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MicroRNA-298 exacerbates myocardial ischemic injury via targeting cyclin D1

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The aim of this study was to explore the role and regulatory mechanism of microRNA-298 (miR-298) in myocardial ischemic injury. H9c2 cardiomyocytes were cultured under hypoxia (3 % O_2) conditions to induce myocardial ischemic injury. Subsequently, the effects of miR-298 overexpression and suppression on hypoxia-induced myocardial damage in H9c2 cells were investigated. Moreover, the target of miR-298 was identified in H9c2 cells and the relationship between miR-298 and the activation of PTEN/PI3K/AKT signaling pathway was explored. miR-298 was upregulated in hypoxia-stimulated H9c2 cells. Overexpression of miR-298 distinctly aggravated hypoxia-induced myocardial damage in hypoxia-treated H9c2 cells, whereas inhibition of miR-298 alleviated hypoxia-induced injury. Moreover, miR-298 negatively regulated the expression of cyclin D1, and cyclin D1 was a target of miR-298 in H9c2 cells. Suppression of cyclin D1 significantly reversed the effects of suppression of miR-298 on hypoxia-induced myocardial damage. Lastly, inhibition of miR-298 activated the PTEN/PI3K/AKT signaling pathway, and this effect could be reversed after suppression of cyclin D1. Our results reveal that miR-298 may exacerbate myocardial ischemic injury by targeting cyclin D1 and regulating the activation of PTEN/PI3K/AKT signaling pathway. miR-298 may serve as a promising targets for reducing myocardial ischemic injury.

1. Introduction

Myocardial ischemic injury is caused by severe impairment of coronary blood supply (Buja 2005) and results in a spectrum of myocardial dysfunctions, such as cardiac dysfunction and myocyte death (Martindale and Metzger 2014). Myocardial infarction is considered as a major cardiovascular disorder resulting from myocardial ischemia (Lindsey et al. 2018), which is a leading cause of death around the world (Do et al. 2015). The therapeutic strategies that protect against myocardial ischemia-reperfusion (I/R) injury can prevent acute myocardial infarction (Hausenloy and Yellon 2013; Ruiz-Meana and García-Dorado 2009). To reduce myocardial ischemic injury, it still imperative to explore the crucial mechanisms underlying myocardial ischemia.

MicroRNAs (miRNAs) are small (about 22 nt) noncoding single-stranded RNAs that downregulate gene expression through binding to 3'-untranslated region (UTR) of target genes (Bartel 2004; Mirzamohammadi et al. 2014). Accumulating studies have reported that miRNAs function as key regulators in modulating myocardial I/R injury (Sala et al. 2014; Ye et al. 2010). For instance, miR-155 is shown to aggravate I/R injury through regulating inflammatory cell recruitment and the generation of reactive oxygen species of macrophages (Eisenhardt et al. 2015). In patients with acute myocardial ischemia, miR-30b can protect myocardial cell function by targeting plasminogen activator inhibitor-1 (Li et al. 2018). However, the crucial miRNAs involving in myocardial ischemia have not been fully investigated.

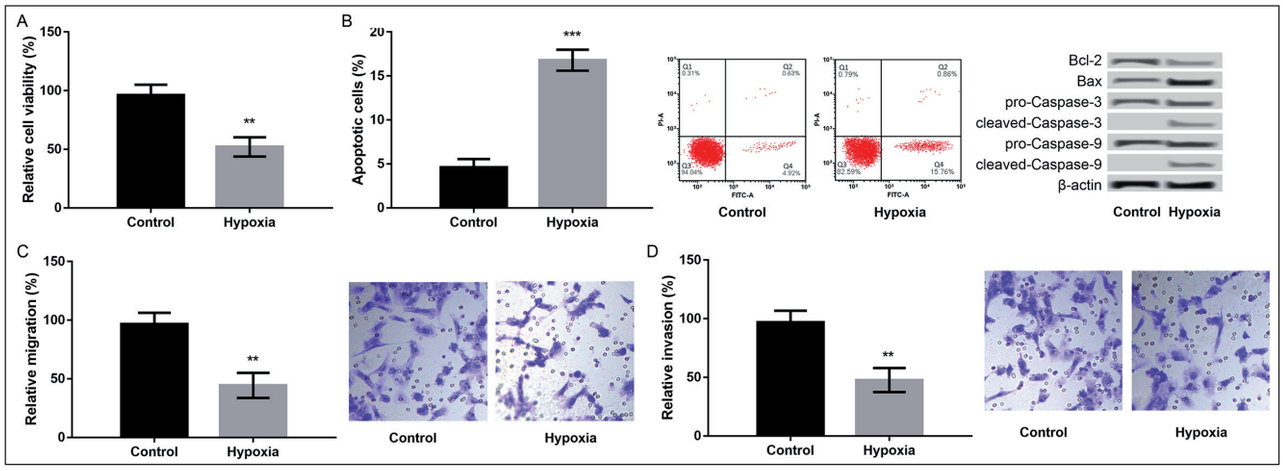

Fig. 1: Hypoxia induced injury in H9c2 cells. A: MTT assay showed cell viability after hypoxia treatment; B: Flow cytometry showed cell apoptosis after hypoxia treatment, and western blot showed the expression of apoptosis-related proteins; C-D: Transwell assay showed cell migration and invasion after hypoxia treatment. All experiments were repeated three times. ** $P < 0.01$ and *** $P < 0.001$ compared to control.

MiR-298 can improve the cardiac function and reduce myocardial apoptosis after myocardial infarction (Zhang et al. 2018). Moreover, miR-298 is found upregulated in the rat model of post-infarction heart failure (Liu et al. 2016). Therefore, we hypothesized that there is the association between dysregulation of miR-298 and myocardial ischemia injury. To verify this, H9c2 cells were cultured under hypoxic conditions to construct a model of myocardial ischemia injury. Subsequently, the effects and regulatory mechanism of miR-298 dysregualtion on hypoxia-induced myocardial damage in H9c2 cells were explored. Our study will provide a new insight for better understanding of the process of myocardial ischemic injury.

2. Investigations and results

2.1. Hypoxia induced injury in H9c2 cells

In this study, we firstly investigated whether hypoxia induced injury in H9c2 cells. In relation to control, hypoxia treatment significantly inhibited cell viability (Fig. 1A), promoted apoptosis (Fig. 1B), and decreased the abilities of migration (Fig. 1C) and invasion (Fig. 1D) in H9c2 cells ($P < 0.01$). These data indicated that hypoxia induced injury in H9c2 cells.

2.2. Suppression of miR-298 alleviated hypoxia-induced myocardial damage

After hypoxia treatment, the expression of miR-298 was remarkably upregulated ($P < 0.01$, Fig. 2A). Moreover, miR-298 was further dysregulated in H9c2 cells to investigate the effects of miR-298 on hypoxia-induced myocardial damage. As presented in Fig. 2B, miR-298 was successfully overexpressed in H9c2 cells after transfection with miR-298 mimic ($P < 0.001$), and that was significantly suppressed after transfection with miR-298 inhibitor ($P < 0.01$). Subsequently, we found that overexpression of miR-298 distinctly aggravated hypoxia-induced myocardial damage in hypoxia-treated H9c2 cells by further decreasing cell viability (Fig. 2C), promoting cell apoptosis (Fig. 2D), and decreasing the abilities of migration (Fig. 2E) and invasion (Fig. 2F) (all $P < 0.05$). However, inhibition of miR-298 alleviated hypoxia-induced myocardial damage in hypoxia-treated H9c2 cells (all $P < 0.05$, Fig. 2C-F).

2.3. MiR-298 negatively regulated cyclin D1 expression, and cyclin D1 was a target of miR-298

Previous studies have shown that cyclin D1 is a crucial regulator in cell cycle that plays a key role in regulating cardiomyocyte proliferation and cardiac regeneration (Mohamed et al. 2018). Therefore, we further investigated that whether cyclin D1 was a potential target of miR-298. As shown in Fig. 3A, the binding sequence of miR-298 and cyclin D1 was predicted by Tagetscan online tool. Moreover, luciferase reporter assay revealed that miR-298 could target the cyclin D1 3'UTR (Fig. 3B). Furthermore, miR-298 mimic significantly decreased the expression of cyclin D1 at mRNA and protein levels, whereas miR-298 inhibitor had opposite effects ($P < 0.05$, Fig. 3C-D). These data indicated that miR-298 negatively regulated cyclin D1 expression, and cyclin D1 was a target of miR-298.

2.4. Suppression of miR-298 alleviated hypoxia-induced myocardial damage by negative upregulation of cyclin D1

Cyclin D1 was further knocked down in H9c2 cells, as a result, the expression of cyclin D1 at mRNA and protein levels were significantly down-regulated in H9c2 cells after transfection

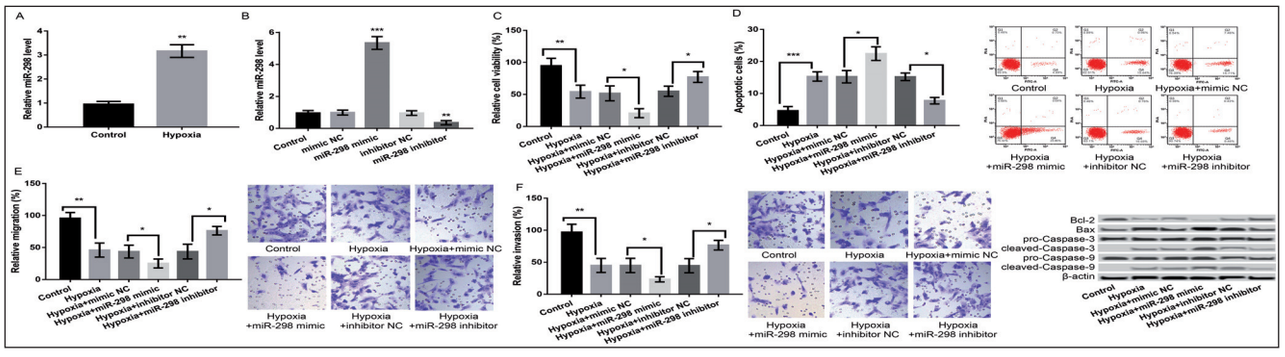

Fig. 2: Overexpression of miR-298 aggravated hypoxia-induced myocardial damage in hypoxia-treated H9c2 cells, whereas inhibition of miR-298 alleviated this injury. A: qPCR showed the expression of miR-298 after hypoxia treatment; B: qPCR showed the dysregulation of miR-298 after transfections. C: MTT assay showed cell viability after different treatments; D: Flow cytometry showed cell apoptosis after different treatments, and western blot showed the expression of apoptosis-related proteins; E-F: Transwell assay showed cell migration and invasion after different treatments. All experiments were repeated three times. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared to control.

with si-cyclin D1 ($P < 0.01$, Fig. 4A). Subsequently, the results showed that the effects of inhibition of miR-298 alone on hypoxia-induced myocardial damage in hypoxia-treated H9c2 cells were significantly reversed after inhibition of miR-298 and suppression of cyclin D1 concurrently, as cell viability was further decreased ($P < 0.05$, Fig. 4B), cell apoptosis was promoted ($P < 0.01$, Fig. 4C), and the abilities of migration and invasion was inhibited ($P < 0.05$, Fig. 4D-E). These data indicated that suppression of miR-298 alleviated hypoxia-induced myocardial damage by negative upregulation of cyclin D1.

2.5. Inhibition of miR-298 activated PTEN/PI3K/AKT signaling pathway via targeting cyclin D1

The downstream mechanism of miR-298/cyclin D1 in regulating hypoxia-induced myocardial damage was investigated. As shown in Fig. 4F, hypoxia treatment significantly increased the expression of PTEN and decreased the expression of p-PI3K/t-PI3K and p-AKT/t-AKT in H9c2 cells. Moreover, inhibition of miR-298 remarkably decreased PTEN expression and increased the expression levels of p-PI3K/t-PI3K and p-AKT/t-AKT in hypoxia-treated H9c2 cells (all $P < 0.05$), indicating that inhibition of miR-298 significantly activated the PTEN/PI3K/AKT signaling pathway. However, suppression of cyclin D1 at the same time clearly reversed the effects of inhibition of miR-298 on the expression changes of these proteins in hypoxia-treated H9c2 cells (all $P < 0.05$). These data indicated that inhibition of miR-298 activated PTEN/PI3K/AKT signaling pathway via targeting cyclin D1.

3. Discussion

Recently, the mechanism of I/R has been explored intensively, but it is still largely unknown. In this study, we studied the role and mechanisms of miR-298 on the hypoxia-induced myocardial injury in H9c2 cells. We found that miR-298 was upregulated in hypoxia-stimulated H9c2 cells. Overexpression of miR-298 distinctly aggravated hypoxia-induced myocardial damage in hypoxia-treated H9c2 cells, whereas inhibition of miR-298 alleviated

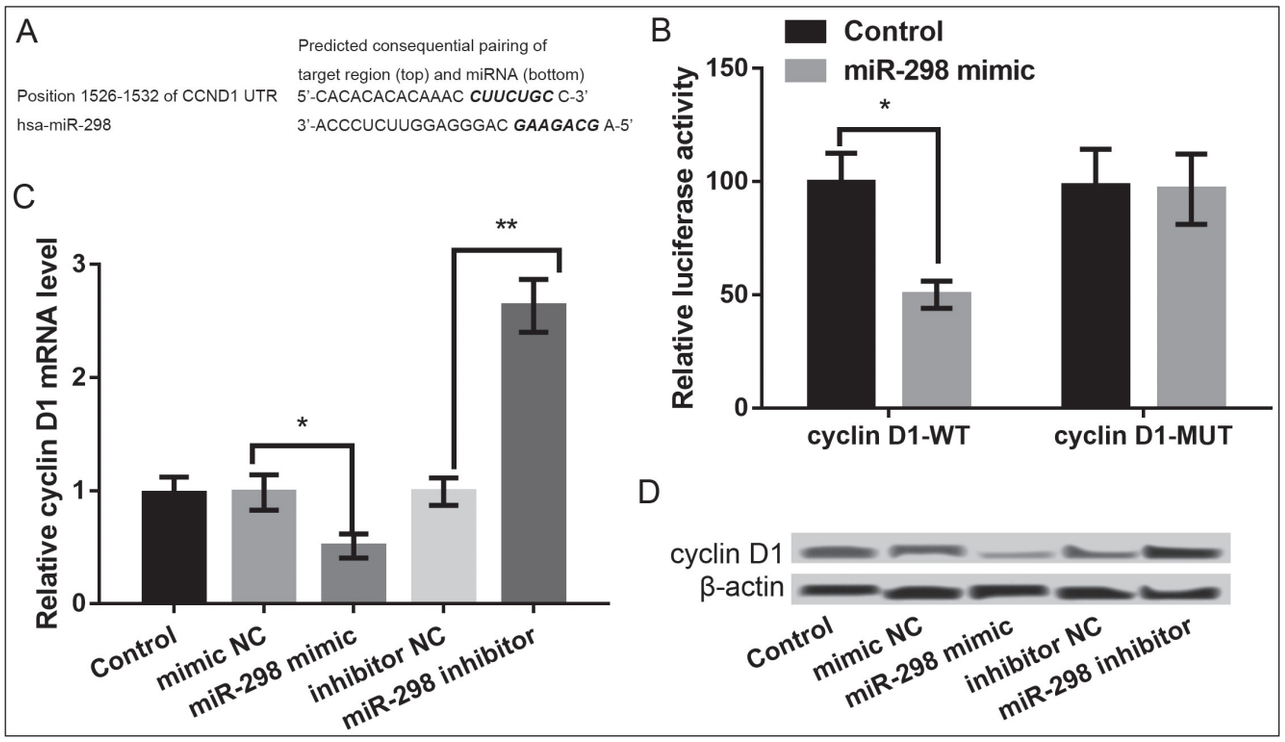

Fig. 3: MiR-298 negatively regulated cyclin D1 expression, and cyclin D1 was a target of miR-298. A: The predicted binding sequence of miR-298 and cyclin D1 by Tagetscan online too; B: Luciferase reporter assay revealed that miR-298 could target the cyclin D1 3'UTR; C-D: qPCR and western blot showed the mRNA and protein expression levels of cyclin D1 after dysregulation of miR-298 by transfection. All experiments were repeated three times. * $P < 0.05$ and ** $P < 0.01$ compared to control.

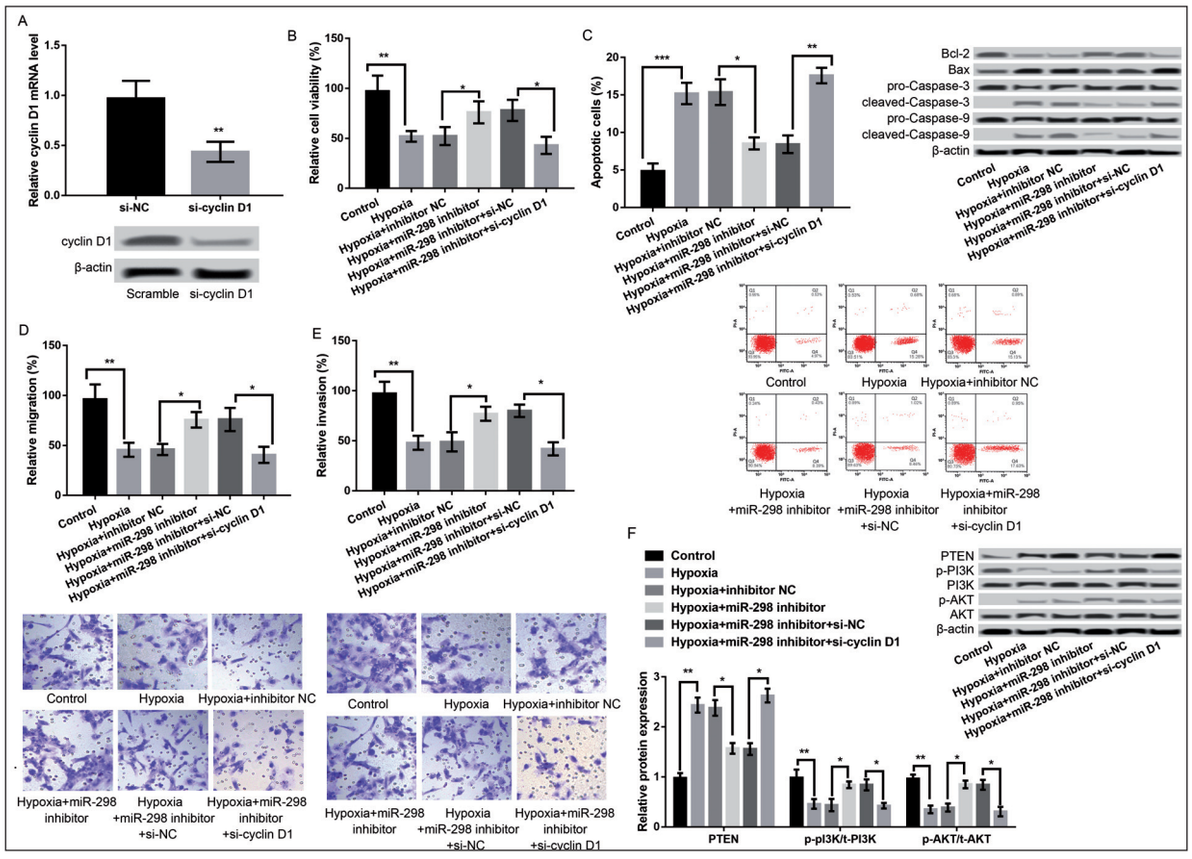

Fig. 4 Suppression of miR-298 alleviated hypoxia-induced myocardial damage by negative upregulation of cyclin D1. A: qPCR and western blot showed the mRNA and protein expression levels of cyclin D1 after transfection with si-cyclin D1 and si-NC. B: MTT assay showed cell viability after different treatments; C: Flow cytometry showed cell apoptosis after different treatments, and western blot showed the expression of apoptosis-related proteins; D-E: Transwell assay showed cell migration and invasion after different treatments. F: The protein expression of PTEN/PI3K/AKT signaling pathway-related proteins after hypoxia, inhibition of miR-298 and/or suppression of cyclin D1. All experiments were repeated three times. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared to control.

hypoxia-induced injury. Moreover, miR-298 negatively regulated the expression of cyclin D1, and cyclin D1 was a target of miR-298 in H9c2 cells. Suppression of cyclin D1 significantly reversed the effects of suppression of miR-298 on hypoxia-induced myocardial damage. Lastly, inhibition of miR-298 activated PTEN/PI3K/AKT signaling pathway, and this effect could be reversed after suppression of cyclin D1. These data indicate that miR-298 may play a key role in hypoxia-induced myocardial damage.

In previous studies, miR-298 is found to be involved in the pathological process of many diseases, such as diverse cancers (Bao et al. 2012; Cao et al. 2018; Yan et al. 2016). In a recent study, miR-298 is shown to play a key role in regulating apoptosis of cardiomyocytes after myocardial infarction (Zhang et al. 2018). Moreover, it is reported that miR-298 exacerbates I/R Injury following ischemic stroke (Sun et al. 2018). In this study, miR-298 was upregulated in hypoxia-stimulated H9c2 cells. Overexpression of miR-298 distinctly aggravated hypoxia-induced myocardial damage in hypoxia-treated H9c2 cells, whereas inhibition of miR-298 alleviated hypoxia-induced injury. We thus speculate that miR-298 can aggravate myocardial ischemic injury.

Furthermore, one important finding of our study was cyclin D1 was a target of miR-298 in H9c2 cells. It has been revealed that the impairment of nuclear expression of cyclin D1 is one of the obstacles to cell cycle in cardiomyocytes (Mimi et al. 2003). Toyoda et al. (2003) demonstrated that cyclin D1 was involved in regulation of cardiac cell proliferation. Moreover, cyclin D1 is shown to regulate the effects of hypoxia-induced miR-191 on hepatic I/R injury (Pan et al. 2019). In this study, suppression of cyclin D1 significantly reversed the effects of suppression of miR-298 on hypoxia-induced myocardial damage. It can therefore be speculated that miR-298 may exacerbate myocardial ischemic injury via targeting cyclin D1. Strikingly, we also found that inhibition of miR-298 activated PTEN/PI3K/AKT signaling pathway, and this effect could be reversed after suppression of cyclin D1. PTEN is shown to play a crucial role in regulating myocardial I/R injury by inhibiting anti-apoptotic survival signals (Ruan et al. 2009). Moreover, PTEN is a major antagonist of PI3K activity (Siddall et al. 2008), which can activate the PI3K activity and attenuate cardiac injury after I/R (Keyes et al. 2010). Moreover, it is shown that the activation of the PI3K/AKT pathway induced by insulin can not only reduce cardiomyocyte apoptosis but also improve cardiac function (Yao and Han 2014). Furthermore, Kong et al. (2016) revealed that HSPA12B could alleviate acute myocardial I/R injury through a PI3K/Akt/mTOR-dependent mechanism. Wang et al. (2018) confirmed that shikonin prevented hypoxia/reoxygenation injury in H9c2 cardiomyocytes via activation of PI3K/Akt signaling pathway. Given the key role of PTEN/PI3K/AKT signaling pathway in myocardial I/R injury, we speculate that miR-298 may exacerbate myocardial ischemic injury *via* suppressing the activation of this pathway.

In conclusion, our results reveal that miR-298 may exacerbate myocardial ischemic injury *via* targeting cyclin D1 and regulating the activation of PTEN/PI3K/AKT signaling pathway. miR-298 may serve as a promising targets for reducing myocardial ischemic injury.

4. Experimental

4.1. Cell culture and treatment

The H9c2 cardiac muscle cell line was cultured in Dulbecco's modified Earl's medium (DMEM) containing 10 % fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in an incubator with 5 % CO_2. To simulate hypoxia, H9c2 cells were then maintained in a hypoxic incubator containing 1 % O_2, 94 % N_2, and 5 % CO_2.

4.2. Quantitative PCR (qPCR)

Total RNA was extracted from different treated H9c2 cells using Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA) and then subjected to reverse transcription for cDNA synthesis. To test the expression of miR-298, real-time qPCR was conducted using the Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II with the TaqMan MicroRNA Assay of miR-298 and U6 (Applied Biosystems, Foster City, CA, USA). U6 was used as the internal control. To determine gene expression, real-time qPCR was conducted using the One Step SYBR® PrimeScript®PLUS RT-RNA PCR Kit (TaKaRa Biotechnology, Dalian, China), and GAPDH served as the inference. Finally, fold changes of gene expression were calculated by relative quantification ($2^{-\Delta\Delta Ct}$) method.

4.3. Cell transfection

Cells transfection was performed using Lipofectamine® RNAiMAX Transfection Reagent (Life technologies, Carlsbad, California, USA). In brief, 1×10^5 H9c2 cells were transfected with miR-298 mimic, mimic, miR-298 inhibitor, inhibitor control (Life technologies), small interfering RNA (siRNA) against cyclin D1 (si-cyclin D1) or negative control (si-NC) (Life technologies) for 72 h.

4.4. Cell viability assay

For cell viability assay, 1×10^5 cells were seeded in duplicate in 60-mm dishes. After different treatments, H9c2 cells were washed, and live cell numbers were determined by trypan blue exclusion.

4.5. Apoptosis assay

After different treatments, H9c2 cells were washed in PBS and then fixed in 70 % ethanol. Subsequently, fixed cells were subjected to propidium iodide (PI) and fluorescein isothiocynate (FITC)-conjugated Annexin V staining in the presence of 50 µg/ml RNase A (Sigma-Aldrich) for 1 h at room temperature in the dark. The apoptotic cells were then detected by flow cytometry using a FACS can (Beckman Coulter, Fullerton, CA, USA), and the obtained data were analyzed using FlowJo software.

4.6. Migration assay

Cell migration was assessed using a modified two-chamber migration assay. Briefly, after different treatments, H9c2 cells suspended in 200 µl of serum-free medium were plated into the upper compartment of 24-well Transwell culture chamber (8 µM pore size; BD Biosciences). The lower compartment was then added with 600 µl of complete medium. After incubation for 48 h at 37 °C, non-migrated cells were removed with a cotton swab. Finally, the migrated cells on the lower side of the filter were fixed, stained with crystal violet, and counted microscopically.

4.7. Invasion assay

Cell invasion was determined using 24-well Millicell Hanging Cell Culture inserts with 8 mm PET membranes (Millipore, Bedford, Massachusetts, USA). Briefly, after different treatments, 5.0×10^4 cells suspended in 200 µl serum-free DMEM medium were plated onto BD BioCoat™ Matrigel ™ Invasion Chambers (8 µM pore size polycarbonate filters; BD Biosciences). Meanwhile, the complete medium was also added to the lower chamber. After incubation for 48 h, the non-invading cells were removed with a cotton swab. The invading cells were fixed in 100 % methanol, stained with crystal violet, and counted microscopically.

4.8. Luciferase reporter assay

To construct the reporter vector cyclin D1-wild-type (cyclin D1-WT), the fragment from cyclin D1 containing the predicted miR-298 binding site was amplified and then cloned into a pmirGlO Dual-luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA). To form the reporter vector cyclin D1-mutated-type (cyclin D1-MUT), the sequence of putative binding site of miR-298 in the cyclin D1 was mutated. Subsequently, HEK 293T cells were co-transfected with the reporter vectors and miR-298 mimics, and the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) were applied to determine the luciferase activity.

4.9. Western blot

Total protein was extracted from cells using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) that was prepared by supplement with protease inhibitors (Roche, Guangzhou, China). The protein quantification was then performed using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The western blot system was established using a Bio-Rad Bis-Tris Gel system. Primary antibodies were purchased from Abcam (Cambridge, UK) and prepared in 5 % blocking buffer at a dilution of 1:1,000. The Polyvinylidene Difluoride (PVDF) membranes were probed with primary antibodies at 4°C overnight, and then incubated with horseradish peroxidase-marked secondary antibody for 1 h at room temperature. After rinsing, the membranes were transferred into the Bio-Rad ChemiDoc™ XRS system, and the protein signals were revealed by incubation with 200 µl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA). Finally, the intensity of the protein bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

4.10. Statistical analysis

All experiments were repeated three times. Statistical analyses for data were performed using Graphpad 6.0 statistical software. The data obtained from multiple experiments are presented as the mean±standard deviation (SD). The data between different groups were compared using a one-way analysis of variance (ANOVA) and $P < 0.05$ was defined as a statistically significant result.

Conflict of interests: None declared.

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