

# Overexpression of lncRNA Dancr inhibits apoptosis and enhances autophagy to protect cardiomyocytes from endoplasmic reticulum stress injury via sponging microRNA-6324

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**Abstract.** Endoplasmic reticulum stress (ERS) contributes to the pathogenesis of myocardial ischemia/reperfusion injury and myocardial infarction (MI). Long non-coding RNAs (lncRNAs) serve an important role in cardiovascular diseases, and lncRNA discrimination antagonizing non-protein coding RNA (Dancr) alleviates cardiomyocyte damage. microRNA (miR)-6324 was upregulated in MI model rats and was predicted to bind to Dancr. The present study aimed to investigate the role of Dancr in ERS-induced cardiomyocytes and the potential underlying mechanisms. Tunicamycin (Tm) was used to induce ERS. Cell viability, apoptosis and levels of associated proteins, ERS and autophagy in Dancr-overexpression H9C2 cells and miR-6324 mimic-transfected H9C2 cells were assessed using Cell Counting Kit-8, TUNEL staining and western blot assay, respectively. The results suggested that Dancr expression levels and cell viability were downregulated by Tm in a concentration-dependent manner compared with the control group. Tm induced apoptosis,

ERS and autophagy, as indicated by an increased ratio of apoptotic cells, increased expression levels of Bax, cleaved (c)-caspase-3/9, glucose-regulated protein 78 kDa (GRP78), phosphorylated (p)-inositol-requiring enzyme-1 $\alpha$  (IRE1 $\alpha$ ), spliced X-box-binding protein 1 (Xbp1s), IRE1 $\alpha$ , activating transcription factor (ATF)6, ATF4, Beclin 1 and microtubule associated protein 1 light chain 3 $\alpha$  (LC3)II/I, and decreased expression levels of Bcl-2, unspliced Xbp1 (Xbp1u) and p62 in the Tm group compared with the control group. Moreover, the results indicated that compared with the Tm + overexpression (Oe)-negative control (NC) group, the Tm + Oe-Dancr group displayed decreased apoptosis, but enhanced ERS and autophagy to restore cellular homeostasis. Compared with the Tm + Oe-NC group, the Tm + Oe-Dancr group decreased the ratio of apoptotic cells, decreased expression levels of Bax, c-caspase-3/9 and Xbp1u, and increased expression levels of Bcl-2, p-IRE1 $\alpha$ , Xbp1s, Beclin 1 and LC3II/I. Dancr overexpression also significantly downregulated miR-6324 expression compared with Oe-NC. The Dual-luciferase reporter assay further indicated an interaction between Dancr and miR-6324. In addition, miR-6324 mimic partially reversed the effects of Dancr overexpression on Tm-induced apoptosis, ERS and autophagy. In conclusion, lncRNA Dancr overexpression protected cardiomyocytes against ERS injury via sponging miR-6324, thus inhibiting apoptosis, enhancing autophagy and restoring ER homeostasis.

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## Introduction

With social and economic development and the aging population, the occurrence of cardiovascular events is increasing each year (1). Among cardiovascular diseases, acute myocardial infarction (AMI), which is associated with high lethality and high disability, has become the leading cause of human mortality globally (2). AMI refers to myocardial necrosis caused by acute or continuous ischemia and hypoxia in the coronary arteries (3). Myocardial ischemia is

a pathological state in which blood perfusion of the heart is decreased, resulting in decreased oxygen supply to the heart and abnormal energy metabolism of the myocardial cells, which cannot support the normal function of the heart (4). Persistent and acute ischemia of the heart can develop into AMI (5).

At present, the treatment strategies for AMI primarily include thrombolysis, percutaneous coronary intervention and surgical bypass surgery. The primary purpose of the therapeutic strategies is to restore the blood supply to the heart, rescue myocardial cells in the infarction area and prevent further damage caused by ischemia (6). However, the current treatment strategies have limited therapeutic effects on damaged cardiomyocytes, as cardiomyocytes in adults cannot be naturally regenerated once they are lost (7). Therefore, rescuing myocardial cells at the near-death state in the marginal area of MI by investigating the function of certain specific long non-coding RNAs (lncRNAs) or proteins is a novel research topic for treating AMI.

lncRNAs are transcripts >200 nucleotides in length, lacking a specific complete open reading frame and protein coding functions (8). lncRNAs function as crucial regulators via regulating gene expression at both the transcriptional and post-transcriptional levels (9). lncRNAs can regulate microRNAs (miRNAs/miRs) by serving as miRNA sponges, adsorbing the corresponding miRNA and exerting transcriptional regulation (10). A number of studies have demonstrated that lncRNAs serve important roles in the occurrence and development of human diseases, including cardiovascular diseases (11,12).

lncRNA discrimination antagonizing non-protein coding RNA (Dancr) encodes human chromosome 4q12, and was first identified as an epidermal cell differentiation suppressor (13). Further investigation revealed that Dancr functions as an oncogene in various types of cancer, including hepatocellular carcinoma (14), non-small cell lung carcinoma (15,16) and osteosarcoma (17). Recently, Dancr has been reported to alleviate hypoxia-induced H9c2 cardiomyocyte damage by upregulating hypoxia inducible factor-1 $\alpha$  (18). In addition, Dancr decreased hypoxia- and hypoglycemia-induced damage of cerebral microvascular endothelial cells via regulating miR-33a-5p/spliced X-box-binding protein 1 (Xbp1s) (19). However, to the best of our knowledge, the effect of Dancr in myocardial ischemia and myocardial infarction has not been previously reported.

In the case of MI/reperfusion (R), endoplasmic reticulum (ER) homeostasis is destroyed, resulting in the accumulation of a large number of unfolded or misfolded proteins in the ER, triggering the unfolded protein response (UPR) and ER stress (ERS) (20). Early ERS exerts a compensatory protective effect, but excessive ERS is involved in the pathophysiological process of various cardiovascular diseases (21). Tunicamycin (Tm) is a commonly used ERS inducer (22). Previous studies have demonstrated that low doses of Tm can produce moderate ERS, which displays a certain protective effect on MI/R (23,24). Moderate ERS can also induce autophagy to help the degradation of unfolded or misfolded proteins, thus alleviating ERS (25). However, excessive ERS may initiate the apoptosis reaction and inflammatory pathways, ultimately participating in the deterioration of cardiovascular

disease (26). ERS in response to various adverse stimuli has been detected and has been reported to be associated with the pathogenesis of MI/R injury, myocardial hypertrophy, ischemic cardiomyopathy, diabetic cardiomyopathy and cardiac fibrosis (27). For example, miR-711 mimic could induce cardiomyocyte apoptosis after ERS-induced MI via upregulating Xbp1 (28). Xu *et al.* (29) demonstrated that inhibition of ERS and the cell apoptosis signaling pathway could protect cardiomyocytes against MI-induced injury.

Autophagy is an important metabolic process that degrades senescent or damaged proteins and organelles into amino groups (30). Autophagy is activated in response to nutritional deficiencies or metabolic stress to maintain tissue function and homeostasis (31). Basic autophagy has been reported to be essential for maintaining normal heart function. Meanwhile, under ischemic stress, autophagy is activated to protect cardiomyocytes from ischemia or I/R injury (32). The beneficial role of autophagy in AMI in the alleviation of MI under ischemic and ischemia/R injuries has been extensively reported (33,34). Therefore, taking advantage of autophagy provides a potential strategy for the development of novel drugs or therapies for AMI (35).

The aforementioned studies indicated that the moderate enhancement of ERS and autophagy, together with the repression of excessive ERS and ERS-mediated apoptosis might serve as a valuable therapeutic strategy for relieving MI-induced injury. The present study aimed to investigate the roles and molecular mechanism underlying Dancr in ERS-induced cardiomyocytes to provide a novel target for the diagnosis and therapy of AMI.

## Materials and methods

**Cell culture and treatment.** The H9c2 rat embryonic cardiomyocyte cell line (American Type Culture Collection) was cultured in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 100 units/ml penicillin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The medium was replaced every other day. At 70-80% confluence, cells were digested with trypsin and EDTA.

Tm (MedChemExpress) was utilized to induce ERS. H9C2 cells were treated Tm (0.1, 0.5, 2.5 or 12.5  $\mu$ M) for 6 h at 37°C and control cells were cultured in normal medium (36).

**Cell transfection.** Prior to transfection, the medium was replaced with serum- and antibiotic-free DMEM. To overexpress lncRNA Dancr, the recombinant full-length rat Dancr cDNA [overexpression (Oe)-Dancr] was cloned into the pcDNA3.1 vector (Thermo Fisher Scientific, Inc.). The pcDNA3.1 empty vector was used as a negative control (NC; Oe-NC). H9C2 cells (60-70% confluence) were transfected with oe-NC, oe-NC, miR-6324 mimic (5'-AGUAGGCCAGACAGCAAGC-3'; Sigma-Aldrich; Merck KGaA) or mimic-NC (5'-GGUUCGUACGUACACUGUUCA-3'; Sigma-Aldrich; Merck KGaA) using Lipofectamine<sup>®</sup> 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Briefly, Lipofectamine 2000 was mixed with 50 nM plasmids or mimics, added to the cells and incubated for 6 h at 37°C. Subsequently, cells were cultured in DMEM for 24 h and then used for subsequent experiments. Transfection

efficiency was evaluated via reverse transcription-quantitative PCR (RT-qPCR).

**Cell Counting Kit-8 (CCK-8).** The CCK-8 assay was performed to assess cell viability. H9c2 cells ( $1 \times 10^4$  cells/well) were cultured in 96-well plates and subjected to corresponding treatments. Subsequently, 10  $\mu$ l CCK-8 reagent (Beyotime Institute of Biotechnology) was added to each well and incubated at 37°C for 2 h in the dark. The optical density of each well was measured at a wavelength of 450 nm using a microplate reader.

**TUNEL staining.** Apoptotic H9c2 cardiomyocytes were visualized by performing TUNEL staining (Nanjing KeyGen Biotech Co., Ltd.) according to the manufacturer's protocol. Briefly, H9c2 cells were cultured on cover slips. After the corresponding treatment, cells were fixed with 4% neutral buffered formalin solution for 30 min at room temperature. Subsequently, 50  $\mu$ l TUNEL reaction mixture was added and incubated for 1 h at 37°C. The nuclei were stained with DAPI (2  $\mu$ g/ml) at room temperature for 5 min. After washing twice with PBS, images were captured from three fields of view using a fluorescence microscope (magnification,  $\times 200$ ).

**Western blotting.** Total protein was extracted from H9c2 cardiomyocytes using lysis buffer (Beyotime Institute of Biotechnology) containing a protease inhibitor and phosphatase inhibitor. After being quantified using a BCA kit (Beyotime Institute of Biotechnology), equal amounts of protein (50  $\mu$ g) were separated via 12% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk at 37°C for 2 h. Subsequently, the membranes were incubated overnight at 4°C with primary antibodies targeted against: Bcl-2 (Abcam; cat. no. ab32124; 1:1,000), Bax (Abcam; cat. no. ab32503; 1:10,000), cleaved (c)-caspase-9 (Abcam; cat. no. ab32539; 1:5,000), caspase-9 (Abcam; cat. no. ab184786; 1:1,000), c-caspase-3 (Abcam; cat. no. ab32042; 1:500), caspase-3 (Abcam; cat. no. ab13847; 1:500), glucose-regulated protein 78 kDa (GRP78; Abcam; cat. no. ab21685; 1:1,000), phosphorylated (p)-inositol-requiring enzyme-1 (IRE1) $\alpha$  (Abcam; cat. no. ab124945; 1:1,000), IRE1 $\alpha$  (Abcam; cat. no. ab37073; 1:1,000), Xbpl1 (ProteinTech Group, Inc.; cat. no. 24868-1-AP; 1:1,000), unspliced Xbp1 (Xbp1u; ProteinTech Group, Inc.; cat. no. 25997-1-AP; 1:1,000), activating transcription factor (ATF)6 (ProteinTech Group, Inc.; cat. no. 24169-1-AP; 1:2,000), ATF4 (ProteinTech Group, Inc.; cat. no. 10835-1-AP; 1:1,000), Beclin 1 (ProteinTech Group, Inc.; cat. no. 11306-1-AP; 1:10,000), microtubule associated protein 1 light chain 3 $\alpha$  (LC3)II/I (ProteinTech Group, Inc.; cat. no. 14600-1-AP; 1:2,000), p62 (Abcam; cat. no. ab56416; 1:1,000) and GAPDH (Abcam; cat. no. ab8245; 1:10,000). Following primary incubation, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG secondary antibodies at room temperature for 2 h. Protein bands were visualized using an enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.) and detection system (Amersham; Cytiva). Protein expression levels were semi-quantified using

Image-Pro Plus software version 6.0 (Media Cybernetics, Inc.) with GAPDH as the loading control.

**RT-qPCR.** Total RNA was extracted from H9C2 cardiomyocytes using an RNA isolation kit (Total RNA Extraction Reagent; Vazyme Biotech Co., Ltd.). Total RNA was reverse transcribed into cDNA using a reverse transcriptase (Vazyme Biotech Co., Ltd.). Subsequently, qPCR was performed using the CFX384 Real-Time System C1000 Thermocycler (Bio-Rad Laboratories, Inc.) and SYBR-Green ROX-mix (Vazyme Biotech Co., Ltd.). The following primers were used for qPCR: Dancr forward, 5'-CTCGGATAGAAGCGCAGGTT-3' and reverse, 5'-AGGCAAGCGGGGTCATTA-3'; miR-6324 forward, 5'-ATAGCTGGGGTCAAGGTGCT-3' and reverse, 5'-CTTGCTGTCTGGCCTACTGA-3'; GAPDH forward, 5'-TTGTGCAGTGCCAGCCTC-3' and reverse, 5'-GGTAACCAGGCGTCCGATAC-3'; and U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTACGAATTTGCGT-3'. The following thermocycling conditions were used for qPCR: Initial denaturation at 95°C for 30 sec then 40 cycles of 95°C for 5 sec and 60°C for 15 sec, followed by default of melt curve. miRNA and mRNA expression levels were quantified using the  $2^{-\Delta\Delta Cq}$  method (37) and normalized to the internal reference genes U6 and GAPDH, respectively.

**Dual-luciferase reporter assay.** The wild-type (WT) and mutated (MUT) Dancr fragments containing the miR-6324 binding sites were synthesized and inserted into the pmirGLO luciferase vector (Promega Corporation) to generate WT-pmirGLO and MUT-pmirGLO, respectively. For the promoter analysis, miR-6324 mimic or mimic NC were cloned into the pGL3 luciferase reporter (Promega Corporation). Cells were co-transfected with WT-pmirGLO or MUT-pmirGLO vectors and miR-6324 mimic or mimic NC. At 48 h post-transfection, luciferase activities were measured using a Dual-Luciferase reporter assay system (Promega Corporation) as previously described (38).

**Statistical analysis.** Data are presented as the mean  $\pm$  standard deviation of from at least three independent experiments. Comparisons between two groups were analyzed using the Student's t-test. Comparisons among multiple groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. Statistical analyses were performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc.).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**lncRNA Dancr is downregulated upon Tm stimulation.** The present study aimed to determine whether Dancr could regulate cardiomyocytes that underwent ERS. Following stimulation with different concentrations of the ERS agonist Tm (0.1, 0.5, 2.5 or 12.5  $\mu$ M), Dancr expression levels and cell viability in H9C2 cells were measured. Compared with the control group, Tm decreased Dancr mRNA expression levels (Fig. 1A) and cell viability (Fig. 1B) in a concentration-dependent manner, suggesting a modulatory effect of Dancr

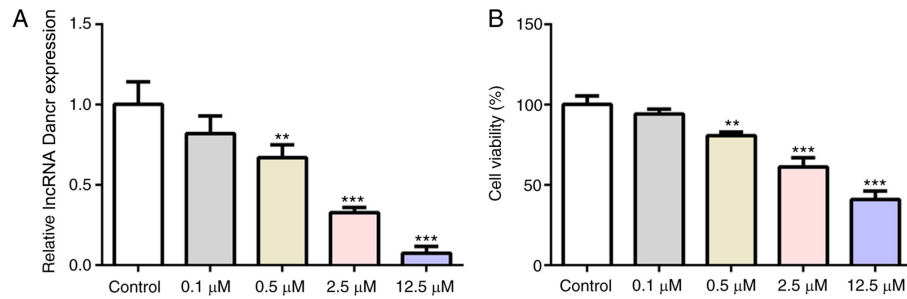


Figure 1. lncRNA Dancr is downregulated by Tm in a concentration-dependent manner. (A) Dancr mRNA expression levels and (B) cell viability in H9C2 cells treated with different concentrations of Tm. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. control. lncRNA, long non-coding RNA; Dancr, discrimination antagonizing non-protein coding RNA; Tm, Tunicamycin.

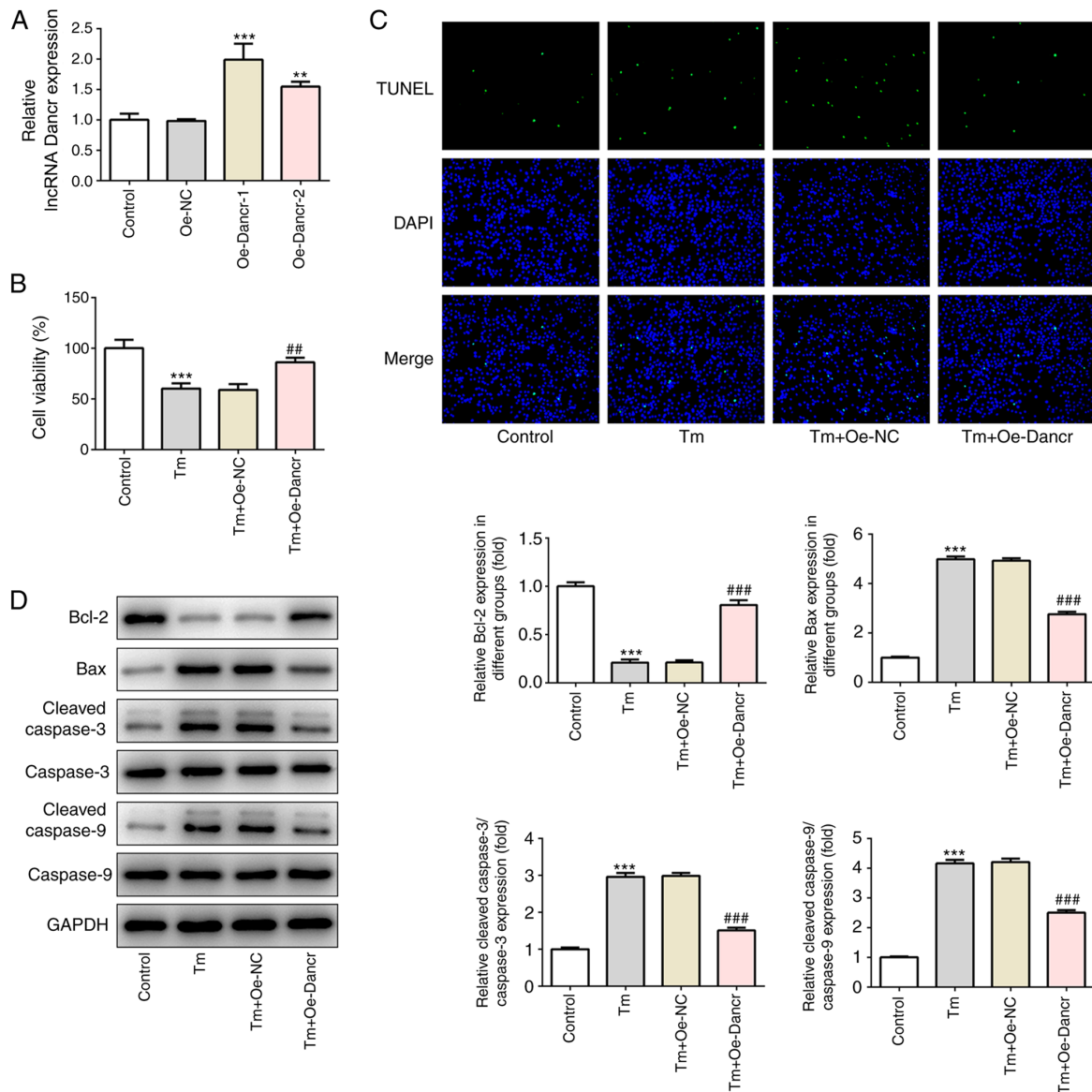


Figure 2. Effects of Dancr overexpression on cell viability and apoptosis in Tm-stimulated H9C2 cardiomyocytes. (A) Transfection efficiency of Oe-Dancr-1 and Oe-Dancr-2. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. Oe-NC. (B) H9C2 cell viability. (C) H9C2 cell apoptosis was measured by performing TUNEL staining. Green represents apoptotic cells and blue represents nuclei (magnification, x200). (D) Western blotting was performed to measure the expression levels of apoptosis-related proteins, including Bcl-2, Bax, cleaved-caspase-3 and cleaved caspase-9. \*\*\* $P < 0.001$  vs. control; ## $P < 0.01$  and ### $P < 0.001$  vs. Tm + Oe-NC. Dancr, discrimination antagonizing non-protein coding RNA; Tm, Tunicamycin; Oe, overexpression; NC, negative control.

on cardiomyocyte ERS. Based on the finding that 2.5  $\mu\text{M}$  Tm resulted in a >50% reduction in Dancr expression but

maintained cell viability at  $\geq 50\%$ , 2.5  $\mu\text{M}$  Tm was selected for subsequent experiments (39).



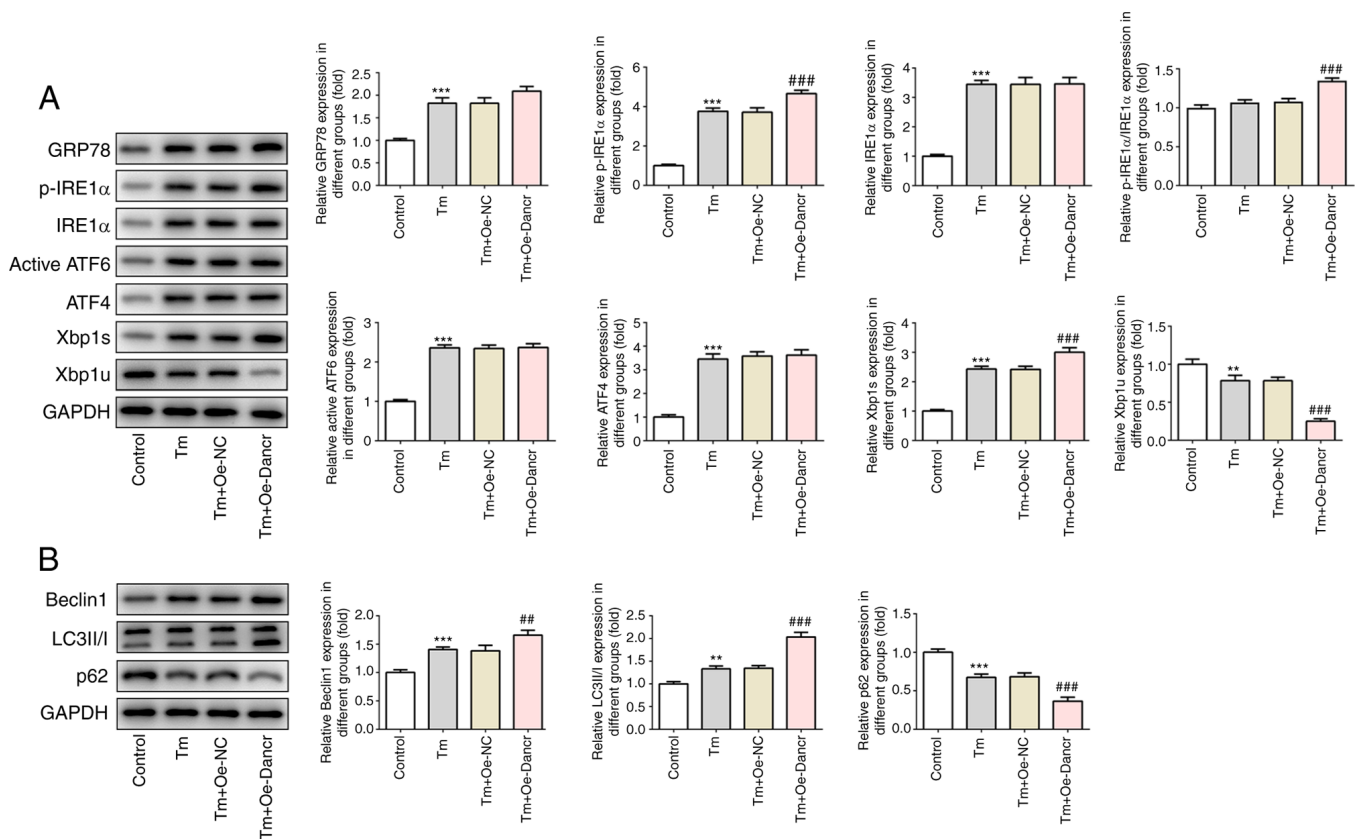


Figure 3. Effects of Dancr overexpression on ERS and autophagy. Western blotting was performed to measure the expression levels of (A) ERS-related proteins, including GRP78, p-IRE1 $\alpha$ , IRE1 $\alpha$ , p-IRE1 $\alpha$ /IRE1 $\alpha$ , ATF6, ATF4, Xbp1s and Xbp1u and (B) autophagy-related proteins, including Beclin1, LC3II/I and p62. \*\*P<0.01 and \*\*\*P<0.001 vs. control; \*\*P<0.01 and \*\*\*P<0.001 vs. Tm + Oe-NC. Dancr, discrimination antagonizing non-protein coding RNA; ERS, endoplasmic reticulum stress; GRP78, glucose-regulated protein 78 kDa; p, phosphorylated; IRE1, inositol-requiring enzyme-1; ATF, activating transcription factor; Xbp1s, spliced X-box-binding protein 1; Xbp1u, unspliced Xbp1; LC3, microtubule associated protein 1 light chain 3 $\alpha$ ; Tm, Tunicamycin; Oe, overexpression; NC, negative control.

*Dancr overexpression enhances cell viability and decreases cell apoptosis in Tm-stimulated H9C2 cardiomyocytes.* To determine the specific role of Dancr in ERS-induced cardiomyocyte injury, Dancr was overexpressed in H9C2 cells. The results demonstrated the successful overexpression of Dancr (Fig. 2A). Oe-Dancr-1 was selected to establish Dancr-overexpression H9C2 cells in subsequent experiments based on its higher efficacy compared with Oe-Dancr-2. Moreover, compared with the control group, 2.5  $\mu$ M Tm treatment significantly decreased cell viability, markedly increased the ratio of apoptotic cells, significantly decreased the expression levels of the antiapoptosis protein Bcl-2, and significantly increased the expression levels of the proapoptosis proteins Bax and c-caspase-3/9 (Fig. 2B-D). However, following Tm stimulation, compared with Oe-NC-transfected cells, Oe-Dancr-transfected cells displayed significantly higher cell viability, a notably lower ratio of cell apoptosis, significantly increased expression levels of Bcl-2, and significantly decreased expression levels of Bax and c-caspase-3/9.

*Dancr overexpression enhances ERS and autophagy in Tm-stimulated H9C2 cardiomyocytes.* The present study analyzed the expression levels of proteins associated with ERS and autophagy. Compared with the control group, Tm treatment resulted in significantly increased expression levels of GRP78, p-IRE1 $\alpha$ , Xbp1s, IRE1 $\alpha$ , ATF6 and ATF4, signifi-

cantly decreased expression levels of Xbp1u and no significant alterations to the p-IRE1 $\alpha$ /IRE1 $\alpha$  ratio, indicating the induction of ERS (Fig. 3A). Compared with the Tm + Oe-NC group, the Tm + Oe-Dancr group displayed significantly enhanced p-IRE1 $\alpha$ , p-IRE1 $\alpha$ /IRE1 $\alpha$  and Xbp1s expression levels and significantly decreased Xbp1u expression levels, but there were no significant alterations to the expression levels of IRE1 $\alpha$ , ATF6 or ATF4, suggesting that Dancr selectively promoted the phosphorylation of IRE1 $\alpha$  and activated the IRE1 $\alpha$  branch in the UPR. Moreover, compared with the control group, Tm treatment induced autophagy, as evidenced by significantly increased expression levels of Beclin 1 and LC3II/I, and significantly decreased expression levels of p62 (Fig. 3B). Compared with the Tm + Oe-NC group, the Tm + Oe-Dancr group displayed increased levels of autophagy, as demonstrated by significantly increased Beclin 1 and LC3II/I expression levels, and significantly lower p62 expression levels.

*miR-6324 mimic partially reverses the effects of Dancr overexpression on Tm-induced apoptosis, ERS and autophagy.* miR-6324 was predicted to bind to Dancr (Fig. 4A). To verify whether miR-6324 was the target of Dancr, the present study constructed two miR-6324 mimics. The results verified the successful transfection of the miR-6324 mimics (Fig. 4B). The direct interaction between Dancr and miR-6324 was assessed by performing a Dual-luciferase reporter assay (Fig. 4C).

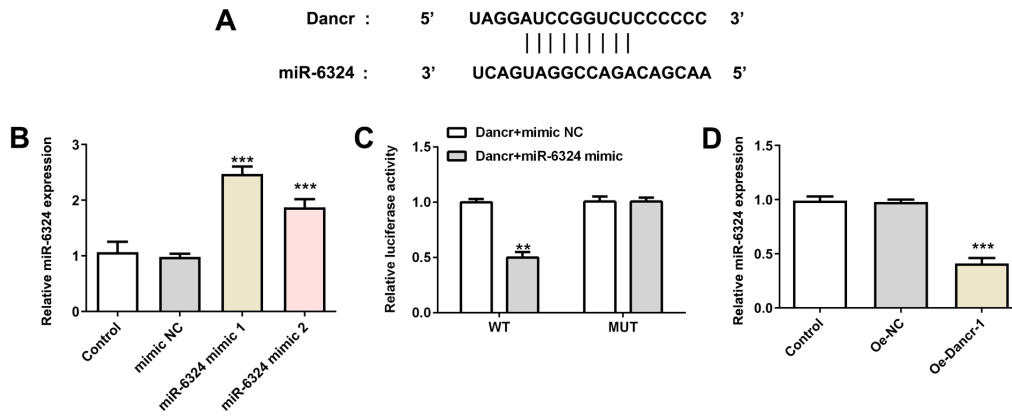


Figure 4. Relationship between Dancr and miR-6324. (A) The predicted binding sites between Dancr and miR-6324. (B) Transfection efficiency of miR-6324 mimic 1 and miR-6324 mimic 2. \*\*\* $P < 0.001$  vs. mimic NC. (C) A Dual-luciferase reporter assay was performed to assess the interaction between Dancr and miR-6324. \*\* $P < 0.01$  vs. Dancr + mimic NC. (D) miR-6324 expression levels following transfection with Oe-Dancr-1. \*\*\* $P < 0.001$  vs. control. Dancr, discrimination antagonizing non-protein coding RNA; miR, microRNA; NC, negative control; Oe, overexpression; WT, wild-type; MUT, mutant.

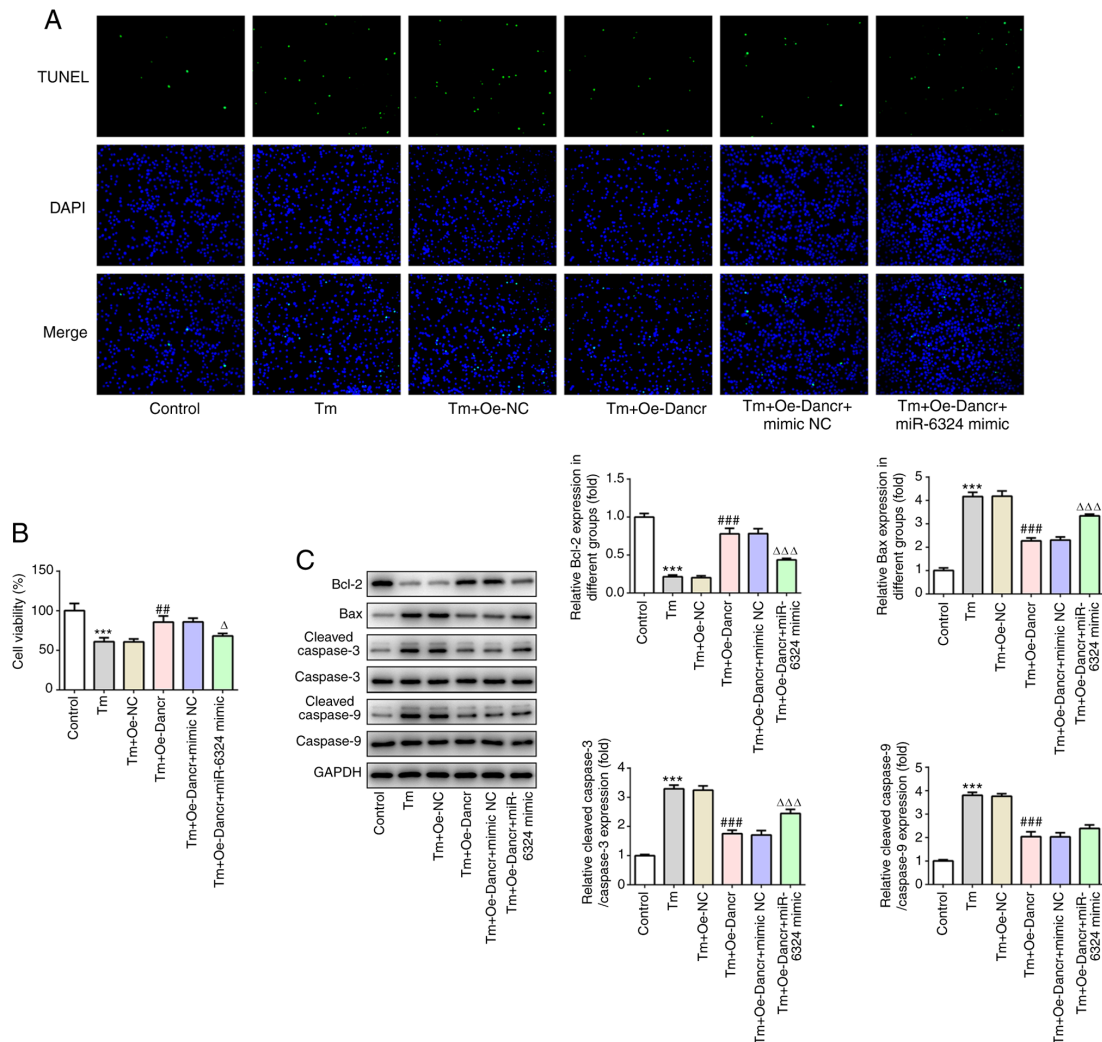


Figure 5. miR-6324 mimic reverses Dancr overexpression-mediated effects on Tm-induced apoptosis. (A) H9C2 cell apoptosis was measured by performing TUNEL staining (magnification, x200). (B) H9C2 cell viability. (C) Western blotting was performed to measure the expression levels of apoptosis-related proteins, including Bcl-2, Bax, cleaved-caspase-3 and cleaved caspase-9. \*\*\* $P < 0.001$  vs. control; ## $P < 0.01$  and ### $P < 0.001$  vs. Tm + Oe-NC; \* $P < 0.05$  and  $\Delta\Delta\Delta P < 0.001$  vs. Tm + Oe-Dancr + mimic NC. miR, microRNA; Dancr, discrimination antagonizing non-protein coding RNA; Tm, Tunicamycin; Oe, overexpression; NC, negative control.

Moreover, Dancr overexpression significantly downregulated miR-6324 expression levels compared with Oe-NC (Fig. 4D).

The results suggested that miR-6324 might participate in the actions of Dancr on ERS-induced cardiomyocyte injury.

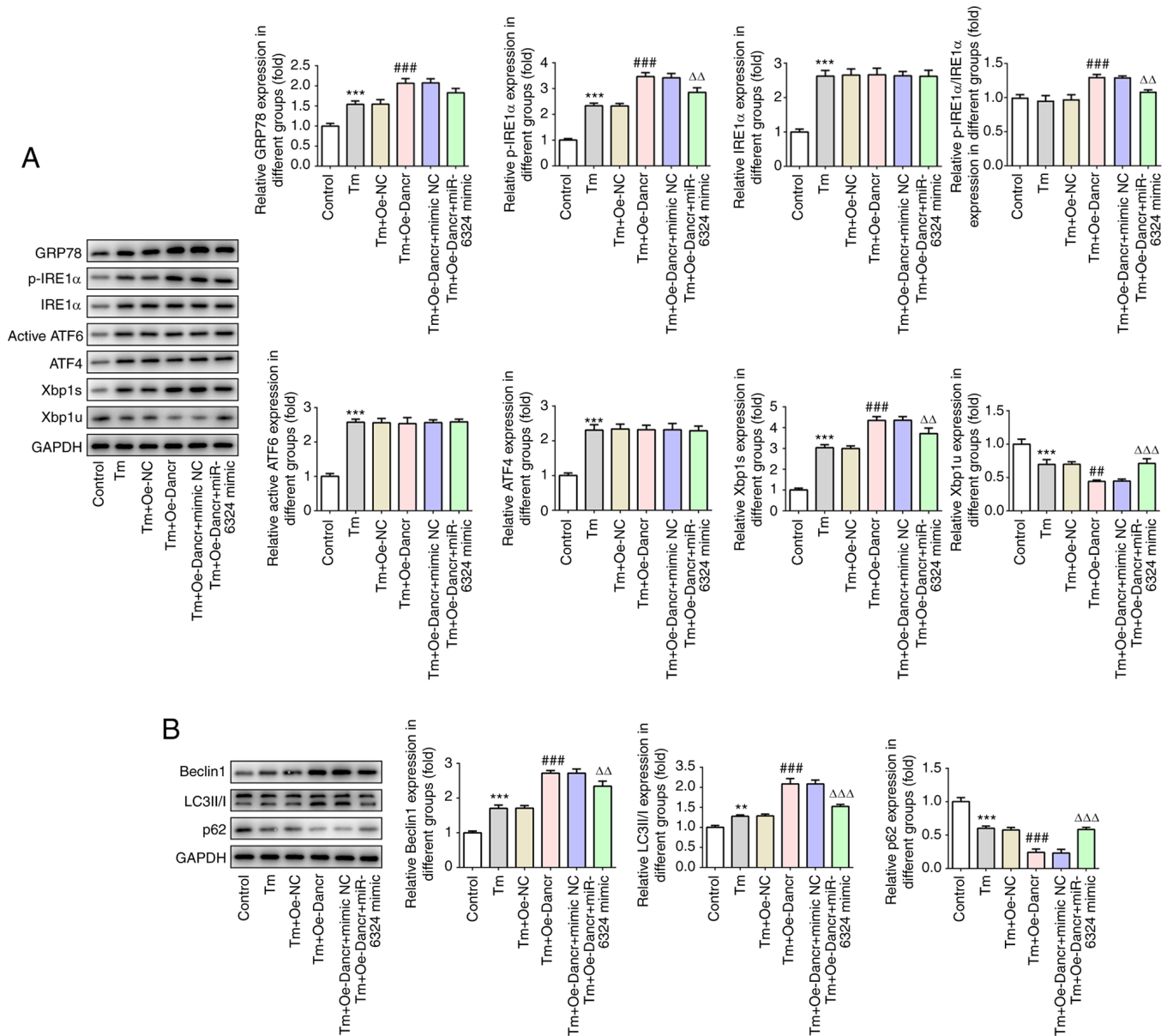


Figure 6. miR-6324 mimic reverses Dancr overexpression-mediated effects on Tm-induced ERS and autophagy. Western blotting was performed to measure the expression levels of (A) ERS-related proteins, including GRP78, p-IRE1 $\alpha$ , IRE1 $\alpha$ , p-IRE1 $\alpha$ /IRE1 $\alpha$ , ATF6, ATF4, Xbp1s and Xbp1u, (B) autophagy-related proteins, including Beclin1, LC3II/I and p62. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. control; ## $P < 0.01$  and ### $P < 0.001$  vs. Tm + Oe-NC;  $\Delta\Delta P < 0.01$  and  $\Delta\Delta\Delta P < 0.001$  vs. Tm + Oe-Dancr + mimic NC. miR, microRNA; Dancr, discrimination antagonizing non-protein coding RNA; Tm, Tunicamycin; ERS, endoplasmic reticulum stress; GRP78, glucose-regulated protein 78 kDa; p, phosphorylated; IRE1, inositol-requiring enzyme-1; ATF, activating transcription factor; Xbp1s, spliced X-box-binding protein 1; Xbp1u, unspliced Xbp1; LC3, microtubule associated protein 1 light chain 3 $\alpha$ ; Oe, overexpression; NC, negative control.

To investigate the modulatory effects of miR-6324 on Dancr-mediated H9C2 ERS, Tm-treated and Dancr-overexpression H9C2 cells were transfected with miR-6324 mimic or mimic-NC. Compared with the Tm + Oe-Dancr + mimic NC group, the Tm + Oe-Dancr + miR-6324 mimic group displayed significantly decreased cell viability and markedly increased numbers of apoptotic cells (Fig. 5A and B). Furthermore, compared with the Tm + Oe-Dancr + mimic NC group, the Tm + Oe-Dancr + miR-6324 mimic group displayed significantly decreased Bcl-2 expression levels, and significantly increased Bax and c-caspase-3/9 expression levels (Fig. 5C).

The expression levels of proteins associated with ERS and autophagy were measured (Fig. 6). Oe-Dancr-mediated upregulation of GRP78, p-IRE1 $\alpha$ , p-IRE1 $\alpha$ /IRE1 $\alpha$ , Xbp1s, Beclin 1

and LC3II/I expression levels was significantly reversed by miR-6324 mimic. Similarly, miR-6324 mimic partially recovered the expression levels of Xbp1u and p62, which were decreased by Tm and further decreased by Dancr overexpression combined with Tm treatment. The aforementioned results indicated that miR-6324 mimic partially abolished Dancr overexpression-mediated effects on Tm-induced apoptosis, ERS and autophagy.

## Discussion

lncRNA Dancr has been reported to protect H9c2 cardiomyocytes against hypoxia-induced damage (18). The present study indicated that Dancr protected H9C2 cells against

ERS-induced apoptosis *in vitro* via the selective activation of the IRE1 $\alpha$ /XBP1 signaling pathway in the UPR, which suggested that Dancr provided cytoprotection in response to ERS.

When the ER senses a wide variety of perturbations, including UPR, the adaptive process of ERS occurs to maintain ER homeostasis and mitigate or eliminate the stress (22). There are three primary ER transmembrane stress sensors that initiate the UPR, including IRE1, protein kinase RNA-like endoplasmic reticulum kinase (PERK) and ATF6, are maintained in an inactive state via binding to their luminal domains with the ER chaperone GRP78. However, in the presence of ERS, GRP78 is released from these complexes and recruited to misfolded proteins, leading to the activation of three distinct UPR branches (40). For instance, the IRE1 branch possesses endoribonuclease activity that splices mRNAs encoding Xbp1u to form mature Xbp1/Xbp1s mRNA, which is then translated to a potent transcription factor that controls the genes encoding proteins that target misfolded proteins for ubiquitination and ER-associated degradation (ERAD). Meanwhile, ATF6 cooperates with IRE1 $\alpha$  for the induction of Xbp1 transcription. The proteins balance the unfolded protein/chaperone system to provide ER homeostasis. If the cell fails to recover from ERS, the UPR represses the adaptive response and triggers apoptosis. Notably, PERK stimulates the expression of the proapoptotic transcription factor C/EBP homologous protein via the mobilization of ATF4 (41). Thus, the ERS serves a dual role, transmitting both adaptive and apoptotic signals. In the present study, compared with the control group, Tm decreased cell viability and induced cell apoptosis, which could be explained by the occurrence of excessive ERS, as evidence by increased expression levels of GRP78, p-IRE1 $\alpha$ , IRE1 $\alpha$ , ATF6, ATF4 and Xbp1s, and decreased expression levels of Xbp1u. As previously described, if ERS is too severe or prolonged, the UPR initiates an apoptotic response (42).

Autophagy is essential for maintaining protein homeostasis and is an evolutionarily conserved protein degradation signaling pathway that removes damaged or expired proteins and organelles by chelating in autophagosomes, which subsequently undergo lysosomal degradation (43). Previous studies have demonstrated that the ERS response can activate the autophagy-lysosome signaling pathway, which serves a major role in the cardiac stress response (44,45). Autophagy functions as a cellular stress signaling pathway and can assist with the degradation of proteins to recover ER homeostasis (44). In the present study, compared with the control group, Tm treatment increased the expression levels of Beclin 1 and LC3II/I, and decreased the expression levels of p62, indicating the induction of autophagy in response to ERS.

lncRNAs have been identified as novel targets for the treatment of cardiovascular diseases. For example, lncRNA CDKN2B antisense RNA 1 has been identified as the most powerful predictor of atherosclerosis (46,47). Chi *et al.* (47) revealed that the lncRNA myocardial infarction associated transcript 1 led to a decrease in the expression of inflammatory factors via inhibition of the NF- $\kappa$ B signaling pathway, thereby decreasing myocardial cell apoptosis and inflammatory cell infiltration to decrease AMI damage. To the best of our knowledge, the present study suggested

the role of Dancr in cardiomyocyte ERS for the first time. Compared with the Tm + Oe-NC group, Dancr overexpression increased cell viability by decreasing cell apoptosis and promoting autophagy in Tm-treated H9C2 cardiomyocytes. Furthermore, compared with the Tm + Oe-NC group, Dancr overexpression enhanced p-IRE1 $\alpha$ , p-IRE1 $\alpha$ /IRE1 $\alpha$  and Xbp1s expression levels, and decreased Xbp1u expression levels, but displayed no significant effects on IRE1 $\alpha$ , ATF6 and ATF4 expression levels in Tm-treated H9C2 cardiomyocytes, suggesting that Dancr selectively activated the IRE1 $\alpha$  branch in the UPR, thus promoting autophagy and ERAD, and ultimately alleviating ERS. It has been reported that enhancing autophagy could protect cardiomyocytes from ERS and apoptosis (48). However, whether the antiapoptotic effect of Dancr is dependent on the autophagy signaling pathway, the ERS signaling pathway or other mediators requires further investigation.

lncRNAs regulate the occurrence and development of human diseases, including cardiovascular diseases, primarily via sponging miRNAs (11). Our previous unpublished data demonstrated that miR-6324 was upregulated in MI model rats. In the present study, online analysis and luciferase reporter assays confirmed that miR-6324 interacted with lncRNA Dancr. Therefore, the present study hypothesized that Dancr displayed cardioprotective effects via sponging miR-6324 and inhibiting its expression. To verify the hypothesis, the present study co-transfected Tm-treated H9C2 cells with Oe-Dancr and miR-6324 mimic. miR-6324 mimic partially reversed Dancr overexpression-mediated effects on cell viability, cell apoptosis, ERS and autophagy. The results indicated that miR-6324 may serve as a downstream target miRNA of Dancr to rescue cell viability, inhibit apoptosis and promote autophagy, thereby relieving cardiomyocyte ERS. However, apoptosis and autophagy are double-edged swords, thus consistent with previous studies (20,24,25), the present study identified Dancr as a potential target to reduce apoptosis and ERS, while enhancing autophagy in Tm-induced H9C2 cardiomyocytes. The enhanced cell viability indicated the protective effect of Dancr, but *in vivo* studies are required to verify the results of the present study. Moreover, as miR-6324 mimic did not completely reverse the effects of Dancr, other downstream targets of Dancr may exist and should be investigated in future studies.

In summary, the present study provided evidence that lncRNA Dancr sponged miR-6324, selectively activated the IRE1 $\alpha$ /Xbp1 signaling pathway of autophagy, repressed apoptosis and enhanced autophagy, leading to amelioration of ERS. The actions of Dancr identified in the present study suggested its potential for the treatment of cardiovascular diseases.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

YHD, DXX, JL and JX conceived and designed the study. JL, JX, YZW, YRG, LW and GWD acquired and analyzed the data. JL and JX drafted the manuscript and figures. YHD and DXX revised the manuscript for critically important intellectual content. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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