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Short communication

Circular RNA expression alterations in extracellular vesicles isolated from murine heart post ischemia/reperfusion injury

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ABSTRACT

Background: Increasing studies indicated the involvement of extracellular vesicles (EVs) in cardiovascular diseases. However, the role of circular RNAs (circRNAs) in cardiac EVs (cEVs) during ischemia/reperfusion (I/R) injury remain unclear.
Methods: We isolated the cEVs from I/R injured hearts and performed RNA sequencing (RNA-seq) to identify the profile of circRNA in cEVs and investigated their potential roles in I/R pathological process.
Results: Cardiac I/R induced a significantly elevated release of EVs in heart within 24 h. RNA-seq of cEVs identified 185 significantly differentially expressed (DE) circRNAs including 119 down-regulated and 66 up-regulated circRNAs in I/R group compared with the sham. GO and pathway analysis showed that these DE-circRNAs were associated with protein binding and kinase activator activity and mainly involved in the metabolic process. The circRNA-miRNA analysis exhibited the broad potentials of the DE-circRNAs to regulate target genes by acting on the miRNAs.
Conclusions: These findings revealed for the first time the specific expression pattern of circRNAs in EVs derived from sham and I/R heart tissues and provided some potential targets and pathways involving in I/R injury which may provide important evidences for the role of both circRNA and EVs in the pathology of cardiac I/R.

1. Introduction

Myocardial ischemia/reperfusion (I/R) injury is one of the most prevalent cardiac disorders which can lead to pathophysiological changes including arrhythmias, myocardial stunning and lethal myocardial reperfusion injury [1]. However, the mechanisms underlying I/R injury remain poorly understood. Extracellular vesicles (EVs) are a class of heterogeneous, nanoscale, cell-derived membranous vesicles and exist in various biological fluids [2]. Previous studies demonstrated the vital roles of EVs in numerous biological functions. Most of these investigations focused on the role of EVs/exosomes isolated from serum/plasma or cell culture medium. It is noteworthy that recent studies confirmed the presence of EVs in the diabetic and infarcted heart [3,4]. However, reports on EVs isolated from heart subjected to I/R remain scarce.

Circular RNAs (circRNAs) are covalently closed RNAs with a feature of abundance, stability and evolutionary conservation [5,6]. Increasing evidences indicated the implication of circRNAs in cardiovascular diseases including myocardial infarction (MI) and heart failure [7]. CircRNAs are enriched and stable in EVs [8] and can be delivered by EVs acting as gene regulators in mammals [9,10]. We therefore speculated that circRNAs in EVs may play important roles in I/R injured conditions. The present study was conducted to identify the trait of circRNAs in EVs from I/R injured heart and provided novel targets for further researches on cardiac I/R injury.

2. Materials and methods

2.1. Establishment of myocardial I/R model

Experiments were performed using 8-week-old specific pathogen-free male C57BL/6 mice (22 ± 1 g) from SLAC Laboratory Animal co., Ltd. (Shanghai, China).

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Myocardial I/R models were set up by ligating the LAD coronary artery for 45 min before reperfusion. All animal procedures were approved by the Institutional Animal Care and Use Committee of Tongji University (Number: TJLAC-018-030).

2.2. EVs Isolation and assessment

The isolation method of EVs from heart was referred to previous studies [4,11] with some modifications (Supplemental Fig. 1). The fresh-isolated EVs were fixed, stained with 2% phosphotungstic acid and then detected using a transmission electron microscope (TEM; Hitachi, HT7700). Size distribution and the concentration of EVs were measured with the ZetaView® NTA technique by Particle Metrix. Confocal imaging was used to confirm the uptake of cEVs by primary cardiomyocytes.

2.3. RNA library construction and circRNA sequencing

Total RNA was isolated from EVs using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). The rRNA in each sample was depleted using the Ribo-Zero

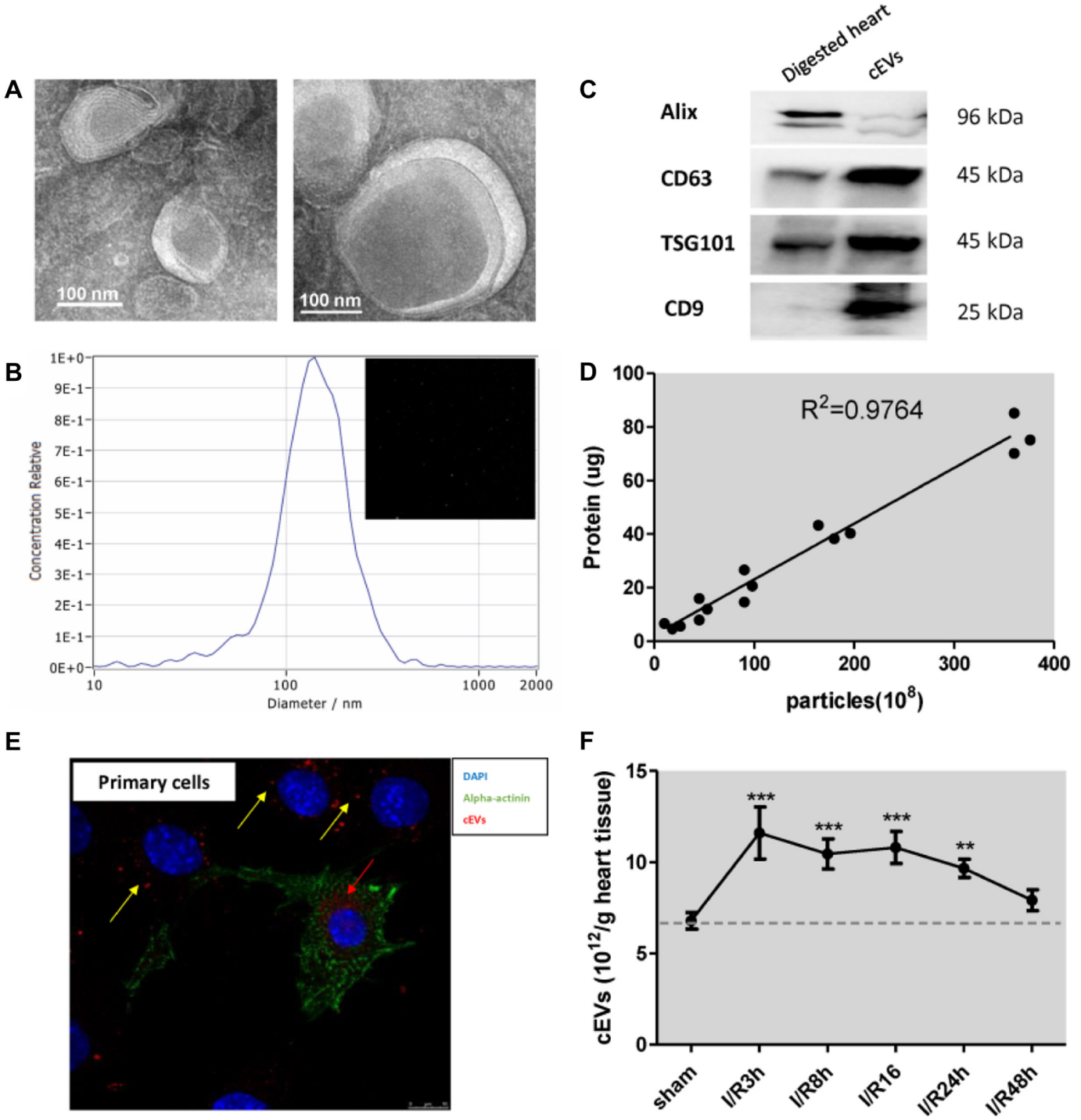


Fig. 1. Characterization of cEVs. (A) A representative TEM image of cEVs. Both small (left) and large (right) EVs were detected in the same sample (bar = 100 nm). (B) Particle size distribution of cEVs was measured using nanoparticle tracking analysis. (C) Protein immunoblots of cEVs, including four typical exosomal markers (CD63, CD9, Alix, and Tsg101). (D) Correlation between particle number and total protein content in serially diluted samples of cEVs. (E) Confocal imaging revealed the recipient cells of the cEVs. Red DiI-labeled cEVs were showed around the nuclei (blue) in the primary cardiomyocytes (red arrow) and non-cardiomyocytes (yellow arrows). (F) Quantification of EVs isolated from I/R injured heart at different time points. The experiment was repeated 3 times and 3 mice in each group. **, $P < 0.01$; ***, $P < 0.001$ compared to the sham group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Magnetic Gold Kit (Epicentre, Inc., Madison, WI, USA) and then RNA libraries were constructed using TruSeq Stranded Total RNA Library Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's directions. The RNA libraries were denatured as single-strand DNA, captured on Illumina Flow Cells (Illumina, San Diego, CA, USA), amplified in situ as clusters and then sequenced with 150 cycles on HiSeq 4000 sequencing system (Illumina, San Diego, CA, USA).

2.4. Statistical analysis

All data were expressed as the mean ± standard deviation (SD) and analyzed using Prism 5.0 (GraphPad Software Inc.). The unpaired Student's *t*-test was used for comparison between two groups. Multiple groups comparison was performed with one-way analysis of variance (ANOVA), followed by the Bonferroni test. Correlation between the particle number and total protein content was assessed using Pearson's linear correlation analysis. A P-value < 0.05 was considered statistically significant.

For expanded detailed information, refer to the supplemental methods.

3. Results

3.1. I/R injury contributed to the release of the EVs in heart tissues

The cEVs exhibited a typical cup-shaped morphology. Both small EVs (around 100 nm) and large EVs (around 200 nm) were captured under TEM (Fig. 1A). The particle size of cEVs was mainly around 130 nm in diameter (Fig. 1B). As shown by Fig. 1C, typical exosome markers including CD63, CD9, Alix, and TSG101 were expressed in cEVs. Moreover, a linear association was found in cEVs between particle number and total protein content (Fig. 1D). Confocal imaging confirmed the transfer of cEVs into the primary cardiomyocytes and non-cardiomyocytes (Fig. 1E).

Next we found the content of cEVs increased significantly 3 h after I/R injury and maintained at high levels even 24 h after reperfusion compared to that in the sham group (Fig. 1F).

3.2. Differential circRNAs profile in cEVs after I/R injury

The number of identified circRNAs was shown in Supplemental table 1. A total of 185 significantly differentially expressed (DE)-circRNAs were identified. Detailed information regarding the up- and down- regulated circRNAs (top 3) was summarized in Supplemental table 2. The heat map and volcano plot of the DE-circRNAs was presented in Fig. 2A and B. Among the 185 DE-circRNAs, 119 circRNAs were down-regulated and 66 circRNAs were up-regulated in I/R group compared with sham group, and 42 of them were newly identified circRNAs (novel circRNAs) (Fig. 2C). The DE-circRNAs was predominantly <2000 nucleotides (nt) (Fig. 2D) and identified as exonic circRNAs (Fig. 2E).

3.3. Predicted pathways of differentially expressed circRNAs

Significantly associated pathways of up-regulated circRNAs included EPH-Ephrin signaling and vesicle biogenesis and budding (Fig. 2F). Down-regulated circRNAs involved pathways (top 3) comprised SMAD2/SMAD3:SMAD4 heterotrimer regulates transcription, signaling by TGF-beta receptor complex and endogenous sterols (Fig. 2F). GO function analysis were shown in Supplemental Fig. 2. Moreover, sequencing analysis confirmed the circRNA profile of cEV in sham operated mice. We also provided the functions and pathways prediction of the high-expressed circRNAs (junction reads ≥ 2) in Supplemental Fig. 3.

3.4. Broad potentials of the circRNAs-miRNA interaction involved in I/R injury

CircRNAs can act as miRNA sponges or competitive endogenous RNAs (ceRNAs) to regulate the gene expression in recipient cells. In the present study, a circRNA-miRNA network constructed with 5 circRNAs (including mmu-circ008351, mmu-circ001007, mmu-circ008228, mmu_circ_0001336 and mmu-circ007845) and each top 5 predicted miRNA targets were showed in Fig. 2G.

3.5. Validation of the circRNA-seq data

To confirm the circRNAs in cEVs, five DE-circRNAs including 3 up-regulated and 2 down-regulated circRNAs were randomly selected and validated. RT-qPCR with divergent primers confirmed the significant differences of the selected circRNAs (Fig. 2H). PCR using divergent primers produced a single distinct band only in cDNA samples rather than gDNA samples implying the presence of back-splicing junctions but not genomic rearrangements (Fig. 2I).

4. Discussion

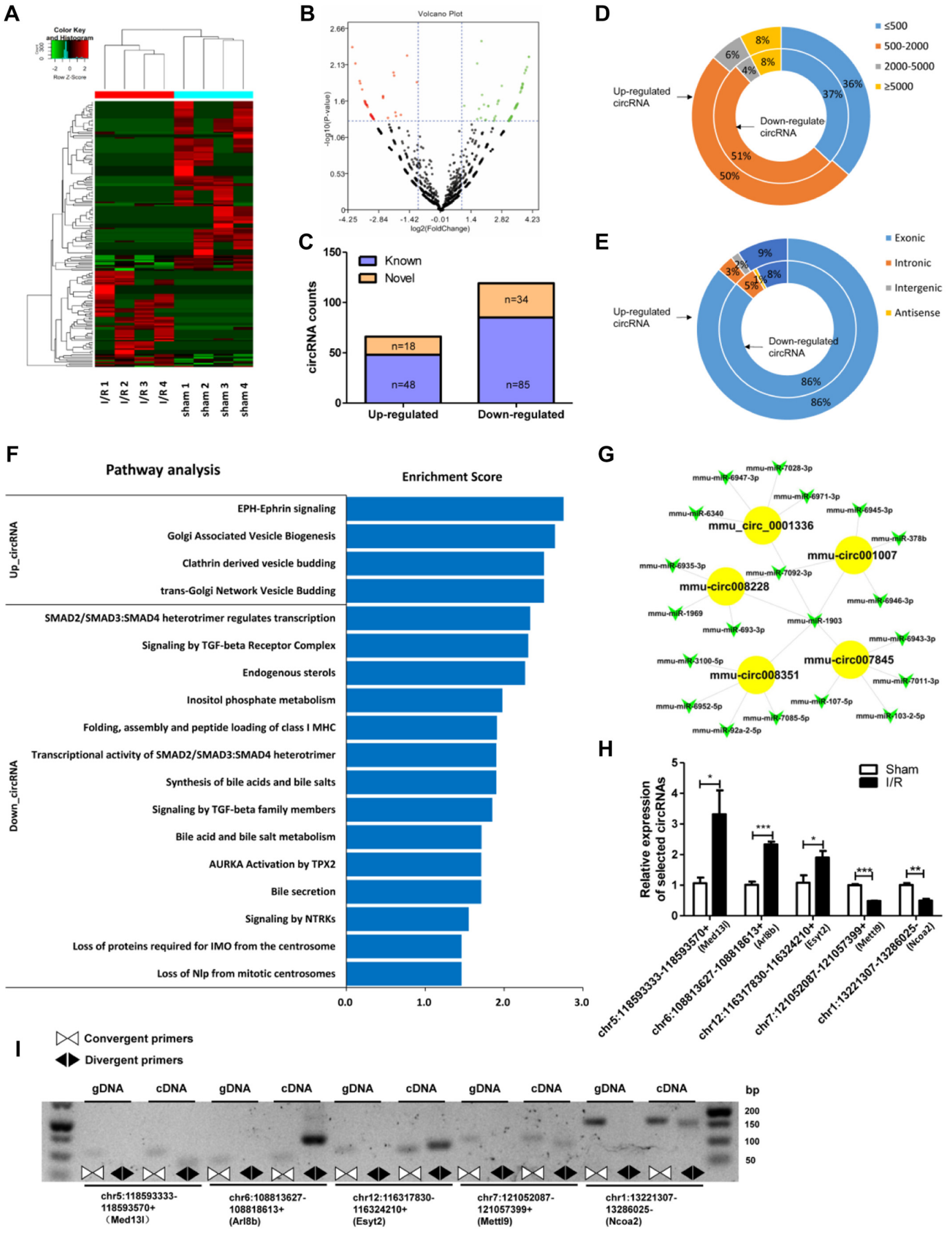
Recently, EVs have received a tremendous amount of attention due to their functions to mediate cell-to-cell communication by transferring multiple RNA species. We firstly exhibited the specific circRNA expression profile in EVs derived from I/R injured heart tissues and provided some potential targets and pathways of circRNA in cEVs involving in the I/R injury. These results provide important clues to reveal the role of both circRNA and EVs in the pathologic process of cardiac I/R.

Reperfusion induces further damage on the myocardium, so called reperfusion injury. The present study found I/R induced increased release of EVs during the first 24 h. As the blood resupplied, the damaged cells were gradually relieved, which possibly contributed to the reduction of cEVs to near baseline levels at 48 h after reperfusion. CircRNAs, as a class of non-coding RNA molecules which are enriched and stable in EVs/exosomes have been confirmed to participate in a variety of pathophysiological processes. Our study identified a total of 185 differentially expressed circRNAs in cEVs post I/R injury which may participate in the development of I/R injury. EPH-Ephrin signaling and vesicle biogenesis and budding were found to be significantly associated with the up-regulated circRNAs in EVs of I/R-injured heart. Previous report revealed the importance of Eph/Ephrin signaling pathway in injury and inflammation [12]. Thus, these up-regulated circRNAs may participate in inflammatory regulation after cardiac I/R injury. The vesicle biogenesis and budding pathways are significantly correlated to the up-regulated circRNAs, which is consistent with our result of the elevated release of cEVs post-I/R injury. The down-regulated circRNAs associated pathways, such as, SMAD2/SMAD3:SMAD4 heterotrimer regulates transcription, signaling by TGF-β receptor complex and endogenous sterols all play important roles in initiating reperfusion-induced pathological events. TGF-β/Smad signaling is a principal mediator of the fibrotic response [13]. Cholesterol, as one of the endogenous sterols contributes to cardioprotection of I/R injury [14]. Thus, the down-regulated circRNAs are possibly involved in regulating fibrotic response and cardiac dysfunction. These novel mechanisms related to the DE-circRNAs in cEVs post I/R need further investigation.

MiRNAs have numerous biological functions and participate in various diseases. Recent studies highlighted the role of circRNAs as miRNA sponges to regulate the biological processes. Our circRNA-miRNA network analysis showed that one circRNA can act on many miRNAs and one miRNA can also combine with multiple circRNAs, which suggested that circRNAs in cEVs may exert important roles via interaction with miRNAs. Further studies are expected to explore the interaction of specific circRNA-miRNAs and investigate their roles in I/R pathological process.

5. Conclusions

The present study firstly identified a group of specific DE-circRNAs in EVs from the murine heart post I/R injury. GO and


A
Color Key and Histogram
Row Z-Score
I/R 1
I/R 2
I/R 3
I/R 4
sham 1
sham 2
sham 3
sham 4
B
Volcano Plot
-log10(P-value)
log2(FoldChange)
C
Known
Novel
circRNA counts
n=18
n=48
n=34
n=85
Up-regulated
Down-regulated
D
≤500
500-2000
2000-5000
≥5000
Up-regulated circRNA
Down-regulate circRNA
8%
6%
4%
8%
36%
37%
51%
50%
E
Exonic
Intronic
Intergenic
Antisense
Up-regulated circRNA
Down-regulated circRNA
9%
2%
3%
1%
5%
8%
86%
86%
F
Pathway analysis
Enrichment Score
Up_circRNA
EPH-Ephrin signaling
Golgi Associated Vesicle Biogenesis
Clathrin derived vesicle budding
trans-Golgi Network Vesicle Budding
Down_circRNA
SMAD2/SMAD3:SMAD4 heterotrimer regulates transcription
Signaling by TGF-beta Receptor Complex
Endogenous sterols
Inositol phosphate metabolism
Folding, assembly and peptide loading of class I MHC
Transcriptional activity of SMAD2/SMAD3:SMAD4 heterotrimer
Synthesis of bile acids and bile salts
Signaling by TGF-beta family members
Bile acid and bile salt metabolism
AURKA Activation by TPX2
Bile secretion
Signaling by NTRKs
Loss of proteins required for IMO from the centrosome
Loss of Nlp from mitotic centrosomes
G
mmu_circ_0001336
mmu-circ001007
mmu-circ008228
mmu-circ008351
mmu-circ007845
H
Sham
I/R
Relative expression of selected circRNAs
chr5:118593333-118593570+ (Med13l)
chr6:108813627-108818613+ (Arl8b)
chr12:116317830-116324210+ (Esyt2)
chr7:121052087-121057399+ (Mettl9)
chr1:13221307-13286025- (Ncoa2)
I
Convergent primers
Divergent primers
gDNA
cDNA
bp
200
150
100
50
chr5:118593333-118593570+ (Med13l)
chr6:108813627-108818613+ (Arl8b)
chr12:116317830-116324210+ (Esyt2)
chr7:121052087-121057399+ (Mettl9)
chr1:13221307-13286025- (Ncoa2)

pathway analysis showed these DE-circRNAs were mainly involved in the metabolic process. The identified circRNAs might serve as novel targets for further potential to prevent myocardial I/R injury.

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Declaration of competing interest

The authors have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcard.2019.08.024.

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Fig. 2. Differential expression of circRNAs in cEVs between sham and I/R group. (A) Clustered heat map of the DE-circRNAs. (B) Volcano plot of the DE-circRNAs. Significantly up- and down-regulated circRNAs were shown as red and green dots respectively. (C) The number of up- and down-regulated circRNAs in cEVs of I/R injured mice vs. the sham group. 42 novel circRNAs were identified (light red). (D) The distribution of DE-circRNAs based on the length of nuclear acids. (E) The distribution of DE-circRNAs based on their categories of circle components. The inner rings in (D-E) exhibits down-regulated circRNAs, and the outer rings in (D-E) exhibits down-regulated circRNAs. (F) Pathway analysis of the DE-circRNAs. (G) The circRNA-miRNA network constructed with 5 selected circRNAs and the top 5 predicted miRNA targets. Yellow cycles represent circRNAs and green arrows represent miRNAs. (H) Relative expressions of 5 DE-circRNA in cEVs were detected by qPCR. (I) PCR amplifcation results for the 5 selected circRNAs in genomic DNA and cDNA samples. Host gene names were indicated in brackets. Divergent primers and convergent primers were used for circRNA validation. The result of qPCR indicated mean ± SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to sham group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)