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Silencing of C3G increases cardiomyocyte survival inhibition and apoptosis via regulation of p-ERK1/2 and Bax

Short title: C3G and cell survival and apoptosis

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Disclosure

The authors report no conflicts of interest in this work.

Summary

Experimental studies have shown that overexpression of Rap guanine nucleotide exchange factor 1 (C3G) plays pro-survival and anti-apoptotic roles through molecule phosphorylated extracellular signal-regulated kinase1/2 (p-ERK1/2) in cardiomyocytes. However, it is still unclear if silencing of C3G may increase cell survival inhibition and apoptosis in cardiomyocytes, and whether C3G silence induced injuries are reduced by the overexpression of C3G through regulation of p-ERK1/2 and pro-apoptotic molecule Bax. In this study, the rat-derived H9C2 cardiomyocytes were infected with C3G small

hairpin RNA interference recombinant lentiviruses, which silenced the endogenous C3G expression in the cardiomyocytes. Then contrary experiments were conducted using C3G overexpression. The cell proliferation and apoptosis were analyzed in the cardiomyocytes which were treated with or without hypoxia/reoxygenation (H/R). Silencing of C3G leaded to significant increase in cell survival inhibition and apoptosis, combined with aggravated the injuries induced by H/R. Overexpression of C3G reduced the injuries induced by the silencing of C3G in the cardiomyocytes via regulation of p-ERK1/2 and Bax. In conclusion, our results provided new experimental evidence that silencing of C3G can increase cell survival inhibition and apoptosis in cardiomyocytes via regulation of p-ERK1/2 and Bax.

KEYWORDS

Rap guanine nucleotide exchange factor 1, apoptosis, survival, Bax, p-ERK1/2, cardiac myocyte, H9C2 cell line

1 INTRODUCTION

Increased survival inhibition and apoptosis in cardiomyocytes are the major causes of ischemia/reperfusion-induced cardiomyocyte losses, myocardial injuries, cardiomyopathy and heart failure after myocardial infarction. Even

modest survival inhibition and apoptosis in cardiomyocytes may have an essential effect on the development and progression of cardiovascular diseases [1]. Integrin receptor heterodimer composed of alpha and beta subunits is located on cardiomyocyte surface. Integrins link extracellular matrix substances into intracellular cytoskeleton in cardiomyocytes, and identify extracellular mechanical stretch, and translate it into intracellular biochemical signaling [1]. Integrin pathway is a possible target for cardiovascular disease treatment due to its components with the effects of cell pro-survival and anti-apoptosis on cardiomyocytes [2-5]. Integrin β1 subunit and its downstream molecules such as integrin-linked kinase (ILK) and focal adhesion kinase (FAK) have been proven to be indispensable to the inhibition of ischemia and reperfusion-induced myocardial injuries, post-infarct cardiac remodeling, ischemic cardiomyopathy and heart failure [6-8] due to their pro-survival and anti-apoptotic effects on cardiomyocytes [7, 8]. Proposed molecular mechanisms are mediated by the upregulation of their downstream pro-survival molecules such as phosphorylated extracellular signal-regulated kinase1/2 (p-ERK1/2) and the downregulation of pro-apoptotic molecules such as Bax [9]. Our previous study has shown that v-crk avian sarcoma virus CT-10 oncogene homolog-like (CrkL) [10], an adaptor protein which can bind Rap guanine nucleotide exchange factor 1 (C3G) in the integrin pathway [1, 11], has the effects of cell pro-survival and anti-apoptosis on H9C2 cardiomyocytes [10, 11]. The CrkL effects on cell pro-survival and anti-apoptosis may be mediated

via C3G. On the other hand, as a guanine nucleotide exchange factor in the integrin pathway [1], C3G was found to be expressed in H9C2 cardiomyocytes and rat peri-infarct myocardium [11, 12]. We have also demonstrated that overexpression of C3G can promote cell survival and alleviate hypoxia and reoxygenation (H/R)-induced survival inhibition and apoptosis in H9C2 cardiomyocytes [11]. However, it is not known if silencing of C3G increases cell survival inhibition and apoptosis, and aggravates the injuries induced by H/R, and whether the C3G silence induced myocyte injuries can be reduced by overexpression of C3G in the cardiomyocytes through regulation of p-ERK1/2 and Bax.

Integrin pathway is a novel therapeutic target for cardiovascular diseases due to its component C3G as a potential intervention molecule. In this experimental study, all 13 endogenous gene transcriptions of C3G isoforms were silenced by a small hairpin RNA interference (shRNA), which targets all of C3G isoform gene transcriptions in rat-derived H9C2 cardiomyocytes. Silencing of C3G was disclosed to increase the cell survival inhibition and apoptosis, and aggravate the injuries induced by H/R in the H9C2 cardiomyocytes via regulation of p-ERK1/2 and Bax. Overexpression of human C3G (hC3G) reduced the injuries induced by C3G silence via regulation of p-ERK1/2 and Bax.

2 RESULTS

2.1 Expression of C3G mRNA

Rat C3G mRNA in the cardiomyocytes was declined after the treatment of H/R in the blank, NT shRNA, C3G shRNA, and C3G shRNA + hC3G groups (All $P < 0.05$, Figure 1A, 1B). Rat C3G mRNA in the cardiomyocytes was significantly lower in the C3G shRNA and C3G shRNA + hC3G groups than in the blank and NT shRNA groups, no matter whether the cardiomyocytes were treated with or without H/R (All $P < 0.05$, Figure 1A, 1B). Furthermore, human C3G mRNA level in the cardiomyocytes was higher in the C3G shRNA + hC3G group as compared with the NT shRNA and C3G shRNA groups, this finding was similar in cardiomyocytes which were treated with or without H/R (All $P < 0.05$, Figure 1B).

2.2 Expression of C3G, p-ERK1/2 and Bax proteins

Decreased C3G and p-ERK1/2 and increased Bax protein levels were found in the cardiomyocytes subjected to H/R treatment in the blank, NT shRNA, C3G shRNA and C3G shRNA + hC3G groups (All $P < 0.05$, Figure 2A, 2B). Decreased C3G and p-ERK1/2 and elevated Bax protein levels of the cardiomyocytes were also found in the C3G shRNA group when compared with the blank and NT shRNA groups (All $P < 0.05$, Figure 2A, 2B). Cardiomyocyte C3G and p-ERK1/2 levels were higher and Bax protein level

was lower in the C3G shRNA + hC3G group than in the NT shRNA and C3G shRNA groups (All $P < 0.05$, Figure 2B). These results were not dependent on the H/R treatment of cardiomyocytes.

2.3 Proliferative rate of H9C2 cardiomyocytes

As indicated in Figure 3A and 3B, H/R reduced the proliferative rate of cardiomyocytes in the blank, NT shRNA, C3G shRNA and C3G shRNA + hC3G groups (All $P < 0.05$, Figure 3A, 3B). In contrast, overexpression of hC3G increased the proliferative rate of cardiomyocytes with or without treatment by H/R (All $P < 0.05$, Figure 3B).

2.4 Apoptotic rate of H9C2 cardiomyocytes

Figure 4A and 4B show that H/R could increase the apoptotic rate of cardiomyocytes in the blank, NT shRNA, C3G shRNA and C3G shRNA + hC3G groups (All $P < 0.05$, Figure 4A, 4B). The apoptotic rate of cardiomyocytes in the C3G shRNA + hC3G group was reduced as compared to the respective apoptotic rate in the NT shRNA and C3G shRNA groups, no matter whether the cardiomyocytes were treated with or without H/R (All $P < 0.05$, Figure 4B).

3 DISCUSSION

This study showed the following main findings: (1) Silencing of C3G mRNA by shRNA decreased C3G protein expression and increased cardiomyocyte survival inhibition and apoptosis. Moreover, it exacerbated injuries induced by H/R in the rat-derived H9C2 cardiomyocytes via downregulation of pro-survival molecule p-ERK1/2 and upregulation of pro-apoptotic molecule Bax. The decrease of C3G expression was associated with the increase of cardiomyocyte survival inhibition and apoptosis. (2) Overexpression of human C3G could alleviate the cardiomyocyte injuries induced by C3G silence through upregulation of p-ERK1/2 and downregulation of Bax.

Rat C3G protein has 13 different isoforms. Each of them are translated from rat *C3G* gene transcript variants correspondingly, and acts as a guanine nucleotide exchange factor in the integrin pathway [13], and could be a part of remodeling mechanisms involved in infarcted myocardium [12]. It is shown that overexpression of C3G can promote cell survival and anti-apoptosis mechanisms in the rat-derived H9C2 cardiomyocytes [11]. However, little is known as to whether silencing C3G could regulate cell survival inhibition and apoptosis, and whether the injuries induced by C3G silence in the cardiomyocytes can be mitigated by overexpression of C3G.

Additionally, there is some available evidence that demonstrates other components of integrin pathway such as integrin β1 subunit [6], FAK [8], ILK [7], CrkL [10] and C3G [11] can prevent cardiomyocyte survival inhibition and apoptosis [4, 5]. The underlying mechanisms of the protective effects of these components are mediated by regulation of their downstream signaling molecules such as ERK1/2 [9, 11, 14, 15] and Bax [10].

Although majority of the embryonic mice with knockout of integrin β1 subunit [16, 17], ILK [18, 19] or FAK [20, 21] exclusively in their cardiomyocytes can still survive to birth, they postnatally develop severe dilated cardiomyopathy and spontaneous heart failure which result from cardiomyocyte survival inhibition and apoptosis [8, 22-24] and reduction of pro-survival molecules such as ERK1/2 and AKT in their cardiomyocytes with knockout of above corresponding genes [25, 26]. When these mice with knockout of integrin β1 subunit or FAK exclusively in their cardiomyocytes were induced by isoproterenol [22], angiotensin II [20], pressure overload [16, 20] or myocardial infarction [6, 8], ischemic cardiomyopathy after myocardial infarction and chronic congestive heart failure in these mice were evidently accentuated. This was due to increased cardiomyocyte survival inhibition and apoptosis modifications and reduced pro-survival molecules such as ERK1/2 and AKT with the gene knockout [25, 26]. Similarly, knockout of integrin β1 subunit [22] or FAK [8] in the cardiomyocytes were disclosed to deteriorate the cell survival inhibition and apoptosis induced by isoproterenol or H/R, because

of decrease in the pro-survival molecules such as ERK1/2 and AKT [9, 25]. Thus, integrin β1 subunit [6, 16], ILK [18] and FAK [8, 20, 21] can indispensably protect cardiomyocytes, and thereafter ameliorate the post-infarct dilative ischemic cardiomyopathy and heart failure through ERK1/2 and AKT.

In contrast, overexpression experiments proved that ILK, CrkL and C3G can also reduce cell survival inhibition and apoptosis in the cardiomyocytes [7, 10, 11] and thus attenuate the post-infarct ischemic cardiomyopathy and development of heart failure [7] via ERK1/2, AKT and downregulation of Bax.

As one of the components of integrin pathway, the GTPase Rap guanine nucleotide exchange factor C3G is a highly conserved protein in the evolutionary process. The amino acid sequence homology of hC3G (NCBI protein accession number: NP_005303.2) shares 83%-92% similarity with each of rat C3G isoform's amino acid sequences (NCBI protein accession numbers: XP_017447591.1, XP_017447592.1, XP_008759905.2, XP_017447593.1, XP_017447594.1, XP_008759906.2, XP_008759907.2, XP_017447595.1, XP_008759908.2, XP_017447596.1, XP_017447597.1, XP_017447598.1 and XP_006233964.1. BLAST from the website: http://www.ncbi.nlm.nih.gov/BLAST were used for the analysis of identity). C3G is one of the direct downstream effectors of adaptor CrkL in the integrin pathway [10]. C3G has been essential for the early embryo development. However, there is no any previous data on C3G role in the postnatal growth,

development, physiology and pathophysiology especially in mammals [27]. Pro-survival molecule ERK1/2, as a mitogen-activated protein kinase can conduct extracellular signals into cytoplasm and nucleus and mediate biochemical reactions. As one of the common effective molecules in the integrin pathway, ERK1/2 has protective effects on cardiomyocytes [10], while pro-apoptotic molecule Bax, as a member of the Bcl-2 family, is involved in mitochondrial apoptosis pathway, and may induce apoptosis in cardiomyocytes by its induction of the release of cytochrome c and activation of caspase proteases [10, 28].

The key findings of our current study are consistent with the previous study results [6, 8, 16, 18, 20, 21]. Nevertheless, like knockout of integrin pathway components such as integrin β1 subunit, FAK and ILK [6, 8, 16, 18, 20, 21], silencing of all C3G isoforms could also lead to cardiomyocyte survival inhibition and apoptosis and exacerbate its injuries induced by H/R via upregulation of pro-apoptotic molecule Bax and downregulation of pro-survival molecule p-ERK1/2. To the best of our knowledge, this is the first study to prove this conclusion. Furthermore, our present study confirmed for the first time that overexpression of human C3G can decrease cell survival inhibition and apoptosis and relieve the injuries induced by C3G silence in the rat-derived H9C2 cardiomyocytes via upregulation of p-ERK1/2 and downregulation of Bax. These new findings are well in line with previously reported results [11]. However, the functional characteristics of each of C3G

isoforms and its downstream relevant survival inhibition and apoptotic signaling molecules need to be clarified in future studies.

In summary, this experimental study demonstrated that silencing of C3G can result in cell survival inhibition and apoptosis and exacerbate the injuries induced by H/R in the H9C2 cardiomyocytes via upregulation of Bax and downregulation of p-ERK1/2. In contrast, overexpression of C3G attenuates the injuries induced by silencing C3G via upregulation of p-ERK1/2 and downregulation of Bax. Our study provided new experimental evidence that silencing of C3G increases cardiomyocyte survival inhibition and apoptosis through upregulation of Bax and downregulation of p-ERK1/2.

4 MATERIALS AND METHODS

4.1 Construction of C3G shRNA lentivirus

Three candidate sequences of *C3G* shRNA lentiviral vectors targeting rat *C3G* mRNA were designed by applying specific software (BLOCK-iT™ RNAi Designer) based on mRNA sequence of each rat *C3G* transcript variants (NCBI GenBank accession numbers: XM_017592102.1, XM_017592103.1, XM_008761683.2, XM_017592104.1, XM_017592105.1, XM_008761684.2, XM_008761685.2, XM_017592106.1, XM_008761686.2, XM_017592107.1, XM_017592108.1, XM_017592109.1 and XM_006233902.3, http://www.ncbi.nlm.nih.gov/gene)

and cloned respectively into GV248-GFP lentiviral vectors (Genechem Co., Ltd, Shanghai, China), which specifically express C3G shRNA. The C3G shRNA targeting sequence listed in Table 1 was the most effective one at suppressing all of the 13 *C3G* transcript variant's mRNA and their corresponding protein expressions which was confirmed by reverse transcription polymerase chain reaction (RT-PCR) and Western blot in rat-derived cardiomyocyte line H9C2 (purchased from the American Type Culture Collection, Manassas, VA, USA), were used in subsequent experiments to silence all of the endogenous *C3G* transcript variant's mRNA. The non-target (NT) sequence listed in Table 1 was also cloned into the GV248-GFP lentiviral vectors and used as a negative control. The C3G shRNA and NT shRNA recombinant lentiviral vectors were packaged into lentiviruses respectively in 293T cells by use of a Lentivector Expression System (Genechem Co., Ltd, Shanghai, China), and selected in the presence of puromycin (1.5 μg/ml) respectively [10]. DNA sequencing results confirmed that the C3G shRNA interference sequence targeting all of the *C3G* transcript variant's mRNA was successfully inserted into the recombinant lentiviruses.

4.2 H9C2 cardiomyocyte culture, treatment with C3G shRNA lentivirus, pCXN2-flag-hC3G plasmid and hypoxia/reoxygenation

H9C2 cardiomyocytes were cultured as described previously [10]. An equal number of the cardiomyocytes at ~60% confluence was infected and transfected respectively with NT shRNA + pCXN2-flag, C3G shRNA + pCXN2-flag, and C3G shRNA + pCXN2-flag-hC3G (hC3G overexpression eukaryotic recombinant plasmid, which was generously provided by Prof. Shinya Tanaka at Hokkaido University in Japan to Dr. Hua Linghu at Chongqing Medical University in China). The methods of infection and transfection were the same as described previously [10]. The H9C2 cells were divided into NT shRNA, C3G shRNA, and C3G shRNA + hC3G groups. Two parts were prepared for each group and treated either with or without H/R. The method of H/R treatment was the same as described previously [10]. Finally, the cells were randomly divided into NT shRNA, C3G shRNA, C3G shRNA + hC3G, NT shRNA + H/R, C3G shRNA + H/R, and C3G shRNA + hC3G + H/R groups. In another protocol, the cells were randomly divided into blank, NT shRNA, C3G shRNA, blank + H/R, NT shRNA + H/R and C3G shRNA + H/R groups. At indicated time points (24 h, 72 h after infection and transfection respectively), the cells were harvested to be analyzed by RT-PCR, Western blot, FCM (flow cytometry) and MTT (tetrazolium-based colorimetric assay) test respectively. The methodology of cell harvest was described previously [10].

4.3 RT-PCR examination of C3G mRNA

Seventy-two hours after infection and transfection, the total RNA was isolated, quantified, transcribed reversely into cDNA [10]. One microliter of cDNA generated was amplified using specific primers designed for hC3G, rat C3G for all its isoforms and rat β-actin genes respectively. The nucleotide sequences and Tm values for all primers are listed in Table 1. The electrophoretic band intensity of RT-PCR products were analyzed as described previously [10].

4.4 Western blot examination of C3G, p-ERK1/2 and Bax proteins

Seventy-two hours after infection and transfection, the cells were collected and lysed. The protein was isolated and its concentration was gauged. An equal amount of protein was separated by electrophoresis, and transferred to a polyvinylidene difluoride membrane. Non-specific sites were blocked as described previously [10]. The membrane was then incubated with corresponding primary antibodies [Anti-rat & human C3G (H-300) rabbit polyclonal antibody: (1:200), No. sc-15359, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; Anti-rat p-ERK1/2 (Thr202/Tyr204) (D13.14.4E) XP® rabbit monoclonal antibody: (1:2000), No. 4370, Cell Signaling Technology Inc., Danvers, MA, USA; Anti-rat Bax rabbit polyclonal antibody: (1:1000), No. 50599-2-Ig, Proteintech Group Inc., Chicago, IL, USA; Anti-rat β-actin (C4) mouse monoclonal antibody: (1:2000), No. sc-47778, Santa Cruz Biotechnology

Inc., Santa Cruz, CA, USA; Anti-flag mouse polyclonal antibody: (1:200), No. AF519, Beyotime Biotechnology Co., Ltd., Shanghai, China] respectively overnight at 4°C, and then with horseradish peroxidase-labeled anti-rabbit or anti-mouse second antibody (IgG, 1:2000, Boster Biosynthesis Biotechnology Co., Ltd., Wuhan, China) for 2 h at 37°C. The immunoreactive band was then watched, and its intensity was determined [10]. The density of C3G, p-ERK1/2 and Bax proteins in relation to β-actin was expressed as C3G/β-actin, p-ERK1/2/β-actin and Bax/β-actin respectively, which represented the relative expression level of C3G, p-ERK1/2 and Bax proteins respectively.

4.5 MTT assay of H9C2 cardiomyocyte viability

At the appointed time points (24 h, 72 h) after infection and transfection, MTT working solution was added to cells in each well plate. The cells continued to be incubated for another 4 h at 37°C. The supernatant was removed. Dimethyl sulfoxide was then added to each cell. The well plate was shaken for 30 min at room temperature. The absorbance of the samples were then detected [10]. The proliferative rate was calculated as (absorbance at 72 h – absorbance at 24 h)/absorbance at 24 h, and expressed as a percentage.

4.6 Flow cytometry examination of H9C2 cardiomyocyte apoptosis

Seventy-two hours after infection and transfection, the apoptotic and necrotic cells were labeled with annexin V-phycoerythrin (PE) and 7-aminoactinomycin D (7-AAD) respectively. Then the fluorescence-labeled cells were sorted by flow cytometry [10]. The cells with annexin-PE positive and 7-AAD negative were determined as the apoptotic cells. The apoptotic rate was calculated as the percentage of the number of the annexin-PE positive and 7-AAD negative cells over the number of the total cells.

4.7 Statistical analysis

Data were expressed as mean values ± standard deviation (S.D.). All analyses were performed using the SPSS22.0 statistical software (IBM Corporation, Armonk, NY, USA). The statistical significance among groups was judged by one-way ANOVA, then, in case of significance, by a two-sided Tukey test for multiple comparisons. A 2-tailed value of $P < 0.05$ was deemed statistically significant.

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DISCLOSURE

None declared.

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Figure legends

Figure 1. The expression of C3G mRNA in H9C2 cardiomyocytes examined by reverse transcription polymerase chain reaction. Figure 1A Note M: DNA marker; Blank: blank group; NT shRNA: non-target small hairpin RNA interference group; C3G shRNA: C3G small hairpin RNA interference group; Blank + H/R: blank + hypoxia/reoxygenation group; NT shRNA + H/R: non-target small hairpin RNA interference + hypoxia/reoxygenation group; C3G shRNA + H/R: C3G small hairpin RNA interference + hypoxia/reoxygenation

group. $^{*}P<0.05$ versus blank and NT shRNA groups; $^{\dagger}P<0.05$ versus C3G shRNA, blank + H/R and NT shRNA + H/R groups. Data were expressed as mean values ± S.D., n=7. **Figure 1B** Note M: DNA marker; NT shRNA: non-target small hairpin RNA interference group; C3G shRNA: C3G small hairpin RNA interference group; C3G shRNA + hC3G: C3G small hairpin RNA interference + human C3G overexpression group; NT shRNA + H/R: non-target small hairpin RNA interference + hypoxia / reoxygenation group; C3G shRNA + H/R: C3G small hairpin RNA interference + hypoxia / reoxygenation group; C3G shRNA + hC3G + H/R: C3G small hairpin RNA interference + human C3G overexpression + hypoxia / reoxygenation group. $^{*}P<0.05$ versus NT shRNA group; $^{\dagger}P<0.05$ versus C3G shRNA group and NT shRNA + H/R group; $^{\ddagger}P<0.05$ versus C3G shRNA + hC3G group; $^{\S}P<0.05$ versus C3G shRNA + H/R group. Data were expressed as mean values ± S.D., n=7.

Figure 2. The expression of C3G, p-ERK1/2 and Bax proteins in H9C2 cardiomyocytes examined by Western blot. Figure 2A Note Blank: blank group; NT shRNA: non-target small hairpin RNA interference group; C3G shRNA: C3G small hairpin RNA interference group; Blank + H/R: blank + hypoxia/reoxygenation group; NT shRNA + H/R: non-target small hairpin RNA interference + hypoxia/reoxygenation group; C3G shRNA + H/R: C3G small hairpin RNA interference + hypoxia/reoxygenation group. $^{*}P<0.05$ versus

blank and NT shRNA groups; $^{\dagger}P<0.05$ versus C3G shRNA, blank + H/R and NT shRNA + H/R groups. Data were expressed as mean values ± S.D., n=7. **Figure 2B** Note NT shRNA: non-target small hairpin RNA interference group; C3G shRNA: C3G small hairpin RNA interference group; C3G shRNA + hC3G: C3G small hairpin RNA interference + human C3G overexpression group; NT shRNA + H/R: non-target small hairpin RNA interference + hypoxia / reoxygenation group; C3G shRNA + H/R: C3G small hairpin RNA interference + hypoxia / reoxygenation group; C3G shRNA + hC3G + H/R: C3G small hairpin RNA interference + human C3G overexpression + hypoxia / reoxygenation group. $^{*}P<0.05$ versus NT shRNA group; $^{\dagger}P<0.05$ versus C3G shRNA group and NT shRNA + H/R group; $^{\ddagger}P<0.05$ versus C3G shRNA + hC3G group; $^{\S}P<0.05$ versus C3G shRNA + H/R group. Data were expressed as mean values ± S.D., n=7.

Figure 3. The proliferative rate of H9C2 cardiomyocytes examined by tetrazolium-based colorimetric assay. Figure 3A Note Blank: blank group; NT shRNA: non-target small hairpin RNA interference group; C3G shRNA: C3G small hairpin RNA interference group; Blank + H/R: blank + hypoxia/reoxygenation group; NT shRNA + H/R: non-target small hairpin RNA interference + hypoxia/reoxygenation group; C3G shRNA + H/R: C3G small hairpin RNA interference + hypoxia/reoxygenation group. $^{*}P<0.05$ versus

blank and NT shRNA groups; $^{\dagger}P<0.05$ versus C3G shRNA, blank + H/R and NT shRNA + H/R groups. Data were expressed as mean values ± S.D., n=10. **Figure 3B** Note NT shRNA: non-target small hairpin RNA interference group; C3G shRNA: C3G small hairpin RNA interference group; C3G shRNA + hC3G: C3G small hairpin RNA interference + human C3G overexpression group; NT shRNA + H/R: non-target small hairpin RNA interference + hypoxia / reoxygenation group; C3G shRNA + H/R: C3G small hairpin RNA interference + hypoxia / reoxygenation group; C3G shRNA + hC3G + H/R: C3G small hairpin RNA interference + human C3G overexpression + hypoxia / reoxygenation group. $^{*}P<0.05$ versus NT shRNA group; $^{\dagger}P<0.05$ versus C3G shRNA group and NT shRNA + H/R group; $^{\ddagger}P<0.05$ versus C3G shRNA + hC3G group; $^{\S}P<0.05$ versus C3G shRNA + H/R group. Data were expressed as mean values ± S.D., n=10.

Figure 4. The apoptotic rate of H9C2 cardiomyocytes examined by flow cytometry. Figure 4A Note Blank: blank group; NT shRNA: non-target small hairpin RNA interference group; C3G shRNA: C3G small hairpin RNA interference group; Blank + H/R: blank + hypoxia/reoxygenation group; NT shRNA + H/R: non-target small hairpin RNA interference + hypoxia/reoxygenation group; C3G shRNA + H/R: C3G small hairpin RNA interference + hypoxia/reoxygenation group. $^{*}P<0.05$ versus blank and NT

shRNA groups; $^{\dagger}P<0.05$ versus C3G shRNA, blank + H/R and NT shRNA + H/R groups. Data were expressed as mean values ± S.D., n=7. **Figure 4B** Note NT shRNA: non-target small hairpin RNA interference group; C3G shRNA: C3G small hairpin RNA interference group; C3G shRNA + hC3G: C3G small hairpin RNA interference + human C3G overexpression group; NT shRNA + H/R: non-target small hairpin RNA interference + hypoxia / reoxygenation group; C3G shRNA + H/R: C3G small hairpin RNA interference + hypoxia / reoxygenation group; C3G shRNA + hC3G + H/R: C3G small hairpin RNA interference + human C3G overexpression + hypoxia / reoxygenation group. $^{*}P<0.05$ versus NT shRNA group; $^{\dagger}P<0.05$ versus C3G shRNA group and NT shRNA + H/R group; $^{\ddagger}P<0.05$ versus C3G shRNA + hC3G group; $^{\S}P<0.05$ versus C3G shRNA + H/R group. Data were expressed as mean values ± S.D., n=7.

TABLE 1. The Target Nucleotide Sequences of C3G shRNA and NT shRNA and The Nucleotide Sequences and Tm Values of The Primers of C3G and β-actin Genes.

shRNA or Genes	Sequences	Size of products (bp)	Tm (°C)
Rat C3G shRNA	sense: 5'-GAAGATCAAGCCTAGGAACAT-3'	-	60
NT shRNA	sense: 5'-TTCTCCGAACGTGTCACGT-3'	-	62
Rat C3G	sense: 5'-CAGGATGGACAGCAGACAGA-3'	384	58
	anti-sense: 5'-CTGCGGTGTCTGGTAGAACA-3'		
Rat β-actin	sense: 5'-AGATGACCCAGATCATGTTTGA-3'	535	55
	anti-sense: 5'-TTGGCATAGAGGTCTTTA-3'		
Human C3G	sense: 5'-CCAGGTCTCAAAGGCAGAAG-3'	297	57
	anti-sense: 5'-CTTCTGAGTTCACGCCTTCC-3'		

Note C3G shRNA: C3G small hairpin RNA interference recombinant lentivirus; NT shRNA: non-target small hairpin RNA interference recombinant lentivirus.