

Article

G3BP2 is involved in isoproterenol-induced cardiac hypertrophy through activating the NF- κ B signaling pathway

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Abstract

The RasGAP SH3 domain-binding proteins (G3BPs) are a family of RNA-binding proteins that can co-ordinate signal transduction and post-transcriptional gene regulation. G3BPs have been shown to be involved in mediating a great diversity of cellular processes such as cell survival, growth, proliferation and apoptosis. But the potential roles of G3BPs in the pathogenesis and progression of cardiovascular diseases remain to be clarified. In the present study, we provide the first evidence that suggests the participation of G3BP2 in cardiac hypertrophy. In cultured neonatal rat cardiomyocytes (NRCMs), treatment with isoproterenol (ISO, 0.1–100 μ mol/L) significantly elevated the mRNA and protein levels of G3BP2. Similar results were observed in the hearts of rats subjected to 7D-injection of ISO, accompanied by obvious heart hypertrophy and elevated the expression of hypertrophy marker genes ANF, BNP and β -MHC in heart tissues. Overexpression of G3BP2 in NRCMs led to hypertrophic responses evidenced by increased cellular surface area and the expression of hypertrophy marker genes, whereas knockdown of G3BP2 significantly attenuated ISO-induced hypertrophy of NRCMs. We further showed that G3BP2 directly interacted with I κ B α and promoted the aggregation of the NF- κ B subunit p65 in the nucleus and increased NF- κ B-dependent transcriptional activity. NF- κ B inhibition with PDTC (50 μ mol/L) or p65 knockdown significantly decreased the hypertrophic responses in NRCMs induced by ISO or G3BP2 overexpression. These results give new insight into the functions of G3BP2 and may help further elucidate the molecular mechanisms underlying cardiac hypertrophy.

Keywords: G3BP2; cardiac hypertrophy; neonatal rat cardiomyocytes; isoproterenol; PDTC; NF- κ B; I κ B α

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Introduction

Cardiac hypertrophy occurs as a response of the heart to various stresses and is characterized by the enlargement of cardiomyocytes, an increase in protein synthesis, and the reactivation of fetal genes. Hypertrophy may initially be adaptive and help preserve systolic function, but sustained pathological hypertrophy will lead to a decompensated phase with deleterious consequences^[1, 2]. Epidemiological studies have revealed that cardiac hypertrophy is an independent risk factor for cardiac dysfunction and sudden death^[3–5]. However, despite the great strides that have been made over the past decades in elucidating the pathogenesis of cardiac hypertrophy, the signaling pathways associated with this complex clinical syndrome are far from fully understood.

The RasGAP SH3 domain binding proteins (G3BPs) are a

highly conserved family of multi-functional RNA binding proteins. In recent years, accumulating evidence has indicated that G3BPs play predominant roles in controlling critical cellular processes that include growth, proliferation and apoptosis^[6–8]. The G3BP family includes two members in mammals, *ie*, G3BP1 and G3BP2. G3BP1 knockout mice exhibit growth retardation, embryonic lethality and increased apoptotic cell death^[9, 10]. In contrast, the overexpression of G3BP1 has been shown to induce S-phase entry with elevated cell proliferation^[11, 12]. The G3BP1 protein is upregulated in a diversity of tumors and may present a novel target for cancer therapy^[13, 14]. Thus far, the mechanisms by which G3BP1 executes its functions remains obscure, but previous studies have implicated G3BP1 in the modulation of Ras signaling, RNA metabolism and the ubiquitin proteasome pathway^[6, 15, 16]. As a close relative of G3BP1, G3BP2 has similar domain structures comprising the NTF2-like domain and two RNA-binding motifs. Similar to G3BP1, G3BP2 is ubiquitously expressed and also contributes to the formation of stress granules

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(SGs), which are translationally silent sites for mRNA sorting^[16, 17]. Nevertheless, a subtle distinction between these two G3BP isoforms is found in the number of proline-rich (PxxP) motifs in the central region of the proteins. PxxP motifs represent the minimal SH3 domain-binding consensus sequences. Because SH3 domain-containing proteins are pivotal signal transducers, the variability in PxxP regions suggests that G3BP1 and G3BP2 may associate with different partners to produce distinct cellular outcomes^[6, 18]. To date, G3BP2 is relatively poorly characterized compared with its counterpart G3BP1, and the role of G3BP2 in the cardiovascular system has not been documented. In this study, we reveal the first evidence that G3BP2 is involved in the cardiac hypertrophy induced by isoproterenol (ISO), which may provide additional insight into the potential functions of G3BP2.

Materials and methods

Primary cell cultures

Primary cell cultures of neonatal rat cardiomyocytes (NRCMs) were produced according to the methods of our previous report^[19]. Cardiomyocytes were isolated and purified from the hearts of 1- to 3-day-old Sprague-Dawley (SD) rats. Next, the cells were seeded into 6-well plates at a density of 1×10^6 cells per well with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.1 mmol/L 5-bromodeoxyuridine. Before treatment, the cells were serum-starved overnight in DMEM with 1% FBS. Subsequently, ISO (Sigma, St Louis, MO, USA) was added to the culture medium to a final concentration of 1 $\mu\text{mol/L}$, and the cells were further incubated for the indicated duration. In the control group, the cells were supplemented with the same volume of DMEM without ISO.

Animal model, echocardiography and morphometric measures

All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 85-23, revised 1996). This research was approved by the Research Ethics Committee of Sun Yat-sen University. SD rats (male, weighing 200–240 g, SPF grade, Certification No 44008500012063) were supplied by the Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). The animals were submitted to subcutaneous (sc) injections of ISO ($1.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, for 7 d). The rats in the control group received normal saline (NS). Two-dimensionally guided M-mode echocardiography was conducted using a Technos MPX ultrasound system (Esaote, Genoa, Italy) as previously described^[20]. After echocardiographic assessment, the rats were sacrificed via exposure to CO_2 . The hearts were rapidly excised. The heart weight (HW) and left ventricle weight (LVW) are expressed as the ratios relative to body weight (BW), and the HW to tibia length (TL) ratio was also calculated. For morphometric measurements, histological cross sections (5 μm thick) from perfuse-fixed hearts were treated with hematoxylin-eosin (HE) staining.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cultured NRCMs or rat cardiac tissues with Trizol reagent (Invitrogen, Carlsbad, CA, USA) in accordance to the manufacturer's instructions. Afterwards, 1 μg of total RNA was quantitatively and reversely transcribed to first strand cDNA using Two-step RT kit (Thermo Fisher Scientific, Rockford, IL, USA). Polymerase chain reaction analyses were used to determine the mRNA levels of the target genes with SYBR-Green Quantitative PCR kits (Toyobo Life Science, Osaka, Japan) and an iCycler iQ system (Bio-Rad, Hercules, CA, USA). Rat-specific primers for G3BP2, atrial natriuretic factor (ANF), brain natriuretic polypeptide (BNP), myosin heavy chain β (β -MHC), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized by Sangon Biotech (Shanghai, China) as listed in Supplementary Material Table S1. GAPDH served as an endogenous control.

Western blot and co-immunoprecipitation (co-IP)

The primary antibodies against G3BP1 (diluted 1:2000) and G3BP2 (diluted 1:2000) were produced by Sigma (St Louis, MO, USA). The primary antibodies against ANF (diluted 1:200), BNP (diluted 1:1000), β -MHC (diluted 1:10000), p65 (diluted 1:1000), p-p65 (diluted 1:1000) and I κ B α (diluted 1:1000) were purchased from Cell Signaling Technology (Beverly, MA, USA). The antibodies against GAPDH (diluted 1:5000) and Lamin B1 (diluted 1:1000) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Nuclear protein was isolated using CellLytic NuCLEAR Extraction Kits (Sigma, St Louis, MO, USA). Western blot analyses were performed following previously described protocol^[21]. The protein band intensities were measured with LabWorks software (Bio-Rad, Hercules, CA, USA). For the co-IP assays, 200 μg of total protein was incubated overnight with 1 μg anti-I κ B α antibody and further treated with protein G-agarose beads (Pierce, Rockford, IL, USA) for 4 h at 4°C. The immunoprecipitates were detected by Western blot.

Plasmid transfection and RNA interference

The NRCMs were transiently transfected with FLAG-tagged G3BP2 or an empty vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Three different small interference RNAs (siRNAs) for G3BP2 and negative control siRNAs were purchased from GenePharma (Shanghai, China). The sequences are provided in Supplementary Material Table S2. RNA interference was conducted using Lipofectamine 2000. At 48 h after transfection, the cells were harvested for RNA or protein extraction to compare the silencing efficacies (Figure S1).

Measurement of the cell surface area

NRCMs in 48-well plates were fixed with 4% paraformaldehyde for 15 min at room temperature. The cells were further incubated for 30 min with 0.1% rhodamine-phalloidin (Invitrogen, Carlsbad, CA, USA) and washed three times with phosphate-buffered saline (PBS). The coverslips were mounted in Prolong

Gold anti-fade reagent with 4', 6-diamidino-2-phenylindole (DAPI, Cell Signaling Technology, MA, USA) and inspected with a High Content Screening system (Thermo Fisher Scientific, Rockford, IL, USA). Cells from randomly selected fields (50 for each group) were examined, and the surface areas were measured using built-in image analysis software.

Immunofluorescence (IF) assay

The NRCMs grown on coverslips were fixed in 4% paraformaldehyde and permeabilized using 0.3% Triton X-100. After three washes with PBS, the cells were blocked with normal goat serum at room temperature for 1 h. Primary p65 antibody (diluted 1:50, Cell Signaling Technology, MA, USA) was applied for 1 h and followed by incubation with Alexa Fluor 488-conjugated secondary antibody (diluted 1:500, Santa Cruz Biotechnology, CA, USA). The coverslips were mounted with DAPI. The images were captured with a confocal microscope (Zeiss, Oberkochen, Germany).

Dual-luciferase reporter gene assay

The NRCMs were seeded into 96-well plates at a density of 5×10^4 cells per well and then co-transfected with NF- κ B luciferase reporter plasmid pGL4.32 (containing the p65 DNA binding sequence GGGACTTTCC, 100 ng per well) and pRL-TK internal control vector (20 ng per well). After 6 h of incubation, the cells were serum-deprived for 12 h and submitted to ISO treatment, RNA interference, or FLAG-G3BP2 transfection. The cells were harvested and lysed in passive lysis buffer. The luciferase activity was measured with a dual-luciferase reporter assay system (Promega, Fitchburg, MA, USA), and the activity was normalized to the Renilla luciferase activity of the pRL-TK.

Statistical analysis

The data are presented as the mean \pm the standard errors of the mean (SEM). The differences between two groups were analyzed with unpaired Student's *t*-tests. The statistical analyses among multiple groups were performed with one-way ANOVAs with Tukey's *post hoc* tests. In all cases, the differences were considered statistically significant at $P < 0.05$.

Results

G3BP2 expression in neonatal rat cardiomyocytes treated with isoproterenol

ISO, a nonselective β -adrenergic receptor (β -AR) agonist, has been widely used to induce cardiac hypertrophy^[22, 23]. Our data revealed that the mRNA expression of G3BP2 in cardiomyocytes was elevated in a dose- and time-dependent manner following ISO treatment (Figure 1A, 1B). ISO stimulation also led to an evident increase in the G3BP2 protein level (Figure 1C, 1D).

G3BP2 expression in ISO-induced cardiac hypertrophy in rats

To further investigate the influence of ISO on G3BP2 expression *in vivo*, SD rats were subjected to ISO injections (1.5 mg $\text{kg}^{-1} \text{d}^{-1}$, sc) for 7 d. The hearts of the ISO-treated rats were

enlarged compared with those from the control group that was treated with normal saline (NS) and exhibited representative hypertrophic pathological changes as shown by HE staining (Figure 2A-C). Echocardiography indicated that the left ventricular anterior wall thickness (LVAW), left ventricular posterior wall thickness (LVPW), fractional shortening (FS) and ejection fraction (EF) were increased, whereas the left ventricular internal diameter (LVID) was decreased in the ISO group (Figure 2D and Supplementary Material Table S3). Additionally, the heart weight-to-body weight (HW/BW) ratio and the left ventricle weight-to-body weight (LVW/BW) ratio were both increased after ISO treatment (Figure 2E). The heart weight/tibia length (HW/TL) ratio was also elevated following ISO treatment (Supplementary Material Figure S1). Additionally, the protein levels of hypertrophic markers, including ANF, BNP and β -MHC, were notably upregulated (Figure 2G). The above results demonstrated that the rat model of cardiac hypertrophy was successfully established. Furthermore, the qRT-PCR and Western blot analyses demonstrated that the expression of G3BP2 was elevated in the cardiac tissues of the ISO-injected rats (Figure 2F and 2G).

G3BP2 knockdown attenuated ISO-stimulated hypertrophy in neonatal rat cardiomyocytes

In this study, ISO treatment resulted in a remarkable upregulation of G3BP2 expression in cultured cardiomyocytes and in rat heart tissues. Subsequently, we employed the approach of RNA interference to explore the role of G3BP2 in hypertrophy stimulated by ISO. Cultured cardiomyocytes were transfected with three different siRNAs termed si-1, si-2, and si-3. Among these siRNAs, si-2 was the most effective for G3BP2 knockdown (Supplementary Material Figure S2). The cell surface areas and levels of ANF, BNP and β -MHC were measured (Figure 3A-3C). The results revealed that the downregulation of G3BP2 with si-2 (siG3BP2) blocked the increase in cell surface area and the expressions of hypertrophic biomarkers triggered by ISO incubation (1 $\mu\text{mol/L}$ for 12 h). In contrast, the overexpression of G3BP2 led to obvious hypertrophic responses in the NRCMs as implied by enlarged cell surface areas and increased levels of hypertrophic biomarkers (Figure 3D-3F).

NF- κ B inhibition ameliorated the cardiomyocyte hypertrophy induced by ISO or G3BP2 overexpression

NF- κ B activation provokes the hypertrophic growth of cardiomyocytes^[24, 25]. To investigate whether NF- κ B signaling is involved in the pro-hypertrophic effects of G3BP2, pyrrolidine dithiocarbamate (PDTC), which is a well-established inhibitor of NF- κ B activity, was added to the NRCMs at a final concentration of 50 $\mu\text{mol/L}$ ^[26, 27]. As illustrated in Figure 4A-4C, both ISO stimulation and G3BP2 overexpression led to obvious increases in cell surface area and the expressions of ANF, BNP and β -MHC, and these increases were suppressed by PDTC. Additionally, PDTC attenuated the nuclear aggregation of p65 and inhibited the NF- κ B-dependent transcriptional activity induced by ISO or G3BP2 overexpression (Figure 4D-4F). Moreover, the elevations of the mRNA levels of the hypertro-

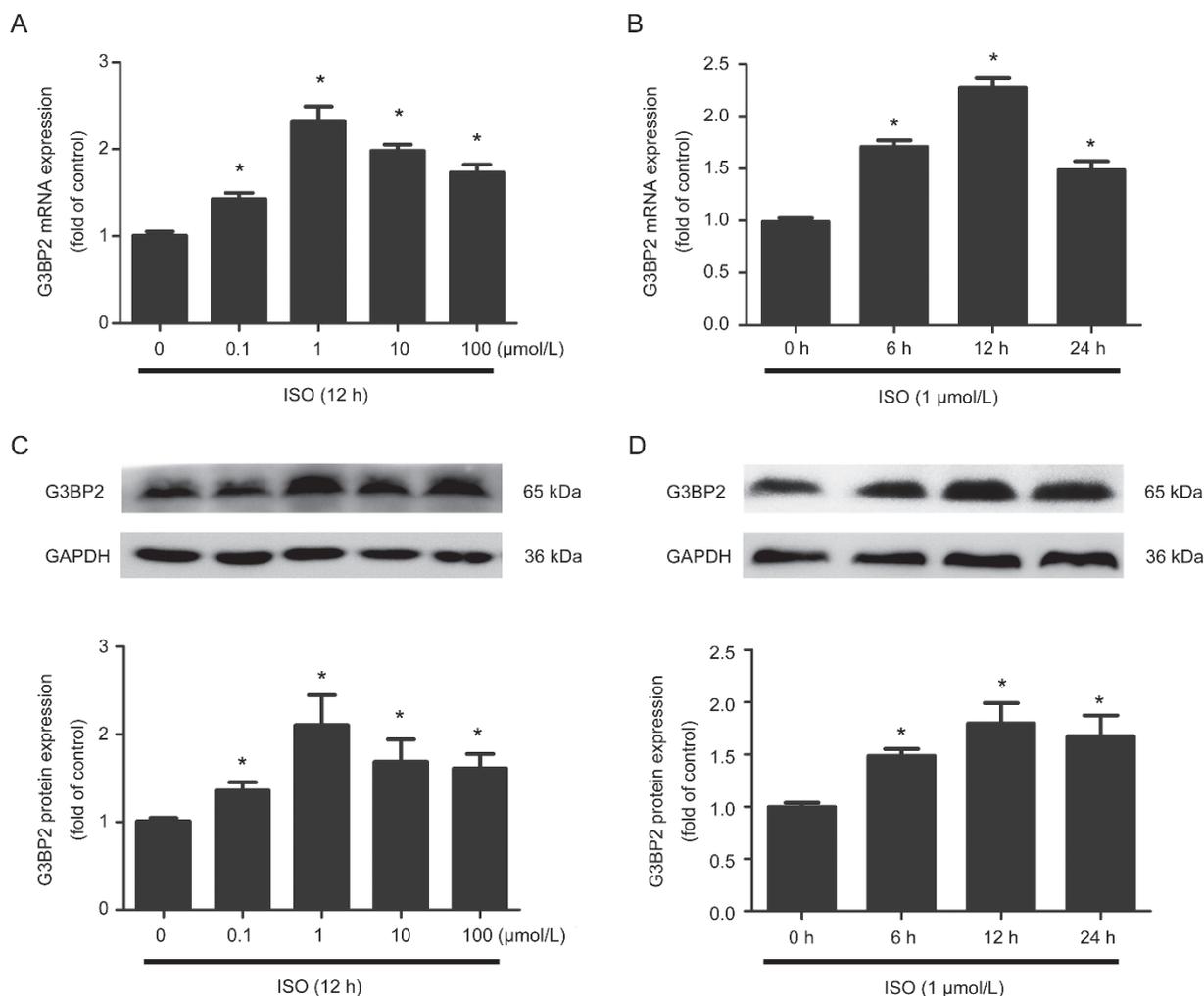


Figure 1. G3BP2 expression was increased in neonatal rat cardiomyocytes following ISO treatment. NRCMs were incubated with various concentrations of ISO for 12 h or with 1 $\mu\text{mol/L}$ ISO for the indicated durations. (A and B) The mRNA levels of G3BP2 were determined by qRT-PCR. (C and D) The protein expression of G3BP2 was measured by Western blot. The results were normalized (by GAPDH) and are presented as the fold-changes relative to the control levels. The data are presented as the mean \pm SEM. * P <0.05 vs control. $n=3$.

phic marker genes were ameliorated by p65 knockdown with siRNA (Figure 4G).

G3BP2 interacted with I κ B α and promoted the nuclear localization of NF- κ B subunit p65 in cardiomyocytes

A previous study showed that in HeLa cells, G3BP2 can combine with I κ B α , which presumably contributes to the activation of the NF- κ B signal transduction cascade^[28]. Consistently, the immunoprecipitation assay revealed a direct interaction between I κ B α and G3BP2 in the NRCMs, and this interaction was strengthened by ISO treatment (Figure 5A). Moreover, the Western blot results showed that ISO stimulation facilitated the nuclear aggregation of NF- κ B subunit p65, and this facilitation was partially inhibited by transfection with G3BP2 siRNA (Figure 5B). The intracellular distribution of p65 in cardiomyocytes was also authenticated with IF assays. As indicated by Figure 5C, p65 was distributed in both the

cytoplasm and the nucleus without stimulation. ISO induced its nuclear translocation, and this process was attenuated by G3BP2 knockdown. In contrast, the overexpression of G3BP2 increased the localization of p65 in the nucleus (Figure 5D, 5E).

Subsequently, the effects of G3BP2 on NF- κ B-related transcriptional activity were evaluated with a dual luciferase reporter gene assay. G3BP2 overexpression gave rise to NF- κ B-Luc reporter gene activity that was similar to the effect of ISO treatment. In contrast, the knockdown of G3BP2 repressed the up-regulation of NF- κ B-Luc reporter gene activity (Figure 5F and 5G). Taken together, these findings suggested that G3BP2 might participate in ISO-induced cardiomyocyte hypertrophy by enhancing NF- κ B signaling.

Discussion

The G3BP family of proteins has been postulated to link signal transduction with RNA metabolism to maintain

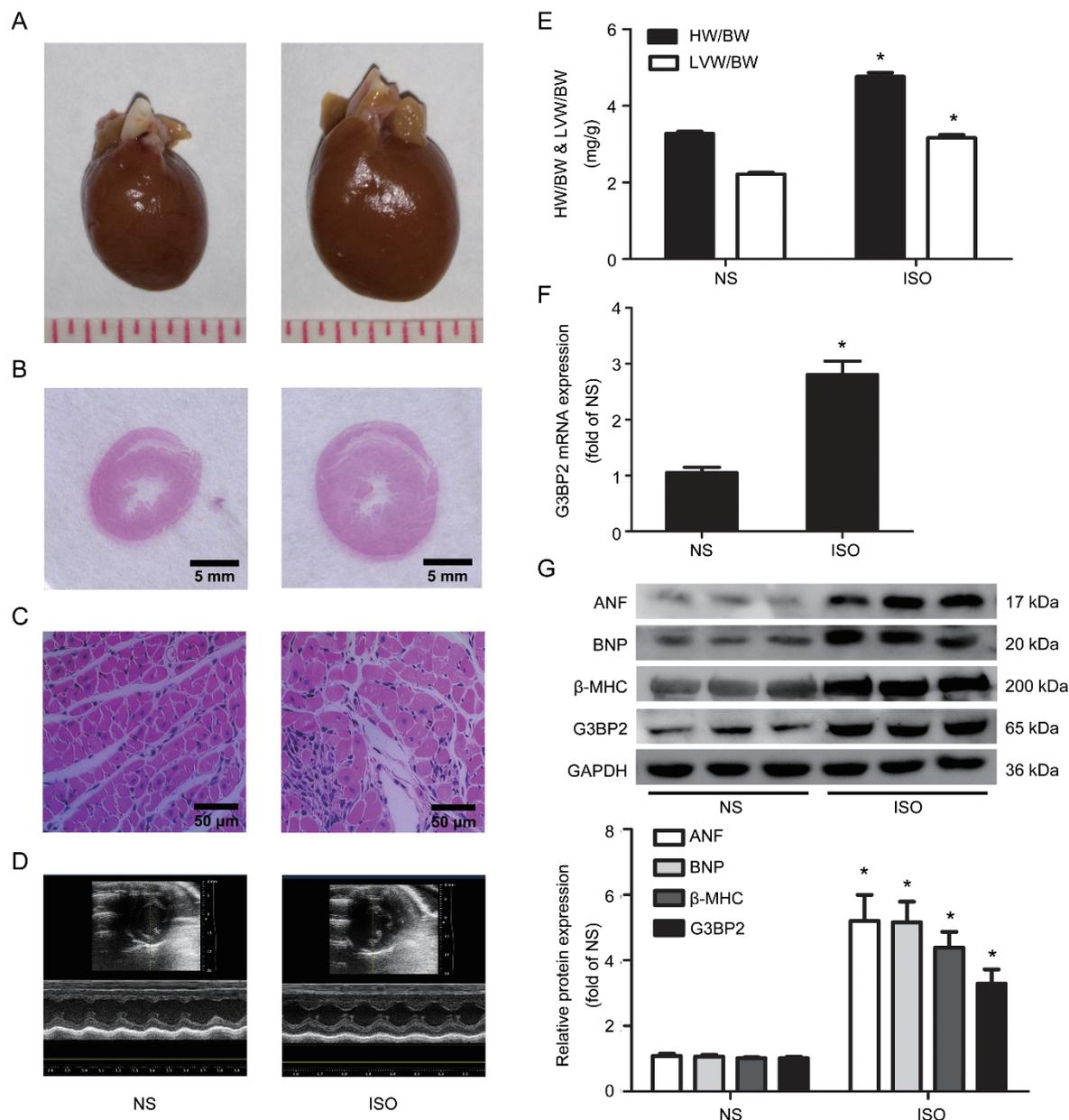


Figure 2. ISO treatment resulted in cardiac hypertrophy accompanied by elevated G3BP2 expression in SD rats. SD rats were subcutaneously injected with ISO ($1.5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) for 7 d. The control animals received normal saline (NS). (A–C) Pathologic changes in the hearts were observed by H&E staining and (D) echocardiography. (E) The HW/BW and LVW/BW ratios were calculated. (F) G3BP2 mRNA expression in heart tissues was detected by qRT-PCR. (G) The protein levels of G3BP2 and hypertrophic markers, including ANF, BNP and β -MHC, were measured by Western blot. The data are presented as the mean \pm SEM. * $P < 0.05$ vs NS group. $n = 8$.

cell survival and homeostasis^[7]. In cardiomyocytes, it has been demonstrated that G3BP1 binds to and stabilizes the mRNA encoding Cdk7, which leads to elevated Cdk7 protein expression and cell growth^[29]. Additionally, G3BP1 mediates the decline of the mature miRNA-1 level, which is necessary for the increase in protein synthesis during cardiac hypertrophy^[30]. However, the potential functions of G3BP2 in the cardiovascular system remain to be determined. Here, we observed that G3BP2 expression was increased in ISO-

treated neonatal rat cardiomyocytes (Figure 1). Upregulations of G3BP2 mRNA and protein levels were also identified in the cardiac tissues of SD rats that received ISO injections (Figure 2). Furthermore, the overexpression of G3BP2 led to cardiomyocyte hypertrophy, but G3BP2 RNA interference significantly attenuated the hypertrophic responses stimulated by ISO (Figure 3). To our knowledge, these data provide previously unreported evidence indicating the participation of G3BP2 in ISO-induced cardiac hypertrophy.

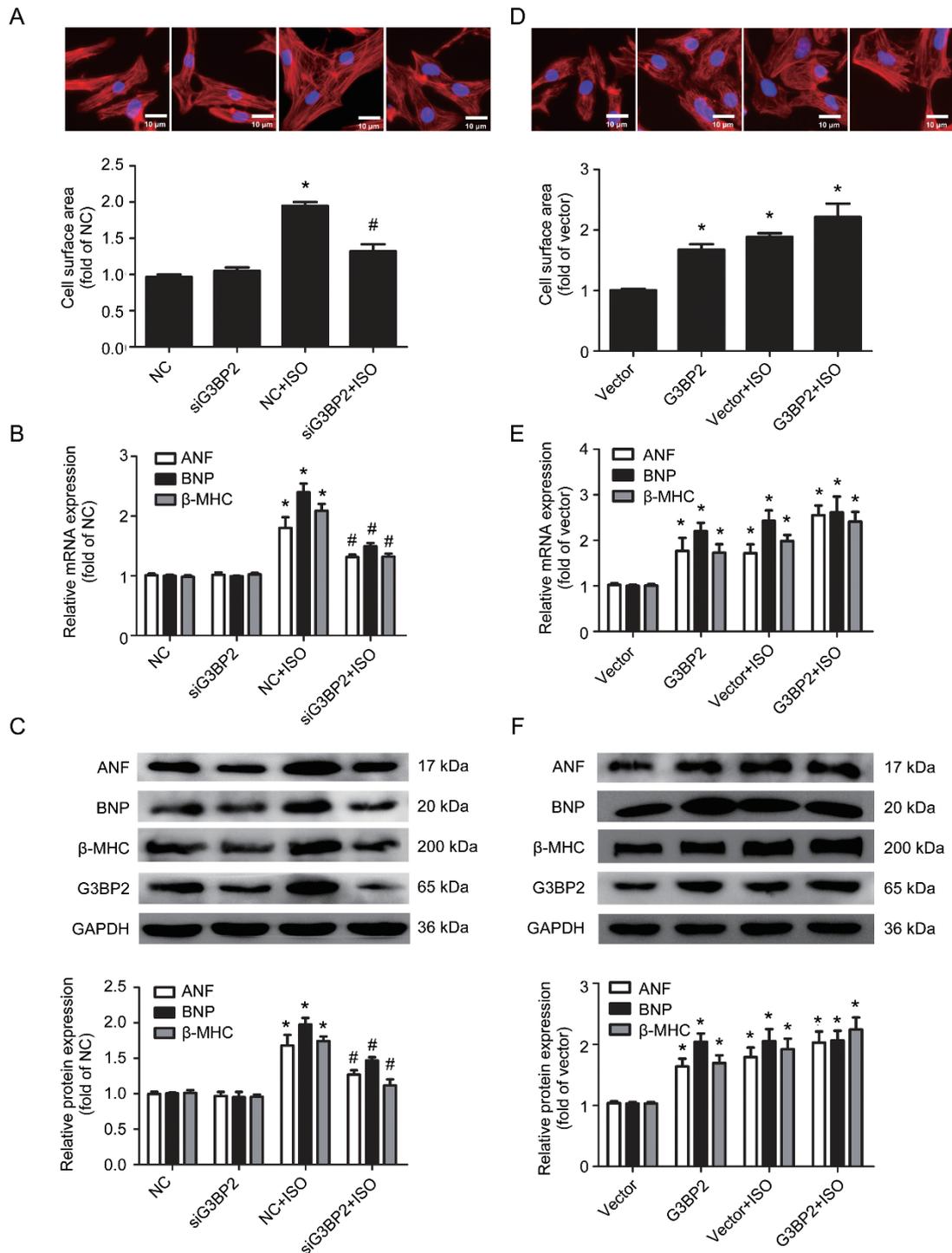


Figure 3. G3BP2 knockdown attenuated ISO-induced hypertrophy, and G3BP2 overexpression led to hypertrophic responses in cardiomyocytes. NRCMs were treated with G3BP2 siRNA (si-G3BP2) or negative control (NC) for 48 h and further incubated with or without 1 μ mol/L ISO for 12 h. (A) The cell surface area was measured with rhodamine-phalloidin staining using a 200 \times objective. (B and C) The mRNA and protein levels of ANF, BNP and β -MHC were detected by qRT-PCR and Western blot, respectively. (D–F) The NRCMs were transfected with G3BP2 plasmid or empty vector for 24 h and subsequently treated with 1 μ mol/L ISO for 12 h. The hypertrophic responses of the cells were determined by measuring the cell surface area and the expressions of the hypertrophic markers. Mean \pm SEM. * P <0.05 vs NC or vector group. # P <0.05 vs NC plus ISO group. n =3.

G3BP1 and G3BP2 are encoded by distinct genes on chromosomes. Structural analysis has revealed that their protein

sequences are 60% identical^[31]. Despite this high homology at the protein level, recent research has shown that the func-

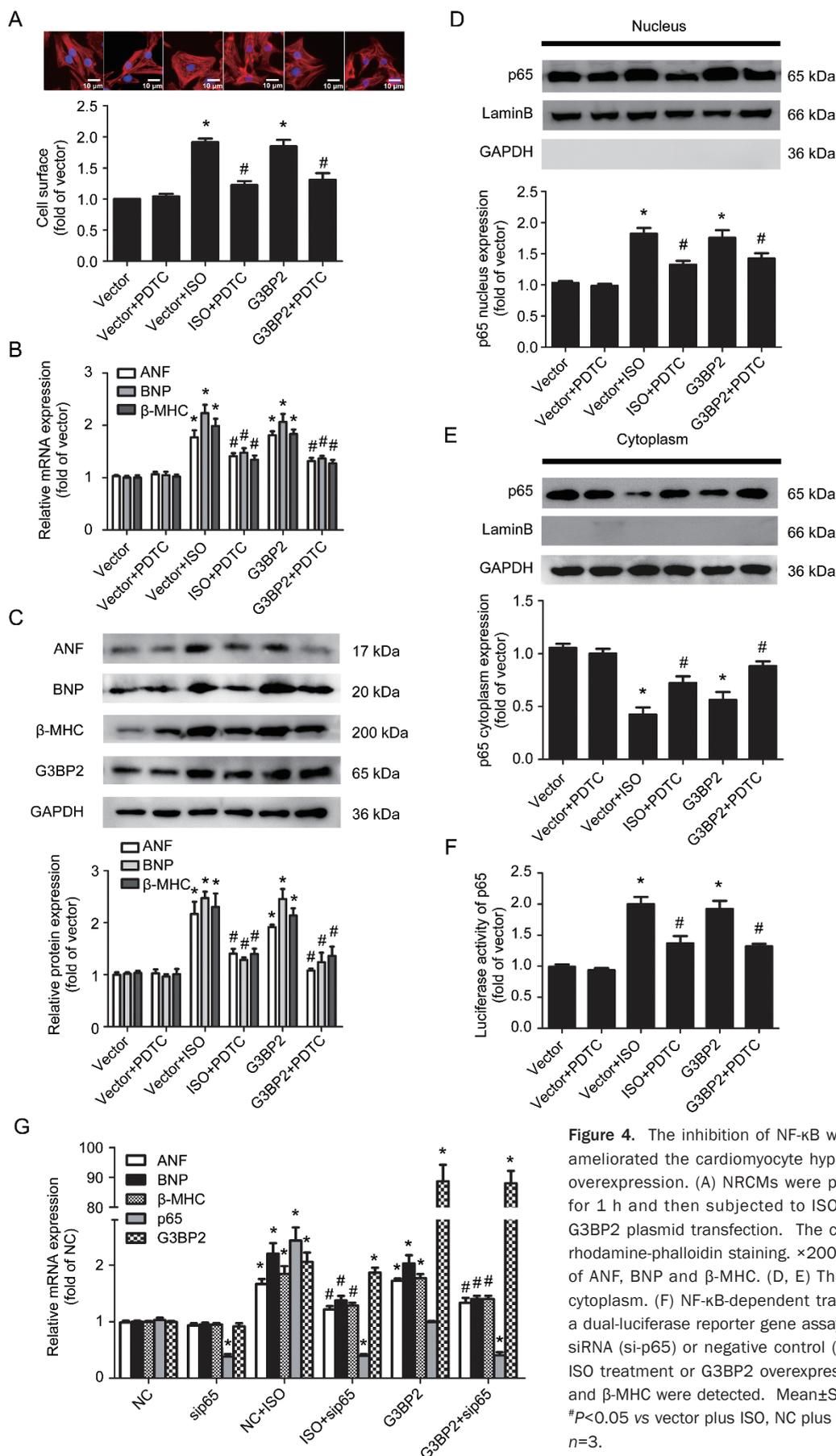


Figure 4. The inhibition of NF- κ B with both PDTC and RNA interference ameliorated the cardiomyocyte hypertrophy triggered by ISO or G3BP2 overexpression. (A) NRCMs were pre-incubated with 50 μ mol/L PDTC for 1 h and then subjected to ISO treatment (1 μ mol/L for 12 h) or G3BP2 plasmid transfection. The cell surface area was measured with rhodamine-phalloidin staining. $\times 200$. (B, C) The mRNA and protein levels of ANF, BNP and β -MHC. (D, E) The levels of p65 in the nucleus and cytoplasm. (F) NF- κ B-dependent transcriptional activity was tested with a dual-luciferase reporter gene assay. (G) NRCMs were treated with p65 siRNA (si-p65) or negative control (NC) for 48 h and then subjected to ISO treatment or G3BP2 overexpression. The mRNA levels of ANF, BNP and β -MHC were detected. Mean \pm SEM. * $P < 0.05$ vs vector or NC group. # $P < 0.05$ vs vector plus ISO, NC plus ISO, or G3BP2 overexpression group. $n = 3$.

tions of the G3BPs do not fully overlap. For example, G3BP1 exhibits phosphorylation-dependent endoribonuclease activity^[7, 8], which has not been reported for G3BP2. Moreover, Wnt-induced gene transcription is attenuated by G3BP2 knockdown, and this effect cannot be rescued by G3BP1 overexpression^[32, 33]. In the present study, we found that following ISO treatment, the mRNA and protein levels of G3BP2 were obviously elevated in the NRCMs, whereas G3BP1 expression remained intact (Supplementary Material Figure S2). Together with previous reports, these findings may elucidate the different functions that are attributed to the G3BP isoforms.

β -adrenergic receptors (β -ARs) are critical for the sympathetic modulation of cardiac performance^[34]. Treatment with ISO, which is a nonselective agonist of β -ARs, can induce cardiac hypertrophy and subsequent heart failure in experimental animals^[22, 23]. Our data revealed that ISO stimulation enhanced G3BP2 expression both *in vitro* and *in vivo* in addition to the development of hypertrophy (Figure 1 and Figure 2E and F), but the underlying mechanisms remain unclear. G3BP is an inducible gene product of human epidermal growth factor receptor 2 (HER2) signaling. G3BP expression is elevated in cells in parallel with the overexpression of HER2^[35]. A recent study showed that in response to β_2 -AR stimulation by ISO, STAT3 is activated and translocates to the nucleus where it binds to the promoter of HER2 and subsequently upregulates HER2 expression^[36]. These findings suggest the possibility that ISO may manipulate G3BP2 expression by modulating STAT3-dependent HER2 expression via β_2 -AR. Further investigations are certainly required to clarify this issue.

Mounting evidence has confirmed a prominent relationship between the NF- κ B signaling pathway and cardiac hypertrophy. The over activation of NF- κ B has been observed in hypertrophic and failing human hearts, whereas the inhibition of NF- κ B can attenuate cardiac hypertrophy^[37, 38]. Moreover, it has been demonstrated that the activation of NF- κ B is required for ISO-induced cardiac hypertrophy^[39]. In line with previous studies, we observed that ISO promoted the aggregation of p65 in the nucleus and enhanced NF- κ B transcriptional activity in cultured cardiomyocytes. Similar results were also observed in G3BP2-overexpressing cells (Figure 4D–F). Treatment with the NF- κ B inhibitor PDTC attenuated the hypertrophic responses and simultaneously suppressed the nuclear localization and transcriptional activity of NF- κ B that was stimulated by ISO or G3BP2 overexpression (Figure 4). These findings suggest that G3BP2 may participate in the development of hypertrophy in a NF- κ B-dependent manner.

Although PDTC is a well-established inhibitor of NF- κ B activation that acts via preventing the phosphorylation and degradation of I κ B α ^[40, 41], some studies have shown that it may lead to off-target effects. As a chelating agent and antioxidant, PDTC induces glutathione synthesis at a concentration lower than that needed to inhibit the biological actions of NF- κ B^[42]. Additionally, PDTC can promote stromelysin expression via the stimulation of the tyrosine kinase-AP-1 pathway in a manner that is independent of its suppression of NF- κ B^[43]. Thus, we performed further experiments using p65 knockdown by

siRNA. The results revealed that both ISO and G3BP2 overexpression-induced expression of hypertrophic marker genes were attenuated following the depletion of p65 (Figure 4G), which further indicates the involvement of NF- κ B signaling in G3BP2-regulated hypertrophy.

In response to stimulation, I κ B α is phosphorylated and subsequently degraded through the ubiquitin-proteasome proteolytic pathway. This process releases p65 to enter the nucleus and then activate the expression of a diversity of genes that are associated with cell survival and growth^[44]. Additionally, unregulated I κ B α translocates into the nucleus, dissociates p65 from DNA, and re-exports it back to the cytoplasm^[45, 46]. The N-terminal region of I κ B α contains a short cytoplasmic retention sequence (CRS) that can be recognized by G3BP2. G3BP2 has been shown to interact directly with I κ B α , and the overexpression of G3BP2 retains I κ B α in the cytoplasm^[28]. Additionally, the I κ B α -interacting domain of G3BP2 has been mapped, and the central acid-rich region of G3BP2 has been indicated to be sufficient for its binding with I κ B α and the promotion of cytoplasmic retention^[28]. These facts suggest a potential link between G3BP2 and NF- κ B signaling because G3BP2 may manipulate the nucleocytoplasmic distribution of I κ B α and thereby influence NF- κ B activity. Here, we found that G3BP2 co-immunoprecipitated with I κ B α , and the association was enhanced by ISO treatment (Figure 5A). G3BP2 overexpression resulted in increased aggregation of p65 in the nucleus accompanied by elevated NF- κ B transcriptional activity. The increased nuclear entry of p65 and NF- κ B activation following ISO stimulation was attenuated by G3BP2 knockdown (Figure 5B and 5C). Although quite preliminary, these results may suggest the possibility that G3BP2 allows NF- κ B to remain active in the nucleus presumably by interacting with I κ B α and retaining it in the cytoplasm. Intriguingly, we also observed that G3BP2 overexpression facilitated p65 phosphorylation and simultaneously downregulated the protein level of I κ B α (Supplementary Material Figure S5), which supports its multiple activation effects on NF- κ B signaling. Nevertheless, the underlying mechanisms still require elucidation.

Despite the evidence presented here concerning the potential connection between G3BP2 and cardiac hypertrophy, there are some obvious limitations to our studies. First, the participation of G3BP2 in the pathogenesis of cardiac hypertrophy should be further confirmed *in vivo*, for example, using rats with cardiac-specific depletion or overexpression of G3BP2. Second, whether the same conclusions can be drawn from the TAC-induced pressure overload hypertrophy animal model is unknown. Additionally, osmotic minipump-based infusion of ISO is preferable to subcutaneous injection because the former ensures constant and sustained drug delivery. Moreover, our experiments also revealed that G3BP2 expression was increased by ISO in cardiac fibroblasts (Supplementary Material Figure S6). As the most prevalent cell type in the heart, fibroblasts are known to contribute to the development of cardiac remodeling and heart failure^[47]. Therefore, the potential influence of enforced G3BP2 expression in cardiac fibroblasts should be considered.

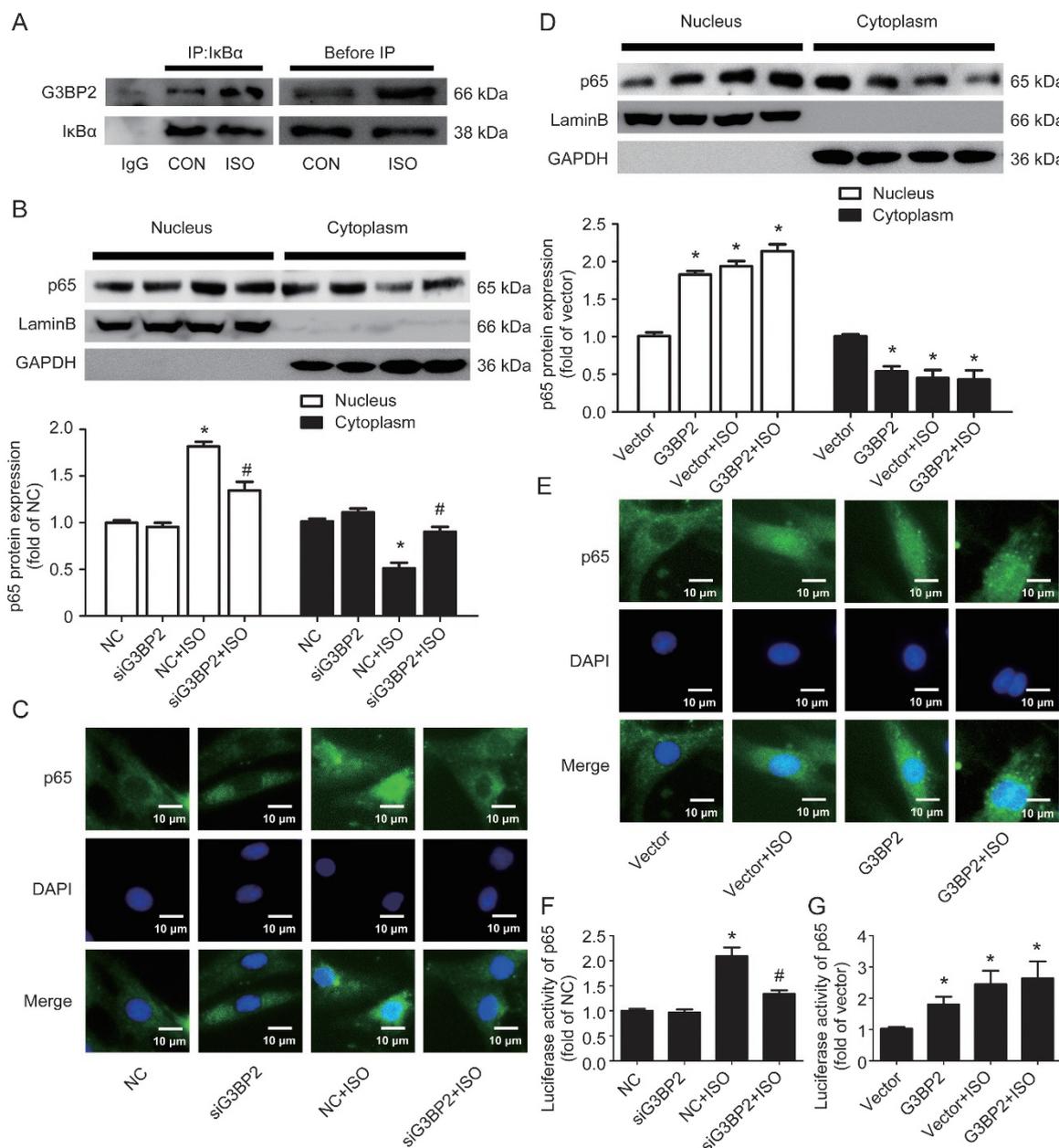


Figure 5. G3BP2 physically interacted with IκBα and increased the nuclear localization of p65. (A) A co-immunoprecipitation assay was performed to show the interaction of G3BP2 and IκBα in NRCMs following ISO incubation. (B and C) Cardiomyocytes were treated with G3BP2 siRNA (si-G3BP2) or negative control (NC) for 48 h and subsequently stimulated without 1 μmol/L ISO for 12 h. Western blotting was conducted to determine p65 protein expression in the nucleus and cytoplasm. The subcellular localization of p65 was observed by confocal immunofluorescence microscopy with a 400× objective. DAPI was used to mark the position of the nuclei. (D and E) Cells were transfected with the G3BP2 plasmid or an empty vector for 24 h and further incubated with 1 μmol/L ISO for 12 h. Western blot and immunofluorescence assays were performed to detect the distribution of p65. The images were obtained via confocal microscopy using a 400× objective. (F and G) A luciferase reporter gene assay was used to evaluate NF-κB-dependent transcriptional activity. The data are presented as the mean±SEM. **P*<0.05 vs NC or vector group. #*P*<0.05 vs NC plus ISO group. *n*=3.

In summary, our present study revealed that G3BP2 is involved in ISO-induced cardiac hypertrophy. G3BP2 expression was significantly increased in cardiomyocytes following ISO treatment and in the heart tissues of rats subjected to ISO injection. The overexpression of G3BP2 led to hypertrophic responses in cultured cardiomyocytes, and RNA

interference with G3BP2 protected the cells from hypertrophy. The pro-hypertrophic effects of G3BP2 could be partially attributed to its positive influence on NF-κB activation. These findings add new information to the limited literature regarding the biological functions of G3BP2 and will help improve the understanding of the molecular mechanisms underlying cardiac

hypertrophy.

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Author contribution

Hui-qi HONG, Jing LU and Pei-qing LIU designed and conducted the experiments and analyzed the data. Xiu-li FANG, Yu-hong ZHANG, Yi CAI, and Jing YUAN partially performed some experiments. Jian-tao YE designed the experiments, analyzed data, wrote the manuscript, and conceived of the whole project.

Supplementary information

Supplementary information is available at the website of Acta Pharmacologica Sinica.

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