European Review for Medical and Pharmacological Sciences 2020; 24: 6980-6993

Hsa-circ-0068566 inhibited the development of myocardial ischemia reperfusion injury by regulating hsa-miR-6322/PARP2 signal pathway

H.-F. ZHOU[1,2], L.-L. XU[3], B. XIE[4], H.-G. DING[2], F. FANG[2], Q. FANG[1]

[1]Intensive Care Unit, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou City, Zhejiang Province, PR. China
[2]Intensive Care Unit, Huzhou Third Municipal Hospital, Huzhou City, Zhejiang Province, PR. China
[3]Electrocardiogram Room, Huzhou Maternity and Child Care Hospital, Huzhou City, Zhejiang Province, PR. China
[4]Intensive Care Unit, Huzhou Central Hospital, Huzhou City, Zhejiang Province, PR. China

Huifei Zhou and Lili Xu contributed to this work equally as co-first authors

Abstract. – OBJECTIVE: In recent years, studies have shown that noncoding RNA (circRNA) is an important regulatory molecule involved in cell physiology and pathology. Herein, we analyzed the role of circRNA-68566 in the regulation of myocardial ischemia-reperfusion (I/R) injury by regulating miR-6322/PARP2 signaling pathway.

MATERIALS AND METHODS: Cell viability was checked by CCK-8; LDH concentration, ROS production, MDA, SOD and GSH-Px were measured by corresponding kits; QPCR was used to inspect the expression of circRNA-0068566 and miR-6322 in I/R injury and H9C2 cells; luciferase reporter assay confirmed the direct target effect of circRNA-0068566 and miR-6322; Western blot was used to investigate PARP2 protein expression in I/R injury and H9C2 cells.

RESULTS: We analyzed the regulatory effect of circRNA-68566 on I/R injury and found that circRNA-68566 promoted the proliferation of injured cardiomyocytes *in vitro* and *in vivo*. circRNA-68566 and miR-6322 were directly combined to regulate the development of I/R injury. We also confirmed that PARP2 was the target of miR-6322 in I/R injury.

CONCLUSIONS: We believed that circRNA-68566 participated in myocardial ischemia-reperfusion injury by regulating miR-6322/PARP2 signaling pathway, which provided a new possible strategy for the treatment of I/R injury.

Key Words:
PARP2, Ischemia-reperfusion injury, MiR-6322, CircRNA-68566, Proliferation.

Introduction

In the past few decades, although the treatment of myocardial ischemia-reperfusion injury has made significant progress[1-3], such as percutaneous coronary intervention[4], thrombolysis [5], various growth factors[6] and drugs[7] were also used to reduce reperfusion injury, it is still the main cause of mortality worldwide[8]. The myocardial blood flow in the ischemic area is restored in time (early reperfusion), but the reperfusion itself may damage the microvascular function and lead to further damage. Myocardial reperfusion could cause the burst of reactive oxygen species (ROS), leading to the death of cardiomyocytes and cardiac dysfunction[9,10]. Excessive ROS led to caspase activation, up-regulation of cytokines, and peroxidation of DNA and protein, which aggravated I/R injury[11,12]. Therefore, more and more new regulation of I/R injury needs to be found, providing a new direction and target for the treatment of I/R injury.

CircRNAs are circular structures without 5-cap and 3-tail[13]. Endogenous noncoding circular RNAs play an important role in the pathological regulation of various diseases[14-17], including I/R injury[18-21]. The Hsa-circ 090021 knockout regulated miR-183/ITGB1 signaling pathway to inhibit the metastasis and growth of GC cells[22]. Hsa-circ 0074834 regulated the expression of ZEB1 and VEGF through microRNA-942-5p, promoting osteogenic differentiation of BMSCs and the repair of bone defects[23]. Additionally, the overexpression of miR-145-5p downregulated the expression of circ 001569 and then activated the mTOR and MEK/ERK pathways, which protected the injury induced by oxygen-glucose development induced injury[24]. Therefore, circRNAs have important biological functions and are an

Corresponding Author: Qiang Fang, MD; e-mail: 1183005@zju.edu.cn

important target of a series of potential diseases. However, the study of circRNAs on I/R injury was rare. Therefore, we aimed at exploring the role of circ-0068566 in I/R injury, providing a new ideal for the treatment of I/R injury.

MicroRNAs (miRNAs) are small noncoding RNA molecules, which contain about 22 nucleotides and can regulate gene expression at the level of transcription and translation[25]. They regulate the proliferation, apoptosis and metabolism of various diseases, including cancer and neurological diseases[26,27], which indicate that miRNAs can be used as a potential new therapeutic target for various diseases. It is indicated that miR-509-5p was involved in the pathogenesis of male infertility and TGCT by regulating cell proliferation and apoptosis[28]; miR-424-5p could promote the proliferation, migration, invasion and adhesion of LSCC cells, and affect the cell cycle process[29]; miRNA-208a could be used as a biomarker to predict the risk of complications after repair of congenital heart disease[30]. Therefore, we would explore the biological function and molecular mechanism of miR-6322 on myocardial ischemia-reperfusion injury.

CircRNA was involved in the regulation of disease development by interacting with miRNA. Of note, circRNA 100269 was negatively correlated with miR-630 to regulate the development of GC [31]; circRNA-5692 enhanced DAB2IP expression by reducing miR-328-5p expression, thus inhibiting the growth of transplanted tumor *in vivo*[32]; circNr1h4 participated in renal damage in salt sensitive hypertensive mice by direct interaction with miR-155-5p[33]. Since the role of circRNA-68566 and its regulatory mechanism have not been evaluated, we speculated that circRNA-68566 regulated I/R injury through miR-6322.

Therefore, the purpose of this study was to evaluate the protective effect of circRNA-68566 on I/R injury and the role of miR-6322/PARP2 signal *in vivo* and *in vitro*.

Materials and Methods

Establishment of I/R Injury Model

The experiment was conducted in accordance with the guidelines of the National Institutes of Health on the use of abortion animals (NIH Publication No. 8523, revised 1996) and was supported by the Ethics Committee of the Medical College of our Institute. Male C57BL/6 mice were anesthetized with 2% isoflurane and the left chest incision temporarily externalized the heart and placed 6-0 silk slip knot around the anterior descending branch of the left coronary artery, resulting in myocardial ischemia. After 45 minutes of myocardial ischemia, ligation was relieved, and reperfusion was performed for 3 or 24 hours. The same procedure was performed in sham operated mice, except that the suture under the left coronary artery was not knotted.

Simulated Ischemia Reperfusion (SIR) Treatment in Vitro

H9C2 cell was used to study myocardial ischemia and purchased from Tiancheng Technology (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) medium containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% (V/V) penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Briefly, H9C2 cells were exposed to ischemia buffer (mM: 10 deoxyglucose, 137 sodium chloride, 12 potassium chloride, 0.49 magnesium chloride, 0.9 calcium chloride, 2.2 H2O, 0.75 sodium sulfite, 20 lactate and 4 HEPES, pH 6.5) for 1 hour in a humidified cell incubator (95% air, 5% CO_2; 37°C). Reperfusion began by returning cells to normal air conditions (95% air, 5% CO_2; 37°C) for 4 h.

Determination of Myocardial Infarct Size

Mice were anesthetized after reperfusion. Left ventricular ejection fraction (LVEF) and left ventricular fraction shortening (LVFS) were calculated by computer algorithm. After ligation of the ligaments around the coronary artery, the infarct area (infarct area/risk area×100%) was determined by Evans blue/TTC double staining and digital imaging.

Determination of Cell Viability

Cells were cultured on 96 well cell culture plates. After the cells were treated and washed with PBS, 10 μl of CCK-8 (Dojindo Molecular Technologies, Kumamoto, Japan) was added to 100 μl culture medium, and the OD was measured at 37°C for 2 hours.

Determination of LDH Concentration, ROS Production, MDA, SOD and GSH-Px

The LDH concentration, ROS production, MDA content, TUNEL, SOD and GSH-Px ac-

tivity levels in heart tissues and cultured cells were determined using commercially available corresponding kits following the manufacturer's instructions.

Transfection

High expression circRNA-0068566 lentivirus, PARP2-si, miR-346 mimics and miR-346 inhibitors were designed and synthesized by Gene Pharmaceutical Co., Ltd. (Shanghai, China) and were transfected into H9c2 cells by liposomes 2000 (Invitrogen, Carlsbad, CA, USA) according to the instructions for 4 hours, and then replaced with normal medium. The transfection efficiency was detected by QPCR or Western blot.

Luciferase Reporter Assay

HEK293T cells were co-transfected with 60 ng miR-6322 or 150 ng circRNA-0068566 (PARP2) 3'-UTR WT or circRNA mut via liposome 2000 (Invitrogen, Carlsbad, CA, USA). After treatment with C for 48 hours, cells were collected and tested according to the instructions of dual luciferase detection kit (Promega, Madison, WI, USA). MiR-6322, circRNA-0068566 (PARP2) 3'-UTR WT and 3' -UTR mut were provided by Gene Pharmacy Co., Ltd. (Shanghai, China)

RNA Pull-Down

MiRNA-6322 was mixed with RNA markers (Roche Diagnostics, Basel, Switzerland and T7RNA polymerase), treated with RNase (Roche Diagnostics, Basel, Switzerland), and purified with RNAesy small Kit (Qiagen, Hilden, Germany). The protein was then mixed with biotinylated RNA, incubated with streptavidin and washed at room temperature. The protein was separated by twelve alkyl sulphate polyacrylamide gel electrophoresis.

RNA Immunoprecipitation

According to the manufacturer's instructions for RNA binding protein immunoprecipitation Kit (Millipore, Billerica, MA, USA), RIPA buffer containing a mixture of protease and phosphatase inhibitor (Sigma-Aldrich) dissolved cells. Primary antibody or anti-rabbit IgG (Cell Signaling Technology, Danvers, MA, USA) was incubated for 45 minutes, and magnetic beads were added at 4°C overnight. RNA was purified from magnetic bead binding RNA protein complex and analyzed by QPCR.

Isolation of RNA and Real-Time PCR

The RNA was extracted from the samples with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was performed according to the instructions of primescript RT Kit (TaKaRa, Dalian, China). PCR was performed using iTaqTM universal SYBR ® green Supermix (Bio-Rad, Hercules, CA, USA). The primer sequences involved were listed in Table I.

Western Blot Analysis

The proteins separated from the cells by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel were transferred to the Immobilon polyvinylidene difluoride (PVDF) membrane (Merck KGaA, Darmstadt, Germany). Then, the membranes were incubated with primary antibodies against cleaved-caspase3(1:1000, Cell Signaling Technology), Bax (1:1000, Cell Signaling Technology) Bcl2 (1:1000, Cell Signaling Technology), PARP2 antibody (1:1000, Cell Signaling Technology) and β-actin (1:1000, Cell Signaling Technology) overnight at 4°C. The next day, rabbit anti IgG (Proteintech, Rosemont, IL, USA) was incubated with horseradish peroxidase (HRP) antibody for 1 hour and the chemiluminescence enhanced detection kit (Cwbio,

Table I. Sequences used for QPCR.

Name	Sequences (5'-3')
circRNA-68566-F	TTTCTTCAACCTCCCCACCC
circRNA-68566-R	TGTAGCAAGCTTTCCCTGTTT
miR-6322-F	TCTGTGTGCCTACGGT
miR-6322-R	CTCAACTGGTGTCGTGGA
U6-F	CTCGCT TCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT
GAPDH-F	TCCAGTACGACTCCACCCAT
GAPDH-R	CGCCTTCTGCCTTAACCTCA

Beijing, China) was used to prepare the membrane and detected by gel imaging system.

Statistical Analysis

The results were showed as mean±SD. The differences were compared by post-mortem *t*-test using ANOVA and subsequent Bonferroni correction and performed with GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). $p < 0.05$ was considered to have statistical significance.

Results

CircRNA-0068566 was Downregulated in Simulated Ischemia-Reperfusion in Vitro

As shown in Figure 1 A, the expression of circRNA-0068566 was significantly decreased in simulated ischemia-reperfusion (SIR) *in vitro*. We then evaluated the effect of circRNA-0068566 on SIR *in vitro* by transfecting lentivirus expressing circRNA-0068566 to increase the expression of circRNA-0068566 in H9C2 cells (Figure 1 B). Our further analysis showed overexpression of circRNA-0068566 significantly increased the survival rate of H9C2 cells injured by SIR (Figure 1 C). Next, compared with the control, it significantly reversed the increase of ROS (Figure 1 D), LDH (Figure 1 E), MDA content (Figure 1 F), Bax protein expression (Figure 1 G-1 H) and cleaved-caspase3 protein expression (Figure 1 I), the decrease of Bcl-2 protein expression (Figure 1 J), the increase of apoptosis rate (Figure 1 K) and the decrease of SOD (intracellular superoxide dismutase) activity and GSH-PX (intracellular glutathione peroxidase) activity (Figure 1 L-1 M) caused by SIR injury. In conclusion, these data suggested that circRNA-0068566 may be an effective regulator in I/R injury.

CircRNA-0068566 Improved I/R Injury

We studied the expression of cirRNA-0068566 *in vivo* to further explore its role in I/R injury. As shown in Figure 2 A, we found that circRNA-0068566 was downregulated in I/R, and then increased the expression of cirRNA-0068566 in mice to study whether cirRNA-0068566 could protect I/R injury. The high expression of cirRNA-0068566 not only promoted the recovery of cardiac function by increasing left ventricular ejection fraction (Figure 2 B) and left ventricular ejection fraction (Figure 2 C), but also reduced the infarct area (Figure 2 D), serum lactate dehydrogenase (Figure 2 E) and MDA content (Figure 2 F) after I/R treatment. In addition, the overexpression of cirRNA-0068566 significantly inhibited the increase of apoptosis rates (Figure 2 G), Bax protein level (Figure 2 H-I), cleaved-caspase3 (Figure 2 J), and reversed the decrease of Bcl2 protein level (Figure 2 K) induced by RI. Moreover, cirRNA-0068566 upregulated increased the anti-oxidative capacity by upregulating the activities of SOD (Figure 2 L) and GSH-Px (Figure 2 M). These data were consistent with our results in vivo. In conclusion, these data suggested that cirRNA-0068566 could effectively inhibit the development of I/R injury.

miR-6322 was a Direct Target of circRNA-0068566

Based on the above results, we further explored the regulatory mechanism of circRNA-0068566 in I/R injury. RIP experimental using anti-AGO2 antibodies findings showed that cirRNA-0068566 regulated miR-6322 in an AGO2-dependent manner in vitro (Figure 3 A). At the same time, the binding sites of circRNA-0068566 and miR-6322 (Figure 3 B) and the results of double luciferase report further indicated (Figure 3 C) that miR-6322 was the direct target of circRNA-0068566. In addition, the low expression of circRNA-0068566 increased the expression of miR-6322 (Figure 3 D), and the opposite conclusion could be drawn (Figure 3 E) with the overexpression of circRNA-0068566. More importantly, miR-6322 was up-regulated during I/R *in vitro* (Figure 3 F). These results showed that miR-6322 may be a direct target of circRNA-0068566 in I/R injury.

Effect of miRNA-6322 Inhibitor on I/R Injury in Vitro

The inhibitor of miRNA-6322 was added to H9C2 cells to study its effect *in* vitro of I/R injury. Interestingly, miRNA-6322 inhibitors significantly reversed the decrease of cell viability (Figure 4 A) and the increase of ROS (Figure 4 B), LDH (Figure 4 C), MDA content (Figure 4 D), Bax (Figure 4 E-4 F), cleaved-caspase3 protein level (Figure 4 G), apoptosis rate (Figure 4 H), and even the decrease of Bcl-2 protein expression (Figure 4 I) and antioxidant activity of SOD (Figure 4 J) and GSH-Px (Figure 4 K). In conclusion, these data suggested that miRNA-6322 involved in the regulation of I/R injury.

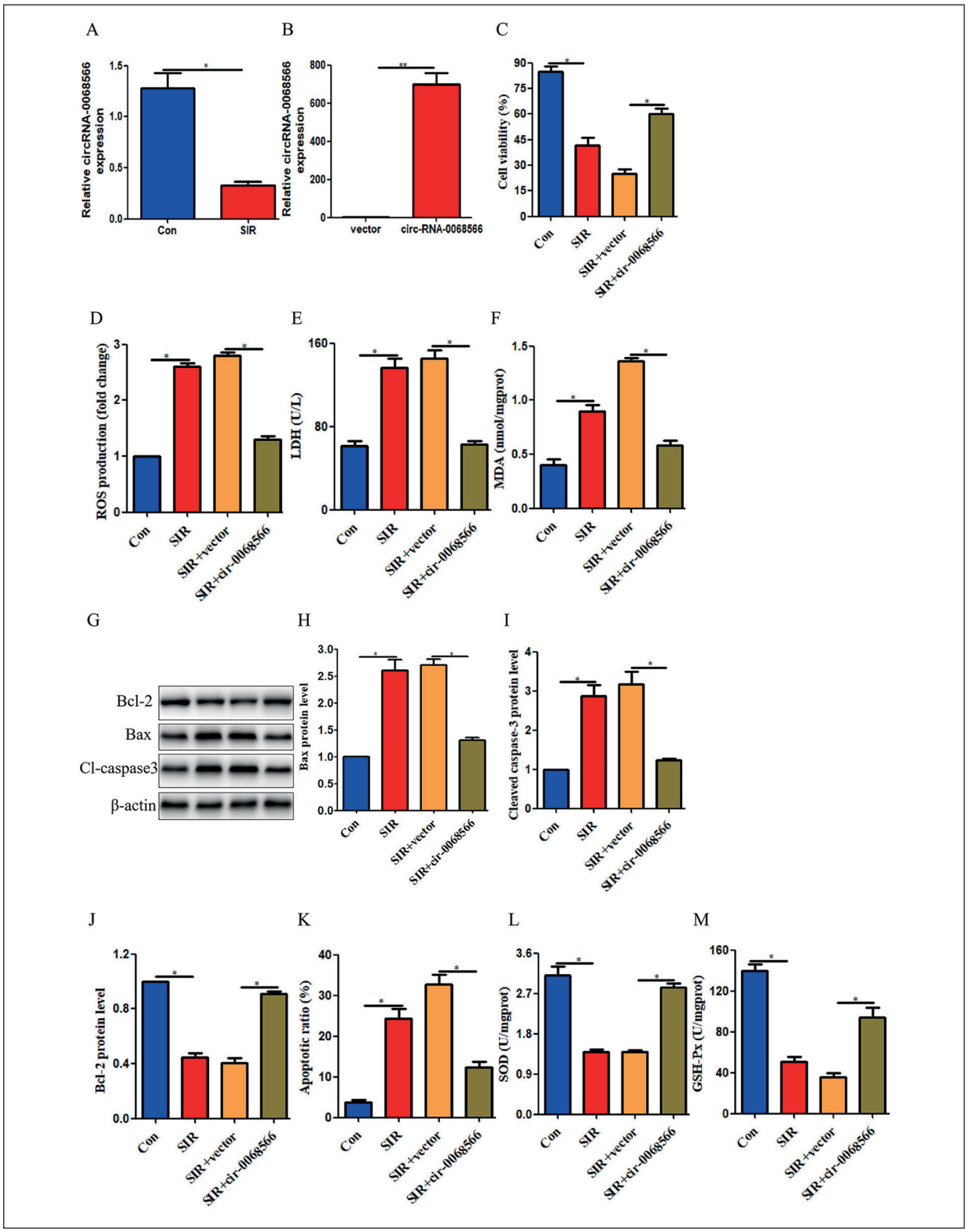

Figure 1. CircRNA-0068566 was downregulated in simulated I/R *in vitro*. **A-B** The expression of circRNA-0068566 was detected by QPCR in H9C2 cells. **C** Cell viability was assessed using the CCK assay. **D** Reactive oxygen species (ROS) production. **E** LDH level. **F** MDA content. **G** Representative blots. **H** Bax protein level. **I** Cleaved-caspase3 protein level. **J** Bcl2 protein level. **K** The apoptotic ratio was detected by TUNEL. **L** Intracellular superoxide dismutase (SOD) activity. **M** Intracellular glutathione peroxidase (GSH-Px) activity. These data were expressed as mean ± SD. n=3 *p <0.05.

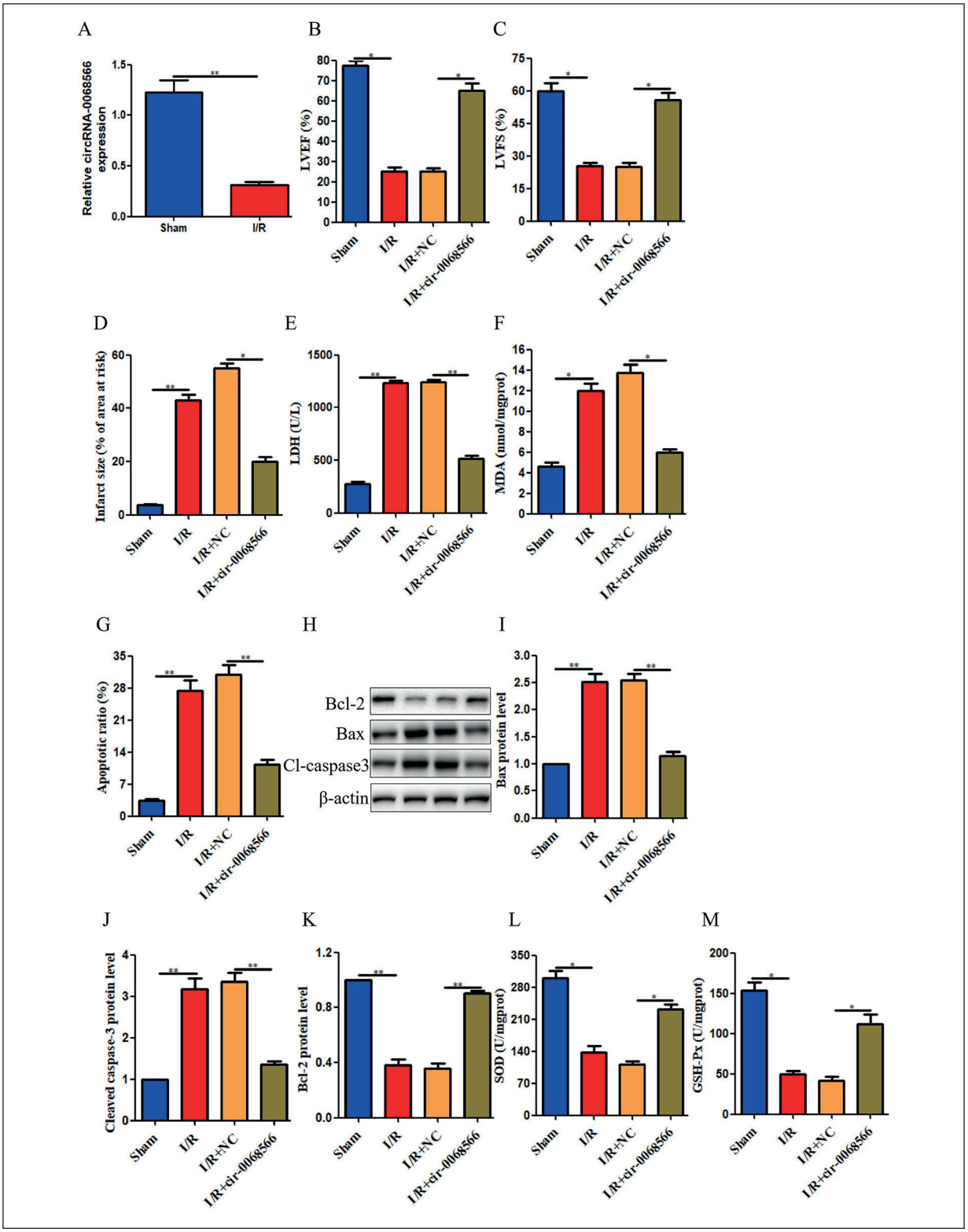

Figure 2. CircRNA-0068566 improved I/R injury. **A** The expression of circRNA-0068566 was detected by QPCR. **B** Left ventricular ejection fraction. **C** Left ventricular fractional shortening. **D** Myocardial infarct size was expressed as the percentage of area at risk. **E** LDH level. **F** MDA content. **G** The apoptotic ratio was detected by TUNEL. **H** Representative blots. **I** Bax protein level. **J** Cleaved-caspase3 protein level. **K** Bcl2 protein level. **L** Intracellular superoxide dismutase (SOD) activity. **M** Intracellular glutathione peroxidase (GSH-Px) activity. These data were expressed as mean ± SD. n=6 **p* <0.05.

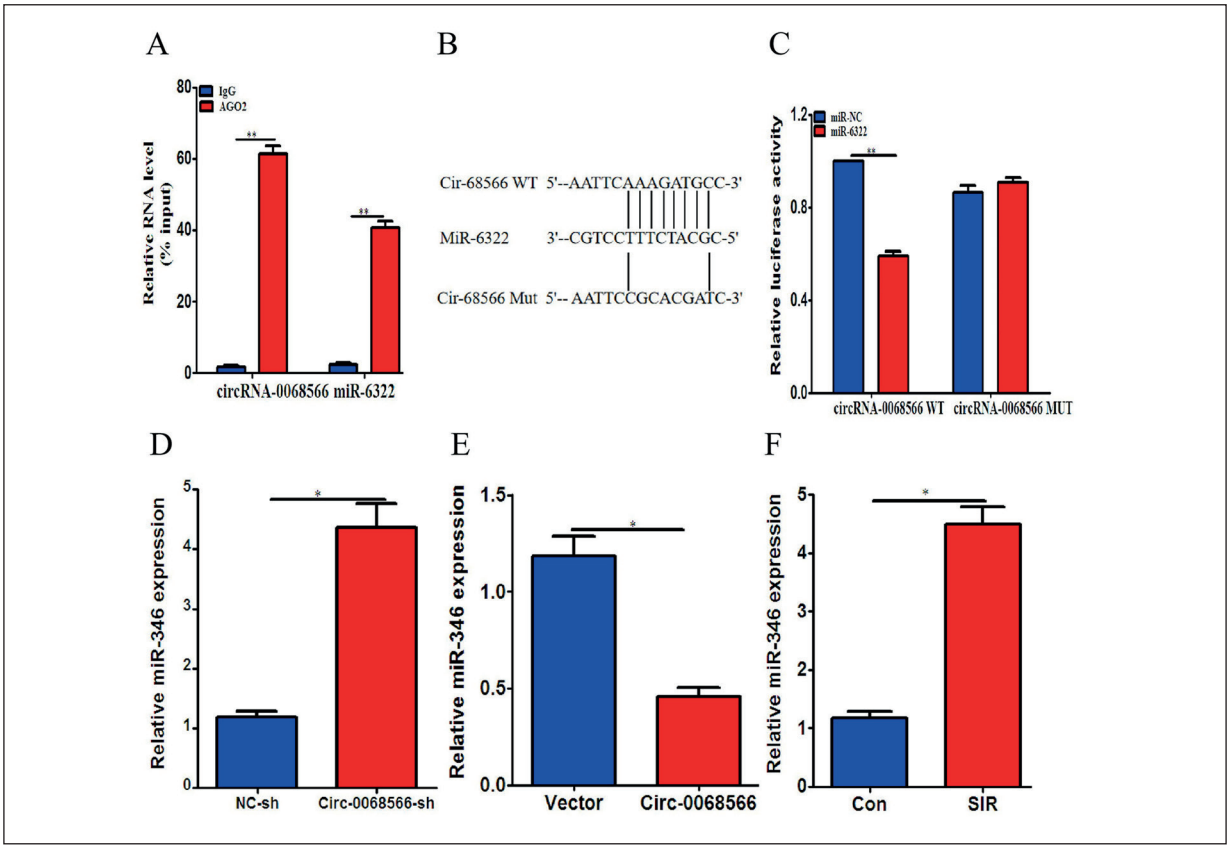

Figure 3. miR-6322 was a direct target of circRNA-0068566. **A** The levels of circRNA-0068566 and miRNA-6322 were detected by QPCR and presented as fold enrichment of AGO2 relative to input. **B** Anti-AGO2 RIP to investigate AGO2 binding to circRNA-0068566 and miR-6322, miRNA-6322 expression and circRNA-0068566 were measured after RIP assay. **C** Target site of miR-6322 and circRNA-0068566. **D** Effect of miR-6322 on the activity of circRNA-0068566 reporter luciferase was checked. **E-G** The levels of miRNA-6322 were detected by QPCR. These data were expressed as mean ± SD. n=3. **p* <0.05.

MiRNA-6322 Involved in I/R Injury

The inhibitor of miRNA-6322 was used to further test the role of miRNA-6322 *in vivo* of I/R injury. As shown in Figure 5 A-5 B, miRNA-6322 inhibitor could reduce the cardiac injury caused by I/R by increasing LVEF and LVFS. Additionally, miRNA-6322 inhibitor decreased infarct area (Figure 5 C), serum LDH level (Figure 5 D), MDA content (Figure 5 E) and apoptosis rate (Figure 5 F) caused by I/R and increased the anti-oxidative capacity by upregulating the activities of SOD (Figure 5 G) and GSH-Px (Figure 5 H). Additionally, as shown in Figure 5I, miRNA-6322 could eliminate I/R-induced apoptosis by increasing Bcl-2 expression (Figure 5 J), decreasing Bax (Figure 5 K) and cleaved-caspase3 protein expression (Figure 5 L). Taken together, these results suggested that miRNA-6322 silencing could alleviate I/R injury.

MiR-6322 Participated in I/R Injury by Interacting with PARP2

Studies have shown that many miRNAs were involved in the regulation of diseases by binding to specific proteins. We discussed whether miR-6322 was also involved in the regulation of I/R injury through this mechanism. Our prediction was confirmed by RNA pull-down test with biotinylated miR-6322 (Figure 6 A) and IP-QPCR assay detection (Figure 6 B) showed that PARP2 might be a potential target of miR-6322. At the same time, the prediction of target binding sites (Figure 6 C) and the report of double luciferase (Figure 6 D) confirmed that PARP2 was the direct target of miR-6322. In addition, miR-6322 inhibitor significantly increased PARP2 protein level, while miR-6322 mimic decreased PARP2 protein level (Figure 6 E). More importantly, we found that the protein expression of PARP2 in I/R

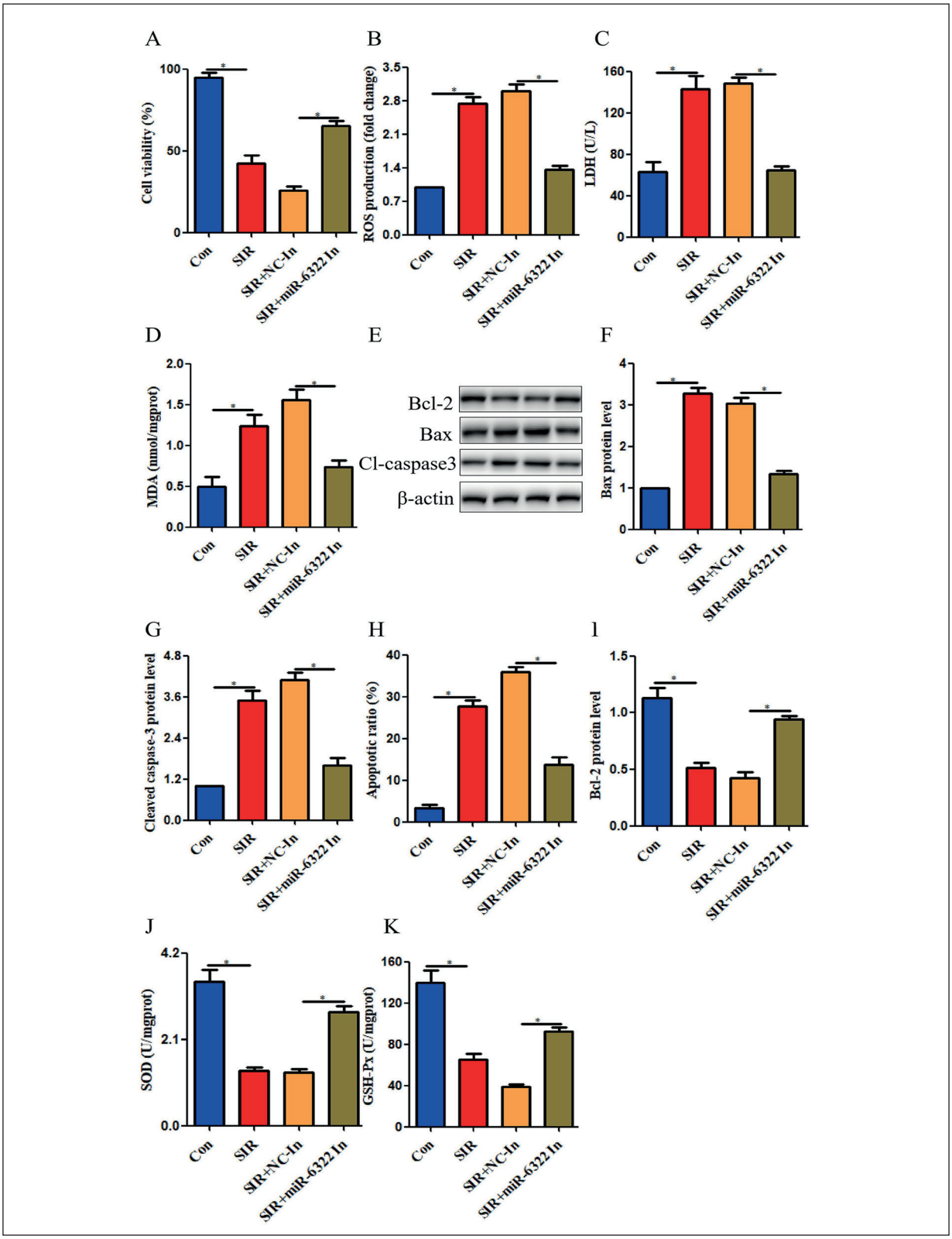

Figure 4. Effect of miRNA-6322 inhibitor on I/R injury *in vitro*. **A** Cell viability was assessed using the CCK assay. **B** Reactive oxygen species. **C** LDH level. **D** MDA content. **E** Representative blots. **F** Bax protein level. **G** Cleaved-caspase3 protein level. **H** The apoptotic ratio was detected by TUNEL. **I** Bcl2 protein level. **J** Intracellular superoxide dismutase (SOD) activity. **K** Intracellular glutathione peroxidase (GSH-Px) activity. These data were expressed as mean ± SD. n=3. *p <0.05.

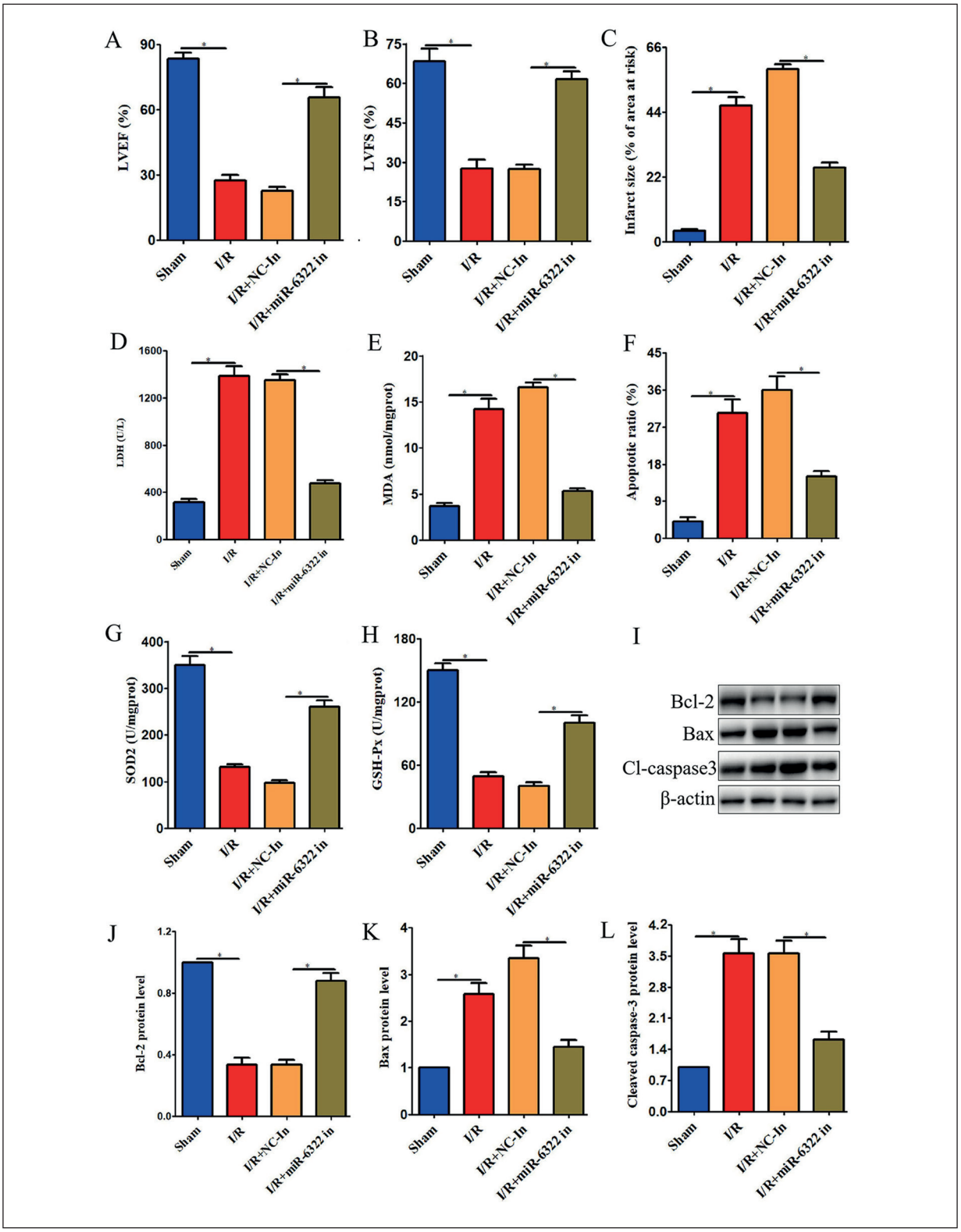

Figure 5. MiRNA-6322 involved in I/R injury. **A** Left ventricular ejection fraction. **B** Left ventricular fractional shortening. **C** Myocardial infarct size was expressed as the percentage of area at risk. **D** LDH level. **E** MDA content. **F** The apoptotic ratio was detected by TUNEL. **G** Intracellular superoxide dismutase (SOD) activity. **H** Intracellular glutathione peroxidase (GSH-Px) activity. **I** Representative blot. **J** Bcl2 protein level. **K** Bax protein level. **L** Cleaved-caspase3 protein level. These data were expressed as mean ± SD. n=6 *p <0.05.

injury and H9C2 was decreased (Figure 6 F-6 G). In conclusion, miR-6322 participated in I/R injury by regulating PARP2.

MiR-6322 Functioned Partly Through PARP2 in Vitro

According to the above conclusion, PARP2 may be one of the targets of miR-6322 involved in I/R injury. Therefore, we further verified the interaction between PARP2 and miRNA-6322 in I/R by overexpression of PARP2 in H9C2 cells (Figure 7 A). The overexpression of PARP2 could reverse the effect treated with miR-6322 mimic by increasing myocardial cell activity (Figure 7 B), decreasing the product of ROS (Figure 7 C), MDA (Figure 7 D) and LDH (Figure 7 E) and increasing the antioxidant activity by upregulating the activities of SOD (Figure 7 F) and GSH-Px (Figure 7 G). In addition, PARP2 upregulated eliminated miR-6322 overexpressing-triggered apoptosis by decreasing apoptosis rate (Figure 7 H), Bax (Figure 7 I-7 J) and cleaved-caspase3 protein expression (Figure 7 K) and increasing Bcl-2 level (Figure 7 L). In general, miR-6322 was at least partially dependent on the role of PARP2 in I/R injury.

Discussion

The results of the present study firstly revealed that circRNA-0068566 has a protective effect on

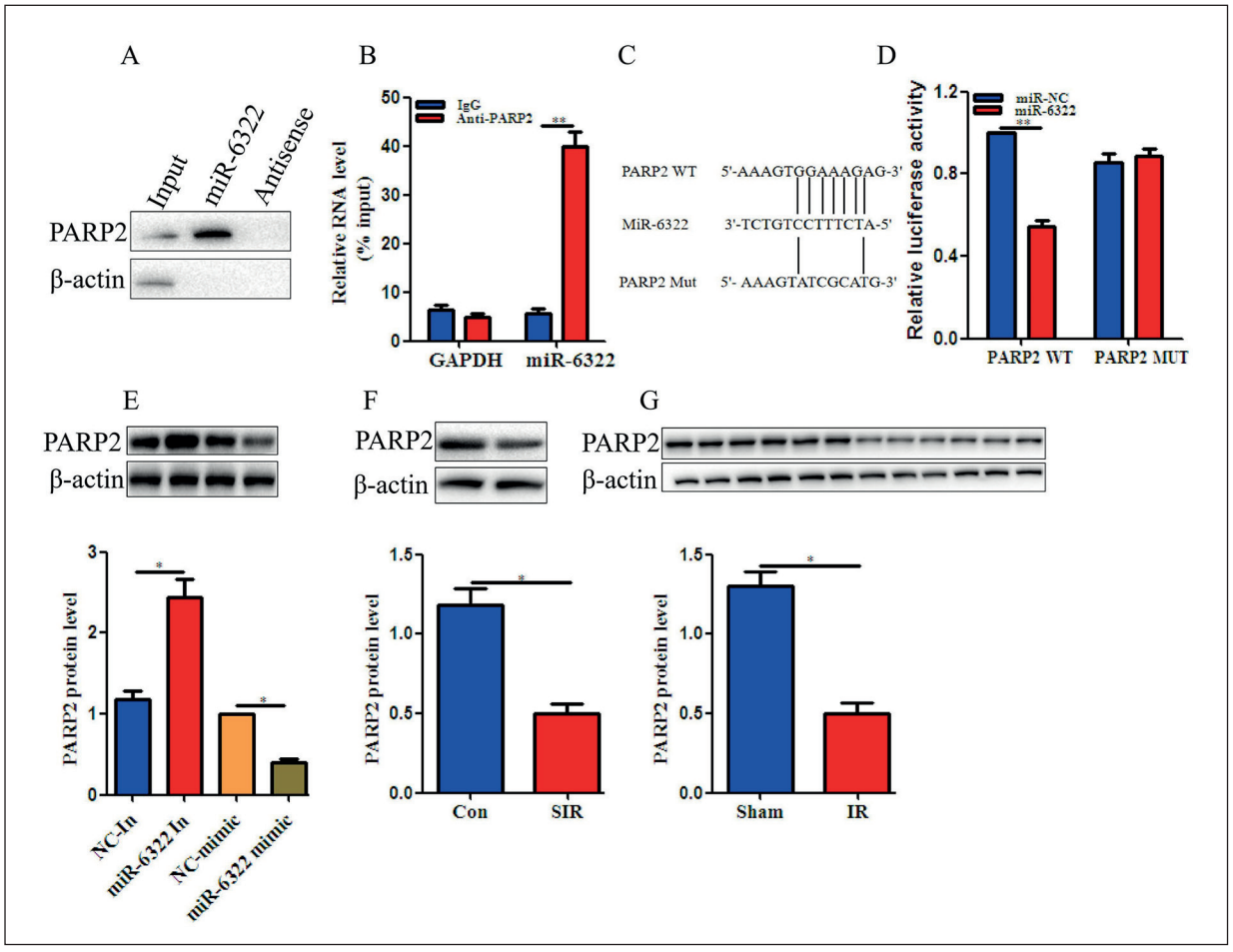

Figure 6. MiR-6322 participated in I/R injury by interacting with PARP2. **A** Western blotting analysis showing the specific interaction of miR-6322 with PARP2. **B** Enrichment of RNA immunoprecipitation was determined as the amount of RNA associated with immunoprecipitation of PARP2 relative to the input control. **C** RIP was performed using anti-PARP2 antibodies and specific primers to detect miR-6322 or GAPDH. **D** Target site of miR-6322 and circRNA-0068566. **E** Effect of miR-6322 on the activity of PARP2 reporter luciferase was checked. **F-H** PARP2 protein level was detected by Western blotting. These data were expressed as mean ± SD. n=3. *p <0.05.

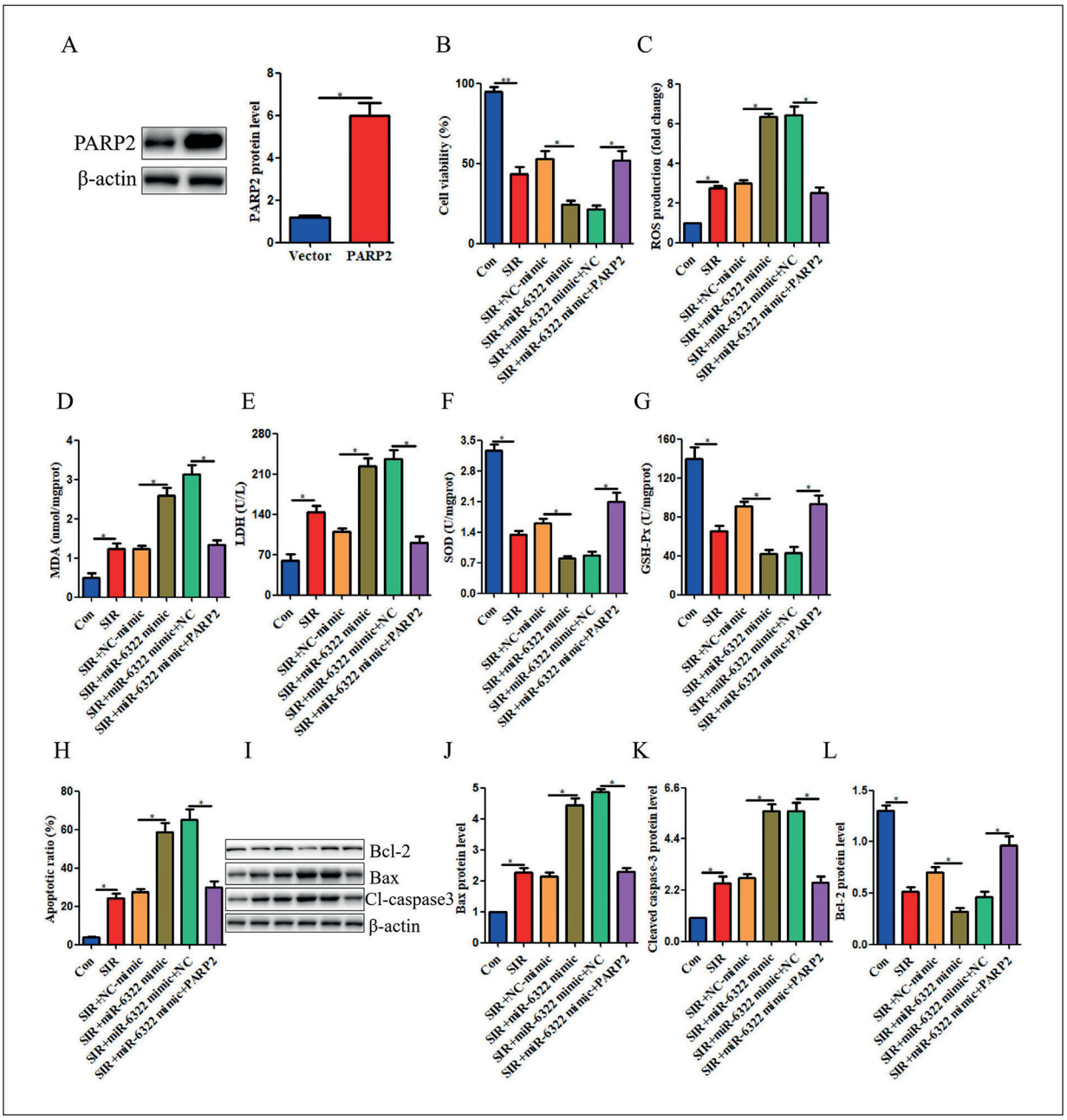

Figure 7. MiR-6322 functioned partly through PARP2 *in vitro*. **A** PARP2 protein levels were investigated by Western blotting. **B** Cell viability was assessed using the CCK assay. **C** Reactive oxygen species. **D** MDA content. **E** LDH level. **F** Intracellular superoxide dismutase (SOD) activity. **G** Intracellular glutathione peroxidase (GSH-Px) activity. **H** The apoptotic ratio was detected by TUNEL. **I** Representative blot. **J** Bax protein level. **K** Cleaved-caspase3 protein level. **L** Bcl2 protein level. These data were expressed as mean ± SD. n=3. *p <0.05.

the heart and H9C2 cells after I/R injury, which was confirmed by improving the recovery of heart function and cell vitality, reducing the area of myocardial infarction, myocardial cell apoptosis, LDH level, MDA content, ROS level and the oxidative stress in heart tissue and myocardial cells. Further, circRNA-0068566 alleviated I/R injury partly by regulating the miR-6322/PARP2 signal pathway.

CircRNA plays an important role in many diseases, such as neurodegenerative diseases, cardiovascular diseases, immune regulation and

cancer and its role in different physiological and pathological conditions is mediated by regulating its expression. However, there are few studies on the relationship between circRNA and I/R injury. We mainly were interested on the role of circRNA-0068566 in cardiovascular reperfusion injury and its regulatory mechanism. Our experiment found that circRNA-0068566 was down-regulated *in vitro* and *in vivo* of I/R injury models and its overexpression clearly improved the recovery of heart function and cell vitality, reduced the area of myocardial infarction, myocardial cell apoptosis, LDH level, MDA content, ROS level and the oxidative stress on the heart and H9C2 cells of I/R injury. These results indicated that circRNA-0068566 could participate in the development of I/R injury.

CircRNA that interacted miRNAs to regulate the development of disease has been reported [24]. However, due to the low abundance of circRNA and the frequent mixing of circRNA-miRNA interactions, the extent to which circRNA function through this mechanism remains open. For circRNA-0068566, through RIP, double luciferase, bioinformatics and other experiments, we found that miRNA-6322 was the target. In addition, we found that the expression of miRNA-6322 was up-regulated in I/R, and the low expression of circRNA-0068566 could increase the expression of miRNA-6322. miRNA-6322 silencing could alleviate I/R injury through improving the recovery of heart function and cell vitality, reducing the area of myocardial infarction, myocardial cell apoptosis, LDH level, MDA content, ROS level and the oxidative stress in heart tissue and myocardial cells. Therefore, we demonstrated that circRNA-0068566 was involved in the regulation of I/R injury by regulating the expression of miRNA-6322.

The regulatory mechanism of miR-6322 involved in I/R injury remained unclear and many studies have shown that miRNAs could participate in the regulation of disease by interacting with specific proteins. Therefore, we tested the interaction between miRNA-6322 and PARP2 by RNA pull-down, IP-QPCR and results showed that PARP2 may be a potential target of miRNA-6322. In addition, bioinformatics analysis and luciferase report revealed that PARP2 was the direct target of miRNA-6322. Meanwhile, the overexpression of miRNA-6322 decreased the expression level of PARP2, while the inhibition of miRNA-6322 increased the expression level of PARP2. Therefore, we speculated that miR-6322 probably interacted with PARP2 to regulate the progression of I/R injury.

Although there are some studies on the function and mechanism of PARP2 [34], there's no research on cardiovascular disease. Therefore, on the basis of the above conclusions, we also found that the expression of PARP2 was down-regulated in the process of I/R injury. More importantly, the down-regulation of PARP2 could reverse the effects induced by miR-6322 overexpression on cell vitality, cell apoptosis, LDH level, ROS level, MDA content, oxidative stress, which expanded our understanding of the mechanism of the interaction between PARP2 and miR-6322 in the process of I/R injury. Overall, these results suggested that the effect of circRNA-0068566 on I/R injury was at least partially mediated by miR-6322/PARP2 signal pathway.

Conclusions

As far as we know, this was the first study showing that circRNA-0068566 has a protective effect on I/R injury and miR-6322 has been used as a target for connecting circRNA-0068566 and PARP2 in I/R injury. Our experiments showed that circRNA-0068566 significantly inhibited I/R induced cardiac dysfunction, oxidative stress and apoptosis by regulating miR-6322/PARP2 signaling pathway, indicating that circRNA-0068566/miR-6322/PARP2 signaling pathway may be a potential target for the treatment of I/R injury.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Sun MS, Jin H, Sun X, Huang S, Zhang FL, Guo ZN, Yang Y. Free radical damage in ischemia-reperfusion Injury: an obstacle in acute ischemic stroke after revascularization therapy. Oxid Med Cell Longev 2018; 2018: 3804979.

2) De Roeck L, Vandamme S, Everaert BR, Hoymans V, Haine S, Vandendriessche T, Bosmans J, Ronsyn MW, Miljoen H, Van Berendoncks A, De Meyer G, Vrints C, Claeys MJ. Adiponectin and ischemia-reperfusion injury in ST segment elevation myocardi-

al infarction. Eur Heart J Acute Cardiovasc Care 2016; 5: 71-76.

3) Koyama T. Lactated Ringer's solution for preventing myocardial reperfusion injury. Int J Cardiol Heart Vasc 2017; 15: 1-8.

4) Hausenloy DJ, Yellon DM. Myocardial ischemia-reperfusion injury: a neglected therapeutic target. J Clin Invest 2013; 123: 92-100.

5) Bawaskar HS, Bawaskar PH, Bawaskar PH. Preintensive care: thrombolytic (streptokinase or tenecteplase) in ST elevated acute myocardial infarction at peripheral hospital. J Family Med Prim Care 2019; 8: 62-71.

6) Dou M, Ma Z, Cheng X, Zou G, Xu Y, Huang C, Xiong W, He S, Zhang Y. Intrathecal lentivirus-mediated RNA interference targeting nerve growth factor attenuates myocardial ischaemia-reperfusion injury in rat. Br J Anaesth 2019; 123: 439-449.

7) Russo I, Penna C, Musso T, Popara J, Alloatti G, Cavalot F, Pagliaro P. Platelets, diabetes and myocardial ischemia/reperfusion injury. Cardiovasc Diabetol 2017; 16: 71.

8) Ziegler M, Wang X, Peter K. Platelets in cardiac ischaemia/reperfusion injury: a promising therapeutic target. Cardiovasc Res 2019; 115: 1178-1188.

9) Ibáñez B, Heusch G, Ovize M, Van de Werf F. Evolving therapies for myocardial ischemia/reperfusion injury. J Am Coll Cardiol 2015; 65: 1454-1471.

10) Zweier JL, Talukder MA. The role of oxidants and free radicals in reperfusion injury. Cardiovasc Res 2006; 70: 181-190.

11) Dröge W. Free radicals in the physiological control of cell function. Physiol Rev 2002; 82: 47-95.

12) Pantazi E, Zaouali MA, Bejaoui M, Folch-Puy E, Ben Abdennebi H, Roselló-Catafau J. Role of sirtuins in ischemia-reperfusion injury. World J Gastroenterol 2013; 19: 7594-7602.

13) Patop IL, Kadener S. circRNAs in Cancer. Curr Opin Genet Dev 2018; 48: 121-127.

14) Xia X, Tang X, Wang S. Roles of circRNAs in autoimmune diseases. Front Immunol 2019; 10: 639.

15) Rong D, Sun H, Li Z, Liu S, Dong C, Fu K, Tang W, Cao H. An emerging function of circRNA-miRNAs-mRNA axis in human diseases. Oncotarget 2017; 8: 73271-73281.

16) 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.

17) Li Y, Lv Z, Zhang J, Ma Q, Li Q, Song L, Gong L, Zhu Y, Li X, Hao Y, Yang Y. Profiling of differentially expressed circular RNAs in peripheral blood mononuclear cells from Alzheimer's disease patients. Metab Brain Dis 2020; 35: 201-213.

18) Altesha MA, Ni T, Khan A, Liu K, Zheng X. Circular RNA in cardiovascular disease. J Cell Physiol 2019; 234: 5588-5600.

19) Zhou LY, Zhai M, Huang Y, Xu S, An T, Wang YH, Zhang RC, Liu CY, Dong YH, Wang M, Qian LL, Ponnusamy M, Zhang YH, Zhang J, Wang K. The circular RNA ACR attenuates myocardial ischemia/reperfusion injury by suppressing autophagy via modulation of the Pink1/FAM65B pathway. Cell Death Differ 2019; 26: 1299-1315.

20) Chen L, Luo W, Zhang W, Chu H, Wang J, Dai X, Cheng Y, Zhu T, Chao J. circDLPAG4/HECTD1 mediates ischaemia/reperfusion injury in endothelial cells via ER stress. RNA Biol 2020; 17: 240-253.

21) Wang S, Chen J, Yu W, Deng F. Circular RNA DLGAP4 ameliorates cardiomyocyte apoptosis through regulating BCL2 via targeting miR-143 in myocardial ischemia-reperfusion injury. Int J Cardiol 2019; 279: 147.

22) Cao C, Han S, Yuan Y, Wu Y, Lian W, Zhang X, Pan L, Li M. Downregulated Circular RNA hsa_circ_0000291 suppresses migration and proliferation of gastric cancer via targeting the miR-183/ITGB1 axis. Cancer Manag Res 2019; 11: 9675-9683.

23) Ouyang Z, Tan T, Zhang X, Wan J, Zhou Y, Jiang G, Yang D, Guo X, Liu T. CircRNA hsa_circ_0074834 promotes the osteogenesis-angiogenesis coupling process in bone mesenchymal stem cells (BMSCs) by acting as a ceRNA for miR-942-5p. Cell Death Dis 2019; 10: 932.

24) Xie H, Ren X, Xin S, Lan X, Lu G, Lin Y, Yang S, Zeng Z, Liao W, Ding YQ, Liang L. Emerging roles of circRNA_001569 targeting miR-145 in the proliferation and invasion of colorectal cancer. Oncotarget 2016; 7: 26680-26691.

25) Cui J, Zhou B, Ross SA, Zempleni J. Nutrition, microRNAs, and human health. Adv Nutr 2017; 8: 105-112.

26) Tüfekci KU, Oner MG, Meuwissen RL, Genç S. The role of microRNAs in human diseases. Methods Mol Biol 2014; 1107: 33-50.

27) Solly EL, Dimasi CG, Bursill CA, Psaltis PJ, Tan JTM. MicroRNAs as therapeutic targets and clinical biomarkers in Atherosclerosis. J Clin Med 2019; 8(12). pii: E2199.

28) Sun J, Niu L, Gao S, Yi X, Chen J. miR-509-5p Downregulation is associated with male infertility and acts as a suppressor in testicular germ cell tumor cells through targeting MDM2. Onco Targets Ther 2019; 12: 10515-10522.

29) Li Y, Liu J, Hu W, Zhang Y, Sang J, Li H, Ma T, Bo Y, Bai T, Guo H, Lu Y, Xue X, Niu M, Ge S, Wen S, Wang B, Gao W, Wu Y. miR-424-5p promotes proliferation, migration and ivasion of laryngeal squamous cell carcinoma. Onco Targets Ther 2019; 12: 10441-10453.

30) Zloto K, Tirosh-Wagner T, Bolkier Y, Bar-Yosef O, Vardi A, Mishali D, Paret G, Nevo-Caspi Y. Preoperative miRNA-208a as a predictor of postoperative complications in children with congenital heart disease undergoing heart surgery. J Cardiovasc Transl Res 2019 Nov 15. doi: 10.1007/s12265-019-09921-1. [Epub ahead of print]

31) Zhang Y, Liu H, Li W, Yu J, Li J, Shen Z, Ye G, Qi X1, Li G. CircRNA_100269 is downregulated in gastric cancer and suppresses tumor cell growth by targeting miR-630. Aging (Albany NY) 2017; 9: 1585-1594.

32) Liu Z, Yu Y, Huang Z, Kong Y, Hu X, Xiao W, Quan J, Fan X. CircRNA-5692 inhibits the progression of hepatocellular carcinoma by sponging miR-328-5p to enhance DAB2IP expression. Cell Death Dis 2019; 10: 900.

33) Lu C, Chen B, Chen C, Li H, Wang D, Tan Y, Weng H. CircNr1h4 regulates the pathological process of renal injury in salt-sensitive hypertensive mice by targeting miR-155-5p. J Cell Mol Med 2020; 24: 1700-1712.

34) Zhou Q, Zhang W, Wang Z, Liu S. Long non-coding RNA PTTG3P functions as an oncogene by sponging miR-383 and up-regulating CCND1 and PARP2 in hepatocellular carcinoma. BMC Cancer 2019; 19: 731.