

## Original Article

# Inhibition of CLU4A exhibits a cardioprotective role in hypoxia/reoxygenation-induced injury by an ERK-dependent pathway

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Received March 14, 2019; Accepted April 19, 2019; Epub June 1, 2019; Published June 15, 2019

**Abstract:** CLU4A is identified as a proto-oncogene in various human cancers. CLU4A was reported to be up-regulated in myocardial ischemia/reperfusion injury, but the precise role of CLU4A played in myocardial ischemia/reperfusion injury remains unknown; and the underlying mechanism of CLU4A in myocardial ischemia/reperfusion injury needs to be investigated. CLU4A expression was measured after myocardial ischemia/reperfusion in mice and in H9C2 cells with hypoxia/reoxygenation treatment by q-PCR and western blotting. The cardioprotective effect of CLU4A inhibition was detected by monitoring the cell viability, cell apoptosis, and LDH activity *in vitro* and *in vivo*, and examining the infarct size and cardiac function *in vivo*. The molecular mechanism was further determined by examining the effects of PD98059, a specific inhibitor of the ERK signaling pathways in H9C2 cells with hypoxia/reoxygenation treatment. CLU4A expression was up-regulated after myocardial ischemia/reperfusion in mice and in H9C2 cells with hypoxia/reoxygenation treatment. Inhibition of CLU4A improved the cell viability, restrained the cell apoptosis, and suppressed LDH activity *in vitro*. Consistently, knockdown of CLU4A reduced the myocardial infarct size and improved cardiac function *in vivo*. si-CLU4A treatment increased phosphorylated ERK (p-ERK) *in vitro*, but the protection role of si-CLU4A was abolished by the ERK inhibitor, PD98059. In conclusion, CLU4A expression was up-regulated in myocardial ischemia/reperfusion. Inhibition of CLU4A exhibited a cardioprotective role by an ERK-dependent pathway.

**Keywords:** CLU4A, apoptosis, ERK signaling pathways, myocardial ischemia/reperfusion injury

## Introduction

Myocardial ischemia caused by myocardial ischemia/reperfusion (I/R) injury remains one of the most fatal diseases worldwide [1]. Myocardial ischemia/reperfusion (I/R) usually occurs when interrupted blood supply to myocardial ischemic regions is restored, such as by coronary artery thrombolytic therapy, heart transplantation and circulatory arrest [2, 3]. It may accelerate inflammatory response and activate oxidative stress, and thus lead to acute myocardial infarction [4, 5]. Hence, understanding the key mechanism of myocardial I/R injury is of great importance for development of promising therapeutic therapy for acute myocardial infarction.

Cullin-RING ligases (CRLs) complexes, which contain cullin, represent a large and diverse

class of ubiquitin ligases. Among all the cullin family members, cullin 4A (CLU4A) functions as a proteolysis mediator and participates in a sequence of cellular processes, including cell cycle progression, transcription, and apoptosis [6-8]. CLU4A also plays an important role in cardiovascular physiologic and pathologic processes. Li *et al.* demonstrated that CLU4A is required for hematopoietic stem-cell engraftment and self-renewal [9]. Waning *et al.* reported that CLU4A is essential for hematopoietic cell viability and its deficiency causes cell apoptosis in the bone marrow [10]. Recently, Zha *et al.* verified that deletion of CLU4A results in cardiac hypertrophy in male mice [11], indicating the importance of CLU4A in heart disease progression. Interestingly, Sigurdsson *et al.* found that CLU4A was differentially expressed in an ischemia model [12], and it was reported that overexpression of CLU4A reduces hypoxia-reox-

xygenation injury in pheochromocytoma (PC12) cells [13]. However, the precise role of CLU4A in myocardial ischemia/reperfusion (I/R) injury remains unclear.

Here, we observed that the mRNA and protein expression of CLU4A in mice cardiomyocytes subjected to ischemia/reperfusion injury were increased. Knockdown of CLU4A attenuates ischemia-reperfusion injury both *in vitro* and *in vivo*. We further found that inhibition of CLU4A restrains ischemia-reperfusion injury through regulating the ERK signaling pathway.

### Materials and methods

#### *Animals and experimental protocols*

Male adult C57BL/6 mice (6-8 weeks, 20-25 g) were obtained from the Laboratory Animal Center of Zhengzhou University (Zhengzhou, China). Mice were raised at room temperature with constant humidity, and had access to food and water *ad libitum*. The present study was approved by the ethics committee of The Second Affiliated Hospital of Air Force Medical University.

The mice were randomly assigned to three groups: (1) the mice in control group accepted the sham operation and were used as normal controls; (2) the mice in I/R + si-NC group were injected with si-NC intraventricularly following the manufacturer's instructions for Entranster *in vivo* RNA transfection reagent (Engreen, Shanghai, China) 24 h before ischemia induction [14]; (3) the mice in I/R + si-CLU4A group were injected with si-CLU4A intraventricularly 24 h before ischemia induction for 3 days before ischemia induction.

#### *Ischemia/reperfusion injury model*

Ischemia/reperfusion injury was conducted as previously described [15] by ligation of the left anterior descending (LAD) coronary artery for 30 min followed by reperfusion. Mice were anesthetized with pentobarbital sodium (60 mg/kg *i.p.*), and the surgical site was disinfected with povidone-iodine and 75% alcohol. Then the chest was opened. A curved needle was passed beneath the LAD coronary artery at a point 1-2 mm inferior to the left auricle. Ischemia was induced by LAD ligation with a 6-0 silk suture for 30 min until the color of myo-

cardium turned to pale and white. Then the silk suture was released for reperfusion. Successful ischemia/reperfusion induction was confirmed by echocardiography after surgery.

#### *Myocardial infarct size*

Myocardial infarct size was detected as previously described [16]. After anesthesia, the heart was immediately harvested and intravenously injected with 2% Evans Blue (Shenggong, Shanghai, China). Along the atrioventricular groove, hearts were cut into 2-mm-thick slices and were treated with 10% paraformaldehyde. The area not stained with a blue color by Evans blue was identified as the area not at risk. The slices were then incubated with 1% 2, 3, 5-triphenyltetrazolium chloride (TTC, Shenggong) to show the ischemic area. The infarct area remained pale in color. The area at risk and the infarct area were quantified using Image J software. Infarct size was identified as a ratio of the infarct area to the area at risk.

#### *Cell culture*

The cardiac muscle cell line H9C2 was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and was maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, MO, USA) which was supplemented with 10% fetal bovine serum (FBS; Gibco Laboratories, Grand Island, NY, USA) and 1% penicillin/streptomycin (Gibco). The cells were cultured in a humidified incubator at 37°C containing 5% CO<sub>2</sub>.

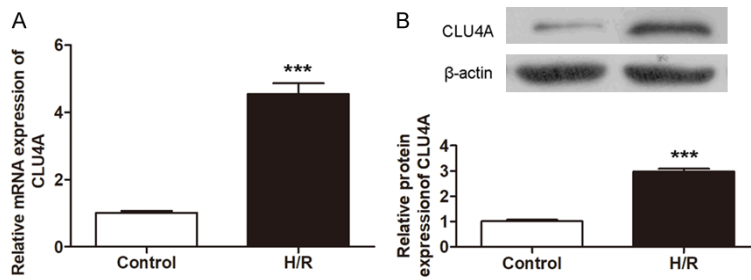
#### *Hypoxia/reoxygenation (H/R) cell model*

H9c2 cells were cultured with glucose-free DMEM and maintained in an oxygen-free atmosphere (95% N<sub>2</sub> and 5% CO<sub>2</sub>) at 37°C for 4 h. Afterwards, the cells were changed to normal culture medium (95% air and 5% CO<sub>2</sub>) at 37°C.

#### *Cell viability assay*

The cell viability was determined by a MTT assay according to standard protocol. In brief, H9c2 cells were planted into a 96-well plate at  $1 \times 10^4$  cells/well and cultured at 37°C for 4 h. Straight after, 5 mg/mL MTT was added into the medium for incubation. Then, the supernatant was discarded and 100  $\mu$ l of Dimethylsulfoxide (DMSO; Sigma) were added the cells.

## CLU4A in myocardial I/R injury



**Figure 1.** Expression of CLU4A in H9c2 cells after simulated ischemia-reperfusion injury. A. mRNA expression of CLU4A was determined by qPCR. B. Protein expression of CLU4A was determined by qPCR. \*\*\* $P < 0.001$ .

The absorbance was measured at 590 nm using a microplate reader (BioTek, Winooski, VT, USA).

### TUNEL assays

Apoptosis in H9c2 cells or myocardial tissues was measured by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay with an In situ Cell Death Detection kit (Roche, Mannheim, Germany) [17]. For analysis of apoptosis myocardial tissues, the frozen myocardial sections were incubated with TUNEL Reaction Mixture at 37°C for 1 h. For measurement of apoptosis in H9c2 cells, cells were fixed with 4% paraformaldehyde, permeated with 0.1% Triton X-100, and then incubated with TUNEL Reaction Mixture in the dark and washed with PBS three times. To visualize the nuclei, cells were counterstained with DAPI for 5 min at room temperature. Cells were pictured with a confocal microscope (FV1000, Olympus, Tokyo, Japan). The percentage of TUNEL-positive cells was determined by ratio of (stained apoptotic cells)/(total number of cells)  $\times 100\%$ .

### Western blot analysis

Western blot analysis was conducted to detect the protein levels in H9c2 cells or myocardial tissues. Briefly, cells were lysed and protein was loaded onto SDS-polyacrylamide gel, and then transferred onto nitrocellulose membranes. Non-fat milk was used to block the membranes, and then the membrane was incubated with specific primary antibody, including anti-CLU4A, anti-phospho-ERK, anti-t-ERK, anti-Bcl-2 and Bax, anti-cleaved caspase-3 and anti- $\beta$ -actin (Cell Signaling Technology Inc. Danvers, Massachusetts) at 4°C for 12 h. After washed

with PBS for three times, the membrane was incubated with peroxidase-conjugated secondary antibodies at 37°C for 2 h.  $\beta$ -actin (Santa Cruz) was used as a loading control.

### qRT-PCR analysis

Total RNAs were extracted using Trizol reagent (Invitrogen, USA) following the manufacturer's protocols, and the isolated RNA samples were reverse-transcribed to cDNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). qRT-PCR was conducted using an ABI-7500 real-time DNA detection system (Applied Biosystems, Waltham, MA, USA) with the MyiQ single-color real-time PCR detection system (Bio-Rad). All samples were analyzed three times, and the data were processed according to the  $2^{-\Delta\Delta Ct}$  method. The relative expression levels were normalized to  $\beta$ -actin. All primers were designed and synthesized by GenePharma (Shanghai, China).

### Statistical analysis

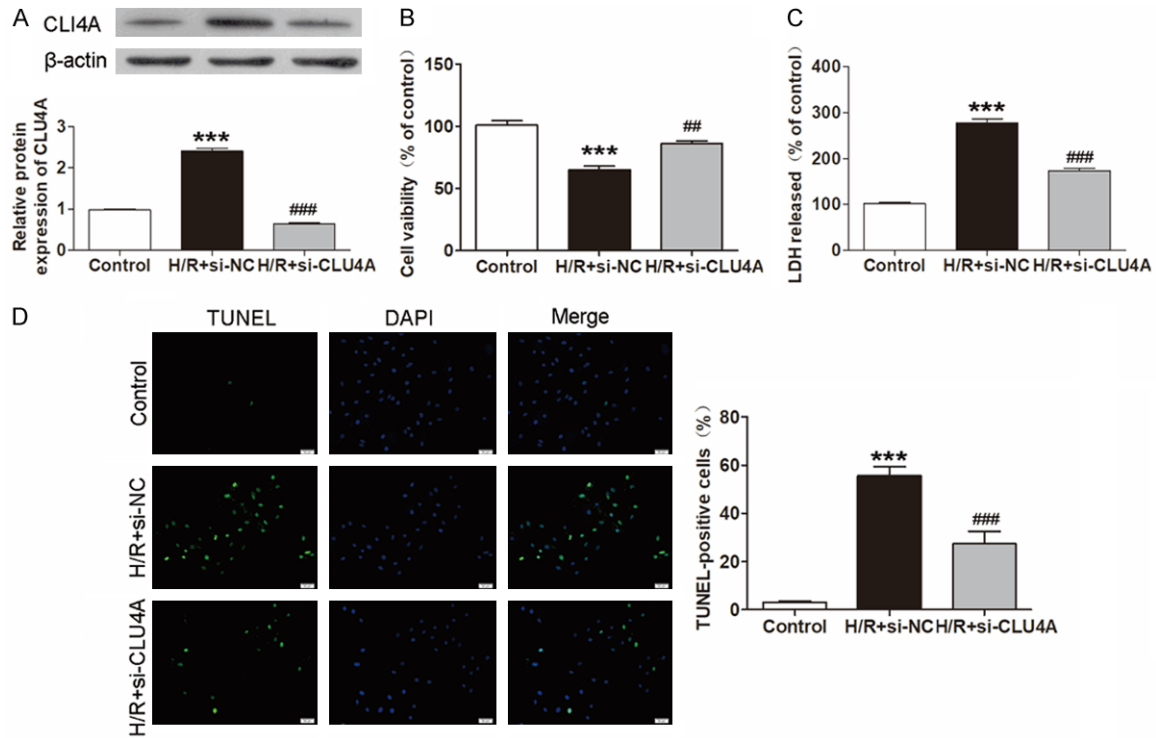
All values are presented as mean  $\pm$  SD. Statistical analyses were performed by Student's t-test or one-way ANOVA followed by Bonferroni post-hoc analysis or the unpaired two-tailed t test as appropriate. A value of  $P < 0.05$  was considered significant. All analyses were performed with GraphPad Prism (San Diego, CA, USA).

## Results

### CLU4A expression is evaluated in cardiomyocytes in ischemia/reperfusion injury

To explore whether CLU4A contributes to modulating cardiomyocyte survival in ischemia/reperfusion injury, we monitored the CLU4A expression in mice hearts subjected to ischemia/reperfusion treatment. As shown in **Figure 1A**, the mRNA expression of CLU4A was increased in mice cardiomyocytes subjected to ischemia/reperfusion injury, as compared to the control group. Consistently, result of western blot verified that the protein expression of CLU4A was also up-regulated in mice cardiomyocytes subjected to ischemia/reperfusion

## CLU4A in myocardial I/R injury



**Figure 2.** Inhibition of CLU4A alleviates hypoxia/reoxygenation injury *in vitro*. H9c2 were transfected with si-NC or si-CLU4A (200 nM), respectively. Twenty four hours later, cells were exposed to hypoxia/reoxygenation treatment. Control: cells were maintained in a standard incubator. A. The efficiency of si-NC or si-CLU4A transfection was confirmed by determining the protein expression of CLU4A by western blotting. B. Cell viability was assessed by MTT assay. C. LDH release was measured. D. Apoptotic cells were measured by TUNEL staining. Representative TUNEL-stained H9c2 cells. TUNEL staining (green) representing apoptotic cells; cell nucleus (blue) (Bar = 50  $\mu$ m). Quantitative analyses of TUNEL-positive cells. Data are shown as the mean  $\pm$  SD for three independent experiments. \*\*\*P < 0.001 vs. Control, ###P < 0.001 vs. H/R + si-NC.

injury (**Figure 1B**). These data demonstrated that CLU4A may play an important role in regulating ischemia/reperfusion injury.

### *Inhibition of CLU4A alleviates hypoxia/reoxygenation injury in vitro*

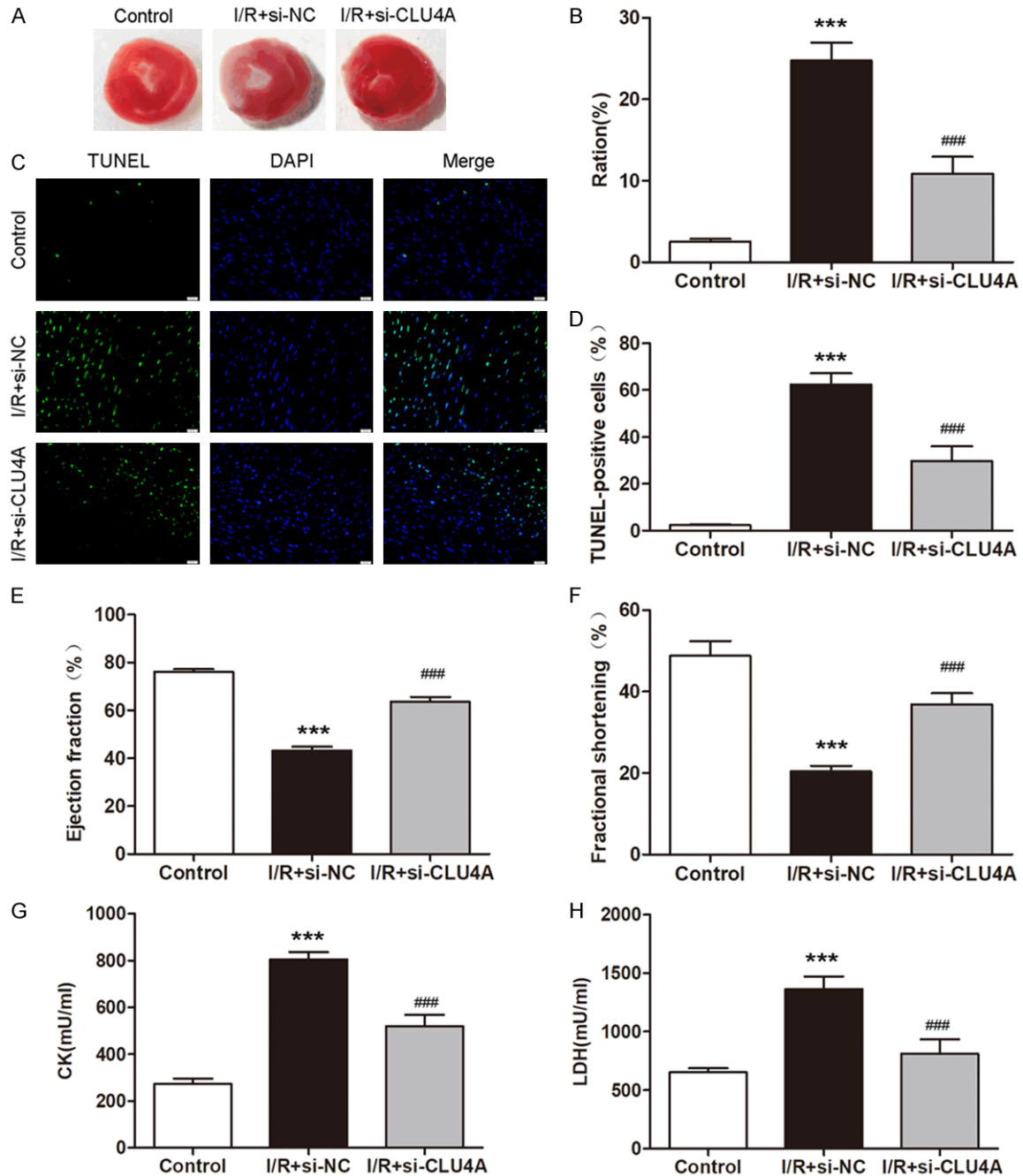
To evaluate whether CLU4A up-regulation contributed to myocardium ischemia/reperfusion injury, a hypoxia/reoxygenation model was established with H9C2 cells, and loss-of-function experiments were performed by transfecting CLU4A siRNA. The results showed that hypoxia/reoxygenation treatment in H9C2 cells increased the CLU4A expression when compared to the control group, and si-CLU4A transfection significantly decreased the expression of CLU4A in hypoxia/reoxygenation H9C2 cells compared with si-NC transfection (**Figure 2A**). MTT assay illustrated that cell viability was observably reduced after hypoxia/reoxygenation stimulation, while si-CLU4A transfection

recovered the decreased cell viability (**Figure 2B**). LDH release assay showed that hypoxia/reoxygenation treatment caused a dramatic increase in LDH release in H9c2 cells, and transfection of si-CLU4A effectively blocked the increase of LDH activities compared with the si-NC group (**Figure 2C**). To determine the effect of CLU4A on cell apoptosis in H9C2 cells, TUNEL assays were performed. **Figure 2D** indicated that cell apoptosis induced by hypoxia/reoxygenation stimulation was inhibited by si-CLU4A transfection. These results demonstrated that inhibition of CLU4A alleviates hypoxia/reoxygenation injury in H9C2 *in vitro*.

### *Knockdown of CLU4A attenuates ischemia-reperfusion injury in vivo*

To investigate whether CLU4A contributes to hypoxia/reoxygenation injury, mice were pre-treated with si-CLU4A or si-NC for 3 days before ischemia induction. TTC staining was applied to

## CLU4A in myocardial I/R injury

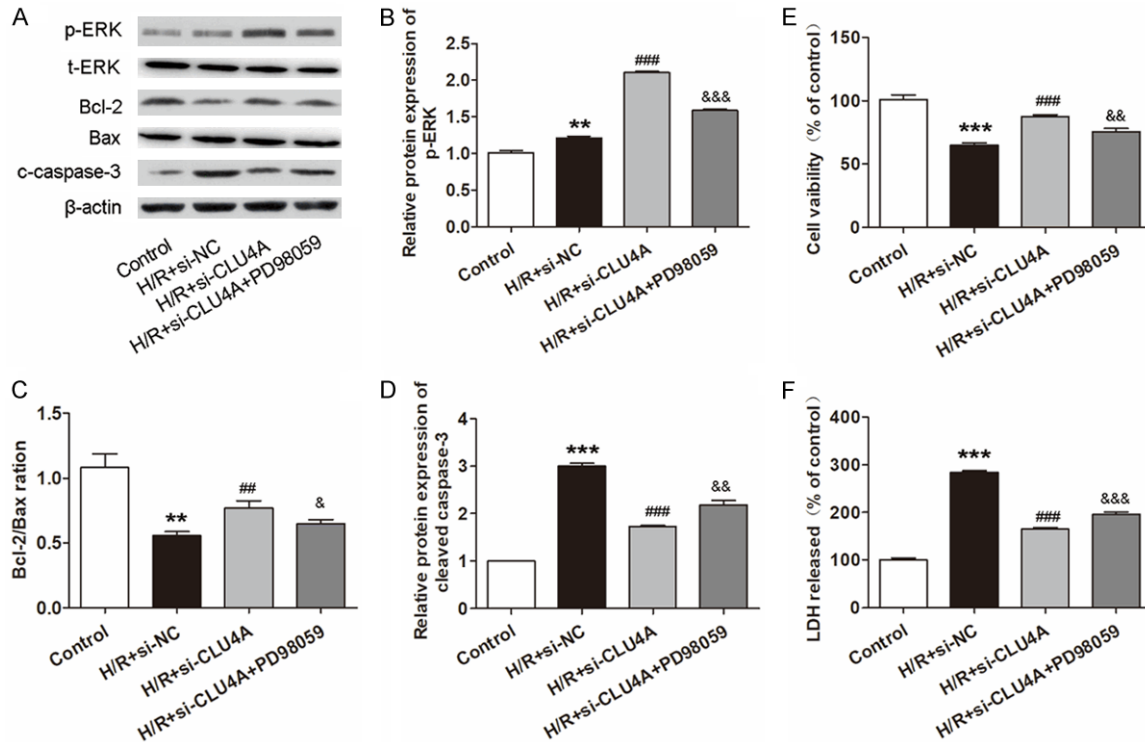


**Figure 3.** Knockdown of CLU4A attenuates ischemia-reperfusion injury *in vivo*. (A) Myocardial infarct sizes were attenuated by si-CLU4A pretreatment in ischemia-reperfusion injury. Representative pictures of myocardial slices stained by TTC. (B) Quantitative analyses of infarct sizes in left ventricle myocardial sections. (C) Myocardial sections were stained with TUNEL (green) and DAPI (blue) (Bar = 20  $\mu$ m). (D) Quantitative analyses of TUNEL-positive cells. Ejection fraction (E) and fractional shortening (F) were detected to reflect the cardiac function. Serum CK (G) and LDH (H) activities were reduced by si-CLU4A pretreatment. Data are shown as the mean  $\pm$  SD for three independent experiments. \*\*\*P < 0.001 vs. Control, ###P < 0.001 vs. I/R + si-NC.

mark the infarcted area in left ventricle myocardial sections 24 hours post-ischemia-reperfusion, and the results manifested that mice pretreated with si-CLU4A had significantly smaller infarct size (Figure 3A and 3B). Also, we exam-

ined myocardial apoptosis by TUNEL assays in mice after ischemia-reperfusion to certify whether CLU4A exert the pro-apoptotic effect in ischemia-reperfusion. We observed that TUNEL-positive cells were observably increased

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**Figure 4.** Inhibition of CLU4A attenuates H9c2 cell hypoxia/reoxygenation injury by the ERK signaling pathway. A. The protein levels of p-ERK, t-ERK, Bcl-2, Bax, and c-caspase-3 were measured by western blotting. B. Quantitative analysis of p-ERK. C. The ratio of Bcl-2/Bax. Effects of UA on the release of LDH in cultured NRCs. D. Quantitative analysis of c-caspase-3. E. Cell viability was determined by MTT assay determined in cultured H9c2 cells. F. LDH release in cultured H9c2 cells. Data are shown as the mean  $\pm$  SD for three independent experiments. \*\*\*P < 0.001 vs. Control, ###P < 0.001 vs. H/R + si-NC, &&&P < 0.001 vs. H/R + si-CLU4A.

in mice subjected to ischemia-reperfusion; whereas pretreatment with si-CLU4A significantly reduced the apoptosis index when compared to the si-NC group (Figure 3C and 3D). In addition, echocardiographic examinations were carried out to verify the effect of CLU4A on cardiac function. As shown in Figure 3E and 3F, LVFS and EF were dramatically decreased after ischemia-reperfusion, which were restored by si-CLU4A pretreatment. Furthermore, serum creatine kinase (CK) and lactate dehydrogenase (LDH) activities of mice suffered ischemia-reperfusion injury were examined. Serum CK and LDH activities were increased by ischemia-reperfusion treatment, while si-CLU4A pretreatment efficiently restrained the enhancement of CK and LDH activities in comparison with si-NC group (Figure 3G and 3H).

### *Inhibition of CLU4A attenuates H9c2 cell hypoxia/reoxygenation injury by activating the ERK signaling pathway*

In order to further study the underlying mechanism by which CLU4A regulates H9c2 cell

hypoxia/reoxygenation injury, we detected the effect of CLU4A on ERK signaling pathway. Results showed no significant change in the protein expression level of t-ERK, but the protein expression level of p-ERK was enhanced in H9c2 cell hypoxia/reoxygenation, and si-CLU4A transfection further increased expressions of p-ERK in H9c2 cells compared with that in si-NC groups. PD98059, an ERK signaling pathway inhibitor, partially abolished the efficacy of si-CLU4A (Figure 4A and 4B). Figure 4A, 4C and 4D depict that the si-CLU4A transfection restrained the cell apoptosis induced by hypoxia/reoxygenation, and co-treatment with PD98059 rescued the inhibitory effect of si-CLU4A on cell apoptosis. MTT assay illustrated that inhibiting ERK with PD98059 abolished the protective effect of si-CLU4A (Figure 4E). Moreover, LDH activity was notably increased in H9c2 cells suffered hypoxia/reoxygenation, and partly restrained by si-CLU4A, but partially rescued by PD98059 treatment (Figure 4F). Taken together, these results showed that inhibition of CLU4A attenuates H9c2 cell hypox-

ia/reoxygenation injury by the ERK signaling pathway.

### Discussion

In the present study, we found that CLU4A was upregulated during myocardial ischemia/reperfusion (I/R) injury. It was indicated that the cytotoxic effect of CLU4A in ischemia/reperfusion (I/R) injury was mainly through activation of apoptosis *in vitro* and *in vivo*. Moreover, it was found that inhibition of CLU4A restrains ischemia-reperfusion injury through regulating the ERK signaling pathway. Therefore, CLU4A might be a promising therapeutic target in the amelioration of ischemia/reperfusion (I/R) injury.

Cullins were identified as pivotal parts of CRLs complexes, a type of ubiquitin ligases with multiple subunits [18]. It has been confirmed that cullins can assemble with various specific target factors, and contribute to a distinct target of ubiquitination. Hence, each cullin is capable of targeting multiple substrates. A large body of research indicates that CLU4A functions as a proto-oncogene in several human cancers [19-21]. However, little was known about CLU4A in myocardial ischemia/reperfusion injury. Here, we showed that the mRNA and protein expression of CLU4A were increased in mice cardiomyocytes subjected to ischemia/reperfusion injury, and hypoxia/reoxygenation treatment in H9C2 cells increased the CLU4A expression. In addition, cell viability was recovered by si-CLU4A transfection after hypoxia/reoxygenation stimulation *in vitro*, and pretreatment with si-CLU4A not only significantly reduced the infarct size, but also restored the cardiac functions compared with si-NC pretreatment *in vivo*. Furthermore, serum CK and LDH activities were efficiently restrained by si-CLU4A pretreatment *in vitro* and *in vivo*, indicating the cytotoxic role of CLU4A in regulating ischemia/reperfusion injury.

Apoptosis is a process of programmed cell death that eliminates the redundant, abnormal, or damaged cells to maintain the function of normal tissues and organs [22]. Apoptosis also plays a vital role in myocardial ischemia/reperfusion injury. It was demonstrated that M2b macrophages ameliorate early myocardial ischemia/reperfusion injury in mice by inhibiting apoptosis [23]. Oligophrenin1 was proved to reduce myocardial ischemia/reperfusion injury by modulating inflammation and myocardial

apoptosis [24]. Vitexin protects against chronic myocardial ischemia/reperfusion injury in rats by inhibiting myocardial apoptosis and lipid peroxidation [25]. In this study, TUNEL assay verified that cell apoptosis induced by ischemia/reoxygenation stimulation was inhibited by si-CLU4A transfection both *in vitro* and *in vivo*, demonstrating that CLU4A promotes ischemia/reperfusion injury by enhancing apoptosis.

ERK signaling pathway was shown to play a regulatory role in myocardial ischemia/reperfusion injury. Trimetazidine has a protective effect on myocardial ischemia/reperfusion injury by activating the AMPK and ERK signaling pathway [26]. Inhibition of Selenoprotein P protects the heart from myocardial ischemia/reperfusion injury through upregulation of the ERK pathway [27]. Evidence also showed that the MEK/ERK pathway is required for protection from myocardial ischemia/reperfusion injury by 3',4'-dihydroxyflavonol [28] Q6. Here, we found that the phosphorylated ERK increased in cardiomyocytes treated with si-CLU4A, but it was blocked by PD98059. Hence, our data indicated that the knockdown of CLU4A protects cardiomyocytes against hypoxia/reoxygenation injury by inhibiting cell apoptosis by activation of ERK signaling pathway, at least in part.

### Conclusion

In summary, we have demonstrated that inhibition of CLU4A attenuated myocardial ischemia/reperfusion injury. Knockout of CLU4A decreased infarct size *in vivo*, and reduced cell apoptosis *in vivo* and *in vitro*. These protective effects of si-CLU4A on cardiomyocytes in myocardial ischemia/reperfusion are mediated by activation of the ERK pathway. These findings provide a new insight that CLU4A may be a potential target for the treatment of myocardial ischemia/reperfusion injury.

### Acknowledgements

This work was sponsored by the National Natural Science Foundation of China (Grant No.: 81470537).

### Disclosure of conflict of interest

None.

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