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Characterization of endonuclease G and mitochondria-sarcoplasmic reticulum-related proteins during cardiac hypertrophy

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Endonuclease G (Endo G) is a novel determinant of cardiac hypertrophy. Here, we report the characterization of Endo G and mitochondria-sarcoplasmic reticulum-related proteins during cardiac hypertrophy, and hypothesize that Endo G regulate mitochondrial function partly through Mfn2 and Jp2 during cardiac hypertrophy. Our results show that Endo G levels gradually increased at the beginning of phenylephrine-induced cardiac hypertrophy, accompanied by an abnormal mitochondrial membrane potential. The up-regulation of Mfn2, Jp2, and Endo G appeared at an early stage of cardiac hypertrophy, whereas PGC1α was not up-regulated until a later stage. Abolishing Endo G with siRNA led to the uncoupling of the mitochondrial electron transport chain from ATP production and decreased PGC1α expression, likely by affecting the juxtaposition of the mitochondria and the sarcoplasmic reticulum *via* Mfn2 and Jp2. Furthermore, abolishing Jp2 altered the expression of Endo G expression and induced mitochondrial dysfunction, suggesting that mitochondrial abnormalities in cardiac hypertrophy are most likely caused by Endo G. Taken together, our study established a link between Endo G and mitochondrial function during cardiac hypertrophy, partly through the effects of Endo G on Mfn2 and Jp2, and revealed a role for Endo G in the crosstalk between the processes controlled by Mfn2 and Jp2 in maladaptive cardiac hypertrophy.

1. Introduction

Cardiac hypertrophy is an adaptive mechanism through which cardiomyocytes respond to various mechanical and neurohormonal stimuli. Although cardiac hypertrophy may initially represent an adaptive response of the myocardium, prolonged hypertrophy ultimately leads to dilated cardiomyopathy, heart failure and even sudden death. Emerging clinical evidence has established the importance and benefit of the regression of hypertrophy in reducing cardiovascular morbidity and mortality (Okin et al. 2013). It is important to explore the key molecular and signaling mechanisms of hypertrophy because the processes underlying the morphology and function of the mitochondria and sarcoplasmic reticulum (SR) during cardiac hypertrophy remain incompletely defined.

It was recently demonstrated that endonuclease G (Endo G), a nuclear endonuclease that belongs to the large family of DNA/RNA-nonspecific nucleases, is a novel determinant of cardiac hypertrophy and mitochondrial function (McDermott-Roe et al. 2011). The inhibition of Endo G in cultured cardiomyocytes results in increases in cell size and hypertrophic biomarkers in the absence of pro-hypertrophic stimulation. In contrast, the maintenance of Endo G levels may play an important role in maintaining mitochondrial energy production and normal cardiac physiology, consistent with the heart's high demand for energy (McDermott-Roe et al. 2011). Unfortunately, little is known about the role of Endo G in mitochondrial function and energy metabolism during cardiac hypertrophy, especially its association with other cardiac hypertrophy-related targets.

Several proteins have been implicated in cardiac hypertrophy. For example, increasing evidence demonstrates that junctophilin 2 (Jp2), which is essential for cellular Ca^{2+} homeostasis and physiological communication in cardiac excitation-contraction (EC) coupling, has been associated with hypertrophic and dilated cardiomyopathies (Beavers et al. 2014; Landstrom et al. 2011, 2007; Zhang et al. 2014). Mitofusin 2 (Mfn2), a mediator of mitochondrial fusion, has been reported to control mitochondrial morphogenesis and the juxtaposition of mitochondria with SR, which provides a specialized pathway for Ca^{2+} shuttling between these organelles (de Brito and Scorrano 2008, 2009). It has been reported that Mfn2 is also associated with cardiac hypertrophy, based on the findings that Mfn2 expression is down-regulated in various models of cardiac hypertrophy (Fang et al. 2007; Yu et al. 2011) and that Mfn2-deficient mice display modest cardiac hypertrophy accompanied by slight functional deterioration (Papanicolaou et al. 2011).

In our previous research, we found that the loss of Jp2 markedly disrupted the juxtaposition of mitochondria and SR, resulting in reduced Mfn2 transcription and ultrastructural abnormalities in mitochondria and SR, and that deficiency of functional crosstalk between mitochondrial mitofusin and SR was partly due to abnormal Ca^{2+} transients caused by siRNA against *Jp2*

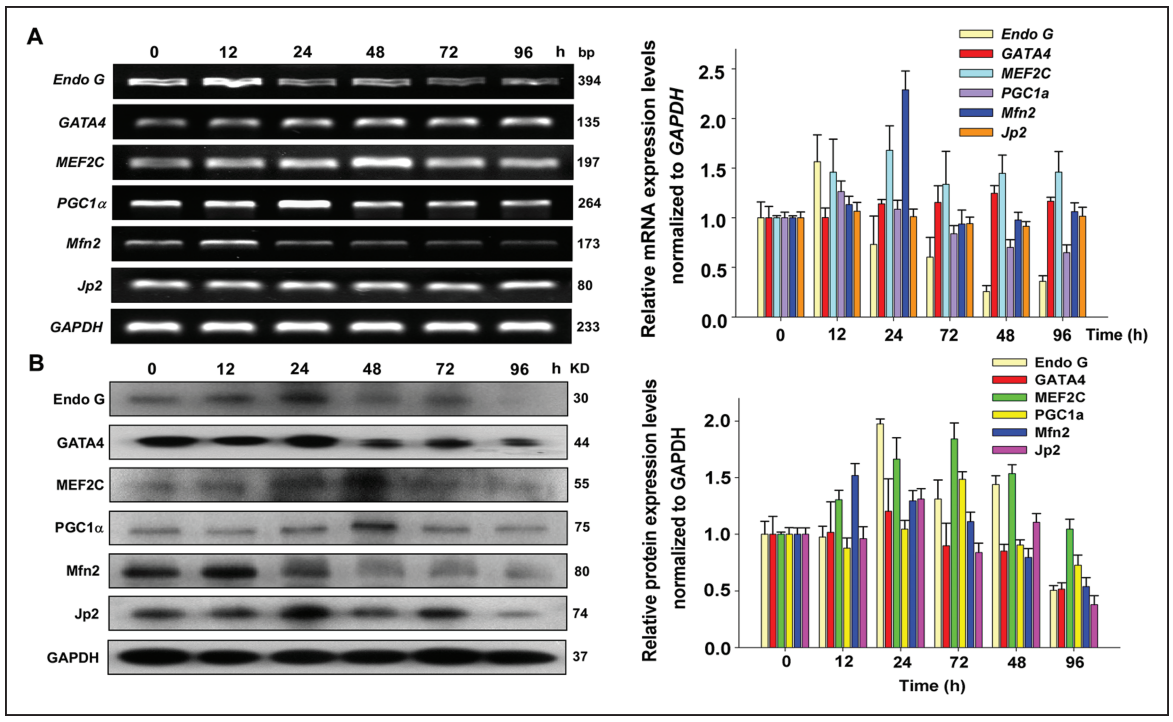

Fig. 1: Time course of mitochondria and SR related gene and protein levels in H9c2 cells following PE stimulation. (A) Expression of mitochondria and SR related genes were analyzed by RT-PCR after the PE treatment of H9c2 cells from 0 h to 96 h. (B) Expression of mitochondria and SR related proteins were analyzed by Western blotting. Quantitative analysis of the bands was performed by densitometric analysis. Data are presented as the means ± S.E.M. of three independent experiments performed in triplicate. Statistical significance was set as $^{**}P < 0.01$, $^{***}P < 0.001$ *vs*. control.

(Liang et al. 2012). Although roles for Endo G in cardiac hypertrophy have been proposed, little is known about its role in mitochondrial function and energy metabolism during cardiac hypertrophy, especially its crosstalk with Jp2 and Mfn2, which are vitally important and closely associated with cardiac hypertrophy.

To explore the basement of molecular mechanisms linking Endo G with cardiac hypertrophy, in the present study, we attempted to characterize the crucial proteins related to cardiac hypertrophy and mitochondria that link the mitochondrial energy metabolism, SR function, and cardiac hypertrophy. We also attempted to establish links between Endo G, Mfn2, Jp2 and to uncover a role for Endo G in the crosstalk between the processes controlled by Mfn2 and Jp2 in maladaptive cardiac hypertrophy.

2. Investigations and results

2.1. Characterization of mitochondria- and SR-related genes and proteins in phenylephrine (PE)-induced cardiomyocyte hypertrophy

To explore the temporal changes in mitochondria- and SR-related genes and proteins, the time course of their expression in H9c2 cardiomyocytes was analyzed by RT-PCR and Western blotting (Fig. 1). Our results show that the expression of the cardiac hypertrophic markers GATA4 and MEF2C initially (0-24 h) increased in a time-dependent manner in H9c2 cells treated with PE, indicating the establishment of hypertrophy. The *Endo G* levels gradually increased to a peak at 48 h and then returned to the basal level within 96 h. Interestingly, the upregulation of Mfn2 (12 h), Endo G (24 h) and Jp2 (24 h) appeared as early as the beginning of cardiac hypertrophy, suggesting that the maintenance of Endo G levels may play an important role in maintaining mitochondrial energy production and normal cardiac physiology, consistent with the heart's high energy demands. These proteins were down-regulated until the later stage (48-96 h), which indicated that the early stage of hypertrophy was a physiological response to the stimulation. However, in the later stage, the mitochondrial functions of energy supply and Ca^{2+} homeostasis could not support the dilated cardiomyocytes. This extended hypertrophy could lead to a pathological response and end with heart failure. The expression of peroxisome proliferator-activated receptor coactivator-1α (PGC1α) did not increase as rapidly as that of the other proteins; it was not up-regulated until the later stage of hypertrophy (48 h). This finding may suggest that mitochondrial dysfunction is followed by the abnormal expression of mitochondria- and SR-related genes and proteins.

2.2. Abolishing Endo G resulted in alteration of mitochondria- and SR-related genes and proteins

To determine whether Endo G is associated with energy metabolism during cardiac hypertrophy, we knocked down Endo G with siRNA in H9c2 cells. The result showed that si*EndoG* sharply decreased expression of Endo G (Fig. 2), both in gene and protein level. Interestingly, si*EndoG* exhibited different effects on mitochondria- and SR-related proteins at different time points. The mitochondrial fusion protein Mfn2, the junctional complex protein Jp2, and the markers of cardiac hypertrophy GATA4 and MEF2C were up-regulated in si*EndoG* H9c2 cells 24 h after transfection (Fig. 2B-1), whereas these proteins were down-regulated 48 h after transfection (Fig. 2B-2); these effects were similar to the results by PE-induced hypertrophy on early and later stage.

2.3. Changes of morphology and mitochondrial $\Delta\Psi m$ in Endo G knockdown and PE-treated H9c2 cardiomyocytes

To explore whether mitochondrial energy production was affected by the disruption of Endo G, H9c2 cells were treated with si*EndoG* and then stained with JC-1. Decreased mitochondrial $\Delta\Psi$m was specifically indicated by a decrease in the red:

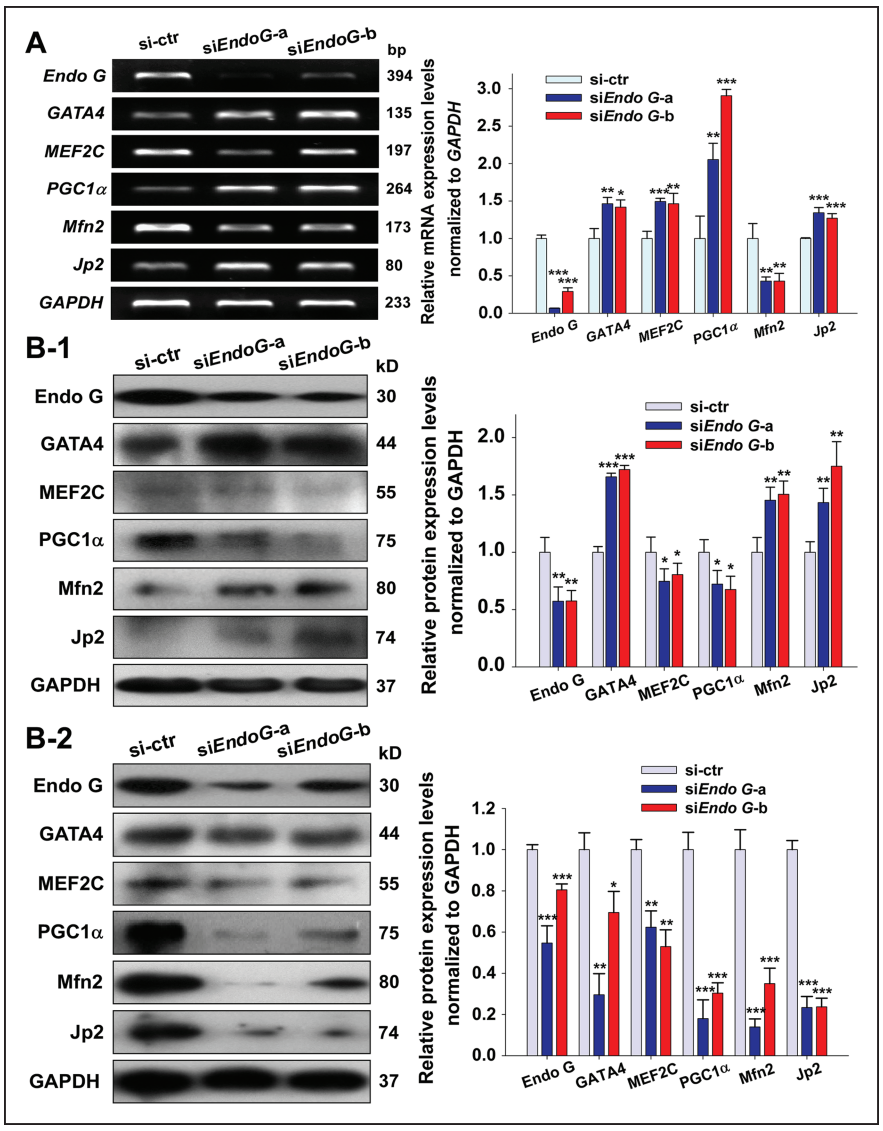

Fig. 2: Abolishing Endo G in H9c2 cells altered the expression level of mitochondria- and SR-related proteins. (A) Expression level of mitochondria- and SR-related genes 48 h after transient transfection with siEndo G was analyzed by RT-PCR. (B) Expression of mitochondria- and SR-related proteins in siEndo G H9c2 cells was analyzed by Western blotting. Data are presented as the means ± S.E.M. of three independent experiments performed in triplicate. Statistical significance was set as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. si-ctr.

green fluorescence intensity ratio (reduced by approximately 50% in si*EndoG* H9c2 cells) (Fig. 3), which was consistent with the $\Delta\Psi$m result of PE treated H9c2 cells (a shift toward low $\Delta\Psi$m after 24 h or 48 h of PE treatment (green) compared with the control group (orange) (Fig. 4C); $\Delta\Psi$m was reduced by more than 25% at 24 h and by more than 60% at 48 h (Fig. 4B)). This result indicated that the si*EndoG* treated H9c2 cells were de-energized, which could be caused by the uncoupling of the electron transport chain from ATP production (Behbahani et al. 2006). Microscopy showed a marked increase in the surface areas of PE stimulated cells: PE enlarged the surface areas of cardiomyocytes 3-fold compared with the control group (Fig. 4A), which was not obviously in si*EndoG* treated H9c2 cells.

2.4. Knockdown of Jp2 altered Endo G expression

To explore whether the knockdown of Jp2 resulted in the alteration of Endo G expression and mitochondrial electron transport chain, we evaluated expression of Endo G and genes related to mitochondria-SR interactions. The result showed that si*Jp2* decreased the level of Endo G (Fig. 5C), indicating the association of Jp2 and Endo G. si*Jp2* also reduced the expression of Mfn2 and PGC1α. Notably, expression of the Ca^{2+}-related hypertrophic marker gene MEF2C was reduced by 80% in si*Jp2* H9c2 cells after 48 h, while, GATA4, another known marker of cardiac hypertrophy, was increased. Furthermore, si*Jp2* decreased $\Delta\Psi$m after 2 h (green) by more than 60% compared to si-ctr (orange) (Fig. 5A, B). This finding indicated that the mitochondria of si*Jp2* H9c2 cells were de-energized. These results supported our previous work in embryonic stem cells, which showed that Jp2 knockdown impairs energy production.

3. Discussion

Endo G is a novel determinant of cardiac hypertrophy. Here, we characterized Endo G and mitochondria- and SR-related proteins during cardiac hypertrophy. We found that abolishing Endo G led to the uncoupling of the mitochondrial electron transport chain from ATP production, as indicated by the altered $\Delta\Psi$m, which suggested that the cardiomyocytes were de-energized. Importantly, PGC1α, which is a master regulator of myocardial biogenesis and energy metabolism and has been implicated in the evolution of pathologic cardiac hypertrophy and heart failure (Pereira et al. 2014), was also down-regulated by si*Endo G*. Pathological hypertrophy leading to maladapted cardiac energy metabolism most likely involves the down-regulation of biogen-

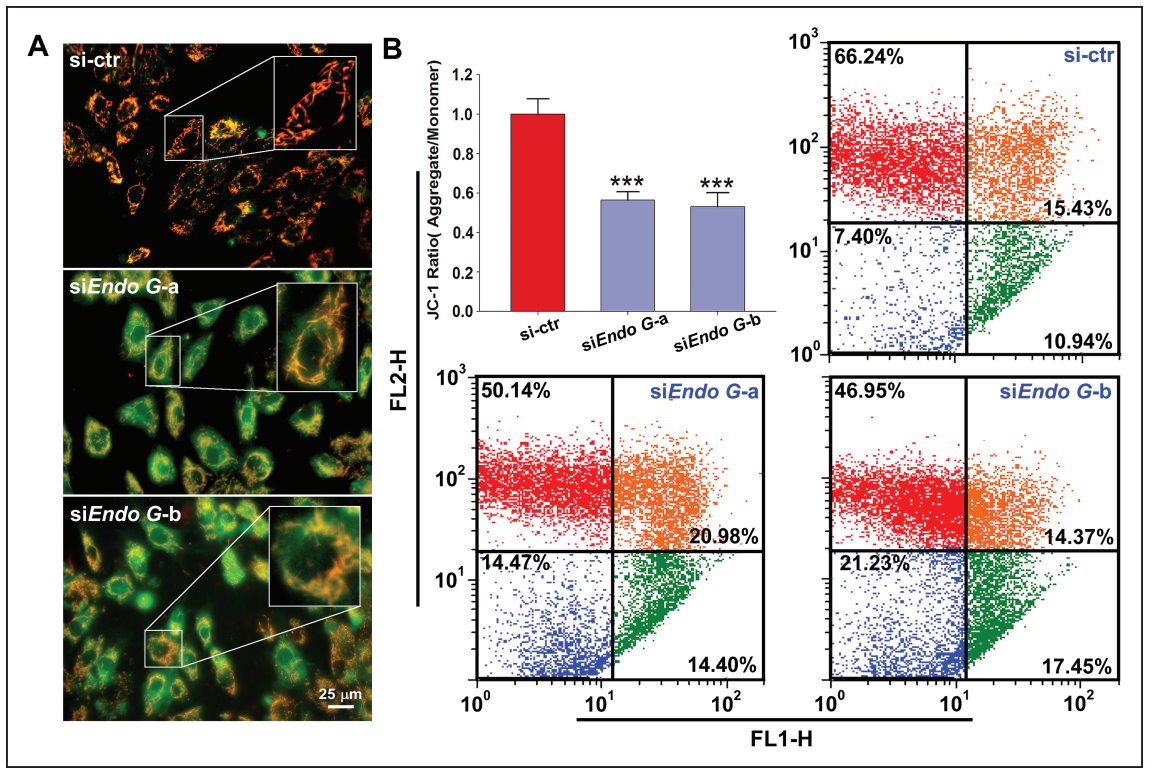

Fig. 3: Mitochondrial $\Delta\Psi$m assessment after knockdown of Endo G in H9c2 cells. (A) $\Delta\Psi$m assessment of H9c2 cells. Cells were treated with siEndo G followed by JC-1 staining. $\Delta\Psi$m was specifically indicated by a decrease in the red: green fluorescence intensity ratio, Scale bar = 25 μm. (B) Quantification of $\Delta\Psi$m by FCM was expressed as the ratio between monomeric and J-aggregate fluorescence (Red/Green). Data are presented as the means ± S.E.M. of three independent experiments. Statistical significance was set as $^{***}P < 0.001$ *vs.* control.

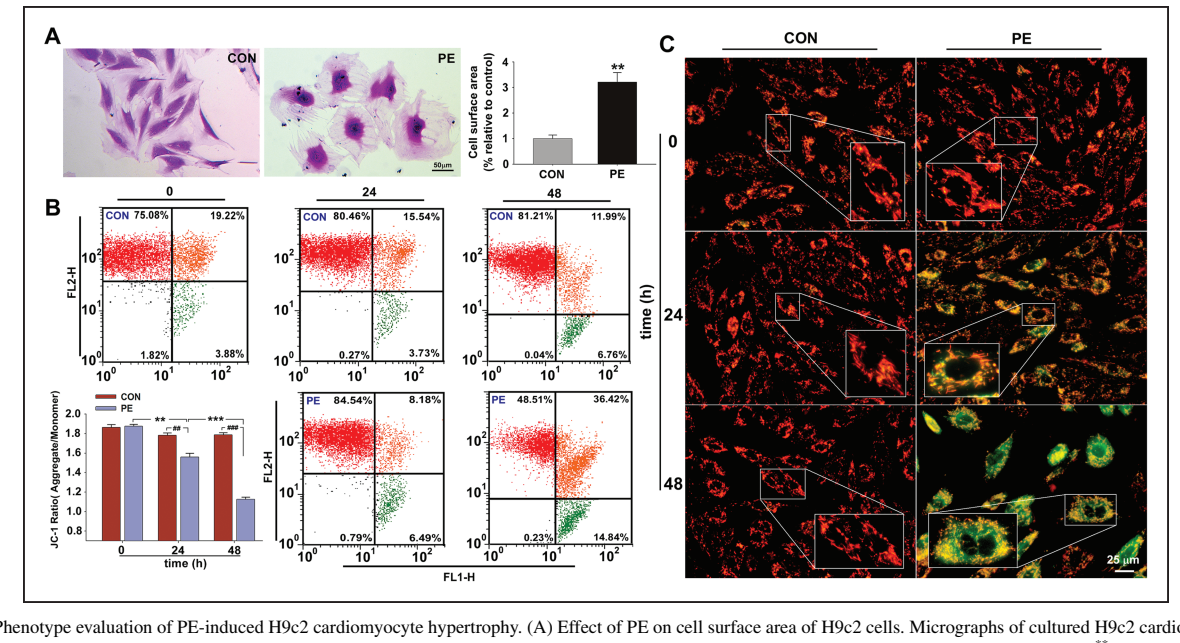

Fig. 4: Phenotype evaluation of PE-induced H9c2 cardiomyocyte hypertrophy. (A) Effect of PE on cell surface area of H9c2 cells. Micrographs of cultured H9c2 cardiomyocytes treated with PE or untreated (control). Scale bars = 50 μm. Cells were imaged after 48 h of treatment. Values shown are the means ± S.E.M., n = 50. $^{**}P < 0.01$ *vs.* control. (B) $\Delta\Psi$m assessment of H9c2 cells by JC-1 staining. H9c2 cells were treated with PE for 24 or 48 h, followed by JC-1 staining. Scale bar = 25 μm. Data are presented as the means ± S.E.M. of three independent experiments. Statistical significance was set as $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* control.

esis and energy metabolism pathways by factors such as Endo G and results in cardiomyocytes with inadequate mitochondrial mass (Goffart et al. 2004).

Mitochondria play essential and versatile roles in cardiomyocyte pathophysiology (Zhou et al. 2013). Mitochondrial energy production is essential for maintaining cardiac function, and the frequent observation of mitochondrial dysfunction in various models of cardiac hypertrophy has led to the general assumption that impaired mitochondrial energetics and the disruption of Ca^{2+} homeostasis contribute to or may even predispose the heart to hypertrophy and failure. Thus, the fact that si*Endo G* de-energized H9c2 cells indicates that mitochondrial energy metabolism is partly associated with Endo G**.**

Mfn2 has been reported to be involved in the rearrangement of the mammalian mitochondrial outer membrane, in tethering mitochondria to the SR, and in establishing the juxtaposition of the two organelles, providing a specialized pathway for shuttling Ca^{2+} without a bulk cytoplasmic Ca^{2+} increase (Parekh

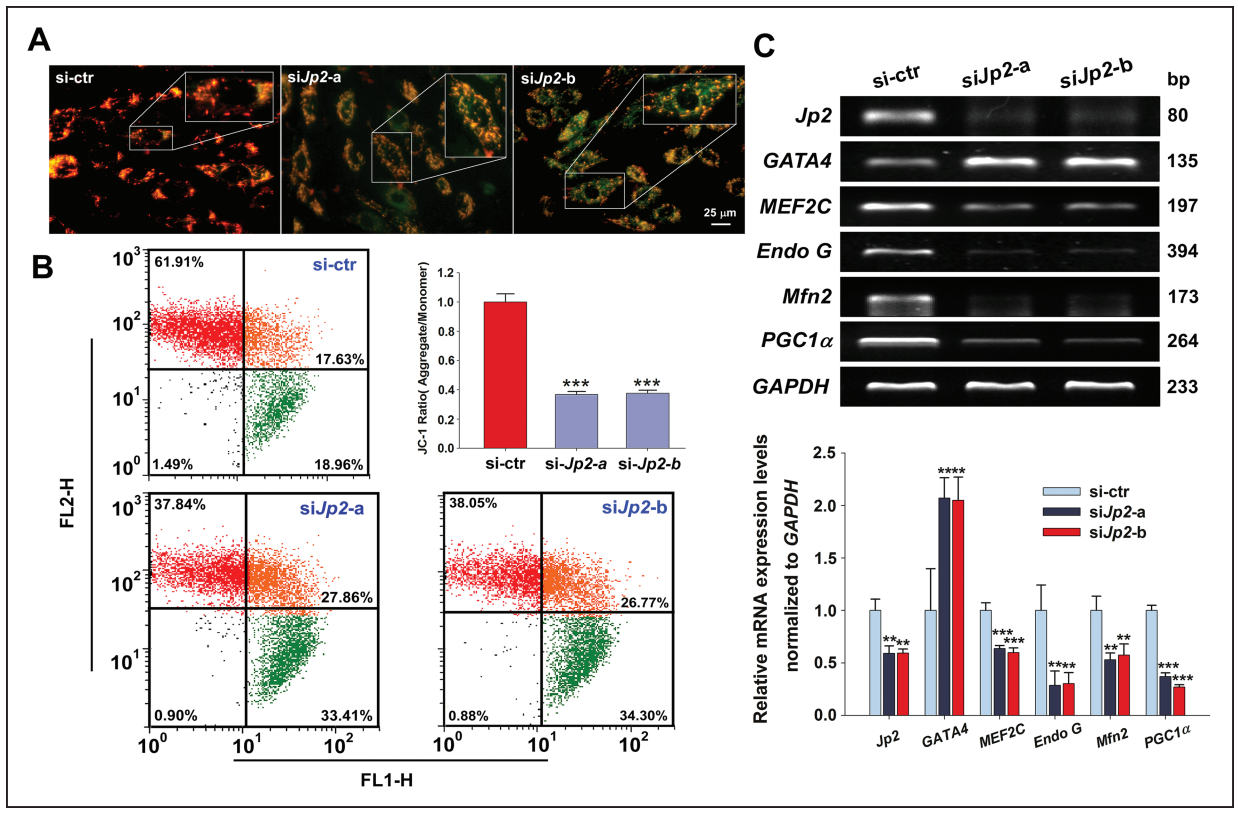

Fig. 5: Abolishing Jp2 in H9c2 cardiomyocytes. (A) Assessment of $\Delta\Psi$m in H9c2 cells by JC-1 staining. H9c2 cells are treated with siJp2 and then stained for JC-1. Scale bar = 25 μm. Quantification of $\Delta\Psi$m by FCM is expressed as the ratio between monomeric and J-aggregate fluorescence (red:green). (B) Expression of mitochondria and SR-related genes at 48 h after transient transfection with siJp2-a, -b, or si-ctr analyzed by RT-PCR. Data are presented as the means ± S.E.M. of three independent experiments. Statistical significance was set as $^{**}P < 0.01$, $^{***}P < 0.001$ *vs*. si-ctr.

2009). Mfn2-deficient mice display modest cardiac hypertrophy accompanied by slight functional deterioration. Therefore, our data suggested that the mitochondrial energy disruption caused by si*Endo G* is partly accomplished through Mfn2.

Furthermore, Jp2, which is essential for the physiological communication between caveolin channels and ryanodine receptor 2 in cardiac EC coupling, mediates Ca^{2+} release for cellular Ca^{2+} homeostasis (Takeshima et al. 2000), was also effected by si*Endo G*. Cellular Ca^{2+} cycling is vital to cardiac cell function and plays an important role in ventricular dysfunctions such as cardiac hypertrophy (Wilkins and Molkentin 2004). The calcium-dependent activation of calcineurin modifies gene expression and induces hypertrophy in cardiomyocytes. It has been reported that cardiac hypertrophy is detrimental to Ca^{2+} handling in the SR, thereby impairing cardiac contraction. In addition, the requirement for balanced steady-state Ca^{2+} flux applies not only to SR Ca^{2+} cycling but also to the uptake and release of Ca^{2+} across the sarcolemma and Ca^{2+} entry into and exit from the mitochondria (Lukyanenko et al. 2009).

Although the transcription of the Ca^{2+}-related hypertrophic marker MEF2C was reduced by 80% in si*Endo G* H9c2 cells after 48 h transient transfection, the expression of another known marker of cardiac hypertrophy, GATA4, was increased; this result is consistent with the report that the inhibition of Endo G in cultured cardiomyocytes resulted in increases in cell size and in hypertrophic biomarkers in the absence of pro-hypertrophic stimulation (McDermott-Roe et al. 2011). MEF2C is a key transcription factor for cardiac hypertrophy that integrates multiple Ca^{2+}/calmodulin-dependent signaling pathways in cardiomyocytes (Xu et al. 2006). In addition, Endo G and mitochondrial dysfunction are closely associated with cardiac hypertrophy (Dai et al. 2011); thus, reduced MEF2C transcription and protein expression support the link between mitochondrial dysfunction and abnormal SR Ca^{2+} transients that may be caused by si*Endo G*. This cascade reaction between Endo G and MEF2C should be further explored in future studies.

Interestingly, we found that the effect of si*Endo G* on mitochondria and SR-related proteins exhibited different behaviors at different time points. Mfn2 and Jp2, which activate genes that promote cardiac hypertrophy, were up-regulated in si*Endo G* H9c2 cells 24 h after transfection, whereas these proteins were down-regulated after 48 h transfection. The result was similar to the expression pattern observed in PE-induced hypertrophy. This finding suggested that at the early stage of si*Endo* G in H9c2 cells, these proteins exhibited compensatory expression, and the energy demand created by si*Endo G* induced hypertrophy in the same manner as the early stage of PE-induced hypertrophy, producing decompensated expression at the later stage.

In addition, si*Jp2* transfection into H9c2 cells resulted in a $\Delta\Psi$m reduction of more than 60% at 24 h after transfection (green) compared with si-ctr cells (orange) (Fig. 5A). This result was consistent with our previous report in embryonic stem cells, which showed that Jp2 knockdown disrupts energy production (Liang et al. 2012). Furthermore, we found that si*Jp2* reduced the expression of Endo G, it suggested that Endo G is associated with Jp2, which was essential for cardiac EC coupling and cellular Ca^{2+} homeostasis (Minamisawa et al. 2004). The expression of the mitochondrial fusion gene Mfn2 and PGC1α was also reduced by si*Jp2* (Fig. 5). These results revealed that the expression of hypertrophy-responsive genes might together contribute to the mechanisms of mitochondrial dysfunction and the juxtaposition of mitochondria with SR.

McDermott-Roe et al. (2011) identified Endo G as a regulator of mitochondrial energetics and convincingly demonstrated the regulation of Endo G expression by PGC1α and ERRα,

Table 1: siRNA sequences

Gene	Primer sequence (5'-3')
si*EndoG*-a	F: CCUGGAACAACCUUGAGAATT
	R: UUCUCAAGGUUGUUCCAGGTT
si*EndoG*-b	F: GCAGCUUGACUCGAACUUATT
	R: UAAGUUCGAGUCAAGCUGCTT
si*Jp2*-a	F: CCAGUGGGAAUACCUUUGATT
	R: UCAAAGGUAUUCCCACUGGTT
si*Jp2*-b	F: GCGAGUGGAAGAACGACAATT
	R: UUGUCGUUCUUCCACUCGCTT

Table 2: Primer sequences

Gene	Primer sequence (5'-3')
Jp2	F: GCCGCTTTGACTTTGATGAT
	R: TCCGTCTGCGTAGGTCTCC
Endo G	F: TTCCGCGAGGATGACTCTGT
	R: CACCTGAGGCGCTACGTTG
MEF2C	F: GATACCCACAACACACCACGCGC
	R: ATCCTTCAGAGAGTCGCATGCGCTT
GATA4	F: TCTCACTATGGGCACAGCAG
	R: GCGATGTCTGAGTGACAGGA
PGC1α	F: AGAAGCGGGAGTCTGAAA
	R: CACAGGTGTAACGGTAGG
Mfn2	F: AAGTCCGGGAAGCTGAAAGT
	R: TCTCGGTTATGGAACCAACC
GAPDH	F: AACTTTGGCATTGTGGAAGG
	R: ACACATTGGGGGTAGGAACA

presumably *via* reactive oxygen species (ROS). Emerging evidence has suggested that Mfn2 is also involved in physiologically relevant processes, such as ROS signaling and mitochondrial Ca^{2+} unloading. The absence of Mfn2 is associated with a marked delay in mitochondrial permeability transition caused by either Ca^{2+} stimulation or by the local generation of ROS. Mfn2-depleted mitochondria are more tolerant of Ca^{2+} overload, and Mfn2 deficiency in myocytes protects isolated cells from ROS stress (Papanicolaou et al. 2011). Unfortunately, we did not determine whether changes in the expression of Endo G played a role in increased ROS production during cardiac hypertrophy. This possibility should be considered in future study.

Taken together, our findings first explored the characterization of Endo G and mitochondria- and SR related proteins during cardiac hypertrophy. In addition, we uncovered a role for Endo G in the crosstalk between the processes controlled by Mfn2 and Jp2 in maladaptive cardiac hypertrophy. Therefore, targeting Endo G and its effectors might represent a new therapeutic strategy for the treatment of cardiac hypertrophy. However, further studies are necessary to elucidate the molecular entities that are directly responsible for cardiac hypertrophy.

4. Experimental

4.1. Culture of H9c2 cardiomyocytes

H9c2 Cardiomyocytes (ATCC, USA) were grown in Dulbecco's modified Eagle high-glucose medium (Gibco, USA) supplemented with 10% (vol/vol) fetal bovine serum (Gibco, USA), 50 U/mL penicillin G, and 50 μg/mL streptomycin (Sigma, USA) in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were grown to ∼50% confluence and synchronized overnight in serum-free medium before treatment.

4.2. Transfection of siRNA against Endo G and Jp2

To knockdown Endo G and Jp2, short interfering RNAs (siRNAs) specifically targeting those genes were transfected into H9c2 cells. siRNA and a validated negative control siRNA were ordered from GenePharma (Shanghai, China). Two siRNA template oligonucleotides containing 21 nt target sequences of rat Endo G and Jp2 were synthesized and then transiently transfected into H9c2 cells (Table 1). Briefly, negative control siRNA, si*EndoG* or si*Jp2* (100 pmol) was complexed with 5 μL of lipofectamine 2000 (Invitrogen, USA) in a final volume of 200 μL Opti-MEM. The complexes were applied to freshly passaged H9c2 cells. 48 h after transfection, knockdown effect was confirmed at gene and protein level (Li et al. 2006).

4.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Gene expression was evaluated by RT-PCR. Total RNA was isolated using TRIzol (Gibco, USA) in accordance with the manufacturer's instructions. To synthesize first strand cDNA, 3 mg total RNA was incubated with 0.5 mg of oligo (dT) (Sangon, China) and 5 mL deionized water at 65 °C for 15 min. Reverse transcription reactions were performed with 200 units of M-MuLV reverse transcriptase (Gibco, USA), 4 μL of 5 μmol/L reaction buffer and 1 mmol/L deoxynucleotide triphosphate (dNTP) mixture for 1 h at 42 °C. The primer sequences were shown in Table 2. The PCR products were analyzed by 1.5% agarose (Biowest, Spain) gel electrophoresis, visualized with ethidium bromide staining, and then quantified using a bio-imaging analyzer (Bio-Rad, USA). The density of the products was quantified using Quantity One software, version 4.2 (Ding et al. 2007b).

4.4. Western blot analysis

Cells were collected in RIPA buffer and lysed for 30 min on ice. The protein concentration was measured using the Bio-Rad protein kit (Hercules, CA), and equal amounts of sample were loaded per well on a sodium dodecyl sulfate (SDS)-polyacrylamide gel. Subsequently, the proteins were transferred onto positively charged nylon membranes with 0.45 μm pores (PVDF, Millipore) and blocked with blotto at room temperature. The membranes were incubated with primary antibody in blotto overnight at 4 °C, washed three times with PBST (0.1% Tween-20), incubated with HRP-conjugated antibodies, and then detected with an enhanced chemiluminescent substrate (Pierce, USA). The primary antibodies used were mouse monoclonal anti-Mfn2 (Santa Cruz, USA) and anti-α-GATA4 (Santa Cruz, USA); rabbit polyclonal anti-Endo G (CST, USA), anti-GAPDH (CST, USA), anti-PGC1α (BioVision, USA), and anti-Jp2 (Santa Cruz, USA), and goat polyclonal anti-MEF2C (Santa Cruz, USA) (Ding et al. 2007a).

4.5. Mitochondrial membrane potential (Δψm) assessment

JC-1 staining was used to assess the mitochondrial ΔΨm in si*Jp2*/si*EndoG* H9c2 cells. JC-1 exhibited potential-dependent accumulation in mitochondria, as indicated by a fluorescence emission shift from green 525 ± 10 nm to red 610 ± 10 nm. Mitochondrial depolarization was specifically indicated by a decrease in the red:green fluorescence intensity ratio (Zuliani et al. 2003).

4.6. Measurement of hypertrophic growth in H9c2 cardiomyocytes

H9c2 cardiomyocytes were used to obtain measurements of hypertrophic growth. Cells grown on glass coverslips were serum starved for 18 h in DMEM containing 1% FBS and treated with PE for 48 h. For size measurement, cells were incubated with 0.1% crystal violet (Sigma, USA) at room temperature for 10 min. After images were acquired under a Nikon microscope, the cell surface area was measured using Image-Pro Plus 5.0 software. In each group, the surface area of 150 cells was measured, calculated as follows: cell surface area (% relative to control) = surface area (after treatment)/surface area (before treatment). The cell size is expressed as the relative surface area standardized to the mean surface area of control cells in each experiment.

4.7. Statistical analysis

SPSS 19 software was used for statistical analyses. The data are expressed as the mean ± standard error of the mean (S.E.M.). Student's *t* test was used for comparing paired data, and one-way analysis of variance (ANOVA) was used for comparisons of multiple groups. A probability of $P < 0.05$ was considered statistically significant.

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