Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;48:1579-1594
DOI: 10.1159/000492280
Published online: August 1, 2018
Accepted: July 22, 2018

Original Paper

Microarray Analysis of Differentially Expressed Profiles of Circular RNAs in a Mouse Model of Intestinal Ischemia/Reperfusion Injury with and Without Ischemic Postconditioning

Dongcheng Feng[a] Zhenlu Li[a] Guangzhi Wang[a] Jihong Yao[b] Yang Li[a] Wasim Qasim[a] Yongfu Zhao[a] Xiaofeng Tian[a]

[a]Department of General Surgery, The Second Hospital of Dalian Medical University, Dalian, [b]Department of Pharmacology, Dalian Medical University, Dalian, China

Key Words
Intestinal ischemia/reperfusion • Ischemic postconditioning • Circular RNA • Microarray • Bioinformatics analysis

Abstract
Background/Aims: Ischemic postconditioning (iPoC) represents a promising strategy to mitigate ischemia/reperfusion (I/R) injury of the intestine, yet the mechanisms of this treatment remain to be elucidated. Circular RNAs (circRNAs), a novel class of endogenous non-coding RNAs, have recently been recognized as important regulators of gene expression and pathological processes. Here, we aimed to investigate the expression patterns of circRNAs after intestinal I/R with and without iPoC and, furthermore, to explore the potential mechanisms of iPoC in relation to the differentially expressed circRNAs. ***Methods:*** The global circRNA and mRNA expression profiles in mouse intestinal mucosa were initially screened by microarray (n = 3 per group) and quantitative real-time PCR was used to validate the expression pattern of circRNAs and mRNAs. Bioinformatics analysis including Gene ontology, KEGG pathway analysis, microRNA binding sites identification and circRNA-miRNA-mRNA network construction were utilized for in-depth mechanism exploration. ***Results:*** There were 4 up- and 58 downregulated circRNAs as well as 322 up- and 199 downregulated mRNAs in the intestinal I/R group compared with the sham group, whereas compared with I/R, iPoC treatment significantly upregulated 12 circRNAs and 129 mRNAs and downregulated 21 circRNAs and 174 mRNAs. The expression levels of a randomly selected set of 6 circRNAs and 5 mRNAs were successfully validated by qRT-PCR. Through a systematic comparison of the direction of circRNA expression changes in all groups, we identified two circRNAs, circRNA_012412 and circRNA_016863, that may be closely associated with the protective

Xiaofeng Tian
Department of General Surgery, The Second Hospital of Dalian Medical University
Dalian 116023 (China)
Tel. +86-0411-86110010, Fax +86-0411-86110010, E-Mail txfdl@dmu.edu.cn

mechanisms of iPoC. Finally, four possible circRNA_012412/circRNA_016863-miRNA-mRNA pathways were predicted, which may play important roles in endogenous protective signaling in iPoC. ***Conclusions:*** This study was the first to comprehensively delineate the expression profiles of circRNAs in a mouse model of intestinal I/R and iPoC and provides novel clues for understanding the mechanisms of iPoC against intestinal I/R injury.

Introduction

Reperfusion injury of the intestine occurs in clinical circumstances including mesenteric artery occlusion, small bowel transplantation, abdominal vascular surgery procedures, hemorrhagic shock, trauma, and sepsis [1, 2]. Although acute intestinal ischemia accounts for only 1% - 2% of all surgical emergencies, the survival rate of in-hospital patients is generally less than 50% [3]. A limited number of pharmacological approaches have been shown to be beneficial in animal studies during the past few decades, but none have demonstrated effectiveness in clinical practice. Therefore, it is clinically very important to find new strategies to reduce intestinal ischemia/reperfusion (I/R) injury and improve patients' outcome.

Ischemic postconditioning (iPoC) refers to a controlled reperfusion process that is performed in the form of a series of repetitive cycles of reperfusion and ischemia with specific timing, applied immediately in the initial reperfusion period after ischemia to increase the resistance of organs against I/R injury. In 2003, Zhao *et al.* first used iPoC as a feasible method to mitigate myocardial infarct size induced by I/R injury [4]. To date, extensive preclinical studies have revealed that iPoC is a safe and clinically applicable approach for limiting I/R injury of several organs [5-8], including the intestine [9, 10]. However, the translation of iPoC results from animal models to a clinical setting has not achieved complete success due to the lack of a standard and optimized iPoC algorithm in clinical trials [11]. Thus, a better understanding of the protective mechanisms underlying iPoC would be helpful in the development of novel and practical therapies for reperfusion injury of the intestine.

With the development of high-throughput technology such as microarrays, the global changes in transcript patterns during special pathophysiologic processes have been widely studied. We have previously identified the expression profiles of microRNAs (miRNAs) in the intestine following I/R injury (Gene Expression Omnibus [GEO] no. GSE83701), supporting the importance of non-coding RNA (ncRNA) regulation in the pathogenesis of intestinal I/R. Recently, a newly discovered type of endogenous ncRNAs, termed circular RNAs (circRNAs), has become a hotspot in RNA research [12]. Unlike linear RNAs, circRNAs form covalently closed loop structures without 5′ or 3′ ends, which increases their stability by conferring resistance to exonuclease degradation [13, 14]. Furthermore, many circRNAs have tissue- and disease-dependent abundance and display conservation across different species [15-17], making them ideal biomarkers for diagnosis and potential therapeutic targets in the future [18]. Increasing evidence has indicated that circRNAs can function as effective sponges for miRNAs, in turn regulating downstream mRNA expression and ultimately participating in various physiological or disease processes [19-21].

Previous research has identified several miRNAs associated with cardioprotection by iPoC [22]. Considering the indispensable role of circRNAs in competing endogenous RNA (ceRNA) networks, we postulated that circRNA alteration may be critically involved in the pathogenesis of intestinal I/R and may, in particular, contribute to iPoC-mediated protection against intestinal I/R injury. In the present study, we investigate the differentially expressed profiles of circRNA in a mouse model of intestinal I/R with or without iPoC and identify circRNAs associated with intestinal protection induced by iPoC through a combination of microarray screening, quantitative real-time PCR (qRT-PCR) verification and a stepwise bioinformatics analysis. Our findings may provide a novel perspective for understanding the protective mechanisms of iPoC and may offer potential candidates for future mechanistic studies.

Materials and Methods

Experimental animals

Eight-week-old male C57BL/6 mice were purchased from the Animal Center of Dalian Medical University (Dalian, China) and were housed in a temperature-controlled room on a 12 h/12 h light/dark cycle. All mice received a standard chow pellet diet and acclimated for 1 week before experimentation. Food was removed 12 h prior to the surgical procedure, but all animals had free access to water. Each experiment was started at the same time of day to avoid the effects of circadian rhythms. This study was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal protocols were approved by the Institutional Ethics Committee of Dalian Medical University (Dalian, China).

Intestinal I/R model construction and iPoC treatment

An intestinal I/R model was established by superior mesenteric artery (SMA) occlusion as described previously by us [23]. Briefly, all the mice were anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg body weight). After abdominal laparotomy, the SMA was temporarily occluded using an atraumatic microvascular clip to cause ischemia. After 45 min, the clip was gently removed to allow reperfusion. Ischemia was recognized by the pulselessness and/or pale color of the small intestine. The return of the pulse and the re-establishment of the pink color were assumed to indicate valid reperfusion of the intestine.

The mice were randomly allocated into three groups (n = 8 in each group): (1) a sham group, in which mice underwent laparotomy and SMA isolation without occlusion; (2) an I/R group, in which mice underwent 45 min of mesenteric ischemia followed by 4 h of reperfusion without iPoC; and (3) an iPoC group, in which iPoC was induced by six alternating cycles of 10 s of reperfusion followed by 10 s of reocclusion, beginning immediately at the onset of reperfusion; this procedure has been demonstrated to be the optimal iPoC algorithm in a small animal model of intestinal I/R injury [24].

Intestinal histological analysis and serum TNF-α, IL-6 and I-FABP measurement

To determine the level of tissue injury, we collected specimens of the ileum from the different groups of animals, fixed the samples, and embedded them in paraffin. The tissue was cut into 4 μm-thick sections, which were then stained with hematoxylin and eosin (H&E). Microscopic histological damage to the intestinal mucosa was evaluated independently by two pathologists who were blinded to the experimental treatments. The degree of injury was graded based on Chiu's scoring system [25]. The serum levels of I-FABP, IL-6 and TNF-α were assayed using an enzyme-linked immunosorbent assay (ELISA) kit (R&D systems) according to the manufacturer's instructions.

Total RNA extraction and quality control

Total RNA was isolated from each intestinal tissue sample using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The purity and concentration of RNA samples were measured by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA integrity and genomic DNA (gDNA) contamination were tested by electrophoresis on a denaturing agarose gel.

RNA labeling and hybridization

Three samples from each group were randomly selected for microarray studies. The sample preparation and microarray hybridization were performed based on Arraystar's standard protocols, and each transcript was represented using 1-5 probes to improve the statistical confidence. For the circRNA study, total RNA from each sample was first digested with RNase R (Epicentre, Madison, USA) to remove linear RNAs and enrich circRNAs. Then, the enriched circRNAs were amplified and transcribed into fluorescent cRNA utilizing a random priming method with an Arraystar Super RNA Labeling Kit (Arraystar, Rockville, USA). The fluorescently labeled cRNAs were hybridized onto the Arraystar Mouse circRNA Array V2.0 (8x15K). After washing the slides, the arrays were scanned by the Agilent Scanner G2505C. For the mRNA study, a whole mouse genome mRNA microarray (Agilent Technology, Santa Clara, CA, USA) was routinely carried out. Briefly, sample labeling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol.

Microarray data analysis

Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the acquired array images. Quantile normalization of raw data and subsequent data processing were performed using the R software limma package. After quantile normalization of the raw data, low-intensity filtering was performed, and the circRNAs that had at least 3 out of 9 samples with flags in "P" or "M" ("All Targets Value") were retained for further analysis. The significantly differentially expressed circRNAs/mRNAs were selected according to the fold change cut-off (fold change≥1.5) and *P*-value < 0.05 or by volcano plot filtering. Hierarchical clustering was performed to show any distinguishable circRNA/mRNA expression patterns among the samples.

Gene Ontology (GO) and pathway analysis

We conducted GO analysis (http://www.geneontology.org) to construct meaningful annotation of genes and gene products based on a wide variety of organisms. The gene functions encompassed biological processes (BP), cellular components (CC) and molecular functions (MF). The $-\log_{10}$ (*P*-value) of any given GO term denoted its enrichment score, representing the significance of its enrichment among differentially expressed genes. We also performed KEGG pathway analysis to harvest pathway clusters based on existing knowledge of the molecular interaction and reaction networks from differentially regulated gene profiling. The $-\log_{10}$ (*P*-value) represented the enrichment score, showing the significance of the correlation within the putative pathway.

Quantitative real-time polymerase chain reaction(qRT-PCR) validation

Total isolated RNAs from each group were reverse transcribed using a PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) according to the manufacturer's instructions. The expression levels of the circRNAs and mRNAs were evaluated by qPCR using a SYBR Green PCR kit (TaKaRa, Dalian, China). Specific divergent primers (instead of commonly used convergent primers) were designed to amplify the circRNA-specific back-splice junctions, while primers for mRNAs were routinely applied. The circRNA sequence results were acquired from the database "circBase" (http://circbase.mdc-berlin.de). The relative expression levels of circRNAs and mRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method by normalizing the expression against that of the housekeeping gene β-actin. Both the target and the reference were amplified in triplicate wells. All primers were designed, verified and synthesized by GenePharma (Shanghai, China), and the sequences are listed in Table 1.

Annotation of circRNA/miRNA interactions

The circRNA/miRNA interactions were predicted with Arraystar's home-made miRNA target prediction software, which is based on the previously established online analytical tools TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/). The targeted miRNAs were ranked according to their miRNA support vector regression (mirSVR) scores, and the five miRNAs with the highest mirSVR scores were identified for further analysis.

Prediction for circRNA miRNA-mRNA pathways

To construct circRNA-miRNA-mRNA networks, we used the Arraystar software to search MREs on circRNA_012412 and circRNA_016863 and selected the top 5 putative target miRNAs according to seed match sequences. Next, target genes of the top 5 miRNAs for each circRNA were predicted based on the online database TargetScan coupled with miRDB (http://mirdb.org/miRDB/). According to the ceRNA regulatory theory, the changes in circRNA and mRNA should be in the same direction. Aiming

Table 1. Primer sequences used for qRT-PCR analysis of circRNA and mRNA levels

Name	Primer F (5'-3')	Primer R (5'-3')
mmu_circRNA_007736	GAGAAGATGGGAGAATTTACTGG	GGGTGATTTGCTAGAGTTTCG
mmu_circRNA_006664	GTCTGAGACTTGGGCCTCC	GCCGAAGAGTGACACGCA
mmu_circRNA_018654	CTGAACGCCACTTGTCCCT	CTGTGATGCCCTTAGATGTCC
mmu_circRNA_011775	GCTTTGCAACCATACTCCCC	CCTGCGGCTTAATTTGACTCA
mmu_circRNA_015231	GCCCTAACCACGTATAAACTCA	GGAACACTGGACGGGAAGA
mmu_circRNA_014406	AAGCTGTTACTCCGTCCTCTG	CGTTCTGGGTGCTGTTTCC
mmu_circRNA_012412	CGAGGGGAAAAGCTGTGG	ACGCAGCCCTCTCCAAGA
mmu_circRNA_016863	CGAGGGCAAAGGGAAAGAGG	CAACCTTTTCCCCTCGGC
Timp1	TCCGTCCACAAACAGTGAGTGTCA	GGTGTGCACAGTGTTTCCCTGTTT
Sumo3	GCAACCATGTCGGAAGAGAAG	TGGTTGTCCATCAAACCGGA
Chac1	GCCCTGTGGATTTTCGGGTA	ATCTTGTCGCTGCCCCTATG
Jam2	TACTGTGAAGCCCGCAACTC	GCAGAAATGACGAAGGCCAC
Basp1	CAAAGACAAGAAGGCCGAAG	CGCGCTGCTAGGTTTAGAGT
β-actin	ACTGCCGCATCCTCTTCCT	TCAACGTCACACTTCATGATGGA

to investigate the underlying protective mechanisms of the two circRNAs in iPoC, we thus identified the intersection between those predicted downstream mRNA targets and the mRNAs that increased in response to iPoC in the mRNA microarray data using a Venn diagram. Finally, the overlapping mRNAs were collected, and the circRNA-miRNA-mRNA axis was constructed.

Statistical analysis

For the microarray results, a fold change of circRNA/mRNA ≥ 1.5 was chosen for further analysis, and $P<0.05$ was considered statistically significant. All other statistical data were analyzed with GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) and were expressed as the mean ± standard deviation. Data with normal distributions were compared using one-way analysis of variance followed by the Student-Newman-Keuls test. Data with non-normal distributions were compared using the Kruskal-Wallis test followed by the Wilcoxon Rank Sum test with Bonferroni adjustments. A two-tailed Student's *t*-test was used to compare means between two groups. $P < 0.05$ was considered statistically significant.

Results

Evaluation of intestinal I/R injury and intestinal protection by iPoC

To confirm the protective effect of iPoC in the present study, we examined intestinal mucosal morphology and blood indexes of gut injury after intestinal I/R. Generally, intestinal I/R causes severe necrosis and ischemic darkening of tissue with vascular congestion throughout the gut tissues in terms of macroscopic appearance. In contrast, mice subjected to iPoC showed only minor signs of necrosis and ischemic congestion, although the gut remained edematous (Fig. 1A). A histological analysis in intestinal mucosa showed that mice receiving iPoC treatment exhibited significantly less intestinal injury than those in the I/R group (Fig. 1B-C). In addition, serum levels of intestinal fatty acid-binding protein (I-FABP) (a sensitive marker of enterocyte damage [26]), TNF-α and IL-6 were increased after intestinal I/R, while iPoC significantly decreased these indicators compared with the I/R group (Fig. 1D-F).These results demonstrated that the current iPoC protocol was effective in protecting the intestine against I/R injury. Therefore, this model is suitable for identifying differentially expressed circRNAs after intestinal I/R with or without iPoC treatment.

Changes in circRNA and mRNA expression patterns in response to intestinal I/R injury and iPoC

To evaluate circRNAs in 1) sham, 2) I/R, and 3) I/R + iPoC groups, we analyzed the expression profiles of circRNAs and protein-coding RNAs in 3 randomly selected samples from each group using microarray analysis. A

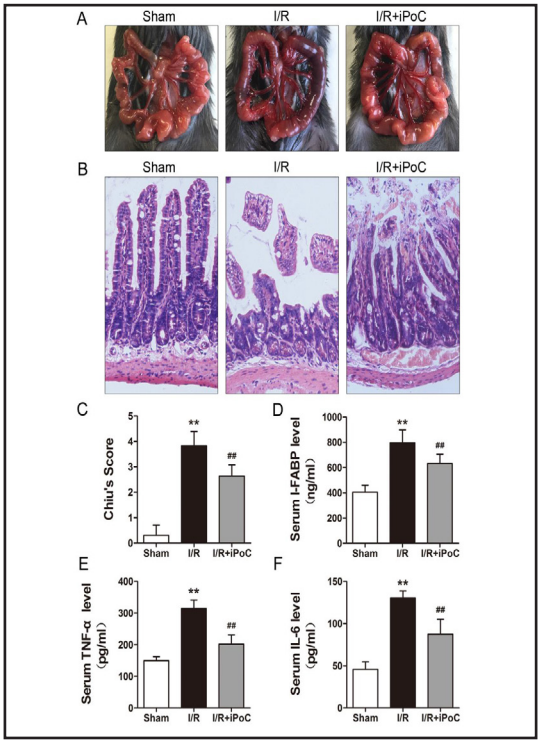

Fig. 1. The iPoC protocol used in the current study was effective in protecting the small intestine from I/R injury. (A) Representative images are shown for the gross morphological appearance of the gut. (B) Representative images are shown for the histopathological changes in the intestinal mucosa (H&E staining; original magnification ×200). (C) Intestinal Chiu's scores. (D-F) Serum levels of (D) I-FABP, (E) TNF-α and (F) IL-6. Data are expressed as the means ± SD, n = 8. **P <0.01 compared with the sham group; ##P <0.01 compared with the I/R group.

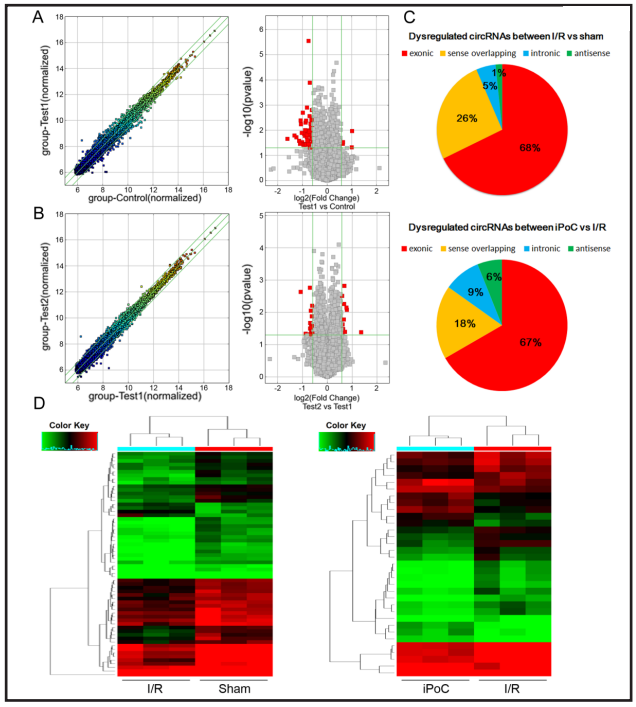

Fig. 2. Differential expression of circRNAs between the I/R and sham groups and between the iPoC and I/R groups. The scatter plots (left panel) and volcano plots (right panel) showed the variation in circRNA expression between I/R and sham groups (A) and between iPoC and I/R groups (B). The values shown on the X- and Y-axes of the scatter plot are the normalized signals of the samples (log_2 scaled). The green lines represent fold change. The circRNAs above the upper green line and below the lower green line are those with expression fold change > 1.5 between the two compared groups. The vertical lines in the volcano plot correspond to 1.5-fold up and down, and the horizontal line represents a P-value of 0.05. The red points in the plot represent the differentially expressed circRNAs with statistical significance. (C) The significantly dysregulated circRNAs were categorized into different subgroups according to their genomic positions and effects. (D) Hierarchical clustering analysis (heat map) revealed different circRNA expression profiles among samples from the microarray data. The expression values are depicted in line with the color scale. Red indicates high relative expression, and green indicates low relative expression. Each circRNA is represented by a single row of colored boxes, and each sample is represented by a single column. 'Control' refers to the sham group, 'test1' refers to the I/R group, and 'test2' refers to the iPoC group.

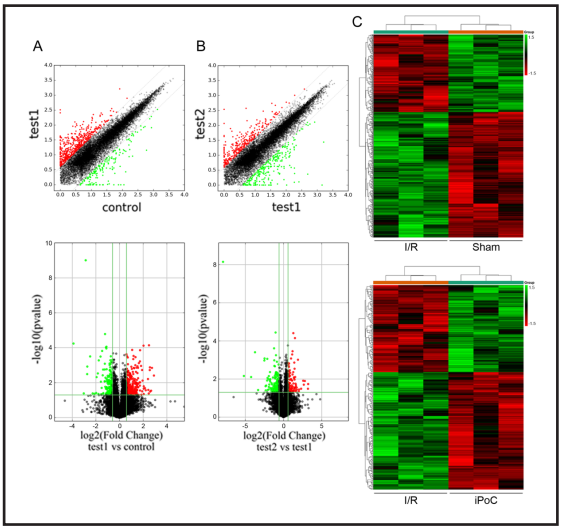

Fig. 3. Differential expression of mRNAs between the I/R and sham groups and between the iPoC and I/R groups. The scatter plots (upper panel) and volcano plots (lower panel) show the variation in mRNA expression between the I/R and sham groups (A) and between the iPoC and I/R groups (B). The red points in scatter plot stand for upregulated mRNAs with fold change ≥ 1.5, and the green points represent downregulated mRNAs. The red or green points in volcano plot represent significantly upregulated or downregulated mRNAs (fold change ≥ 1.5, P-value < 0.05), respectively. (C) Hierarchical clustering analysis (heat map) revealed different mRNA expression profiles among samples from the microarray data. Green and red denoted high and low expression, respectively. 'Control' refers to the sham group, 'test1' refers to the I/R group, and 'test2' refers to the iPoC group.

total of 9, 821 circRNA and 12, 689 mRNA targets in the intestine were detected by microarray probes. As a result, 62 circRNAs and 521 mRNAs in the I/R group showed more than 1.5-fold change with statistical significance ($P< 0.05$) compared with those in the sham group. Among them, 4 circRNAs and 322 mRNAs were upregulated, while 58 circRNAs and 199 mRNAs were downregulated (Fig. 2A and Fig. 3A). The number of down-expressed circRNAs was much

greater than the number of up-expressed circRNAs. Furthermore, 33 circRNAs and 303 mRNAs were detected to be differentially expressed (fold change ≥1.5, $P< 0.05$), of which 12 circRNAs and 129 mRNAs were upregulated and 21 circRNAs and 174 mRNAs were downregulated in the iPoC group compared with the I/R group (Fig. 2B and Fig. 3B). The top 10 dysregulated circRNAs and mRNAs are summarized in Table 2 and Table 3 based on fold change. The significantly dysregulated circRNAs were further classified into different subgroups as described below. Among the circRNAs that were dysregulated between the I/R group and the sham group, there were 42 exonic, 16 sense overlapping, 3 intronic, and 1 antisense. Among the circRNAs that were dysregulated between the iPoC group and the I/R group, there were 22 exonic, 6 sense overlapping, 3 intronic, and 2 antisense (Fig. 2C). We also extracted and clustered the differentially expressed circRNAs and mRNAs in both comparison groups for better observation of expression patterns. Hierarchical clustering analysis clearly showed that the circRNA and mRNA expression profiles in I/R intestines were different from those in normal intestines, whereas iPoC treatment markedly affected the I/R-induced circRNA and mRNA expression patterns (Fig. 2D and Fig. 3C).

Table 2. Top 10 significantly dysregulated circRNAs and mRNAs ranked by fold change between I/R and sham groups

CircRNA Name	circRNA_type	GeneSymbol	Fold Change	Regulation	P-value
mmu_circRNA_30227	exonic	Pacrg	2.016	up	0.011
mmu_circRNA_41543	exonic	A330076H08Rik	1.996	up	0.048
mmu_circRNA_41532	intronic		1.565	up	0.029
mmu_circRNA_37502	exonic	Szt2	1.555	up	0.035
mmu_circRNA_20066	sense overlapping	DQ715306	3.053	down	0.022
mmu_circRNA_23861	exonic	Myo1d	2.504	down	0.016
mmu_circRNA_41204	sense overlapping	Calm3	2.370	down	0.019
mmu_circRNA_012412	sense overlapping	Rn45s	2.195	down	0.026
mmu_circRNA_001769	sense overlapping	Rn45s	2.143	down	0.034
mmu_circRNA_43788	exonic	4931406C07Rik	2.107	down	0.004

mRNA Gene Name	Fold Change	Regulation	P-value
Irf2bp1	7.513	up	0.004
Slc26a2	6.694	up	0.001
Thbs1	6.362	up	0.029
Cebpd	6.040	up	0.032
Fosl1	5.931	up	0.001
Cldn3	14.925	down	5.72069E-05
Gpx2	8.130	down	0.041
Mcts2	7.246	down	9.84522E-10
Cldn15	6.667	down	0.003
Chac1	6.623	down	0.001

Table 3. Top 10 significantly dysregulated circRNAs and mRNAs ranked by fold change between iPoC and I/R groups

CircRNA Name	circRNA_type	GeneSymbol	Fold Change	Regulation	P-value
mmu_circRNA_34414	exonic	Map1a	2.564	up	0.041
mmu_circRNA_31111	exonic	Msh2	1.721	up	0.008
mmu_circRNA_016863	sense overlapping	Rn45s	1.703	up	0.007
mmu_circRNA_012412	sense overlapping	Rn45s	1.635	up	0.040
mmu_circRNA_28662	intronic	Kcnq3	1.605	up	0.002
mmu_circRNA_18944	sense overlapping	Wdfy1	2.105	down	0.002
mmu_circRNA_018352	exonic	Hnrnpll	1.831	down	0.046
mmu_circRNA_21870	exonic	Fig4	1.631	down	0.048
mmu_circRNA_25735	exonic	Slc24a4	1.629	down	0.002
mmu_circRNA_45613	sense overlapping	Maged1	1.620	down	0.032

mRNA Gene Name	Fold Change	Regulation	P-value
Suox	9.317	up	0.018
Defa-rs1	9.145	up	0.032
Cyp24a1	6.880	up	0.013
Itln1	6.274	up	0.020
Clps	5.929	up	0.012
Rpl23a-ps3	243.902	down	7.0669E-09
Gm21002	37.037	down	0.007
Gm15308	18.868	down	0.008
Basp1	13.333	down	0.000
Clec2e	10.030	down	0.038

qRT-PCR validation of differentially expressed circRNAs and mRNAs

We further confirmed the microarray results by qRT-PCR. Six circRNAs and five mRNAs were chosen based on the fold change, raw data and *P*-value. As shown in Fig. 4, qRT-PCR results indicated that the expression levels of Timp1 and Sumo3 were upregulated, while the expression levels of circRNA_007736, circRNA_006664 and Chac1 were observed to be downregulated significantly after intestinal I/R compared with sham treatment. Meanwhile, compared with I/R, iPoC stimuli markedly increased the expression of circRNA_018654, circRNA_011775 and Jam2, whereas circRNA_015231, circRNA_014406 and Basp1 were all decreased. The above results were consistent with the microarray assay, thus demonstrating the high reliability of the microarray data.

Computational analysis of significantly dysregulated mRNAs

To understand the behavior of these differentially expressed circRNAs, we explored how the patterns of coding gene expression change upon I/R and iPoC because biologically related gene groups can share the same patterns of change. Gene Ontology (GO) enrichment analysis of differentially expressed mRNAs may reveal the role of differentially regulated circRNAs. Our data showed that the upregulated mRNAs in response to intestinal I/R, associated to biological processes, were involved in the macromolecule metabolic processes and regulation of cell death. Meanwhile, the downregulated transcripts were most relevant to cellular response to endogenous stimuli and protein complex biogenesis and assembly (Fig. 5A). Compared with the I/R group, iPoC significantly upregulated the mRNAs associated with regulation of cell communication and negative regulation of the reactive oxygen species (ROS) biosynthetic process, whereas the downregulated mRNAs in the iPoC group were involved in the monocarboxylic acid, carboxylic acid and cellular lipid metabolic processes (Fig. 5B).

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis revealed that pathways such as the HIF-1 signaling pathway and the nuclear factor-kappa B

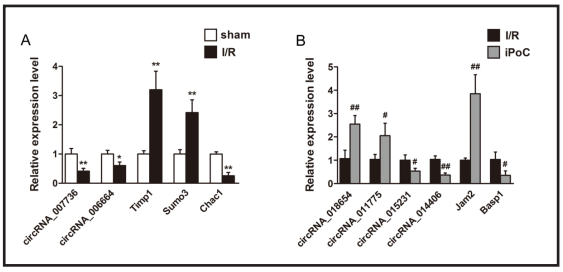

Fig. 4. qRT-PCR validation of the selected circRNAs and mRNAs between the I/R group and the sham group (A) and between the iPoC group and the I/R group (B). Data are expressed as the means ± SD, n = 6. *P <0.05, and **P <0.01 compared with the sham group; #P <0.05 and ##P <0.01 compared with the I/R group.

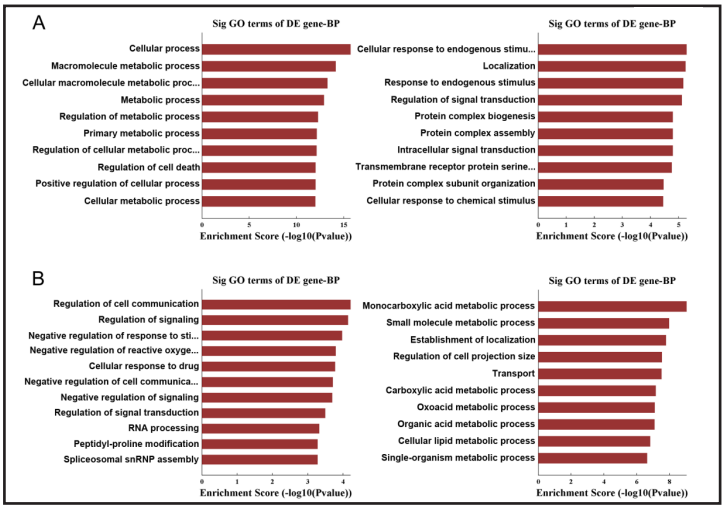

Fig. 5. Gene Ontology (GO) analysis of the biological functions of differentially expressed mRNAs. GO annotation of the top ten most enriched mRNAs that were significantly up- (left panel) and down-regulated (right panel) between the I/R and sham groups (A) and between the iPoC and I/R groups (B), covering the theme of biological processes.

(NF-κB) signaling pathway were related to the upregulated mRNAs, while pathways such as the sphingolipid signaling pathway and the phospholipase D signaling pathway were related to the downregulated mRNAs in the I/R group (Fig. 6A). Furthermore, some vital pathways could also be influenced by the altered mRNAs in the iPoC group. The T cell receptor signaling pathway and the cGMP-PKG signaling pathway were the top pathways of the upregulated mRNAs, whereas starch and sucrose metabolism was the top enriched KEGG pathway for the downregulated mRNAs in the iPoC group compared with the I/R group (Fig. 6B). The above results suggest that these GO terms and pathways might contribute significantly to the pathogenesis and development of intestinal I/R injury and might be associated with iPoC-mediated protection.

Identification of circRNAs associated with intestinal protection by iPoC

IPoC can be considered as an effective "inhibitor" of I/R injury. By comparing circRNA expression changes and the direction of the changes (i.e., up- or downregulation) among the three groups, possible causal relationships between circRNA changes and endogenous intestinal protection can be revealed. Therefore, we firstly selected differentially expressed circRNAs that exhibited the opposite direction of the changes between the two comparison groups (sham *vs* I/R and I/R *vs* iPoC). By following this screening strategy, we identified two circRNAs, circRNA_012412 and circRNA_016863, that were downregulated by I/R compared

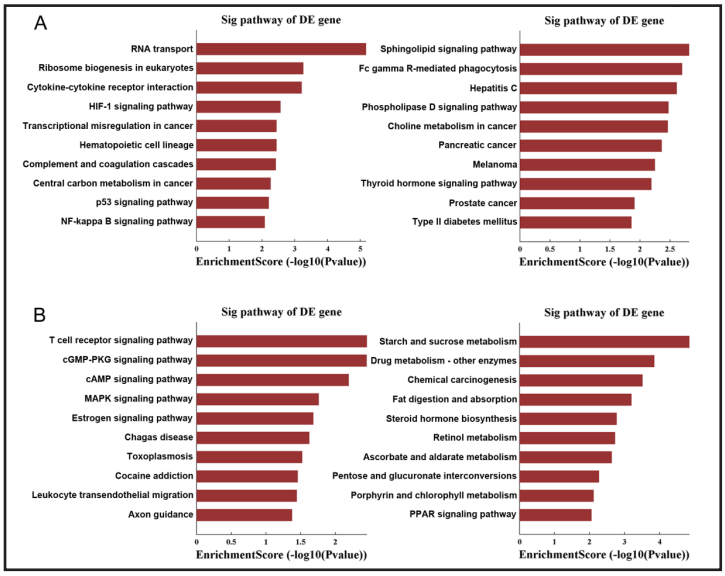

Fig. 6. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the differentially expressed mRNAs. KEGG pathway enrichment analysis of the top ten most enriched mRNAs that were significantly up- (left panel) and downregulated (right panel) between the I/R and sham groups (A) and between the iPoC and I/R groups (B).

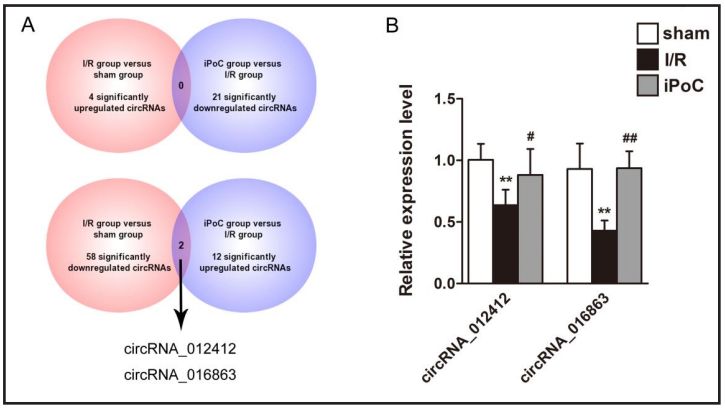

Fig. 7. Identification and qRT-PCR validation of circRNAs associated with intestinal protection by iPoC. (A) The Venn diagram presents the overlap of differentially expressed circRNAs that showed opposite directions of change between the two comparison groups. (B) Expression levels of circRNA_012412 and circRNA_016863 in mouse intestines among the three groups, as detected by qRT-PCR. Data are expressed as the means ± SD, n = 6. **P <0.01 compared with the sham group; #P <0.05 and ##P <0.01 compared with the I/R group.

with sham treatment but had their downregulation significantly inhibited by iPoC (Fig. 7A). These two circRNAs were considered closely associated with the protective mechanisms of iPoC. The genomic locus of circRNA_012412 and circRNA_016863 is on chromosome 17, and both of them are spliced from *Rn45s*. The expression levels of circRNA_012412 and circRNA_016863 were then validated by qRT-PCR. As shown in Fig. 7B, the expression levels of the two circRNAs were reduced considerably in the I/R intestine, while iPoC treatment markedly restored their expression levels. These results suggest that circRNA_012412 and circRNA_016863 may act as protective factors during intestinal I/R, which prompted us to continue investigating the functions of these two circRNAs.

MiRNA prediction and circRNA-miRNA-mRNA pathway construction for circRNA_012412 and circRNA_016863

As reported, one of the well-known regulatory functions of circRNA is to interact with miRNAs via miRNA response elements (MREs), thereby serving as miRNA sponges and competitively suppressing their activity. To determine the functions of circRNA_012412 and circRNA_016863, we searched for their putative target miRNAs using Arraystar's miRNA target prediction software, which is based on the TargetScan and miRanda databases. The five highest-ranking candidate miRNAs for each circRNA are listed in Table 4, and detailed annotation of their circRNA/miRNA interactions is presented in Fig. 8A.

To further elucidate the underlying protective mechanisms of the two circRNAs in iPoC, we predicted the circRNA_012412/circRNA_016863-miRNA-mRNA signaling pathways. Through miRNA target gene prediction using TargetScan and miRDB database, 722 genes were enriched in connection with the 10 miRNAs mentioned above. Since the ceRNA hypothesis proposes that circRNAs may positively regulate their downstream mRNAs, we thus generated Venn diagrams to show the intersections of the 129 mRNAs significantly

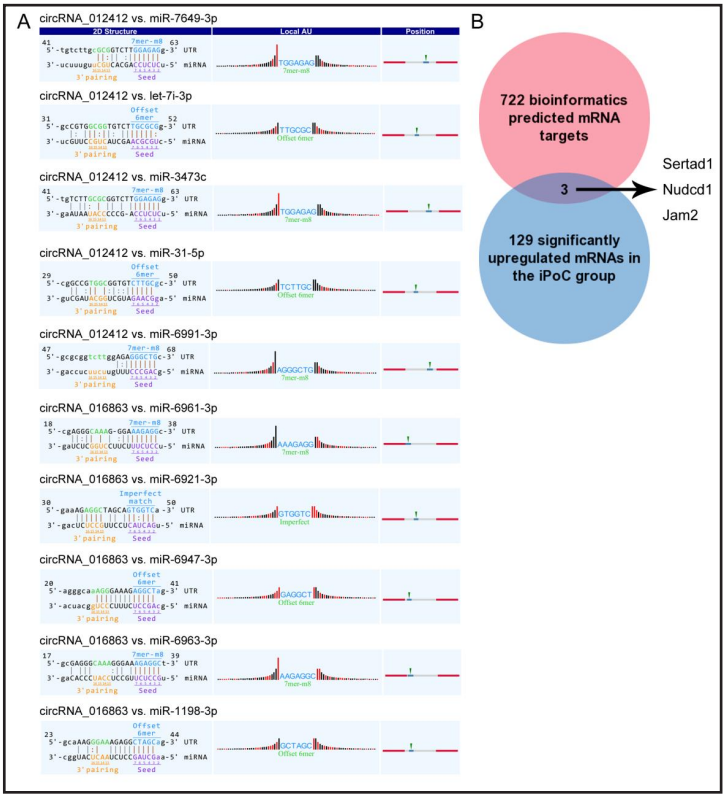

Fig. 8. Prediction of circRNA-miRNA-mRNA associations. (A) Detailed annotation of circRNA/miRNA interactions. The 2D structure displays the MRE sequence, the target miRNA seed type (7mer-m8, 8mer, 6mer, offset 6mer, imperfect) and the pairing of target miRNA nucleotides 13-16. The precise base positions are shown in the alignments in the upper left and right corners. The "local AU" displays the 30 nucleotides upstream and downstream of the seed sequence. Black bars stand for G/C and low accessibility. Red bars stand for A/U and high accessibility of the seed. The height of the bar represents the extent of accessibility. The position column displays the most likely relative MRE position on the linearized circRNA sequence. (B) The Venn diagram presents the overlap of the predicted downstream mRNA targets associated with circRNA_012412 and circRNA_016863 and the significantly upregulated mRNAs induced by iPoC in the microarray data.

Table 4. The two identified circRNAs and their predicted miRNA response elements (MREs)

CircRNAs	Alias (circBase)	Chrom	Gene Symbol	MRE1	MRE2	MRE3	MRE4	MRE5
mmu_circRNA_012412	mmu_circ_0000772	chr17 +	Rn45s	mmu-miR-7649-3p	mmu-let-7i-3p	mmu-miR-3473c	mmu-miR-31-5p	mmu-miR-6991-3p
mmu_circRNA_016863	mmu_circ_0000776	chr17 +	Rn45s	mmu-miR-6961-3p	mmu-miR-6921-3p	mmu-miR-6947-3p	mmu-miR-6963-3p	mmu-miR-1198-3p

increased by iPoC from the mRNA microarray and the 722 predicted mRNA targets related to circRNA_012412 and circRNA_016863 (Fig. 8B). Using this analytical approach, we identified three overlapping mRNAs and ultimately constructed the following four circRNA-miRNA-mRNA pathways: circRNA_012412-miR-7649-3p-Sertad1, circRNA_012412-miR-3473c-Sertad1, circRNA_012412-miR-6991-3p-Nudcd1 and circRNA_012412-miR-6991-3p-Jam2. These ceRNA regulatory pathways may play pivotal roles in endogenous protective signaling in iPoC and warrant further intensive investigation.

Discussion

I/R injury of the small intestine is a life-threatening clinical event requiring urgent treatment. One of the most investigated methods for mitigating lethal reperfusion injury is iPoC. Despite intensive research in recent decades, the exact biological mechanisms underlying the intestinal protective effect of iPoC remain largely unclear, thus hampering its clinical application. Previous molecular research seeking to characterize the protective mechanisms of iPoC mainly focused on protein-coding gene regulation until the discovery of numerous ncRNAs including long non-coding RNAs (lncRNAs) and circRNAs. The importance of circRNAs as potential molecular markers for disease diagnosis and treatment and as powerful miRNA sponges has gradually been recognized. Since no data have been reported to date regarding the roles of circRNAs in intestinal I/R and iPoC, here, we utilized circRNA microarray analysis to acquire that during the early phase of I/R injury (i.e., at 4 h of reperfusion), circRNA expression profiles are altered in the mouse intestine and that these profiles are significantly influenced by subsequent iPoC stimuli. Moreover, by a systematic comparison of circRNA expression changes and the direction of the changes induced by I/R with or without iPoC, we identified two possible circRNAs associated with endogenous intestinal protection by iPoC. Through bioinformatic prediction of the functions of the two circRNAs, we constructed four circRNA-miRNA-mRNA pathways that may contribute to the protective mechanisms of iPoC against intestinal I/R injury.

The microarray expression profiles identified a total of 4 up- and 58 downregulated circRNAs as significantly differentially expressed after intestinal I/R, whereas 33 circRNAs in the iPoC group were significantly differentially expressed (12 upregulated and 21 downregulated) compared with those of the I/R group. Approximately 2/3 dysregulated circRNAs were exonic, which is in accordance with previous research that circRNAs mainly arise from back-spliced exons [27]. Since the functional study of circRNAs began only in recent years, it is not surprising that none of the top dysregulated circRNAs have been reported in the literature. However, some mRNAs that acted as the linear alternative transcript of those circRNAs were found to participate in various cellular processes associated with I/R injury. For instance, Calm3 is the linear transcript of circRNA_41204 (one of the top 5 decreased circRNAs in intestinal I/R) and it encodes calmodulin (CaM), a ubiquitous Ca^{2+}-binding protein that regulates a vast array of Ca^{2+}-sensitive biological events, which play important roles in the pathogenesis of I/R injury [28, 29]. Wdfy1 is the linear transcript of circRNA_18944 (one of the top 5 decreased circRNAs in iPoC), and its protein product was found to recruit the signaling adaptor protein TRIF to Toll-like receptors (TLRs) 3 and 4, thereby mediating the activation of NF-κB and induction of inflammatory cytokines [30]. Interestingly, the TLR signaling pathway was reported to be involved in the ischemic tolerance induced by preconditioning or postconditioning [31]. As circRNAs may regulate the transcription of their parent genes [32], those circRNAs described above may play roles in intestinal I/R injury

and iPoC by regulating the expression of their linear counterparts.

The data from the mRNA microarray were also encouraging. Of the top 10 dysregulated mRNAs modulated by intestinal I/R injury, Cldn3 (claudin-3) had the highest fold change among the downregulated mRNAs and was previously identified as a key tight junction protein that helps establish the paracellular barrier of the intestinal epithelia [33]. The significantly decreased gene expression of Cldn3 and Cldn15 indicated a defect in intestinal epithelial barrier function after I/R injury [34, 35], while decreased Gpx2 expression implied a low intracellular antioxidant level in intestinal I/R progression, as suggested by previous studies [36]. Our data also provided some new clues for understanding the molecular mechanisms whereby iPoC confers protection. Among the increased mRNAs induced by iPoC, Itln1 (intelectin-1), also known as omentin-1, is a GPI-linked protein that is expressed particularly by intestinal Paneth cells and goblet cells [37]. Intelectin-1 has been shown to limit multiple aspects of cardiovascular pathology following ischemic injury [38], indicating that it might be a potential factor mediating iPoC-induced intestinal protection. Furthermore, the expression of Basp1 was downregulated by iPoC, and it was previously identified as a pro-apoptotic factor in tubular cells [39]. All these consistencies reinforce the reliability of our microarray data. However, the roles of other mRNAs in the protective effect of iPoC against intestinal I/R injury are still unclear and need further study in the future.

GO terms and KEGG pathway analysis for the differentially expressed mRNAs revealed some biological processes and pathways that could play vital roles in the pathogenesis of intestinal I/R injury, including metabolic processes, cell death, endogenous stimulus response, and the HIF-1, NF-κB and sphingolipid signaling pathways. Recent study has demonstrated that I/R injury altered sphingolipid metabolism in the intestine [40]. Moreover, regulation of cell communication, ROS biosynthesis and multiple signaling pathways such as cGMP-PKG, cAMP, MAPK and PPAR were identified in the iPoC group compared with the I/R group, suggesting that related coding genes may play crucial roles in iPoC-mediated protection against intestinal I/R injury through regulating the above mentioned biological mechanisms and signal transduction pathways. This observation was partially in line with previous studies in ischemic heart, which showed that the cardioprotective effects of iPoC were associated with the reduction in oxidative stress levels and the activation of certain pro-survival protein kinase cascades, including those of protein kinases A, C, and G and members of the MAPK family (Erk1/2, p38 and JNK) [41-43].

A finding worth noting is that two circRNAs identified in the present study exhibited converse alteration in response to I/R injury and iPoC. Microarray data combined with qRT-PCR validation showed that compared with the levels in the sham group, the expression levels of circRNA_012412 and circRNA_016863 were both downregulated upon I/R injury. However, their expressions were upregulated with iPoC post-treatment compared to I/R injury. We presume that decreased levels of these two circRNAs may contribute to intestinal dysfunction upon I/R injury, while iPoC may restore normal intestinal function by upregulating their expression. Accordingly, the two circRNAs may be potential molecular targets of iPoC, and we consider them possible protective factors against intestinal I/R injury.

The functional mechanisms of most circRNAs are still being discovered. Recent studies have reported that circRNAs contain multiple conserved miRNA target sites and can serve as efficient miRNA sponges to regulate gene expression in many physiological and pathological conditions. Hansen et al. were the first to demonstrate that the circular transcript ciRS-7 (circular RNA sponge for miR-7, also known as CDR1as), one of the most representative circRNAs, contained more than 70 selectively conserved miR-7 binding sites and that it strongly suppressed miR-7 activity, resulting in increased levels of miR-7 targets [20]. Similarly, the circRNA MFACR can act as a sponge for miR-652-3p, in turn increasing the expression of the miRNA target gene MTP18 and regulating mitochondrial fission and apoptosis in cardiomyocytes [44]. Here, with bioinformatic methods, we preliminarily sought potential miRNA targets of circRNA_012412 and circRNA_016863 based on sequence pairing and listed the five highest-ranking candidate miRNAs. Then, we constructed several circRNA-miRNA-mRNA regulatory axes through a combination of miRNA target prediction

software and mRNA microarray results; these regulatory axes provide evidence regarding the protective mechanisms of the two identified circRNAs. Notably, although none of the four circRNA-miRNA-mRNA pathways have been reported previously, several molecules were found to be involved in certain pathophysiological processes when separately analyzed. For example, Sertad1 (also known as Sei-1 or TRIP-Br1) is able to inhibit ROS- or nutrient/serum deprivation-induced apoptosis in cancer cells [45, 46], while Jam2 belongs to the junctional adhesion molecule (JAM) family and is involved in the formation of tight junctions in both endothelial and epithelial cells [47, 48]. Recently, Zhong et al. predicted a circTCF25-miR-103a-3p/miR-107-CDK6 pathway in bladder cancer by multiple bioinformatical approaches. They further demonstrated that circTCF25 over-expression could promote cancer cell proliferation and migration through miR-103a-3p/miR-107-CDK6 axes [49]. Thus, we speculate that modulation of specific circRNAs in intestinal I/R and iPoC may affect the downstream pathway function in which they are involved via miRNA-dependent regulation of target gene expression.

The current study has some limitations that should be acknowledged. First, we performed only a time-point analysis in this study. Recent studies showed that circRNAs and protein-coding genes were dynamically regulated and their expression levels changed with reperfusion time during I/R and iPoC [50, 51]. In addition, iPoC induces time-dependent changes in ROS generation, calcium overload and protein kinases activation [42, 43, 52]. Further study regarding circRNA expression pattern at different time of reperfusion following intestinal ischemia will be conducted to better elucidate the correlation between circRNA dysregulation and cellular processes alterations induced by iPoC. Second, the two circRNAs (circRNA_012412 and circRNA_016863) identified by microarray analysis need their exact roles in intestinal I/R injury and iPoC confirmed via loss- and gain-of-function experiments. Third, our results were obtained in an animal model; whether they could be translated into human beings merits further exploration. There has been accumulating evidence that circRNA expression appears to be conserved across mammals [53]. In our microarray results, we also discovered that many differentially expressed circRNAs were conserved between mouse and human (data not shown). We infer that parts of the circRNA expression in our study are similar and of interest for humans. Furthermore, some other factors should be considered in translation of intestinal protection for patient benefit. The complexity of disease processes that result in intestinal ischemia, aging-related comorbidities, co-medications as well as methodological gaps must be taken into consideration when translating intestinal protective effects of iPoC into the clinical setting [1, 11, 54].

Conclusion

Our study reveals that numerous circRNAs and mRNAs are dysregulated in response to intestinal I/R and subsequent iPoC treatment. Bioinformatic analysis predicted several potential circRNA-miRNA-mRNA regulatory pathways, which may enrich our understanding of the molecular mechanisms whereby iPoC confers protection and may also offer promising therapeutic targets for intestinal I/R injury.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (No. 81372037 and No. 81671954 to Xiaofeng Tian and No. 81600411 to Guangzhi Wang).

Disclosure Statement

The authors declare to have no conflict of interests.

References

1 Gonzalez LM, Moeser AJ, Blikslager AT: Animal models of ischemia-reperfusion-induced intestinal injury: Progress and promise for translational research. Am J Physiol Gastrointest Liver Physiol 2015;308:G63-G75.
2 Lenaerts K, Ceulemans LJ, Hundscheid IH, Grootjans J, Dejong CH, Olde DS: New insights in intestinal ischemia-reperfusion injury: Implications for intestinal transplantation. Curr Opin Organ Transplant 2013;18:298-303.
3 Grootjans J, Lenaerts K, Buurman WA, Dejong CH, Derikx JP: Life and death at the mucosal-luminal interface: New perspectives on human intestinal ischemia-reperfusion. World J Gastroenterol 2016;22:2760-2770.
4 Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA, Vinten-Johansen J: Inhibition of myocardial injury by ischemic postconditioning during reperfusion: Comparison with ischemic preconditioning. Am J Physiol Heart Circ Physiol 2003;285:H579-H588.
5 Hausenloy DJ, Yellon DM: Ischaemic conditioning and reperfusion injury. Nat Rev Cardiol 2016;13:193-209.
6 Zhao ZQ: Postconditioning in reperfusion injury: A status report. Cardiovasc Drugs Ther 2010;24:265-279.
7 Theodoraki K, Karmaniolou I, Tympa A, Tasoulis MK, Nastos C, Vassiliou I, Arkadopoulos N, Smyrniotis V: Beyond preconditioning: Postconditioning as an alternative technique in the prevention of liver Ischemia-Reperfusion injury. Oxid Med Cell Longev 2016;2016:8235921.
8 Wang JY, Shen J, Gao Q, Ye ZG, Yang SY, Liang HW, Bruce IC, Luo BY, Xia Q: Ischemic postconditioning protects against global cerebral ischemia/reperfusion-induced injury in rats. Stroke 2008;39:983-990.
9 Wen SH, Ling YH, Li Y, Li C, Liu JX, Li YS, Yao X, Xia ZQ, Liu KX: Ischemic postconditioning during reperfusion attenuates oxidative stress and intestinal mucosal apoptosis induced by intestinal ischemia/reperfusion via aldose reductase. Surgery 2013;153:555-564.
10 Cheng CH, Lin HC, Lai IR, Lai HS: Ischemic postconditioning attenuate reperfusion injury of small intestine: Impact of mitochondrial permeability transition. Transplantation 2013;95:559-565.
11 Feyzizadeh S, Badalzadeh R: Application of ischemic postconditioning's algorithms in tissues protection: Response to methodological gaps in preclinical and clinical studies. J Cell Mol Med 2017; 21:2257-2267.
12 Qu S, Yang X, Li X, Wang J, Gao Y, Shang R, Sun W, Dou K, Li H: Circular RNA: A new star of noncoding RNAs. Cancer Lett 2015;365:141-148.
13 Chen LL, Yang L: Regulation of circRNA biogenesis. RNA Biol 2015;12:381-388.
14 Suzuki H, Tsukahara T: A view of pre-mRNA splicing from RNase R resistant RNAs. Int J Mol Sci 2014;15:9331-9342.
15 Guo JU, Agarwal V, Guo H, Bartel DP: Expanded identification and characterization of mammalian circular RNAs. Genome Biol 2014;15:409.
16 Salzman J, Chen RE, Olsen MN, Wang PL, Brown PO: Cell-type specific features of circular RNA expression. PLoS Genet 2013;9:e1003777.
17 Jeck WR, Sorrentino JA, Wang K, Slevin MK, Burd CE, Liu J, Marzluff WF, Sharpless NE: Circular RNAs are abundant, conserved, and associated with ALU repeats. RNA 2013;19:141-157.
18 Meng S, Zhou H, Feng Z, Xu Z, Tang Y, Li P, Wu M: CircRNA: Functions and properties of a novel potential biomarker for cancer. Mol Cancer 2017;16:94.
19 Greene J, Baird AM, Brady L, Lim M, Gray SG, McDermott R, Finn SP: Circular RNAs: Biogenesis, function and role in human diseases. Front Mol Biosci 2017;4:38.
20 Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, Kjems J: Natural RNA circles function as efficient microRNA sponges. Nature 2013;495:384-388.
21 Memczak S, Jens M, Elefsinioti A, Torti F, Krueger J, Rybak A, Maier L, Mackowiak SD, Gregersen LH, Munschauer M, Loewer A, Ziebold U, Landthaler M, Kocks C, le Noble F, Rajewsky N: Circular RNAs are a large class of animal RNAs with regulatory potency. Nature 2013;495:333-338.
22 Varga ZV, Zvara A, Farago N, Kocsis GF, Pipicz M, Gaspar R, Bencsik P, Gorbe A, Csonka C, Puskas LG, Thum T, Csont T, Ferdinandy P: MicroRNAs associated with ischemia-reperfusion injury and cardioprotection by ischemic pre- and postconditioning: ProtectomiRs. Am J Physiol Heart Circ Physiol 2014;307:H216-H227.
23 Wang G, Yao J, Li Z, Zu G, Feng D, Shan W, Li Y, Hu Y, Zhao Y, Tian X: MiR-34a-5p inhibition alleviates intestinal Ischemia/Reperfusion-Induced reactive oxygen species accumulation and apoptosis via activation of SIRT1 signaling. Antioxid Redox Signal 2016;24:961-973.

24 Rosero O, Onody P, Stangl R, Turoczi Z, Fulop A, Garbaisz D, Lotz G, Harsanyi L, Szijarto A: Postconditioning of the small intestine: Which is the most effective algorithm in a rat model? J Surg Res 2014;187:427-437.
25 Chiu CJ, McArdle AH, Brown R, Scott HJ, Gurd FN: Intestinal mucosal lesion in low-flow states. I. A morphological, hemodynamic, and metabolic reappraisal. Arch Surg 1970;101:478-483.
26 Piton G, Capellier G: Biomarkers of gut barrier failure in the ICU. Curr Opin Crit Care 2016;22:152-160.
27 Ashwal-Fluss R, Meyer M, Pamudurti NR, Ivanov A, Bartok O, Hanan M, Evantal N, Memczak S, Rajewsky N, Kadener S: CircRNA biogenesis competes with pre-mRNA splicing. Mol Cell 2014;56:55-66.
28 Zhang Y, Lin R, Tao J, Wu Y, Chen B, Yu K, Chen J, Li X, Chen LD: Electroacupuncture improves cognitive ability following cerebral ischemia reperfusion injury via CaM-CaMKIV-CREB signaling in the rat hippocampus. Exp Ther Med 2016;12:777-782.
29 Woods C, Shang C, Taghavi F, Downey P, Zalewski A, Rubio GR, Liu J, Homburger JR, Grunwald Z, Qi W, Bollensdorff C, Thanaporn P, Ali A, Riemer K, Kohl P, Mochly-Rosen D, Gerstenfeld E, Large S, Ali Z, Ashley E: In vivo Post-Cardiac arrest myocardial dysfunction is supported by ca^{2+}/Calmodulin-Dependent protein kinase II-Mediated calcium Long-Term potentiation and mitigated by alda-1, an agonist of aldehyde dehydrogenase type 2 Circulation 2016;134:961-977.
30 Hu YH, Zhang Y, Jiang LQ, Wang S, Lei CQ, Sun MS, Shu HB, Liu Y: WDFY1 mediates TLR3/4 signaling by recruiting TRIF. EMBO Rep 2015;16:447-455.
31 Wang PF, Xiong XY, Chen J, Wang YC, Duan W, Yang QW: Function and mechanism of toll-like receptors in cerebral ischemic tolerance: From preconditioning to treatment. J Neuroinflammation 2015;12:80.
32 Li Z, Huang C, Bao C, Chen L, Lin M, Wang X, Zhong G, Yu B, Hu W, Dai L, Zhu P, Chang Z, Wu Q, Zhao Y, Jia Y, Xu P, Liu H, Shan G: Exon-intron circular RNAs regulate transcription in the nucleus. Nat Struct Mol Biol 2015;22:256-264.
33 Garcia-Hernandez V, Quiros M, Nusrat A: Intestinal epithelial claudins: Expression and regulation in homeostasis and inflammation. Ann N Y Acad Sci 2017;1397:66-79.
34 Li Q, Zhang Q, Wang C, Liu X, Qu L, Gu L, Li N, Li J: Altered distribution of tight junction proteins after intestinal ischaemia/reperfusion injury in rats. J Cell Mol Med 2009;13:4061-4076.
35 Rosero O, Onody P, Kovacs T, Molnar D, Lotz G, Toth S, Turoczi Z, Fulop A, Garbaisz D, Harsanyi L, Szijarto A: Impaired intestinal mucosal barrier upon ischemia-reperfusion: "patching holes in the shield with a simple surgical method". Biomed Res Int 2014;2014:210901.
36 Bhattacharyya A, Chattopadhyay R, Mitra S, Crowe SE: Oxidative stress: An essential factor in the pathogenesis of gastrointestinal mucosal diseases. Physiol Rev 2014;94:329-354.
37 Komiya T, Tanigawa Y, Hirohashi S: Cloning of the novel gene intelectin, which is expressed in intestinal paneth cells in mice. Biochem Biophys Res Commun 1998;251:759-762.
38 Kataoka Y, Shibata R, Ohashi K, Kambara T, Enomoto T, Uemura Y, Ogura Y, Yuasa D, Matsuo K, Nagata T, Oba T, Yasukawa H, Numaguchi Y, Sone T, Murohara T, Ouchi N: Omentin prevents myocardial ischemic injury through AMP-activated protein kinase- and Akt-dependent mechanisms. J Am Coll Cardiol 2014;63:2722-2733.
39 Sanchez-Nino MD, Fernandez-Fernandez B, Perez-Gomez MV, Poveda J, Sanz AB, Cannata-Ortiz P, Ruiz-Ortega M, Egido J, Selgas R, Ortiz A: Albumin-induced apoptosis of tubular cells is modulated by BASP1 Cell Death Dis 2015;6:e1644.
40 Hoehn RS, Seitz AP, Jernigan PL, Gulbins E, Edwards MJ: Ischemia/Reperfusion Injury Alters Sphingolipid Metabolism in the Gut. Cell Physiol Biochem 2016;39:1262-1270.
41 Inserte J, Hernando V, Vilardosa U, Abad E, Poncelas-Nozal M, Garcia-Dorado D: Activation of cGMP/protein kinase G pathway in postconditioned myocardium depends on reduced oxidative stress and preserved endothelial nitric oxide synthase coupling. J Am Heart Assoc 2013;2:e5975.
42 Penna C, Mancardi D, Rastaldo R, Pagliaro P: Cardioprotection: A radical view Free radicals in pre and postconditioning. Biochim Biophys Acta 2009;1787:781-793.
43 Hausenloy DJ, Yellon DM: Survival kinases in ischemic preconditioning and postconditioning. Cardiovasc Res 2006;70:240-253.
44 Wang K, Gan TY, Li N, Liu CY, Zhou LY, Gao JN, Chen C, Yan KW, Ponnusamy M, Zhang YH, Li PF: Circular RNA mediates cardiomyocyte death via miRNA-dependent upregulation of MTP18 expression. Cell Death Differ 2017;24:1111-1120.
45 Li C, Jung S, Yang Y, Kim KI, Lim JS, Cheon CI, Lee MS: Inhibitory role of TRIP-Br1 oncoprotein in hypoxia-induced apoptosis in breast cancer cell lines. Int J Oncol 2016;48:2639-2646.

46 Jung S, Li C, Duan J, Lee S, Kim K, Park Y, Yang Y, Kim KI, Lim JS, Cheon CI, Kang YS, Lee MS: TRIP-Br1 oncoprotein inhibits autophagy, apoptosis, and necroptosis under nutrient/serum-deprived condition. Oncotarget 2015;6:29060-29075.
47 Aurrand-Lions M, Duncan L, Ballestrem C, Imhof BA: JAM-2, a novel immunoglobulin superfamily molecule, expressed by endothelial and lymphatic cells. J Biol Chem 2001;276:2733-2741.
48 Bazzoni G: The JAM family of junctional adhesion molecules. Curr Opin Cell Biol 2003;15:525-530.
49 Zhong Z, Lv M, Chen J: Screening differential circular RNA expression profiles reveals the regulatory role of circTCF25-miR-103a-3p/miR-107-CDK6 pathway in bladder carcinoma. Sci Rep 2016;6:30919.
50 Mehta SL, Pandi G, Vemuganti R: Circular RNA expression profiles alter significantly in mouse brain after transient focal ischemia. Stroke 2017;48:2541-2548.
51 Pignataro G, Esposito E, Cuomo O, Sirabella R, Boscia F, Guida N, Di Renzo G, Annunziato L: The NCX3 isoform of the Na^+/Ca^{2+} exchanger contributes to neuroprotection elicited by ischemic postconditioning. J Cereb Blood Flow Metab 2011;31:362-370.
52 Cohen MV, Yang XM, Downey JM: Acidosis, oxygen, and interference with mitochondrial permeability transition pore formation in the early minutes of reperfusion are critical to postconditioning's success. Basic Res Cardiol 2008;103:464-471.
53 Rybak-Wolf A, Stottmeister C, Glazar P, Jens M, Pino N, Giusti S, Hanan M, Behm M, Bartok O, Ashwal-Fluss R, Herzog M, Schreyer L, Papavasileiou P, Ivanov A, Ohman M, Refojo D, Kadener S, Rajewsky N: Circular RNAs in the mammalian brain are highly abundant, conserved, and dynamically expressed. Mol Cell 2015;58:870-885.
54 Ferdinandy P, Hausenloy DJ, Heusch G, Baxter GF, Schulz R: Interaction of risk factors, comorbidities, and comedications with ischemia/reperfusion injury and cardioprotection by preconditioning, postconditioning, and remote conditioning. Pharmacol Rev 2014;66:1142-1174.