for 24 h at room temperature, dehydrated and embedded in paraffin, as previously described (Zhao et al., 2011). After the treatment described above, the labeled myocytes were examined using light microscopy and evaluated by blinded investigators.

2.5 | Transmission electron microscopy

The tissues were fixed with 2.5% glutaraldehyde at 4 °C overnight and then postfixed with 1% osmium tetroxide. After dehydration, the
ventricular tissues were embedded in epoxy resin, and ultrathin sections were stained with uranyl ethanoate and lead citrate and observed by transmission electron microscopy.

2.6 | RNA extraction

The microarray work was performed by Kangchen Biotech (Shanghai PR China). Six samples (three samples for the CsA group and three for the control group) were selected randomly for the microarray analysis. Total RNA was extracted using TRlzol reagent (Invitrogen) following the manufacturer's instructions. The purity and concentration of total RNA from each sample were quantified and verified using a NanoDrop ND-1000 system. Additionally, the RNA integrity was assessed by electrophoresis on a denaturing agarose gel.

2.7 | Labeling and hybridization

Sample preparation and microarray hybridization were performed based on Arraystar's standard protocols. Briefly, total RNA was digested with RNase R (Epicenter, Inc.) to remove linear RNAs and enrich circRNAs. Then, the enriched circRNAs were amplified and transcribed into fluorescent cRNA using a random priming method (Arraystar Super RNA Labeling Kit; Arraystar). The labeled cRNAs were hybridized onto the Arraystar Rat circRNA Array (8x15K, Arraystar) and incubated for 17 h at 65°C in an Agilent Hybridization Oven. After the slides were washed, the arrays were scanned by an Agilent G2505C scanner.

2.8 | Microarray and quality control

Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed using the R software package. Differentially expressed circRNAs between the two groups were identified through fold change filtering and Student's t test.

2.9 | Quantitative PCR analysis

Briefly, total RNA from cardiac tissues was extracted using TRlzol reagent (Invitrogen) according to the manufacturer's protocols. The circRNA was measured according to the manufacturer's instructions (Arraystar). All experiments were performed in triplicate, and the median was used to calculate the relative circRNA concentrations. All samples were normalized to GAPDH. The primers are listed in Table 3.

2.10 | GO and KEGG pathway analysis

Gene Ontology (GO) analysis was applied to analyze the functional categories of all differentially expressed mRNAs. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed to determine whether the genes share a similar biological role. The enriched genes were determined by hyper-geometric distribution.

2.11 | Annotation for circRNA/miRNA interaction

The circRNA/miRNA interaction was predicted with Arraystar's homemade miRNA target prediction software. All the differentially expressed circRNAs were annotated in detail with the circRNA/miRNA interaction information.

2.12 | Statistical analysis

Scanned images were imported into Agilent Feature Extraction software for raw data extraction. All data are expressed as the means ± SEM. Statistical analysis was performed with unpaired two-tailed Student's t tests between two groups. A value of P < 0.05 was considered significant. The threshold set for deregulated circRNAs in the microarray data was a fold change ≥ 1.2 and a P value < 0.05.

3 | RESULTS

3.1 | Successful establishment of CsA-mediated cardiotoxicity in rats

3.1.1 | H&E staining

Light microscopic examination showed the changes induced by CsA in the rat myocardial cells (Figure 1A). No obvious pathological findings were detected in the control heart tissues. In the CsA group, the myocardial cells shrink and appeared disorderly, and the number of collagen fibers increased between the cells.

3.1.2 | Changes in TUNEL staining

Low levels of TUNEL-positive cells were detected in the control group (7.8 ± 0.75%). However, the apoptotic cells increased dramatically in the CsA group compared with the control group (27.7 ± 1.05%, P < 0.01 vs. control). TUNEL assays indicated that CsA induced clear cardiomyocyte apoptosis in rats (Figure 1B,D).
FIGURE 1  Morphological characterization of CsA cardiotoxicity rat model. (A) HE staining in rat tissues (400×).
(B) Representative illustration of TUNEL staining, nuclei with brown staining, indicate TUNEL-positive cells (400×) (black short arrows for apoptotic nuclei).
(C) Ultrastructural changes if cardiomyocyte apoptosis. Magnification is 10,000× (control), 5000× (CsA) (black long arrows for apoptotic bodies). (D) Quantitative results of the TUNEL staining for each group. Data are shown as the means ± SEM. **P < 0.01 versus control group.
3.1.3 | Ultrastructural changes

Transmission electron microscopy revealed ultrastructural changes in cardiomyocytes (Figure 1C). No significant ultrastructural changes were detected in the control group, which displayed plentiful muscle fibers, evenly distributed chromatin and clear mitochondrial cristae. In the CsA group, the ultrastructural changes of the cardiomyocytes included the formation of apoptotic bodies, an increased density of the mitochondrial matrix, and damage to the myofilaments.

3.2 | Expression patterns of circRNAs in rats with CsA-induced cardiotoxicity

A rat Arraystar circRNA microarray was carried out for three randomly selected samples from each group. From the circRNA expression profiles, differentially expressed circRNAs were identified between the CsA group and the control group. Hierarchical clustering (heat map) was performed (Figure 2A), and volcano (Figure 2B), scatter (Figure 2C), and box plots (Figure 2D) were generated based on the
| circRNA          | P value       | FDR            | FC (abs) | Regulation | circRNA_type | chrom | strand | best_transcript | GeneSymbol |
|------------------|---------------|----------------|----------|------------|--------------|-------|--------|----------------|------------|
| rno_circRNA_014540 | 0.003306356   | 0.999536243    | 2.1833678 | Up         | Exonic       | chr6  | -      | NM_01010951    | Prepl      |
| rno_circRNA_006822 | 0.037361753   | 0.999536243    | 1.6312778 | Up         | Exonic       | chr17 | +      | NM_011106100   | Agtbp1     |
| rno_circRNA_014890 | 0.03746584    | 0.999536243    | 1.606299  | Up         | Exonic       | chr7  | -      | NM_001130574   | Tctf20     |
| rno_circRNA_008315 | 0.021764214   | 0.999536243    | 1.601282  | Up         | Exonic       | chr2  | -      | NM_001109558   | Setd7      |
| rno_circRNA_012213 | 0.046827317   | 0.999536243    | 1.4623267 | Up         | Sense overlapping | chr4  | -      | NM_001108622   | Hipk2      |
| rno_circRNA_005048 | 0.042821439   | 0.999536243    | 1.44736   | Up         | Exonic       | chr14 | +      | XM_573620      | RGD1561241 |
| rno_circRNA_012347 | 0.002858764   | 0.999536243    | 1.4120673 | Up         | Sense overlapping | chr5  | +      | NM_053935      | Sh3gl2     |
| rno_circRNA_000823 | 0.0017652772  | 0.999536243    | 1.3795088 | Up         | Sense overlapping | chr1  | -      | XM_003749000   | LOC100909406 |
| rno_circRNA_002481 | 0.03794571    | 0.999536243    | 1.376932  | Up         | Exonic       | chr10 | +      | NM_022193      | Acaca      |
| rno_circRNA_011242 | 0.02515943    | 0.999536243    | 1.371773  | Up         | Exonic       | chr4  | -      | NM_022243      | Hibadh     |
| rno_circRNA_002472 | 0.03030252    | 0.999536243    | 1.367146  | Up         | Exonic       | chr2  | -      | NM_001107710   | Lrig2      |
| rno_circRNA_005241 | 0.014996901   | 0.999536243    | 1.358259  | Up         | Exonic       | chr8  | +      | NM_001170531   | Rasgrf1    |
| rno_circRNA_000974 | 0.01477705    | 0.999536243    | 1.344752  | Up         | Intergenic   | chr14 | -      | -              | -          |
| rno_circRNA_0000794 | 0.01290031    | 0.999536243    | 1.3446038 | Up         | Exonic       | chr1  | +      | NM_001191631   | Ppapdc1a    |
| rno_circRNA_003028 | 0.011975283   | 0.999536243    | 1.3268494 | Up         | Sense overlapping | chr11 | +      | NM_012791      | Dyrk1a     |
| rno_circRNA_011402 | 0.006979777   | 0.999536243    | 1.3170874 | Up         | Exonic       | chr4  | +      | NM_012491      | Add2       |
| rno_circRNA_006659 | 0.044238784   | 0.999536243    | 1.3087337 | Up         | Exonic       | chr17 | +      | NM_001110860   | Crem       |
| rno_circRNA_000234 | 0.029291408   | 0.999536243    | 1.2996294 | Up         | Sense overlapping | chr1  | -      | NM_019248      | Ntrk3      |
| rno_circRNA_008352 | 0.013719082   | 0.999536243    | 1.2951467 | Up         | Sense overlapping | chr3  | +      | XM_229993      | Csrnp3     |
| rno_circRNA_008240 | 0.006954501   | 0.999536243    | 1.2898375 | Up         | Exonic       | chr2  | -      | NM_001009653   | Mccc1      |

Abbreviations: FC, fold change; FDR, false discovery rate.
| circRNA         | P value  | FDR        | FC (abs) | Regulation | circRNA_type | chrom | strand | best_transcript | GeneSymbol |
|-----------------|----------|------------|----------|------------|--------------|-------|--------|----------------|------------|
| rno_circRNA_005771 | 0.022340653 | 0.999536243 | 1.5675106 | Down       | Sense overlapping | chr15 | –      | NM_001107281 | Klf12      |
| rno_circRNA_015072 | 0.037089011 | 0.999536243 | 1.5108253 | Down       | Exonic       | chr7   | +      | NM_134364     | Atp5b      |
| rno_circRNA_007681 | 0.007212199 | 0.999536243 | 1.4605777 | Down       | Exonic       | chr19  | –      | NM_001108439 | Large      |
| rno_circRNA_009424 | 0.03617954  | 0.999536243 | 1.3999727 | Down       | Exonic       | chr2   | –      | XM_215491    | Parp8      |
| rno_circRNA_007680 | 0.017723448 | 0.999536243 | 1.3805144 | Down       | Exonic       | chr19  | –      | NM_001108439 | Large      |
| rno_circRNA_013860 | 0.04207048  | 0.999536243 | 1.3662102 | Down       | Intergenic   | chr6   | +      | –             | –          |
| rno_circRNA_003481 | 0.016647763 | 0.999536243 | 1.35493  | Down       | Exonic       | chr12  | –      | XM_346914    | Pan3       |
| rno_circRNA_000004 | 0.046703097 | 0.999536243 | 1.3487289 | Down       | Exonic       | chr1   | –      | XM_341852    | Pr12       |
| rno_circRNA_003412 | 0.021512539 | 0.999536243 | 1.3329613 | Down       | Exonic       | chr11  | +      | NM_022301    | Pi4ka      |
| rno_circRNA_003995 | 0.005752417 | 0.999536243 | 1.3319859 | Down       | Exonic       | chr13  | +      | NM_001105988 | Plxna2     |
| rno_circRNA_011303 | 0.039665774 | 0.999536243 | 1.3007254 | Down       | Exonic       | chr4   | +      | XM_001072014 | Prdm5      |
| rno_circRNA_013859 | 0.018455014 | 0.999536243 | 1.2968118 | Down       | Intergenic   | chr6   | +      | –             | –          |
| rno_circRNA_005476 | 0.026259298 | 0.999536243 | 1.2952471 | Down       | Exonic       | chr15  | +      | NM_001108868 | Rnf31      |
| rno_circRNA_013858 | 0.020203693 | 0.999536243 | 1.2749468 | Down       | Intergenic   | chr6   | +      | –             | –          |
| rno_circRNA_002208 | 0.032135506 | 0.999536243 | 1.2720941 | Down       | Exonic       | chr10  | –      | XM_002727722 | Dnah9      |
| rno_circRNA_013861 | 0.022954888 | 0.999536243 | 1.2714683 | Down       | Intergenic   | chr6   | +      | –             | –          |
| rno_circRNA_007925 | 0.022035638 | 0.999536243 | 1.2712163 | Down       | Exonic       | chr19  | –      | NM_001107430 | Pdpr       |
| rno_circRNA_015071 | 0.004273977 | 0.999536243 | 1.2695557 | Down       | Exonic       | chr7   | +      | NM_134364    | Atp5b      |
| rno_circRNA_002774 | 0.006607468 | 0.999536243 | 1.2652571 | Down       | Exonic       | chr10  | +      | NM_001191653 | Tanc2      |
| rno_circRNA_010988 | 0.044088316 | 0.999536243 | 1.2624671 | Down       | Intergenic   | chr3   | –      | –             | –          |

Abbreviations: FC, fold change; FDR, false discovery rate.
circRNA levels among the samples. We found 104 differentially expressed circRNAs: 67 upregulated circRNAs and 37 downregulated circRNAs (fold change ≥ 1.2, \( P < 0.05 \)). The top 20 upregulated circRNAs and the top 20 downregulated circRNAs are summarized in Tables 1 and 2 based on fold change.

3.3 | qRT-PCR verification of selected circRNAs

We randomly selected 12 dysregulated circRNAs: six upregulated circRNAs (rno_circRNA_002481, rno_circRNA_008315, rno_circRNA_011402, rno_circRNA_014540, rno_circRNA_014890, and rno_circRNA_016761) and six downregulated circRNAs (rno_circRNA_003481, rno_circRNA_003511, rno_circRNA_003995, rno_circRNA_000367, rno_circRNA_005502, and rno_circRNA_011350) for verification in these samples. Similar trends were observed between the quantitative PCR and microarray analyses. Three selected upregulated circRNAs (Figure 3A) and three selected downregulated circRNAs (Figure 3B) were confirmed. Specific circRNA primers for quantitative PCR analysis are listed in Table 3.

3.4 | GO and KEGG pathway analysis

To explore how circRNAs regulate parental gene expression, we used GO analysis to annotate the host genes of the differentially expressed
circRNAs in biological processes, cellular components, and molecular function (Figure 4). GO analyses of the differentially expressed circRNAs revealed that these circRNAs might play critical roles in cellular metabolism, protein modification, and biosynthetic processes in CsA-induced cardiotoxicity. Finally, KEGG pathway enrichment analysis confirmed that seven pathways involved in metastasis were associated with the dysregulated circRNAs (Figure 5). The involved pathways were GABAergic synapse, morphine addiction, human papillomavirus infection, Hippo signaling pathway-multiple species, autophagy-other, HTLV-I infection, and Hippo signaling pathway. KEGG pathway analyses demonstrated that the differentially expressed circRNAs may be related to autophagy or the Hippo signaling pathway in CsA-induced cardiotoxicity.

### 3.5 Prediction of circRNA/miRNA interactions

In this study, two confirmed circRNAs (rno_circRNA_014540 and rno_circRNA_003481) were selected to identify potential miRNA targets. The potential miRNA targets of rno_circRNA_014540 include miR-329-5p, miR-376c-3p, and miR-877 (Figure 6A). For rno_circRNA_003481, the potential miRNA targets include miR-667-5p, miR-135b-5p, and miR-133c (Figure 6B).

### Table 3 Specific circRNA primers for quantitative PCR analysis

| Name                   | Sequence                      | PS (bp) |
|------------------------|-------------------------------|---------|
| GAPDH (RAT)            | F:5' GCTCTCTGCTCCTCCCTGTTCTA3' R:5' TGGTAACCAGGGCTCCGATA 3' | 124     |
| rno_circRNA_000367     | F:5' AACTCCTTTACCCCCACTACCA 3' R:5' CAATTTCTGTTCCATTGACCTG 3' | 73      |
| rno_circRNA_002481     | F:5' ACCATGTTGGAGTTGTGTGT 3' R:5' TAGACAGCTCCCTCCTAATGCCA 3' | 121     |
| rno_circRNA_003481     | F:5' CATCTCGTTTAAATGACTCTCGG 3' R:5' GGAATCTGTGAGGCTTGTGTC 3' | 100     |
| rno_circRNA_003511     | F:5' GAAGAACCTTGGGTCGAAGAC 3' R:5' GGAGAGAGCCTCCGTGTGG 3' | 87      |
| rno_circRNA_003995     | F:5' AGGGGAATACATCATCGCTGA 3' R:5' GTGGACAGCTCTGCAAACATTG 3' | 149     |
| rno_circRNA_005502     | F:5' TGTTGGAGACAGACAATGAGG 3' R:5' TGTTTGGTGTAGGAGAAGGG 3' | 53      |
| rno_circRNA_008315     | F:5' CTTATGAGTCGCAGAGCACC 3' R:5' TTGCAGAAACCCCTCGTGCCTTGC 3' | 95      |
| rno_circRNA_011350     | F:5' GGACAACTGAGATGTCTTCAAA 3' R:5' GAGGGTTCTCTCTGTTTCC 3' | 132     |
| rno_circRNA_011402     | F:5' GGTTAAACAATCTCCACCATCACC 3' R:5' GCCACACCACACACAGCCG 3' | 119     |
| rno_circRNA_014540     | F:5' TGGTTCCTCCCTCTTAAAGG 3' R:5' GGAATTTTCCACTCTCTGCTTA 3' | 54      |
| rno_circRNA_014890     | F:5' TTACCGTGCTGGCATTACAGG 3' R:5' TTGTGCCTCAGCAGACTGC 3' | 81      |
| rno_circRNA_016761     | F:5' TATCCAATGTTCCAGTGAGACC 3' R:5' ACGAATCTCGTGATGTTAAG 3' | 147     |

Abbreviation: PS, product size.

4 DISCUSSION

CsA is a frequently used immunosuppressive drug in the treatment of autoimmune diseases and graft rejection. However, many studies have suggested that CsA can induce cardiotoxicity (Rezzani et al., 2006; Rezzani et al., 2009), which limits its clinical applications. The histopathological changes associated with CsA-induced cardiotoxicity include alterations in the shape, size, and structure of the cardiac muscle in both the atria and ventricles (Ozkan et al., 2012; Rezzani, 2004). Selcoki et al. (2007) showed that CsA increased infiltrated inflammatory cells, disorganization of the myocardial fibers, and myocardial fibrosis in rats. Our previous studies found that CsA induced cardiomyocyte apoptosis in vivo and in vitro (Tang et al., 2011; Zhao et al., 2011). CsA-induced cardiotoxicity cannot be neglected. To date, few studies have examined the molecular mechanism of CsA-induced cardiotoxicity. Several experimental studies have shown that CsA-mediated cardiotoxicity may be due to mitochondrial damage (Jurado et al., 1998), oxidative stress (Rezzani, 2006; Sagiroglu et al., 2014), intracellular calcium overload (Florio et al., 2003; Tang et al., 2011; Zhao et al., 2011), and cell apoptosis (Rezzani et al., 2009; Tang et al., 2011). The molecular mechanism of CsA-induced cardiotoxicity still needs to be fully investigated.
FIGURE 4 GO analysis of increased and decreased mRNAs according to the values in the enrichment score under the theme of BP, CC, and MF. BP, biological processes; CC, cellular component; GO, Gene Oncology; MF, molecular function
In the present study, CsA-induced cardiotoxicity was evaluated by TUNEL assay, H&E staining, and transmission electron microscopy in the CsA and control groups. In the CsA group, the cardiomyocytes showed slight mitochondrial swelling, cellular apoptosis, and damage to the myofilaments. Our study further confirmed that a conventional dose of CsA could induce cardiomyocyte toxicity in rats.

CircRNAs, initially considered to be byproducts of linear mRNA splicing and “junk,” have been reported to be an evolutionally conserved class of RNA molecules in living organisms that are highly stable and abundantly expressed (Jeck et al., 2013; Memczak et al., 2013). Thus, they are better biomarkers and therapeutic targets than linear RNAs in clinical applications (Kun-Peng et al., 2018). Recently, emerging data have shown that circRNAs play an essential role in the initiation and development of cardiovascular diseases and are becoming an active research area (Li et al., 2018). Heart-related circRNA (HRCR) could act as a miR-223 sponge to regulate the expression of miR-223 and protect against cardiac hypertrophy and heart failure (Wang et al., 2016). Furthermore, Zhao et al. (2017) found that hsa_circ_0124644 was upregulated significantly in patients compared with healthy controls and could be employed as a potential diagnostic biomarker for coronary artery disease. CircRNAs were also reported to be involved in cardiomyocyte death and myocardial infarction (MI). Geng et al. (2016) reported that the circRNA Cdr1as could regulate the expression of its target genes (PARP and SP1) as a powerful miR-7a sponge in cardiomyocytes and promote MI in mice. However, the mechanisms by which circRNAs function in CsA-induced cardiotoxicity have not been fully elucidated. Here, we combined a microarray assay, qRT-PCR verification, and bioinformatics to elucidate the molecular mechanisms of CsA-induced cardiotoxicity. Overall, 104 circRNAs were detected. We identified 67 upregulated and 37 downregulated circRNAs and summarized their general characteristics. Thus, our study could provide a comprehensive understanding of circRNAs in CsA-induced cardiotoxicity.

To confirm the results of the microarray analysis, we performed qRT-PCR analysis. We randomly selected 12 dysregulated circRNAs—six upregulated circRNAs and six downregulated circRNAs—for verification in these samples. Three selected upregulated circRNAs and three selected downregulated circRNAs were confirmed. GO analysis was performed to annotate genes targeted by the differentially expressed circRNAs in the domains of cellular metabolism, protein modification, and biosynthetic processes. Finally, KEGG pathway enrichment analysis indicated that seven pathways involved in metastasis were associated with the dysregulated circRNAs, suggesting that these pathways might significantly contribute to CsA-induced cardiotoxicity. KEGG pathway analyses showed that the differentially expressed circRNAs may be related to autophagy or the Hippo signaling pathway in CsA-induced cardiotoxicity.

Increasing evidence has indicated that circRNAs can absorb miRNAs like a sponge and are critical in regulating miRNA levels. Their interactions with disease-associated miRNAs demonstrate that circRNAs play a crucial role in disease regulation (Hansen, Jensen, et al., 2013). A landmark study from Hansen et al. showed that the circRNA ciRS-7 (circRNA sponge for miR-7), also known as Cdr1as, has over 70 miR-7 binding sites and could inhibit the activity of miR-7, providing the first evidence that circRNAs can function as miRNA sponges (Hansen, Jensen, et al., 2013; Hansen, Kjems, et al., 2013). In this study, we found many dysregulated circRNAs in rats with CsA-induced cardiotoxicity and predicted the
circRNA/miRNA interaction with Arraystar's homemade miRNA target prediction software based on TargetScan and miRanda. For example, among the identified potential circRNA/miRNA interactions, the upregulated circRNA rno_circRNA_014540 can act as a sponge of miR-376C-3p. Overexpression of hsa-miR-376c-3p promoted cell apoptosis in vitro and inhibited gastric tumor growth in vivo (Tu et al., 2016). The circRNA rno_circRNA_014890 can absorb miR-298-5p, and this miRNA can improve myocardial apoptosis after MI through its target gene BAX (Zhang et al., 2018). The downregulated circRNA rno_circRNA_003481 can bind miR-135b-5p. Suppression of miR-135b-5p can protect against myocardial ischemia/reperfusion injury by activating the JAK2/STAT3 signaling pathway in mice under inhalation anesthesia with sevoflurane (Xie et al., 2017). The downregulated circRNA rno_circRNA_000367 can bind miR-30c-2-3p and miR-30c-1-3p. The miR-30 family is highly expressed in H9c2 cardiomyocytes. The upregulated circRNA rno_circRNA_002481 can bind miR-150-5p, and miR-150-5p can inhibit the progression of myocardial fibrosis by targeting early growth response 1 (EGR1) in myocardial cells (Shen et al., 2019). The upregulated circRNA rno_circRNA_014890 can absorb miR-298-5p, and this miRNA can improve myocardial apoptosis after MI through its target gene BAX (Zhang et al., 2018). The downregulated circRNA rno_circRNA_003481 can bind miR-135b-5p. Suppression of miR-135b-5p can protect against myocardial ischemia/reperfusion injury by activating the JAK2/STAT3 signaling pathway in mice under inhalation anesthesia with sevoflurane (Xie et al., 2017). The downregulated circRNA rno_circRNA_000367 can bind miR-30c-2-3p and miR-30c-1-3p. The miR-30 family is highly expressed in
cardiomyocytes and has been shown to act as an autophagy inhibitor in some biological processes (Zhang et al., 2015). Doxorubicin caused downregulation of the miR-30 family through GATA-6 in doxorubicin-induced cardiomyocyte injury, while overexpression of miR-30 protected cardiomyocytes from doxorubicin-induced apoptosis (Roca-Alonso et al., 2015). Our study shows that circRNAs may act as miRNA sponges to regulate cardiac autophagy and apoptosis processes in CsA-induced cardiotoxicity. However, due to the limited known functions of circRNAs and miRNAs, more studies are needed to determine their relationships and understand the biological and molecular mechanisms of circRNAs in CsA-induced cardiotoxicity.

In summary, our study is the first to explore the expression profiles of circRNAs in a small number of CsA-treated samples and paired normal heart tissue samples based on a small sample size. We performed a comprehensive analysis of the dysregulated circRNAs during CsA-induced cardiotoxicity. Therefore, our study could help elucidate the molecular mechanisms of CsA-mediated cardiotoxicity. We speculate that circRNAs may serve as promising diagnostic or prognostic biomarkers and potential therapeutic targets for patients with CsA-induced cardiotoxicity; this possibility needs to be studied further.

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CONFLICT OF INTEREST
The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT
The data used to support the findings of this study are available from the corresponding author upon request.

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