

## Original Article

# Interleukin-1 receptor-associated kinase 4 overexpression attenuates angiotensin II-induced cardiomyoblast hypertrophy

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**Abstract:** Interleukin-1 receptor-associated kinase 4 (IRAK4) plays a key role in oncology diseases and inflammation. The aim of this study was to investigate the role of IRAK4 in cardiomyoblast hypertrophy induced by angiotensin II and elucidate the underlying mechanisms. In vitro studies were performed using H9c2 cardiomyoblasts with IRAK4 overexpression. Models of cardiomyoblast hypertrophy were induced by angiotensin II treatment. Cardiomyoblast hypertrophy was evaluated by immunofluorescence staining of  $\alpha$ -actin. mRNA and protein expression were detected by real-time PCR and Western Blot analyses. IRAK4 overexpression significantly attenuated cardiomyoblast hypertrophy and decreased activation of the ERK1/2, p38 and NF $\kappa$ B pathways in angiotensin II-treated H9c2 cardiomyoblasts. Moreover, IRAK4 overexpression led to down-regulated expression levels of IL-1 $\beta$ , TNF $\alpha$ , TNF receptor-associated factor 6 (TRAF6), and up-regulated expression levels of Myeloid differentiation factor 88 (MyD88) and phosphorylated TGF $\beta$ -activated kinase 1 (p-TAK1). Our results suggest that IRAK4 may serve as a new therapeutic target for treating cardiomyocyte hypertrophy, and may act as a regulator of MyD88 and Traf6.

**Keywords:** IRAK4, angiotensin II, cardiomyoblast hypertrophy

## Introduction

Cardiac hypertrophy is an important adaptive response of cardiomyocytes to a variety of mechanical stimuli such as pressure overload [1] and neurohormonal stimuli [2] like angiotensin II, endothelin-1, and adrenaline, which eventually leads to heart failure. It is known that pressure overload can activate the renin-angiotensin system (RAS) [3] and induce the release of angiotensin II (Ang II), which activates the Galpha (q) protein-coupled receptor (GPCR) signaling pathway. Therefore, Ang II-induced cardiomyocyte hypertrophy model is an in vitro model that has been broadly used [4].

Innate immunity and cardiovascular diseases are connected with each other closely [5, 6]. Toll-like receptors (TLRs) play critical roles in the innate immune systems, and also are involved in the adaptive immune systems. Take TLR4 for example, once activated, the TLRs

cluster go through a chain of changes. The downstream signalosome architecture includes Myeloid differentiation factor 88 (MyD88), TNF receptor-associated factor (TRAF) 3, TRAF6, Fas-associated protein with death domain (FADD) and Interleukin-1 receptor-associated kinase 4 (IRAK4). Some studies indicated that almost all parallel pathways of TLR4 signaling are competitive [4]. Making the roles of these signaling clear means a lot to the researches of TLRs. IRAK4, one member of Toll-like receptor 4, has been reported to play a role in several inflammatory responses [7, 8] and oncology disorders [9]. We have previously shown that IRAK4 deficiency aggravates pathological cardiac hypertrophy, cardiac dysfunction and fibrosis associated with the ERK, p38 and NF $\kappa$ B signaling cascades [10]. Here, we investigated the effect of IRAK4 overexpression in Ang II-induced cardiomyoblast hypertrophy.

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## Materials and methods

### *Recombinant overexpressed plasmid construction*

According to the instructions on the Addgene website, the pDONR2-IRAK4 plasmid (Addgene 23749) was transformed to pcDNA3.1-HA-IRAK4 plasmid to increase the IRAK4 expression in H9c2 cardiomyoblasts. Briefly, Vector NTI was used to design the special primer of IRAK4 (Forward primer: CGCGGATCCGCCACC-ATGAACAAACCCATAACACCATCAA, Reverse primer: CCGGAATTCTTAAGCGTAATCTGGAACATC-GTATGGGTAAGAAGCTGTCATCTCTTGACGC). The genetic segment of IRAK4 was expanded by RT-PCR. The augment segment of IRAK4 and plasmid pcDNA3.1 were digested with endonuclease BamH I and EcoR 1, respectively, and then connected to establish the recombinant plasmid pcDNA3.1-HA-IRAK4 (human IRAK4 overexpression eukaryotic recombinant plasmid).

### *Cell culture and transfection*

Rat heart-derived H9c2 cardiomyoblasts were cultured in the high-glucose Dulbecco's modified Eagle's medium (GIBCO, C11995) supplemented with 10% (v/v) fetal bovine serum (GIBCO, 10099), penicillin (100 U/ml) and streptomycin (100 mg/ml) (GIBCO, 15140) in humidified CO<sub>2</sub> incubator (SANYO 18 M) with 5% CO<sub>2</sub> at 37°C [11]. Cells were seeded in six-well culture plates for mRNA and protein extraction, in 24-well culture plates for cell area analysis, or in 96-well plates for CCK-8 assay.

The transfection was mediated by FuGENE<sup>®</sup> HD transfection reagent (Promega E2311). All the procedures of transfection were performed according to the manufacturer's instructions. Briefly, add 2 µg or 4 µg of plasmid DNA to a sterile tube, and vortex, for a 3:1 FuGENE<sup>®</sup> HD Transfection Reagent: DNA ratio, add 6 µl or 8 µl of FuGENE<sup>®</sup> HD Transfection Reagent directly to medium, and mix immediately. Incubate for 0-15 minutes. Add the mixture to H9c2 cardiomyoblasts in six-well plate, evaluate the transfection efficiency 36 hours later using Western Blot. Finally, 4 µg plasmid DNA was used in further experiments.

For further experiments, twelve hours after pcDNA3.1-HA-IRAK4 or pcDNA3.1-HA plasmid

transfection, the medium was replaced with serum-free medium, and the cells were cultured for another 24 hours. Then each group was treated with Ang II or not, respectively.

### *Cell viability*

Cell viability was analyzed using the Cell Counting Kit-8 (CCK-8) assay. 0.08 µg plasmid DNA: 0.24 µl FuGENE<sup>®</sup> HD Transfection Reagent or 0.16 µg plasmid DNA: 0.48 µl FuGENE<sup>®</sup> HD Transfection Reagent per well were added to H9c2 cardiomyoblasts in 96-well plate. Following pcDNA3.1-HA-IRAK4 or pcDNA3.1-HA plasmid transfection for 48 hours, 10 µl CCK-8 solution was added to each well of the 96-well plate and then incubated for an additional 7 hours. Absorbance was measured at 450 nm using a microplate reader (Synergy HT; BioTek, Winooski, VT, USA). The percentage of cell viability was calculated according to the following formula: Cell viability (%) = optical density (OD) of the treatment group/OD of the control group × 100%.

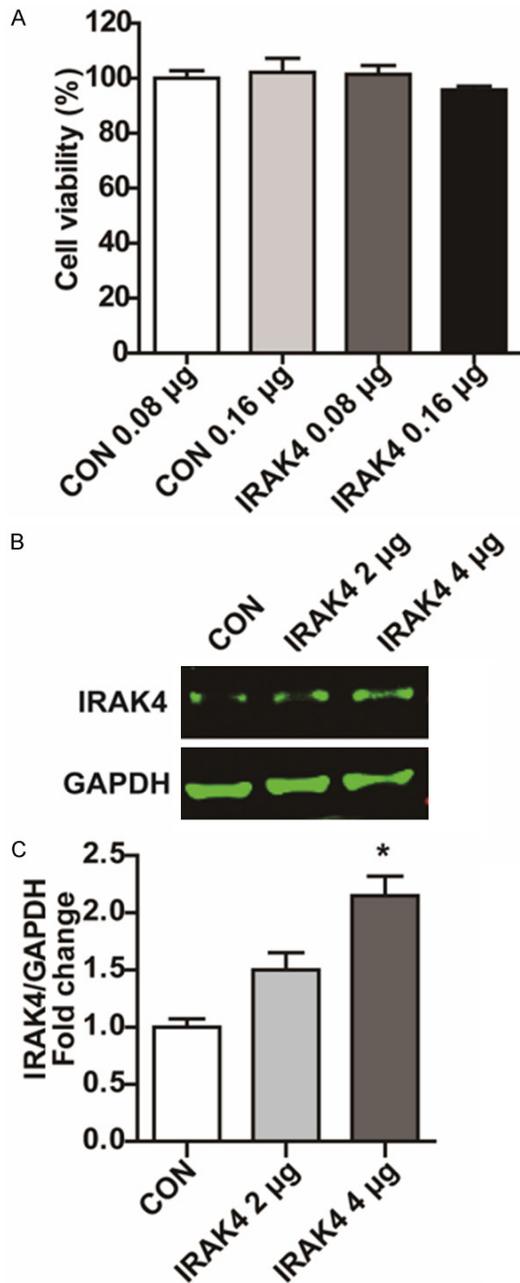
### *Cell area analysis*

1.6 µg plasmid DNA: 4.8 µl FuGENE<sup>®</sup> HD Transfection Reagent per well were added to H9c2 cardiomyoblasts in 24-well plate. To assess cell area, cells were stained by immunofluorescence for cardiac α-actin (Abcam, ab7817). The cells were washed with phosphate-buffered saline (PBS), fixed with RCL2 fixing liquid and permeabilized in 0.1% Triton X100 in PBS. The cells were stained with anti-α-actin at a dilution of 1:100 in 1% goat serum overnight at 4°C, and then incubated with Alexa Fluor<sup>®</sup> 488 goat anti-mouse IgG for 1 hour at 37°C. Cells on the coverslips were mounted onto glass slides with SlowFade Gold antifade reagent with DAPI and the cell areas were measured using a quantitative digital image analysis system (Image Pro-Plus version 6.0; Media Cybernetics, Inc., Rockville, MD, USA).

### *Quantitative real-time RT-PCR*

The mRNA expression levels of hypertrophic marker atrial natriuretic peptide (ANP) was detected by RT-PCR. Total RNA was extracted using Tripure Isolation Reagent (Roche, 116-67165001), and the yield and purity of the samples were spectrophotometrically estimated using the A260/A280 and A260/A230 ratios

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**Figure 1.** Measurement of IRAK4 Expression in H9c2 Cardiomyoblasts by Western Blot. A. Effect of plasmid transfection on cell viability, as determined by the CCK-8 assay. B and C. Western blot analysis of IRAK4 expression in H9c2 cardiomyoblasts in the indicated groups. No significant differences between CON and IRAK4 2 µg group or between IRAK4 2 µg and 4 µg group. \* $P < 0.05$  vs control (CON) group (empty vector pcDNA3.1-transfected H9c2 cardiomyoblasts).

via a SmartSpec Plus Spectrophotometer (Bio-Rad). cDNA was synthesized from 2 µg RNA of each sample using the Transcripto First Strand cDNA Synthesis Kit (Roche, 04896966001).

The PCR amplifications were quantified using LightCycler 480 SYBR Green 1 Master Mix (Roche, 04707516001) and the result was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression.

### Western blot

Samples were lysed in RIPA lysis buffer, and the total protein concentration was detected using BCA protein assay kit (Thermo, 23227) by ELISA (Synergy HT, Bio-tek). The cell lysate (50 µg) was used for SDS/transfer membranes (Millipore, IPFL00010). The primary antibodies included antibodies specific for GAPDH (Cell Signaling Technology, 2118), IRAK4 (Cell Signaling Technology, 4363), phosphorylated (p)-extracellular signal-related kinase 1/2 (ERK1/2) (Cell Signaling Technology, 4370), total (T)-ERK1/2 (Cell Signaling Technology, 4695), p-p38 (Cell Signaling Technology, 4511), T-p38 (Cell Signaling Technology, 9212), p-NFκBp65 (Bioworld, BS4135), NFκBp65 (Cell signaling Technology, 8242), IL-1β (R&D, AF-401-NA), TNFα (Cell signaling Technology, 11948P), p-TGFβ-activated kinase 1 (TAK1) (Cell signaling Technology, 4508), T-TAK1 (Cell signaling Technology, 5206), MyD88 (Cell signaling Technology, 4283), and Traf6 (Santa Cruz Biotechnology, sc-7221). The secondary antibody was goat anti-rabbit (LI-COR, 926-32211) IgG. The blots were scanned by a two-color infrared imaging system (Odyssey, LICOR). Specific protein expression levels were normalized to GAPDH protein.

### Statistical analysis

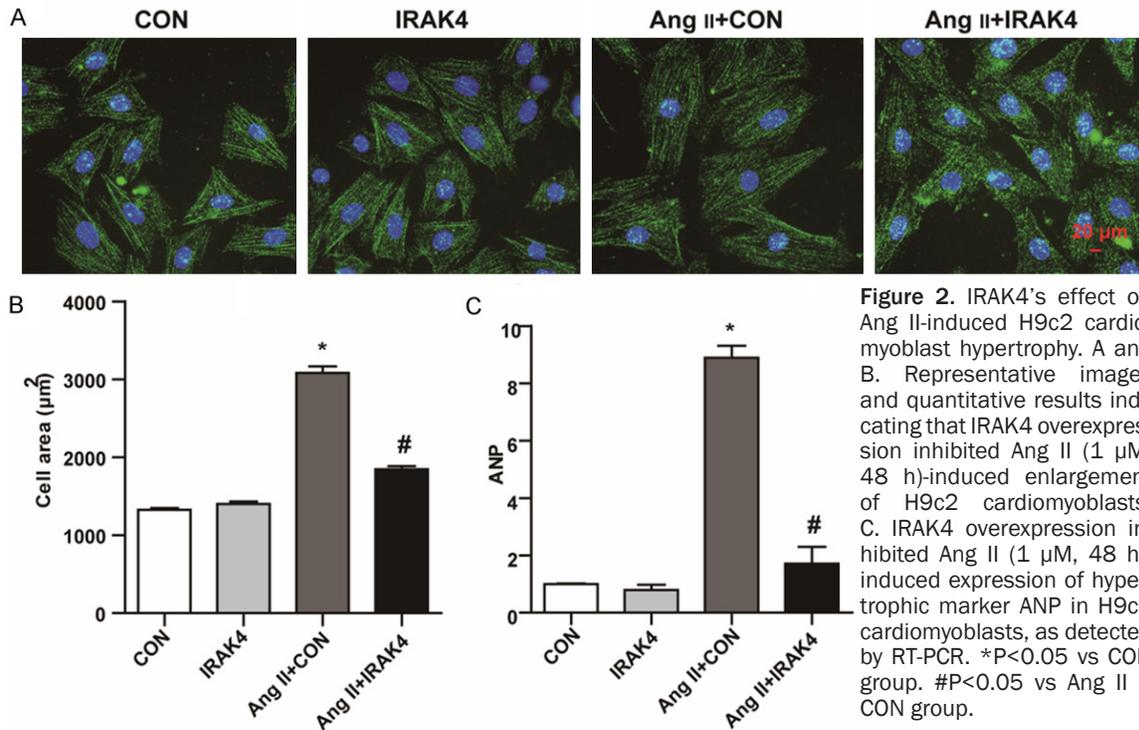
Data are presented as the means ± SEM. Statistical analysis was performed using SPSS 13.0 (SPSS, Inc) software. Differences among the groups were determined by two-way ANOVA followed by Tukey's multiple-comparison test. Student's t-tests were used to compare means between the two groups.  $P < 0.05$  was considered to be significantly different.

## Results

### Plasmid transfection exhibits no significant effect on cell viability

The potential cytotoxicity of plasmid transfection was analyzed using CCK-8 assay. H9c2 cells were transfected with two concentrations of pcDNA3.1-HA-IRAK4 plasmids for 36 hours.

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**Figure 2.** IRAK4's effect on Ang II-induced H9c2 cardiomyoblast hypertrophy. A and B. Representative images and quantitative results indicating that IRAK4 overexpression inhibited Ang II (1 µM, 48 h)-induced enlargement of H9c2 cardiomyoblasts. C. IRAK4 overexpression inhibited Ang II (1 µM, 48 h)-induced expression of hypertrophic marker ANP in H9c2 cardiomyoblasts, as detected by RT-PCR. \* $P < 0.05$  vs CON group. # $P < 0.05$  vs Ang II + CON group.

Cell viability exhibited no significant differences in the indicated groups, as shown in **Figure 1A**.

### Measurement of IRAK4 expression in H9c2 cardiomyoblasts by Western blot

The results showed that a small amount of endogenous IRAK4 was expressed in empty vector pcDNA3.1-transfected H9c2 cardiomyoblasts. However, in H9c2 cardiomyoblasts that had been transfected with the IRAK4 recombinant expression vector pcDNA3.1-IRAK4 (4 µg) in six-well culture plates, the expression of IRAK4 was significantly increased ( $P < 0.01$ ) (**Figure 1B** and **1C**). So, we chose 4 µg for transfection in H9c2 cardiomyoblasts.

### Measurement of IRAK4's effect on Ang II-induced H9c2 cardiomyoblast hypertrophy

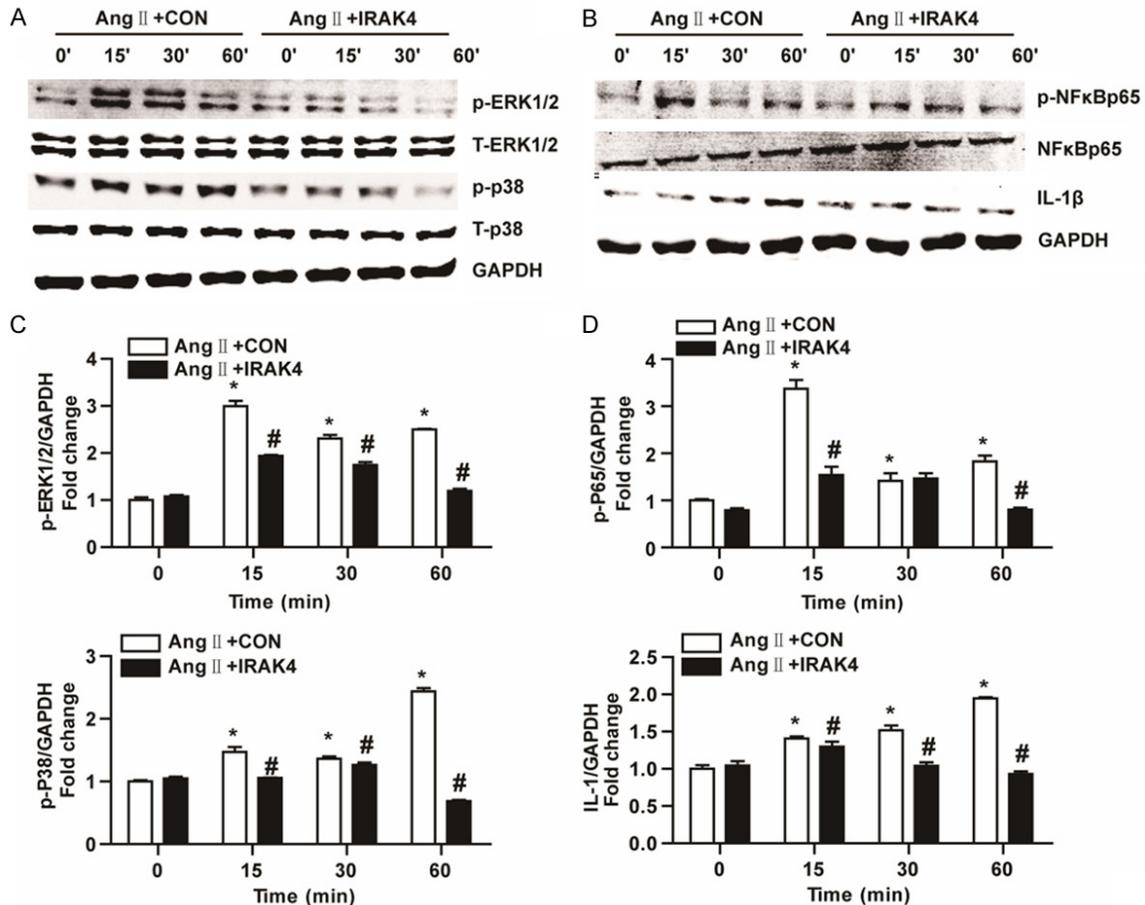
Cell areas of H9c2 cardiomyoblasts were determined by  $\alpha$ -actin staining to further evaluate the effect of IRAK4 overexpression after Ang II stimulation. Ang II stimulation for 48 hours resulted in a significant increase of the cell areas of H9c2 cardiomyoblasts. IRAK4 overexpression markedly attenuated the increase (**Figure 2A** and **2B**), indicating that Ang II-induced enlargement of H9c2 cardiomyoblasts was suppressed by IRAK4 overexpres-

sion. Treatment of H9c2 cardiomyoblasts with Ang II (1 µM, 48 h) up-regulated mRNA expression of the hypertrophic marker ANP. The induction of ANP in response to Ang II (1 µM) was inhibited significantly by IRAK4 overexpression (**Figure 2C**). It indicates that IRAK4 overexpression attenuates Ang II-induced cardiomyoblast hypertrophy.

### IRAK4 overexpression blocks the activation of ERK1/2, P38 and NF $\kappa$ B signaling pathways in response to Ang II in vitro

Several studies indicated that IRAK4 is involved in inflammatory MAPK signaling pathways activation [12, 13]. To investigate the molecular mechanism by which IRAK4 mediates its beneficial effect on cardiomyoblasts, we examined MAPKs and NF- $\kappa$ B signaling, which are pivotal contributors to the development of cardiac hypertrophy. Western blot was used to detect the protein levels of p-ERK1/2, p-P38 and p-NF $\kappa$ B. We found that their expressions were inhibited by IRAK4 overexpression after Ang II treatment at the indicated time points (**Figure 3A** and **3B**). It was also confirmed that the pro-inflammatory cytokines TNF $\alpha$  and IL-1 $\beta$  were also down-regulated in Ang II-treated cardiomyoblast with IRAK4 overexpression (**Figures 3B, 4A** and **4B**).

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**Figure 3.** IRAK4 overexpression blocks the activation of ERK1/2, P38 and NFκB signaling pathways in response to Ang II in vitro. A and C. The levels of total and phosphorylated ERK1/2, P38 in empty vector pcDNA3.1-transfected H9c2 cardiomyoblasts and pcDNA3.1-IRAK4-transfected H9c2 treated with Ang II at the indicated time points, including representative blots and quantitative results. B and D. The levels of p-NFκBp65, NFκBp65 and IL-1β in empty vector pcDNA3.1-transfected H9c2 cardiomyoblasts and pcDNA3.1-IRAK4-transfected H9c2 treated with Ang II at the indicated time points, including representative blots and quantitative results. \* $P < 0.05$  vs Ang II + CON group at the 0 time point. # $P < 0.05$  vs Ang II + CON group at the same time point.

*IRAK4 overexpression leads to expression changes of Traf6, MyD88 and phosphorylated TAK1 in response to Ang II in vitro*

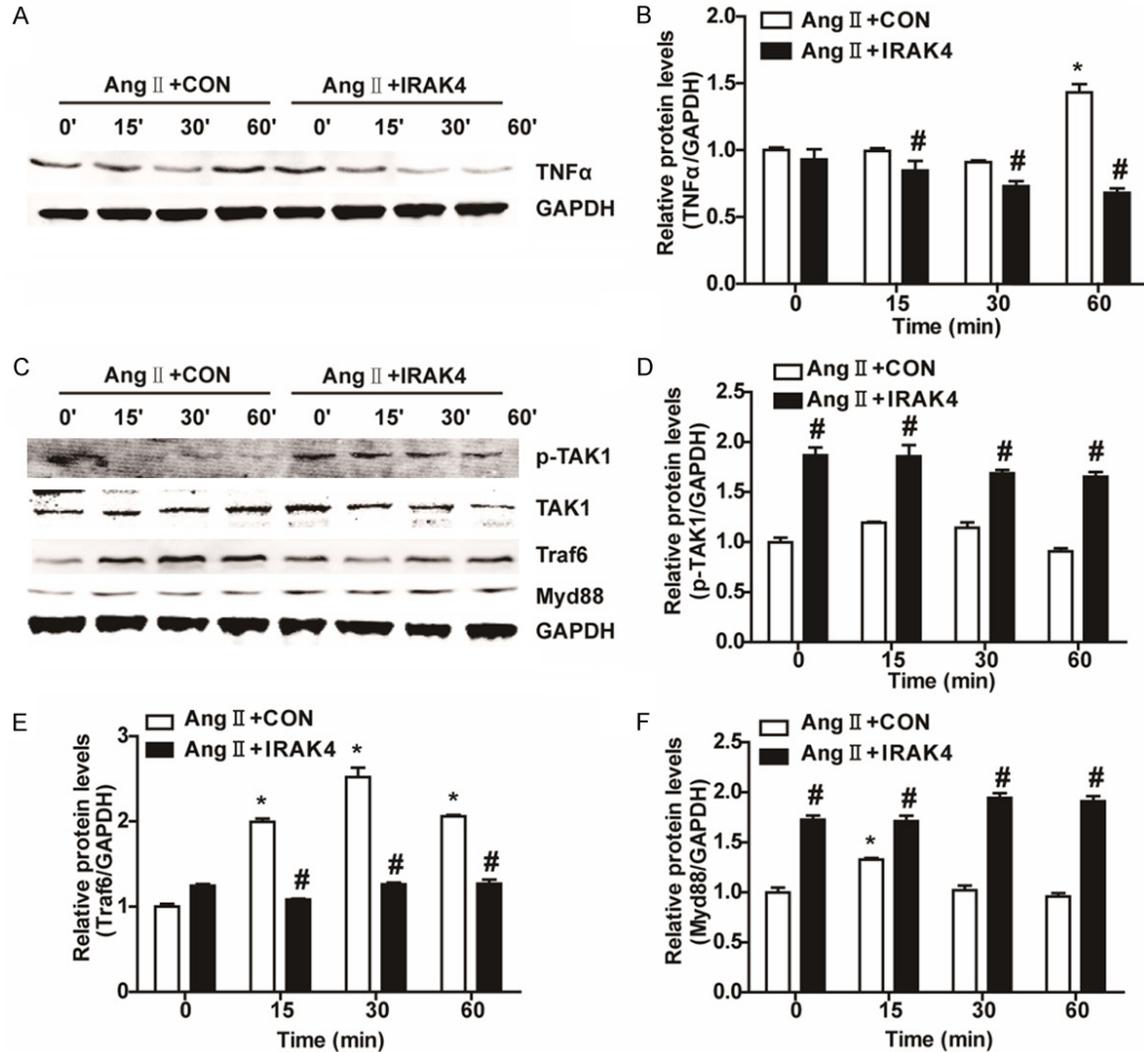
To clarify the mechanism by which IRAK4 specifically blocks ERK, p38 and NFκB pathways, further detection was done to uncover the expression level changes of Traf6, MyD88 and phosphorylated TAK1. We found that IRAK4 overexpression suppressed the expression of Traf6 (Figures 4C and 4E). However, we also got an impressive discovery that while IRAK4 was overexpressed in the cardiomyoblasts, the expressions of MyD88 and phosphorylated TAK1 increased (Figures 4C, 4D and 4F).

### Discussion

A plethora of signal transduction events are involved in the cardiovascular diseases, includ-

ing some in the innate immune systems [6]. In this study, we demonstrate the role of IRAK4, which is a ubiquitously expressed kinase involved in the regulation of innate immunity, in Ang II-induced cardiomyoblast hypertrophy using H9c2 cardiomyoblasts with IRAK4 overexpression. We found that (i) IRAK4 overexpression significantly attenuated cardiomyoblast hypertrophy induced by Ang II. (ii) IRAK4 overexpression significantly attenuated the activation of the ERK1/2, p38 and NFκB pathways in Ang II-treated cardiomyoblasts accompanying with down-regulated TNFα and IL-1β expression. (iii) IRAK4 overexpression led to increased MyD88 expression and decreased Traf6 expression in Ang II-treated cardiomyoblasts. Together, we demonstrate that the regulation of IRAK4 expression in the cardiomyoblasts may affect the responses of them to Ang II stimulation.

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**Figure 4.** IRAK4 overexpression Leads to Expression changes of Traf6, MyD88 and Phosphorylated TAK1 in Response to Ang II in vitro. A and B. The TNF $\alpha$  protein expression level in empty vector pcDNA3.1-transfected H9c2 cardiomyoblasts and pcDNA3.1-IRAK4-transfected H9c2 treated with Ang II at the indicated time points, including representative blots and quantitative results. C-F. The levels of p-TAK1, TAK1, Traf6 and MyD88 in empty vector pcDNA3.1-transfected H9c2 cardiomyoblasts and pcDNA3.1-IRAK4-transfected H9c2 treated with Ang II at the indicated time points, including representative blots and quantitative results. \* $P < 0.05$  vs Ang II + CON group at the 0 time point. # $P < 0.05$  vs Ang II + CON group at the same time point.

Ang II is considered as a hypertrophic agent on cardiomyocyte independent of hypertension through the Angiotensin II receptor type 1 (AT1R) [14, 15]. Ang II could induce cardiomyocyte hypertrophy as assessed via cardiomyocyte morphology and expression of hypertrophic marker ANP [16]. Original studies indicated that the expression of p-ERK1/2, p-P38 and p-NF $\kappa$ B were all increased in Ang II-treated cardiomyocytes [17, 18]. In our study, Ang II-treated cardiomyoblasts show the same changes including increased cell size, ANP mRNA expression and cell signaling changes.

Inhibition of IRAK4 is suggested to be beneficial in some autoimmune related disorders including arthritis [19], innate immunity-related endotoxic shock and acute liver injury [20]. Surprisingly, here in our study, we found that up-regulation of IRAK4 in the cardiomyoblasts could protect the cells from Ang II-induced hypertrophy. It demonstrates the complexity of IRAK4's function in different types of organs or cells, which may be due to the complexity of the TLR signaling pathway architecture in which IRAK4 is an important member [2]. Traditionally, stimulation of TLRs propagates pro-inflamma-

tory pathways and anti-inflammatory pathways. In the pro-inflammatory pathways, MyD88 recruits IRAKs or Traf6, further stimulates MAPKs and NF $\kappa$ B, which ultimately results in the transcription of pro-inflammatory cytokines, such as TNF $\alpha$  and IL-1 $\beta$ . In the anti-inflammatory pathways, MyD88 recruits Traf3, which ultimately results in the transcription of anti-inflammatory cytokines IL-10 [21]. In our study, after Ang II stimulation, exogenous IRAK4 overexpression led to decreased activation of the ERK1/2, p38 and NF $\kappa$ B pathways in cardiomyoblasts accompanying with down-regulated TNF $\alpha$  and IL-1 $\beta$  expression. Moreover, we detected increased MyD88 expression and decreased Traf6 expression. Since Traf6 and Traf3 competitively bind to overlapping interfaces on MyD88. We make a wild guess that in Ang II-treated cardiomyoblasts, IRAK4 overexpression may lead to more MyD88 recruiting Traf3, and meanwhile suppress the Traf6/MAPKs/NF $\kappa$ B pathway, although we did not test the Traf3 pathway. Thus, it is very likely that IRAK4 is the key protein regulating the compete binding of Traf6 and Traf3 to MyD88. A previous study suggests that in the context of Ang II-induced hypertension and cardiac hypertrophy, MyD88 exerts a negative regulatory role through regulating the inflammatory process [22]. However, mice lacking MyD88 adaptor protein has significantly improved survival and reduced inflammation, cardiac fibrosis, and cardiac hypertrophy after myocardial infarction [23]. The role of MyD88 in cardiovascular diseases seems controversial. It demonstrates that under different conditions, different adaptor proteins-mediated pathways are activated. Further studies will be needed to clarify the role of IRAK4-associated proteins in experimental models of heart diseases. Cardiac-specific ablation of TAK1 induces spontaneous apoptosis and necroptosis that leads to adverse remodeling and heart failure [24]. In our study, IRAK4 overexpression also led to increased TAK1 phosphorylation, which may represent a beneficial effect at this condition. But the underlying mechanisms are still unclear. It may be associated with the FADD protein, which is also associated with MyD88 and apoptosis [25].

This study suggests that IRAK4 may serve as a new therapeutic target for treating cardiomyocyte hypertrophy. It will be important to further

evaluate IRAK4-associated proteins in experimental models of heart diseases.

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### Disclosure of conflict of interest

None.

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