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Original Article

Dihydromyricetin Alleviates H9C2 Cell Apoptosis and Autophagy by Regulating CircHIPK3 Expression and PI3K/AKT/mTOR Pathway*

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ABSTRACT **Objective:** To investigate the effect and potential mechanism of dihydromyricetin (Dmy) on H9C2 cell proliferation, apoptosis, and autophagy. **Methods:** H9C2 cells were randomly divided into 7 groups, namely control, model, EV (empty pCDH-CMV-MCS-EF1-CopGFP-T2A-Puro vector), IV (circHIPK3 interference), Dmy (50 μmol/L), Dmy+IV, and Dmy+EV groups. Cell proliferation and apoptosis were detected by cell counting kit-8 assay and flow cytometry, respectivley. Western blot was used to evaluate the levels of light chain 3 Ⅱ/Ⅰ (LC3Ⅱ/Ⅰ), phospho-phosphoinositide 3-kinase (p-PI3K), protein kinase B (p-AKT), and phospho-mammalian target of rapamycin (p-mTOR). The level of circHIPK3 was determined using reverse transcriptase polymerase chain reaction. Electron microscopy was used to observe autophagosomes in H9C2 cells. **Results:** Compared to H9C2 cells, the expression of circHIPK in H9C2 hypoxia model cells increased significantly (P<0.05). Compared to the control group, the cell apoptosis and autophagosomes increased, cell proliferation rate decreased significantly, and the expression of LC3Ⅱ/Ⅰ significantly increased (all P<0.05). Compared to the model group, the rate of apoptosis and autophagosomes in IV, Dmy, and Dmy+IV group decreased, the cell proliferation rate increased, and the expression of LC3Ⅱ/Ⅰ decreased significantly (all P<0.05). Compared to the control group, the expressions of p-PI3K, p-AKT, and p-mTOR in the model group significantly reduced (P<0.05), whereas after treatment with Dmy and sh-circHIPK3, the above situation was reversed (P<0.05). **Conclusion:** Dmy plays a protective role in H9C2 cells by inhibiting circHIPK expression and cell apoptosis and autophagy, and the mechanism may be related to PI3K/AKT/mTOR pathway.

KEYWORDS dihydromyricetin, circHIPK, autophagy, cell apoptosis, PI3K/AKT/mTOR

Myocardial infarction (MI) is the death of myocardial cells due to prolonged myocardial ischemia resulting from insufficient blood flow and oxygen supply.[1] Hypoxia is one of the factors that affect physiological processes including energy metabolism, autophagy, cell motility, angiogenesis, and erythropoiesis.[2] Study has shown that hypoxia is a potential pathogenic factor for myocardial ischemia. Acute or persistent hypoxia can cause cardiomyocyte apoptosis and autophagy, resulting in cardiomyocyte damage, which progresses to MI.[3] Currently, MI treatment focuses on angiotensin converting enzyme inhibitors/angiotensin receptor blockers, statins, and β blockers,[4] but the efficacy is not ideal. Therefore, there has been an increasing number of approaches for treating MI such as protection against hypoxia-induced myocardial cell injury.[5]

circRNA is a special subclass of ncRNA, which

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is characterized by linking of 3' and 5' ends to form a covalent closed loop structure. It has been shown that varieties of circRNAs are involved in regulation of myocardial cell injury repair. CircNfix can promote cardiac regeneration and repair and functional recovery after MI.[4] CircMFACR,[6] circFndc3b,[7] circITCH,[8] and circ_0010729[9] can regulate myocardial cells apoptosis and improve myocardial function. Wang, et al[10] found that circHIPK3 was overexpressed in myocardial ischemia/reperfusion (I/R) model cells and could regulate the oxidative damage of cardiac microvascular endothelial cells through mir-29a/IGF-1 pathway. Chen, et al[11] found that circHIPK3 could regulate autophagy in non-small cell lung cancer through miR-124-3P-STAT3-PRKAa/AMPKα signaling pathway.[11] Therefore, the ability of circHIPK3 to regulate myocardial cell injury by influencing autophagy needs to be studied further.

Dihydromyricetin (Dmy) is an important plant flavonoid isolated from the Chinese medicinal plant, *Ampelopsis Grossedentata*,[12] which has heart protection, anti-diabetes, liver protection, neuroprotection, anti-tumor, and skin protection benefits.[13] Studies have shown that Dmy can protect vascular endothelial cells from oxidative stress injury by activating PI3K/AKT signaling pathway and increasing the expression of PI3K/AKT pathway, which plays a protective role against myocardial ischemia-reperfusion injury.[14,15] Tan, et al[16] found that Dmy can eliminate TFEB-dependent cell autophagy in cutaneous squamous cell carcinoma cells by reducing the expression of lncRNA MALAT1; however, the effect of Dmy on autophagy in PI3K/AKT/mTOR pathway by regulating circRNA remains unclear. Thus, in this study, we constructed H9C2 cell injury model, and then observed the changes in cell proliferation, apoptosis, and autophagy after Dmy intervention, and further studied the potential mechanism of Dmy to provide a theoretical basis for Dmy in clinical treatment of MI.

METHODS

Cell Culture and Cell Hypoxia Injury Model Construction

H9C2 cells were obtained from Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, SH30022.01B, HyClone) supplemented with 10% fetal bovine serum (FBS, No. 10270-106, Gibco, USA) in an atmosphere containing 5% CO_2 and 95% air at 37 ℃. The medium was replaced every 24 h, and the cells were subcultured or cryopreserved when the confluence reached 80%.

Cell hypoxia injury model was constructed according to references.[17,18] Precisely, H9C2 cells were cultured in serum-free DMEM in a constant incubator temperature at 37 ℃, with 94% N_2, 5% CO_2, and 1% O_2 for 24 h.

si-circHIPK3 Construction and Treatment

pCDH-CMV-MCS-EF1-CopGFP-T2A-Puro vector (ZY5BB-1, ZYbscience, China) with Green fluorescent protein (GFP) tag was used for circHIPK3 interference vector and the host bacteria was DH5α. The cells' RNAs were isolated using RNA extraction kit (Wuhan Hualian Biotechnology LTD, Co. China), and reverse transcription was conducted followed by polymerase chain reaction (PCR) amplification using circHIPK3-F and circHIPK3-R primers (Wuhan Hualian Biotechnology Co., Ltd, China). PCR products were collected using gel extraction kit (NPK-600, TOYOBO, China) and pCDH-CMV-MCS-EF1-CopGFP-T2A-Puro vector was linearized using restriction enzymes, XhoI and BamHI at 37 ℃ for 2 h, and the linearized vectors were then recycled using purified column. After the digested PCR gene fragments were inserted into the linearized vectors, they were incubated at 16 ℃ overnight, and the acquired circHIPK3 interference vectors were transformed into competent DH5α cells. Then, target plasmids were extracted from the bacterial liquid according to the instructions. Cells were transfected with pCDH-CMV-MCS-EF1-CopGFP-T2A-Puro-circHIPK3-shRNA1 (short hairpin RNA sequence: 5'-GCAAACCAACATGGGAAATCC-3'), pCDH-CMV-MCS-EF1-CopGFP-T2A-Puro-circHIPK3-shRNA2 (short hairpin RNA sequence: 5'-GCAGCCTTACAGGGTTAAAGT-3'), or empty pCDH-CMV-MCS-EF1-CopGFP-T2A-Puro vectors (sh-circHIPK3 negative control, NC) using Lipofectamine 2000 (No. 11668-027, Invitrogen, USA) according to the manufacturer's instruction. Non-transfected H9C2 cells were served as controls. After 48 h of transfection, the transfection rate was evaluated.

H9C2 cells were divided into 7 groups, namely control (not subjected to any transfection), model (not subjected to any transfection), EV (empty pCDH-CMV-MCS-EF1-CopGFP-T2A-Puro vector), IV (circHIPK3 interference), Dmy (Dihydromyricetin, 50 μmol/L),[19] and Dmy+IV, Dmy+EV groups respectively.

Reverse Transcription Quantitative PCR

The whole RNA of the tissue and cell samples were extracted using Trizol reagent according to the manufacturer's procedures, and cDNA was synthesized using reverse transcriptase kit (Takara, USA). Quantitative polymerase chain reaction (qPCR) was performed in real-time system (BIO-RAD) using YBR Green PCR kit (KM4101, KAPA Biosystems, USA). Each qPCR reaction was performed in duplicates. The results were analyzed using $2^{-\triangle\triangle Ct}$ method. The primers were designed and configured by Wuhan Tianyi Huayu Gene Technology Co., Ltd, China (Table 1).

Table 1. Primer Sequences

Primer	Sequence (5'–3')	Size
circHIPK3	Forward: CGACAGCCATACAGGGTTA	156 bp
	Backward: AAGGCACTTGACTGAGTTTGA	
GAPDH	Forward: CAAGTTCAACGGCACAG	138 bp
	Backward: CCAGTAGACTCCACGACAT	

Cell Counting Kit-8

Cells were seeded in a 96-well plate at 5×10^3 cells/mL using RPMI 1640 medium containing 10% FBS and treated for 24 h. To evaluate cell proliferation, 10 μL of cell counting kit-8 (CCK-8) solution (CA1210, Solarbio, China) was added to each well and the cells were cultured at 37 ℃ for 4 h. The optical density was measured using a microplate reader (Multiskan FC, Thermo, USA) at 450 nm.

Flow Cytometry

The cells in each group were cultured for 24 h and then harvested, added to 1 mL pre-cooled phosphate-buffered saline (PBS), and centrifuged at $1000\times g$ for 5 min. The apoptosis and data of H9C2 cells were analyzed using flow cytometry (Beckman Coulter, USA) according to the manufacturer's instructions.

Transmission Electron Microscopy

Cells were pre-fixed with 2.5% glutaraldehyde (10–20 times the tissue volume) at 4 ℃ for 30 min, fixed with 1% osmic acid for 1 h, dehydrated, soaked in a 1:1 mixture of acetone:epoxy at 40 ℃ for 6 h, fixed with pure epoxy resin at 40 ℃ for 4 h, and embedded. The samples were then sliced and subjected to double staining and lead citrate staining for 15 min. After rinsing with double-distilled water, the ultrastructure of mitochondria was observed using transmission electrom microscopy (TEM, HT7700, Hitachi, Janpan).

Western Blot

Protein extracts (20 μg) prepared from cells were separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to polyvinylidene fluoride membranes (Millipore, MA, USA). The membranes were blocked with 5% milk in Tris-buffered saline (pH 7.6) containing 0.1% Tween-20, incubated with specific primary antibodies overnight at 4 ℃, and further incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at 4 ℃. The primary antibodies used were anti-LC3 (1:1,000, No. PAB30697, Bioswamp, China), anti-PI3K (1:1,000, No. PAB30084, Bioswamp), anti-p-PI3K (1:1,000, ab182651, Abcam, USA), anti-AKT (1:500, PAB30596, Bioswamp), anti-p-AKT (1:1,000, No. 4060T, CST), anti-mTOR (1:1,000, No. PAB30674, Bioswamp), anti-p-mTOR (1:1,000, No. 5536T, CST), and anti-β-actin (1:1,000, No. PAB36265, Bioswamp). After 3 washes with PBS/Tween 20, the membranes were incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit IgG (1:20,000, SAB43714, Bioswamp) for 2 h at 4 ℃. Protein bands were visualized through enhanced chemiluminescence detection (Tanon-5200, TANON, China) and analyzed using AlphaEase FC gel image analysis software (Alpha Innotech, USA).

Statistical Analysis

All values are presented as the mean ± standard deviation ($\bar{x}\pm s$). One-way analysis of variance followed by Tukey's *post hoc* test was performed to compare the differences among multiple groups using SPSS 19.0 software (IBM Corp., Armonk, USA). $P<0.05$ was considered statistically significant.

RESULTS

Sh-circHIPK3 Inhibits Apoptosis and Promotes Cell Proliferation

Figure 1A shows that the expression level of circHIPK3 in the model group was significantly higher than that in control group ($P<0.05$). Compared to the control and EV groups, the levels of circHIPK3 in sh-circHIPK3-1 and sh-circHIPK3-2 groups were significantly decreased ($P<0.05$, Figure 1B). Compared to the control group, the cell proliferation in the model group were significantly reduced ($P<0.05$), while cell proliferation in IV group were significantly increased compared to model and EV groups ($P<0.05$, Figure 1C). Flow cytometry was used to examine the effect of sh-circHIPK3 on cell apoptosis, and the results showed

that compared to control group, cell apoptosis rate was significantly increased in the model group (P<0.05), while cell apoptosis the significantly reduced after treatment with sh-circHIPK3 (P<0.05, Figure 1D).

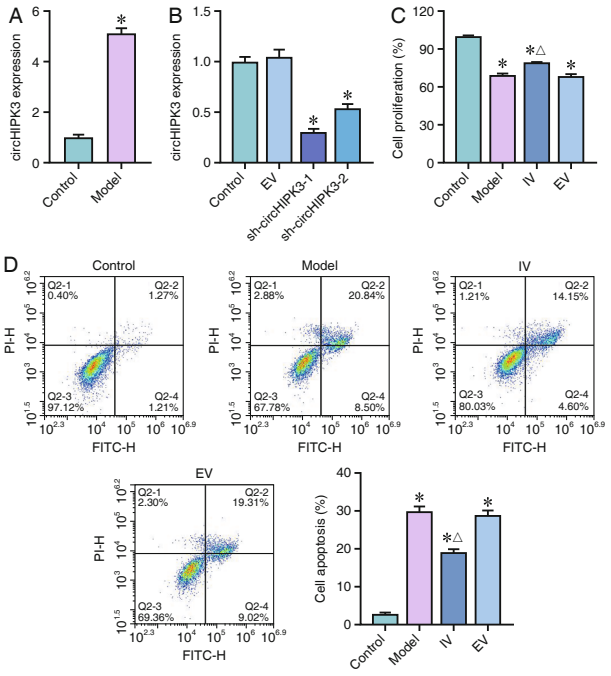

Figure 1. Sh-circHIPK3 Inhibited Apoptosis and Promoted Cell Proliferation in H9C2 Cells

Notes: (A) CircHIPK3 expression detected by RT-qPCR. (B) Transfection efficiency detection. (C) H9C2 cell proliferation detected by CCK8. (D) Cell apoptosis was detected using flow cytometry. $^{*}P$<0.05 *vs*. control group, $^{\triangle}P$<0.05 *vs*. model group, $\bar{x}\pm s$, n=3

Sh-circHIPK3 Inhibits Cell Autophagy

Compared to the control group, autophagosomes increased in the model group, and LC3Ⅱ/Ⅰ protein expression increased obviously (P<0.05). Compared to model and EV groups, autophagosomes in IV group reduced, and the protein expression of LC3Ⅱ/Ⅰ significantly decreased (P<0.05, Figures 2A and 2B).

Effect of sh-circHIPK3 on PI3K/AKT/mTOR Pathway

The results of Western blot showed that compared to the control group, the expressions of p-PI3K, p-AKT, and p-mTOR in the model group significantly decreased (P<0.05). Compared to the model and EV groups, the expressions of p-PI3K, p-AKT, and p-mTOR in the IV group were significantly increased (P<0.05, Figure 3).

Dmy Exerts Its Protective Effects by Regulating PI3K/AKT/mTOR Pathway

Compared to the control group, the expression

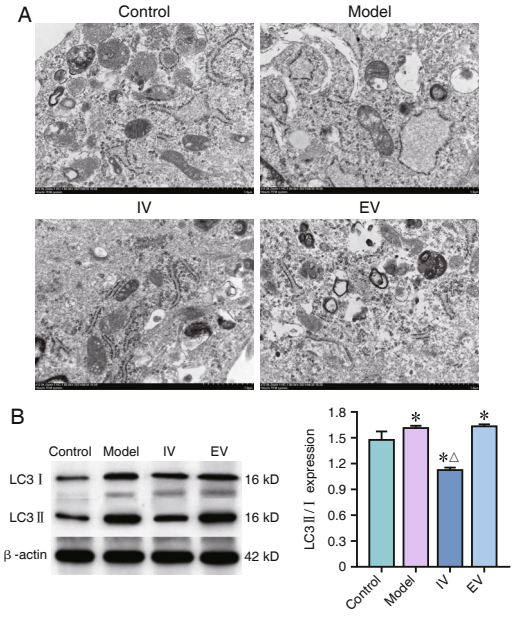

Figure 2. Effect of sh-circHIPK3 on Cell Autophagy in H9C2 Cells

Notes: (A) Autophagosomes examined using transmission electron microscopy in H9C2 cells (magnification: 12,000×, scale bar=1 μm). (B) Western blot was used to detect LC3Ⅱ/Ⅰ expression. $^{*}P$<0.05 *vs*. control group, $^{\triangle}P$<0.05 *vs*. model group, $\bar{x}\pm s$, n=3

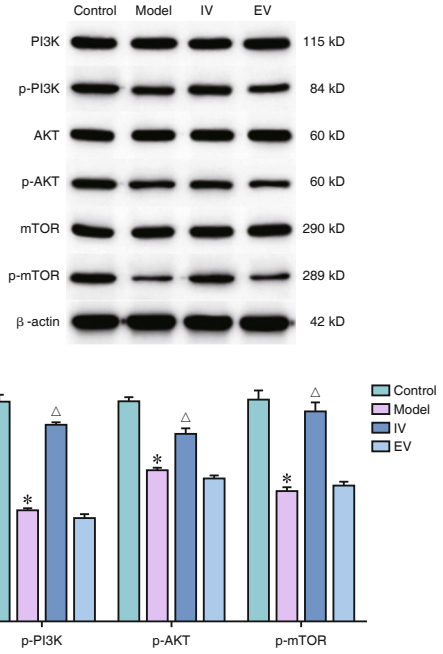

Figure 3. Effect of sh-circHIPK3 on PI3K/AKT/mTOR Pathway in H9C2 Cells by Western Blot

Notes: $^{*}P$<0.05 *vs*. control group, $^{\triangle}P$<0.05 *vs*. model group, $\bar{x}\pm s$, n=3

of circHIPK3 in the model group were increased (P<0.05), and the level of circHIPK3 in the Dmy group were significantly decreased compared to the model group (P<0.05, Figure 4A). As shown in Figure 4B, compared to the control group, cell proliferation rate

in the model group were significantly decreased (P<0.05), and that in the Dmy and Dmy+IV groups significantly increased (P<0.05), among which the cell proliferation rate in Dmy+IV group was the highest. Compared to the control group, the expressions of p-PI3K, p-AKT, and p-mTOR in the model group were decreased significantly (P<0.05). Compared to the model group, the expressions of p-PI3K, p-AKT, and p-mTOR in Dmy and Dmy+IV groups were increased significantly (P<0.05, Figure 4C).

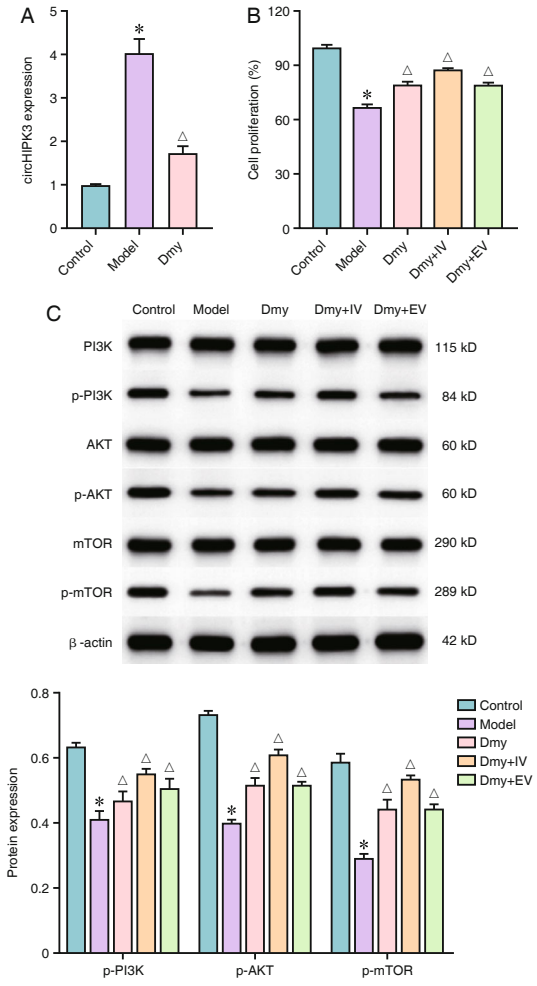

Figure 4. Dmy Exerts Its Protective Effects by Regulating PI3K/AKT/mTOR in H9C2 Cells

Notes: (A) circHIPK3 expression detected using RT-PCR. (B) Cell proliferation was measured using CCK8. (C) p-PI3K, p-AKT, and p-mTOR expressions were evaluated using Western blot. *P<0.05 *vs*. control group, △P<0.05 *vs*. model group, $\bar{x} \pm s$, n=3

DISCUSSION

Oxygen is necessary for human body functions. When hypoxia reaches a certain degree, it will inevitably lead to irreversible damage and death of body cells. Most of the energy supply for sustaining life is generated by oxidation reaction. Oxygen can be considered as one of the most basic substances that sustain life activities. Therefore, hypoxia can cause a variety of physiological and pathological reactions in the body, and MI is one of them. circRNAs, a special subclass of ncRNAs, maintain ncRNA stability due to their circular structure, reduce exonuclease sensitivity, and lead to longer half-life compared to linear RNA molecules.[20] Study has shown that cirRNA can bind to specific proteins or small RNA to function as ceRNA.[21] Compared to normoxic cardiomyocytes, circHIPK3 was overexpressed in myocardial I/R model cells and could regulate oxidative damage of cardiac microvascular endothelial cells through miR-29a/IGF-1 pathway.[10] However, there are few studies on the role of circHIPK3 in injured myocardial cells. Therefore, in this study, we first observed the expression of circHIPK3 in injured H9C2 cells, and the results showed that the expression of circHIPK3 in injured H9C2 cells significantly increased compared to normal H9C2 cells. We further observed the effect of circHIPK3 on proliferation of injured H9C2 cells, and the results showed that compared to the control group, cell proliferation rate was significantly reduced in the model group, which improved after treatment with sh-circHIPK3.

Autophagy is a protective mechanism of the human body, and can cause the reuse of organelles that have lost their functions. The maturation of autophagosomes is a sign and a key product of autophagy.[22,23] For intracellular degradation process, autophagy is critical for the survival of eukaryotic cells and mammals. In the process of autophagy, PI3K/AKT/mTOR signaling axis plays a core role. Under normal circumstances, PI3K and AKT in cells promote the accumulation and activation of mTOR to form mTOR complex by phosphorylation of TSC1/2 and binding of Ras family member Rheb to GTTP enzyme. Once the cell is damaged, mTOR complex dissociates, inhibits the activity of mTOR, forms autophagosomes, and induces autophagy.[24] In this study, we observed that apoptosis and autophagy was significantly increased after H9C2 cell injury, and the activity of PI3K/AKT/mTOR pathway was significantly decreased. However, the situation was reversed after treatment with sh-circHIPK3, suggesting that sh-circHIPK3 could alleviate apoptosis and autophagy after H9C2 cell injury.

Dmy protects the skeletal muscle, cardiomyo-

cytes, nerve cells, and other cells from cell damage caused by hypoxia/hypoxia by regulating mitochondrial function, affecting energy metabolism, and inhibiting apoptosis. Previous studies have shown that Dmy not only protects vascular endothelial cells from oxidative stress injury by activating PI3K/AKT signaling pathway but also prevents the development of diabetic nephropathy by regulating miR-155-5p/PTEN and PI3K/AKT/mTOR signaling pathways.[14,15,25] We further observed in this study, the effect of Dmy on circHIPK3 expression and PI3K/AKT/mTOR pathway in injured H9C2 cell, and the results showed that Dmy could suppress circHIPK3 expression and promote PI3K/AKT/mTOR signaling pathway activation in injured H9C2 cell, suggesting that Dmy could exert its protective effect by suppressing circHIPK3 expression and activating PI3K/AKT/mTOR pathway (Figure 5).

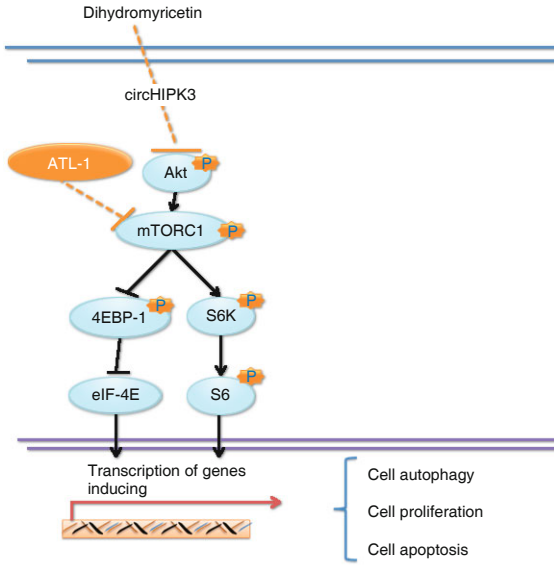

Figure 5. Main Molecular Mechanism Diagram

Lack of clinical sample validation is one of the shortcomings of this study, but the main purpose of this study is to investigate the effect and mechanism of Dmy on cell apoptosis and autophagy in H9C2 cells. We think that the exist experiment may not be optimal, but should be sufficient to draw the conclusion, and we will verify our study in clinical samples in subsequent studies.

In conclusion, the inhibition of circHIPK3 expression can alleviate apoptosis and autophagy in H9C2 cells and promote cell proliferation, and Dmy can inhibit circHIPK3 expression. Therefore, the protective effect of Dmy on H9C2 cells may be achieved by inhibiting circHIPK3 expression and H9C2 autophagy and apoptosis, and by regulating the activity of PI3K/AKT/mTOR pathway.

Conflict of Interest

There are no conflicts of interest.

Author Contributions

Zhang ZY, Liu C, and Wang PX drafted the manuscript. Han YW and Zhang YW reviewed and modified the manuscript. Hao ML, Song ZX, and Zhang XY revised the manuscript. All authors contributed to the article and approved the submitted version.

Data Availability Statement

The data used to support the findings of this study can be obtained from the corresponding author upon request.

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