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REDD1 attenuates cardiac hypertrophy via enhancing autophagy

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ABSTRACT

Cardiac hypertrophy is a major risk factor of cardiovascular morbidity and mortality. Autophagy is established to be involved in regulating cardiac hypertrophy. REDD1, a stress-responsive protein, is proved to contribute in autophagy induction. However, the role of REDD1 in cardiac hypertrophy remains unknown. Our study demonstrated that REDD1 knockdown by RNAi exacerbated phenylephrine (PE)-induced cardiac hypertrophy, manifested by increased hypertrophic markers such as ANP and cell surface area. In addition, we discovered that ERK1/2 signaling pathway was involved in the effect of REDD1 on hypertrophy. Moreover, our study showed that REDD1 knockdown impaired autophagy in hypertrophied cardiomyocytes. mTOR, a signaling molecule governing autophagy induction, was activated by the knockdown of REDD1 under PE stress. Importantly, the pro-hypertrophic effect of REDD1 knockdown was significantly reversed by the autophagy enhancer rapamycin. Taken together, we firstly prove that REDD1 is essential for inhibiting cardiac hypertrophy by enhancing autophagy.

1. Introduction

Cardiac hypertrophy is a maladaptive response to cardiac overload or injury, and it is acknowledged to be a major risk factor for adverse clinical outcomes in patients with cardiovascular diseases [1]. Cardiac hypertrophy is characterized by increased cell surface area, protein synthesis and hypertrophic mRNA markers at cellular and molecular levels. Anti-hypertrophic treatment significantly improves clinical syndrome and increases survival rates. Considering that cardiac hypertrophy is modifiable, discovering target genes that modulate this process is crucial.

REDD1 (also known as RTP801, DDIT4 and Dig2) is a 25 kDa protein which is ubiquitously expressed in various human tissues. Its expression is sharply induced by a series of cellular stress such as hypoxia [2], endoplasmic reticulum stress [3], DNA damage [4] etc. REDD1 is identified to function as an inhibitor of mTOR and thereby exerts its biological influence tightly related to the inhibition of mTOR in different settings. In cardiovascular field, REDD1 expression in heart increased in acute alcohol intoxication [5]; decreased cardiac protein synthesis caused by chronic alcohol consumption was associated with inactivation of mTOR mediated by increased REDD1 expression [6]. Moreover, REDD1 is one of the proteins that modulate mTOR activity when the heart underwent ischemia/reperfusion (I/R) injury [7]. However, how REDD1 functions in the process of pathological cardiac hypertrophy remains unknown.

Autophagy is a catabolic pathway to degrade the mis-folded or dysfunctional intracellular components and thereby maintaining cellular homeostasis [8,9]. Autophagy is established to be an important mechanism regulating a variety of cardiovascular diseases [10]. As for pathological cardiac hypertrophy, appropriate autophagy induction might play a protective role [11,12]. Molitoris et al. demonstrated that REDD1 contributed to autophagy activation in lymphocytes treated with dexamethasone [13]. Moreover, it was reported that REDD1 was responsible for pharmacological autophagy induction by the inhibition of mTOR [14]. Considering the regulatory role of autophagy in cardiac hypertrophy and the reported pro-autophagic effect of REDD1, we tested our hypothesis

Abbreviations: PE, phenylephrine; REDD1, regulated in DNA damage and development 1; DDIT4, DNA-damage-inducible transcript 4; Dig2, dexamethasone-induced gene 2; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; ERK1/2, extracellular signal-regulated protein kinase; JNK1/2, c-Jun N-terminal protein kinase.

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that REDD1 might protect cardiomyocytes from hypertrophic stress via the induction of autophagy.

2. Materials and methods

2.1. Drug treatments and reagents

PE was purchased from Tokyo Chemical Industry. Rapamycin was purchased from Cell Signaling Technology and it was applied at a concentration of 100 nM 1 h before PE stress to induce autophagy. Chloroquine (CQ) obtained from Sigma–Aldrich (C6628) was applied to cardiomyocytes at 10 μM. The following primary antibodies obtained from Cell Signaling Technology were used: anti-phosphorylated-ERK1/2, anti-total-ERK1/2, anti-phosphorylated-JNK1/2, anti-total-JNK1/2, anti-phosphorylated-p38, anti-total-p38, anti-phosphorylated-mTOR (Ser2448), anti-total-mTOR, anti-phosphorylated-p70S6K, anti-total-p70S6K, anti-phosphorylated-Akt (Ser473), anti-total-Akt and anti-GAPDH antibodies. Anti-REDD1 antibody was obtained from Proteintech Group. Anti-LC3B monoclonal antibody was obtained from Novus Biologicals. Anti-p62 monoclonal antibody was purchased from Sigma–Aldrich. Anti-Cathepsin D was purchased from Santa Cruz.

2.2. Cardiomyocyte cultures and transfections

Primary neonatal ventricular cardiomyocytes were prepared as previously described [15]. The knockdown of REDD1 was performed using LipofectamineTM RNAiMAX according to the manufacturer's instructions. The sequence of siRNA targeting REDD1 was the following: 5′ GUGCCCACCUUUCAGUUGA dTdT 3′. Thirty-six hours after seeding, the cardiomyocytes were transfected with REDD1-specific siRNA (50 nmol/L) and scramble siRNA (50 nmol/L) in serum-free medium for 12 h. After another 12 h of serum deprivation, the cardiomyocytes were exposed to PE at 100 μM to induce cardiac hypertrophy for indicated time.

2.3. RNA isolation and quantitative real-time PCR (q-PCR)

RNA isolation and q-PCR were performed as previously described [15]. The primers used were as follows: ANP, 5′-GAGCCAGCAGATTGAGAACAT-3′ (forward) and 5′-TACTCTCCGCTCTTCCAGTCA-3′ (reverse); and GAPDH, 5′-ACAGCAACAGGGTGGTGGAC-3′ (forward) and 5′-TTTGAGGGTGCAGCGAACTT-3′ (reverse).

2.4. Western blotting analysis

The Western blotting was performed according to a previously reported method [15]. The quantitative analysis was performed using the Quantity One software.

2.5. Immunofluorescence staining

Immunofluorescence staining was performed as previously described [16]. The primary antibody was rabbit polyclonal anti-troponin I (1:50, Santa Cruz), and the immune complexes were detected by Cy3-conjugated secondary antibodies (1:100, Proteintech Group). The nuclei were stained with DAPI (0.5 mg/mL, Sigma). The images were obtained at 400× using a CKX41 Olympus microscope.

2.6. Measurement of the cell surface area

Twenty-four hours after PE incubation, the cardiomyocytes were fixed with 4% paraformaldehyde. The relative cell surface area was analyzed using the Image-Pro Plus software. 50–100 cardiomyocytes in each group were randomly selected and examined in each experiment.

2.7. Statistical analysis

All of the data are expressed as the mean ± s.e.m. from at least three independent experiments. The differences between the means were evaluated using one-way or two-way ANOVA. The statistical significance was established at $p < 0.05$. All of the statistical analyses were performed using the SPSS13.0 software.

3. Results

3.1. Changes of REDD1 expression in cardiac hypertrophy induced by PE

To preliminarily study whether REDD1 plays a role in the process of PE-induced cardiac hypertrophy, we detected the protein expression after 6, 12 and 24-h incubation of PE. As shown in Fig. 1, REDD1 protein expression declined 12 h after PE stress compared to vehicle, and further decreased after another 12 h. The significant alteration of REDD1 indicated that down-regulation of REDD1 might be involved in the development of cardiac hypertrophy.

3.2. Knockdown of REDD1 exacerbated the cardiomyocyte hypertrophy induced by PE

To determine whether REDD1 is crucial in the protection against hypertrophic stress, we examined the effect of REDD1 knockdown on hypertrophic markers including relative mRNA expression of ANP and cell surface area in the presence of PE. We achieved the knockdown of REDD1 in cardiomyocytes through siRNA transfection and the silencing effect was approximately 50% (Fig. 2D). As shown in Fig. 2A, knockdown of REDD1 did not affect the basal expression of ANP. Compared to control group transfected with scramble siRNA (si-Scramble), cardiomyocytes with REDD1 knockdown (si-REDD1) exhibited an exacerbated hypertrophic phenotype, featured as increased expression of ANP (Fig. 2A)

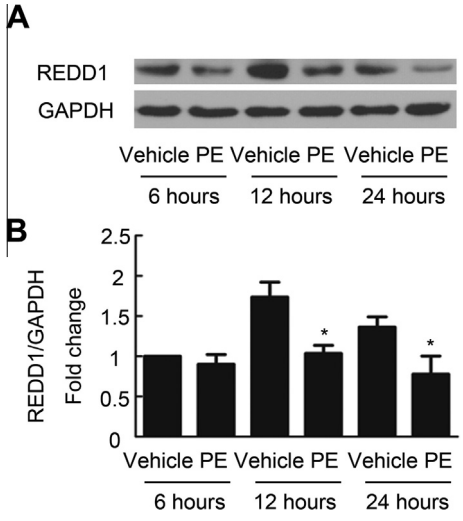

Fig. 1. Effect of PE on the REDD1 expression in cardiomyocytes. (A) Representative immunoblots of the REDD1 and GAPDH levels after vehicle or PE treatments for the indicated time. (B) Quantitative analysis of data shown in (A). GADPH was used as an internal control. $^{*}p < 0.05$ versus the corresponding vehicle groups $n = 4$ (n represents independent experiments).

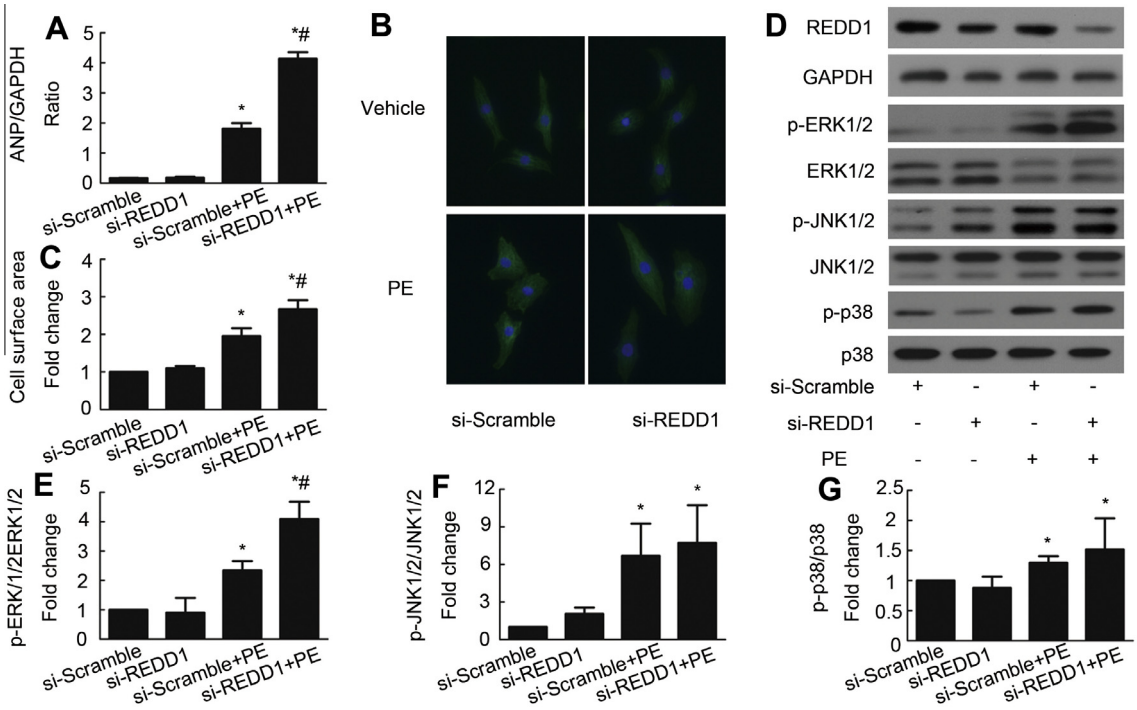

Fig. 2. Effect of REDD1 knockdown on PE-induced cardiomyocytes hypertrophy. After siRNA transfection and serum deprivation, cardiomyocytes were treated with or without PE for 24 h. The relative level of ANP (A) mRNA expression was determined by q-PCR and GAPDH was used as an internal control. (B) Cardiomyocytes were stained with Troponin I and the nuclei were stained with DAPI. (C) The effect of REDD1 knockdown on the cell surface area after 24 h of PE incubation was quantified. $^{*}p < 0.05$ versus the corresponding control group without PE, $^{\#}p < 0.05$ versus si-Scramble PE group. (D) The knockdown effect of siRNA on REDD1 was compared with scramble siRNA. GADPH was used as an internal control. The levels of MAPK signaling were detected by Western blotting analysis 30 min after PE incubation. Representative immunoblots of phospho- and total ERK1/2, JNK1/2 and p38-MAPK. (E–G) Quantitative analysis of phosphorylated and total ERK1/2, JNK1/2 and p38. $^{*}p < 0.05$ versus the corresponding control group without PE, $^{\#}p < 0.05$ versus si-Scramble PE group $n = 3–5$.

and cell surface area (Fig. 2B and C). Therefore, our data indicated that REDD1 is essential to resist hypertrophic stress.

The MAP kinases (MAPK) are the fundamental signaling pathways contributing to cardiac hypertrophy. To further explore how REDD1 affect cardiac hypertrophy, we examined the effect of REDD1 knockdown on the activation of MAPK (including ERK1/2, JNK1/2 and p38MAPK) (Fig. 2D–G). Thirty minutes after PE incubation, MAPK was significantly phosphorylated. And our data revealed that REDD1 knockdown further increased the level of phosphorylated ERK1/2 under PE stress (Fig. 2D and E), which is the most crucial signaling of MAPK mediating cardiac hypertrophy. However, the activation of JNK1/2 (Fig. 2D and F) and p38 (Fig. 2D and G) remained unchanged in cardiomyocytes with REDD1 knockdown. Taken together, ERK1/2 could be a pivotal signaling pathway in the REDD1-mediated inhibition of hypertrophy.

3.3. Knockdown of REDD1 impaired autophagy in hypertrophied cardiomyocytes

Basal autophagy is essential to maintaining cellular homeostasis. We discovered that autophagy was down-regulated in cardiomyocytes treated with PE, as evidenced by decreased autophagic markers including LC3BII (Fig. 3A and C) and degradation of p62 (Fig. 3B and D). Moreover, we examined the autophagy by transfecting an adenovirus harboring tandem fluorescent mRFP-GFP-LC3 [17]. The numbers of LC3 dots with fluorescence reflected the level of autophagy. GFP loses its fluorescence in the acidic environment of the lysosomes when autophagosomes fuse with the lysosomes while mRFP doesn't. Thus, the green dots indicate autophagosomes, while the red dots indicate both autophagosomes and autolysosomes. In the overlaid images, the yellow dots indicate autophagosomes, and the free red dots indicate autolysosomes (Fig. 3F–H). We observed significant decline in the numbers of yellow dots and free red dots per cell after PE treatment, which was in consistent with the alteration of autophagic biomarkers indicating that PE impaired autophagy. We next examined the effect of REDD1 knockdown on autophagy. Importantly, without affecting basal level of autophagy, REDD1 knockdown directly exacerbated the impairment of autophagy in the presence of PE, manifested by more decreased levels of LC3BII (Fig. 3A and C), degradation of p62 (Fig. 3B and D) and numbers of autophagosomes and autolysosomes (Fig. 3F–H). However, LC3BII alone might not be the ideal marker evaluating the level of autophagy [18]. Both the generation of autophagic cargos and lysosomal degradation might contribute to alteration of LC3BII. And therefore we applied CQ, an inhibitor of the fusion between autophagosomes and lysosomes, to further access the initial step of autophagy without disturbance of lysosomal degradation. As shown in Fig. 3A and C, CQ enhanced the levels of LC3BII in all groups. In addition, in the presence of CQ, LC3BII decreased due to PE treatment and REDD1 knockdown further reduced it. Moreover, we discovered that the lysosome activity marker, cathepsin D, remained unchanged after the knockdown of REDD1 (Fig. 3B and E), indicating that the contribution of lysosomal degradation to LC3BII could be excluded.

We further explored the signaling mechanism involved in autophagy regulated by REDD1. Thus, phosphorylation of mTOR (Ser2448), which governs the induction of autophagy, was examined. PE significantly induced the phosphorylation of mTOR, and REDD1 knockdown further enhanced its phosphorylation (Fig. 3I and J). mTOR consists of two kinase complexes: mTORC1 and mTORC2, both of which were reported to play a certain role in the regulation of autophagy [19,20]. And therefore, we examined the effect of REDD1 knockdown on mTORC1 and mTORC2 activation. We discovered that in the presence of PE, REDD1 knockdown

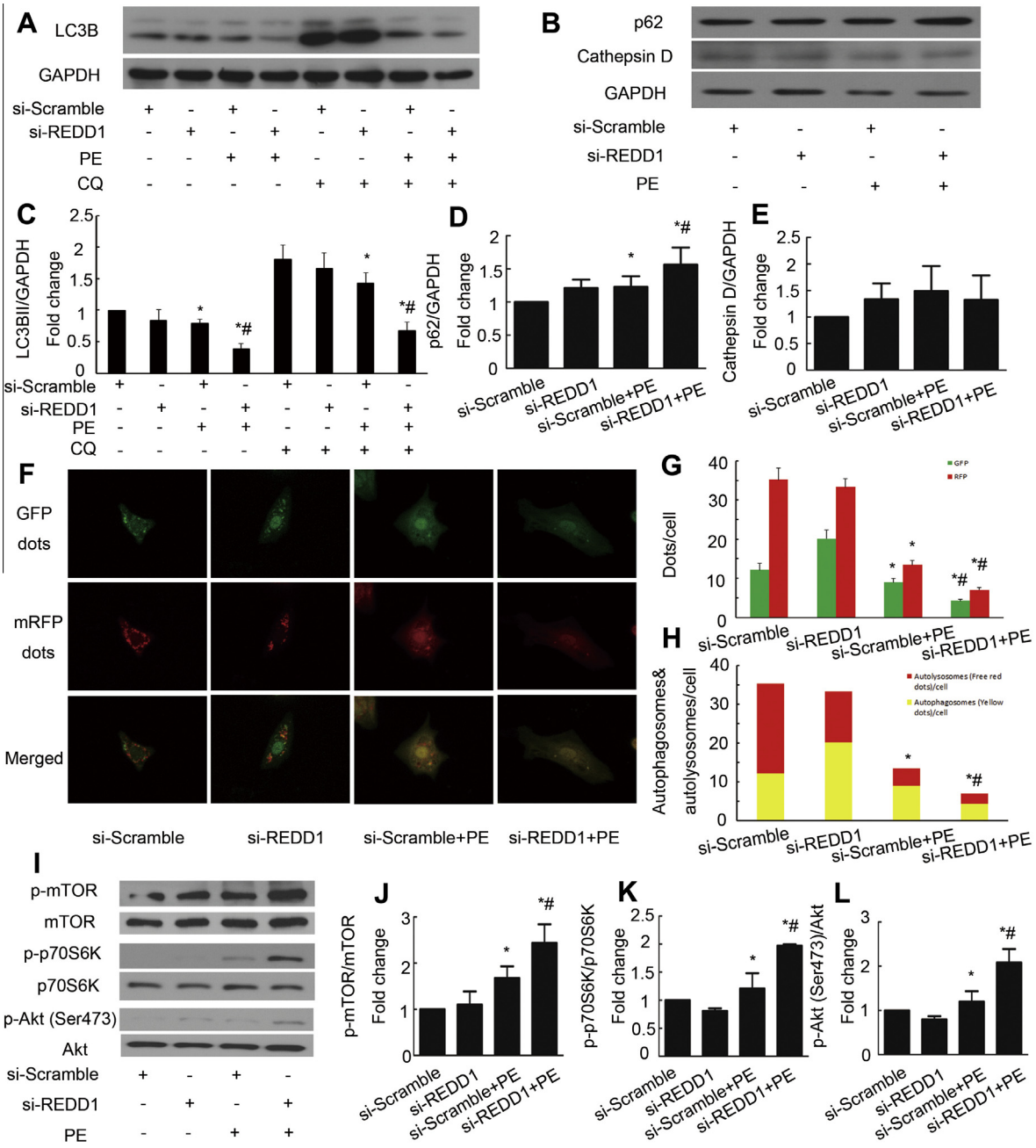

Fig. 3. Effect of REDD1 knockdown on autophagy. Cardiomyocytes were treated with or without PE for 12 h. (A) The representative immunoblots and graphs (C) show the changes in the levels of LC3BII in the absence and the presence of CQ. (B) The representative immunoblots and graphs show the changes in the levels of p62 (D) and cathepsin D (E). GAPDH was used as an internal control. $^{*}p < 0.05$ versus the corresponding control group without PE, $^{\#}p < 0.05$ versus si-Scramble PE group. (F) Cardiomyocytes were transfected with adenovirus harboring mRFP-GFP-LC3 at an MOI of 100 for 6 h. After another 24 h of serum-deprivation, the cardiomyocytes were treated with or without PE for 12 h. Representative images of fluorescent LC3 dots are shown. (G) Mean number of GFP and mRFP dots per cell. $^{*}p < 0.05$ versus the numbers of GFP or RFP dots in corresponding control group without PE. $^{\#}p < 0.05$ versus the numbers of GFP or RFP dots in si-Scramble PE group. (H) Mean numbers of autophagosomes (dots with both red and green fluorescence; yellow fluorescence in the merged images) and autolysosomes (dots with only red fluorescence; free red fluorescence in the merged images). $^{*}p < 0.05$ versus the numbers of autophagosomes and autolysosomes in corresponding control group without PE, respectively. $^{\#}p < 0.05$ versus the numbers of autophagosomes and autolysosomes in si-Scramble PE group, respectively. (I) Representative immunoblots of the expressions of phospho- and total mTOR, p70S6K and Akt (Ser473). (J–L) Quantitative analysis of the levels of phospho- and total mTOR, p70S6K and Akt (Ser473). $^{*}p < 0.05$ versus the corresponding control group without PE, $^{\#}p < 0.05$ versus the si-Scramble PE group $n = 3–5$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

significantly promoted the activation of mTORC1, an effect that was manifested by increased phosphorylation of p70S6K (Fig. 3I and K). Moreover, phosphorylation of Akt (Ser473), downstream of mTORC2, was also enhanced due to REDD1 knockdown in the presence of PE (Fig. 3I and L). Therefore, these data suggested that REDD1 negatively modulates autophagy in the presence of PE, accompanied by the inhibition of mTORC1 and mTORC2.

3.4. Exacerbation of cardiac hypertrophy due to REDD1 knockdown can be reversed by rapamycin

We next verified whether autophagy participates in the pro-hypertrophic effect of REDD1 knockdown. Cardiomyocytes transfected with si-Scramble and si-REDD1 were treated with rapamycin, an autophagy enhancer. As shown in Fig. 4, rapamycin could

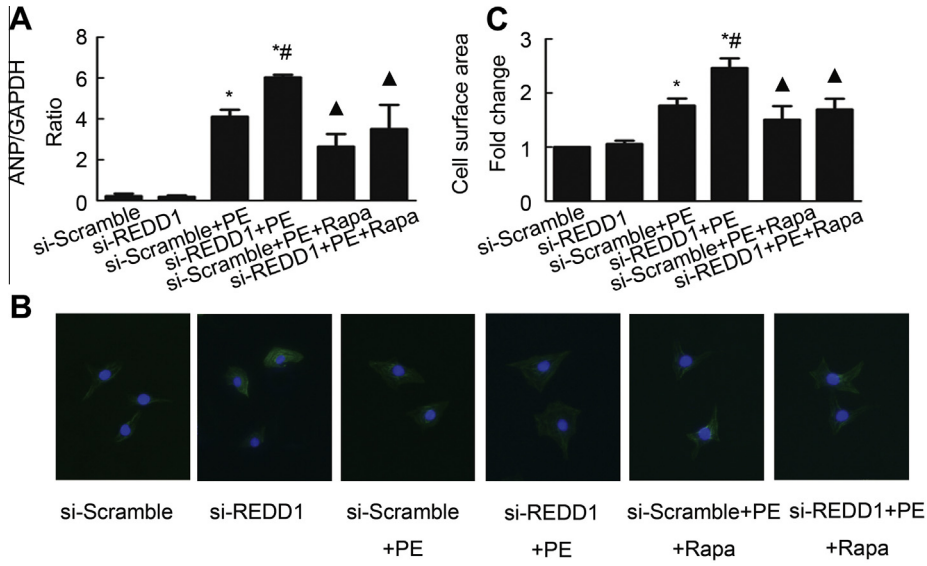

Fig. 4. Rapamycin ameliorated the pro-hypertrophic effect resulted from REDD1 knockdown. The cardiomyocytes treated with PE were co-incubated with or without rapamycin for 24 h. (A) The mRNA expression levels of ANP in cardiomyocytes with different treatments are shown. (B) Images of cells stained with Troponin I. (C) Quantification of cardiomyocyte surface area. $^{*}p < 0.05$ versus the corresponding control group without PE, $^{\#}p < 0.05$ versus si-Scramble PE group without rapamycin, $^{\blacktriangle}p < 0.05$ versus the corresponding si-Scramble or si-REDD1 PE group without rapamycin $n = 3$ or 5.

attenuate cardiac hypertrophy induced by PE, featured as decreased levels of ANP (Fig. 4A) and cell surface area (Fig. 4B and C). In the absence of rapamycin, REDD1 knockdown resulted in a more hypertrophied phenotype when treated with PE. Treatment with rapamycin significantly ameliorated the hypertrophic response resulted from REDD1 knockdown in PE- stressed cardiomyocytes. Nevertheless, rapamycin could not completely block the pro-hypertrophic effect due to REDD1 knockdown, although the disparity between si-Scramble group and si-REDD1 group with rapamycin did not reach statistically significance. These results demonstrated that autophagy could be one of the most important mechanisms mediating cardiac hypertrophy regulated by REDD1.

4. Discussion

In our present study, we discovered that knockdown of REDD1 deteriorated PE-induced cardiac hypertrophy accompanied by ERK1/2 activation. Moreover, REDD1 knockdown resulted in dysfunctional autophagy, with the participation of mTOR signaling pathway. Importantly, the pro-hypertrophic effect of REDD1 knockdown could be reversed by rapamycin, indicating that autophagy plays a pivotal role in the negative regulatory effect of REDD1 on cardiac hypertrophy.

REDD1 has emerged as a stress responsive protein, the expression of which sharply increases during many stresses. It functions as an inhibitor of mTOR which regulates cell growth and metabolism. In cardiovascular field, studies about the role of REDD1 limit in how the expression of REDD1 reacts in different settings including acute and chronic alcohol consumption [5,6], diet-induced obesity [21] and ischemia/reperfusion injury [7]. However, the role of REDD1 in pathological cardiac hypertrophy and the related mechanisms remain unknown. In the present study, we discovered significant decline of the protein expression of REDD1 in cardiomyocytes after 6 h, 12 h and 24 h of PE incubation. Combined with previous studies illustrating that REDD1 expression is induced by cellular stress, our findings indicated that REDD1 might participate in resisting hypertrophic stress and decay when the stress is persistent. We further explored how REDD1 functioned in cardiac hypertrophy by RNAi-mediated knockdown of REDD1. In the hypertrophied cardiomyocytes, REDD1 knockdown resulted in further increased hypertrophic markers (including mRNA expression of ANP and cell surface area) compared with control. Based on the phenotype we discovered, we showed that REDD1 exhibits the protective function in the process of cardiac hypertrophy. It is essential to resist cardiac hypertrophic stress and therefore it is an anti-hypertrophic protein.

MAPK is a classic and crucial signaling pathway mediating cardiac hypertrophy [22]. To further examine the effect of REDD1 on cardiac hypertrophy, we detected the three components of MAPK including ERK1/2, JNK1/2 and p38-MAPK. We found that PE induced the phosphorylation of ERK1/2, JNK1/2 and p38. Moreover, further enhanced ERK1/2 phosphorylation was found in cardiomyocytes with REDD1 knockdown exposed to PE. Since ERK1/2 cascade is essential for the initial of pathological cardiac hypertrophy induced by PE [23], our data solidly verified that REDD1 functioned as an anti-hypertrophic effector through inhibiting ERK1/2. There is no study about the relation between REDD1 and ERK1/2, JNK1/2, whereas a study reported that physical exercise decreased the REDD1 protein expression and increased levels of p38 phosphorylation [21]. Herein, we found that REDD1 didn't exert its anti-hypertrophic effect via JNK1/2 and p38. It might be contributed to the controversial roles of p38 and JNK1/2 in cardiac hypertrophy [24]. Our data indicated that ERK1/2 signal is involved in the pro-hypertrophic effect of REDD1 knockdown.

Autophagy is a common biological phenomenon that is responsible for eliminating dysfunctional substrates and thereby maintaining cellular homeostasis [25]. Dysfunctional autophagy is adverse to normal biological function and results in serious diseases such as congenital myopathies [26] and Parkinson disease [27]. Cardiac-specific Atg5-deficient mice rapidly developed into cardiac dysfunction 1 week after transverse aortic constriction [28]. And appropriate activation of autophagy is favorable for the protection against cardiac hypertrophic stress such as pressure overload and neurohumoral stimuli [11,12]. In our present study, we discovered that PE significantly decreased the autophagic indicators such as LC3BII in the absence and presence of CQ, degradation of p62 and mRFP-GFP-dots, which suggested that impaired autophagy was related to the pathological mechanism of cardiac hypertrophy. Recent years, REDD1 has emerged to be a mediator

of autophagy, whereas researches of REDD1 with focus of autophagy in cardiovascular diseases especially in cardiac hypertrophy are lacking. We demonstrated that REDD1 knockdown deteriorated the impairment of autophagy due to PE stress without affect basal autophagy level. In consistence with the previous studies, our study suggests that REDD1 is essential for autophagy restoration and it acts as a positive regulator of autophagy during the process of hypertrophy.

mTOR is a highly-conserved modulator that governs a variety of biological function such as autophagy, cell growth, metabolism, etc. [29]. It is well-established that mTOR is an inhibitor of autophagy induction [30,31]. In our study, PE significantly induced the phosphorylation of mTOR, an effect that directly led to down-regulated autophagy. Furthermore, our data confirmed that REDD1 is an upstream inhibitor of mTOR in the process of cardiac hypertrophy. REDD1 knockdown further activated mTOR phosphorylation in the presence of PE, leading to a blockade of autophagy initiation. mTOR consists of two kinase complexes: mTORC1 and mTORC2. We discovered that the phosphorylation of p70S6K and Akt (Ser473), the downstream effectors of mTORC1 and mTORC2, respectively, were both significantly enhanced by REDD1 knockdown. mTORC1 is verified to be an inhibitor of autophagy. It mainly regulates the initiation of autophagy, and the inhibition of mTORC1 directly starts up autophagy [32]. Considering the proven anti-autophagic role of mTORC1, its activation by REDD1 knockdown contributed to the impaired autophagy and acceleration of hypertrophy induced by PE. To confirm whether REDD1 promoted autophagy through mTORC1 and thereby attenuating cardiac hypertrophy, we applied rapamycin, an enhancer of autophagy and an mTORC1 inhibitor. Rapamycin significantly blunted the pro-hypertrophic effect due to REDD1 knockdown. Namely, when autophagy was recovered by mTORC1 inhibition, the detrimental effect on cardiac hypertrophy due to REDD1 knockdown was weakening. However, we observed the fact that rapamycin could not completely reverse pro-hypertrophic effect induced by REDD1 knockdown, even though it didn't show statistical significance. It might suggest that the mTORC1-autophagy pathway was a pivotal mechanism involved in the regulation of cardiac hypertrophy mediated by REDD1, but other than that, there might be other potential mechanism to be explored. On the other hand, the effect of mTORC2 on autophagy is controversial [20,33], although it was reported that mTORC2 suppressed autophagy in skeletal muscle *in vivo* [33]. Considering the major role of REDD1-mTORC1-autophagy pathway in the regulation of cardiac hypertrophy, the involvement of mTORC2 pathway remains a question mark.

In summary, our study revealed that REDD1 attenuates cardiac hypertrophy via the restoration of autophagy. Our demonstrations suggested that REDD1 might be a potential target gene for future therapeutic strategy of cardiac hypertrophy.

Acknowledgments

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