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ZAK induces cardiomyocyte hypertrophy and brain natriuretic peptide expression via p38/JNK signaling and GATA4/c-Jun transcriptional factor activation

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Abstract Cardiomyocyte hypertrophy is an adaptive response of heart to various stress conditions. During the period of stress accumulation, transition from physiological hypertrophy to pathological hypertrophy results in the promotion of heart failure. Our previous studies found that ZAK, a sterile alpha motif and leucine zipper containing kinase, was highly expressed in infarcted human hearts and demonstrated that overexpression of ZAK induced cardiac hypertrophy. This study evaluates, cellular events associated with the expression of two doxycycline (Dox) inducible Tet-on ZAK expression systems, a Tet-on ZAK WT (wild-type), and a Tet-on ZAK DN (mutant, Dominant-negative form) in H9c2 myoblast cells; Tet-on ZAK WT was found to increase cell size and hypertrophic marker BNP in a dose-dependent manner. To ascertain the mechanism of ZAK-mediated hypertrophy, expression analysis with various inhibitors of the related upstream and downstream proteins was performed. Tet-on ZAK WT expression triggered the p38 and JNK pathway and also activated the expression and nuclear translocation of p-GATA4 and p-c-Jun transcription factors, without the involvement of p-ERK or NFATc3. However, Tet-on ZAK DN showed no effect on the p38 and JNK signaling cascade. The results showed that the inhibitors of JNK1/2 and p38 significantly suppressed ZAK-induced BNP expression. The results show the role of ZAK and/or the ZAK downstream events such as JNK and p38 phosphorylation, c-Jun, and GATA-4 nuclear translocation in cardiac hypertrophy. ZAK and/or the ZAK downstream p38, and JNK pathway could therefore be potential targets to ameliorate cardiac hypertrophy symptoms in ZAK-overexpressed patients.

Keywords Cardiac hypertrophy · Sterile alpha motif and leucine zipper containing kinase · Mixed lineage kinase

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Introduction

Cardiovascular disease represents pathological syndromes of the heart and adjacent tissues, mainly blood vessel, in response to pressure overload [1, 2]. During the process of cardiac remodeling, myocardial hypertrophy develops as an adaptive response to hemodynamic overload and various other neurohumoral factors. But prolonged hypertrophy ultimately leads to deterioration of cardiac function

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and chronic heart failure [3–9]. Myocardial hypertrophy is one of the most frequent causes of heart failure in most heart disease conditions among humans and is a critical sign for negative prognostic [1, 2]. In the early stages of cardiac hypertrophy, frequently designated as adaptive response to stress, the heart walls thicken to compensate for the increased stress [9].

Signaling cascades of the mitogen-activated protein kinases (MAPKs) such as the extracellular signal-regulated protein kinase (ERK) pathway, the p38 kinase pathway and the c-Jun N-terminal protein kinase (JNK) pathway, are known to regulate a wide array of cellular events by phosphorylation in response to extracellular stimuli [10]. Activated p38, JNKs, and ERKs phosphorylate multiple intracellular targets, including numerous transcription factors that induce the reprograming of cardiac gene expression [11].

MAPK pathways are stimulated in response to various extracellular stimuli and they modulate the function of several transcription activators. Activation of p38 kinase is necessary for hypertrophic agonist-induced GATA-4 binding to B-type natriuretic peptide (BNP) gene and sufficient for GATA-dependent B-type natriuretic peptide gene expression [12, 13]. In addition, early mediators of hypertrophic transcriptional program include the transient activation of immediate-early genes, which encode transcription factors, such as c-Jun [14, 15].

In our previous studies, a novel gene that encodes a serine/threonine kinase was discovered and designated as ZAK for leucine zipper (LZ) and sterile alpha motif (SAM) kinase [16]. ZAK is classified into ZAKα form and ZAKβ form, both forms harbor a kinase catalytic domain (KD) and an LZ domain. The most prominent difference between them is that the ZAKα contains SAM motif which the ZAKβ lacks. ZAK belongs to the mixed lineage kinase (MLK) family which comprises a group of closely related serine/threonine kinases that function as mitogen-activated protein kinase kinase kinase (MAP3K) [17]. MAP3K is an upstream kinase which activates downstream proteins by serine/threonine phosphorylation, after which active downstream proteins would induce different cellular response in various cells [18].

Our previous results revealed that ZAK is expressed abundantly in heart than in other organs [16]. ZAK expression in mammalian cells specifically leads to the activation of JNK pathway and further activates the NF-κB transcription factor. ZAK can also activate MKK7, an activator of JNK [19]. Moreover, ZAK leads to increase in the protein levels of TIMP-1 and TIMP-2 resulting in the reduction of MMP-9 activity and activation of JNK1/2 and p38 signaling pathways to increase the MMP-2 activity that contributes to cardiac fibrosis [20]. In H9c2 cardiomyoblast cells, MKK7 induces the characteristic features of hypertrophy, which suggests that ZAK itself may be involved in signal transduction for the regulation of cardiac hypertrophy [21]. Further, overexpressed ZAK not only induces cell hypertrophy but also increases the hypertrophic marker ANF expression in the H9c2 cardiomyoblast cells. Interestingly, the inhibition in the ZAK kinase activity rescues cardiomyocytes from TGF-β-induced cell hypertrophy and increased ANF expression [22]. All of these studies indicate that overexpression of ZAK would cause H9c2 cardiomyoblast cell hypertrophy.

In this study, we further investigated the mechanisms and signaling pathways involved in ZAK-induced BNP and neonatal cardiomyocyte hypertrophy using Tet-on ZAK transformant H9c2 cells.

Methods

Construction of Tet-on gene expression system

The ZAKα form used in this study was obtained from Dr. Yang, Chung Shan Medical University as a Tet-on ZAK in H9c2 cells. In this system, two different ZAK genes with distinct protein activities were used; a wild-type ZAK (ZAK WT) and dominant-negative ZAK (ZAK DN) with a K45M point mutation in the kinetic domain. The ZAK gene expression was induced by the addition of doxycycline (Dox). ZAK being a protein kinase, the difference between ZAK WT and ZAK DN is identified from their kinase activities. ZAK DN had the weakest activity. ZAK WT had the normal activity without modification.

Cell culture

H9c2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 2 mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 1 mM pyruvate in 5 % CO_2 at 37 °C. After transfection, cells were cultured in serum-free medium for 12 h and subsequently treated with Dox to induce the expression of ZAK genes. After further incubation for 23 h, the cells were harvested and extracted for analysis.

Cardiomyocyte culture

Neonatal cardiomyocytes were isolated and cultured using the commercial Neonatal Cardiomyocyte Isolation System Kit according to manufacturer's directions (Cellutron Life Technologies, Highland Park, New Jersey, USA). Briefly, hearts from 1- to 2-day-old Sprague-Dawley rats were removed, the ventricles were pooled, and the ventricular cells were dispersed by digestion solution at 37 °C. Ventricular cardiomyocytes were isolated and cultured in DMEM

containing 10 % fetal bovine serum, 100 μg/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine. After 3–4 days, cells were incubated in serum-free essential medium overnight.

Transient transfection

H9c2 cells were seeded into 10 cm dishes in DMEM containing 10 % FBS and on the next day medium was replaced with fresh DMEM medium with penicillin 2 h before transient transfection, and JNK/p38 siRNA was introduced into cells by using PureFection™ Nanotechnology-based Transfection Reagent according to the manufacturer's guidelines. After 12 h, medium was replaced with serum-free DMEM medium with penicillin and cells were prepared for following treatments. Also neonatal primary culture cells were seeded into 24-well in DMEM containing 10 % FBS and, on the next day, medium was replaced with fresh DMEM medium with penicillin 2 h before transient transfection, and JNK/p38 siRNA was co-transfected into cells with pEGFPC1-ZAK DNA plasmid by using PureFection™ Nanotechnology-based Transfection Reagent according to the manufacturer's guidelines. After 12 h, medium was replaced with serum-free DMEM medium with penicillin and cells were prepared for following treatments.

Whole cell extraction

Cultured H9c2 cells were scraped and washed once with PBS, then cell suspension was spun down, and cell pellets were lysed on ice for 30 min in lysis buffer [50 mmol/L Tris-base (pH 7.4), 0.5 mol/LNaCl, 1 mol/L ethylenediaminetetraacetic acid (EDTA), 1 mM/L beta-mercaptoethanol (BME), 1 % NP-40, 10 % glycerol, IGEPAL CA-630 (Sigma-Aldrich) and protease inhibitor cocktail tablets (Roche)] and spun down at 4 °C, 12,000 rpm for 20 min, the supernatant was collected in new eppendorf tube and stored at −80 °C. Protein quantification was done using the Bio-Rad protein assay reagent (Bio-Rad Hercules, CA, USA) which is based on the Bradford dye-binding method.

Western blot analysis

The Western blotting for protein expression analysis was as described previously with slight modifications [23]. Proteins were separated in SDS-PAGE (8 or 12 %) and transferred to nitrocellulose membranes. Non-specific protein binding was prevented by incubating the membranes in blocking buffer (5 % milk, 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1 % Tween-20) and the proteins were blotted with specific antibodies at 4 °C overnight. After incubation with secondary antibody for 2 h, densitometric analysis of immunoblots was performed using Fuji LAS 3000 imaging system. For repeated blotting, nitrocellulose membranes were stripped with Western blot stripping buffer (GMbiolab Co. Ltd. Taichung, Taiwan) at room temperature for 30 min.

Cytoplasmic and nuclear fractionations

Cell cytoplasmic and nuclear fractions were collected with the extraction reagent, lysis buffer A (50 mM Tris-base, 0.5 M NaCl, 1.0 mM EDTA, 1 % NP-40, 1 % Glycerol, 1 mM Mercaptoethanol, Proteinase k inhibitor, and lysis buffer B (50 mM Tris-base, 0.5 M NaCl, 1.0 mM EDTA, 1 % Glycerol, and Proteinase k inhibitor). In brief, 5×10^6 cells were trypsinized (0.05 % trypsin/0.53 mM EDTA) and re-suspended in 100 μl lysis buffer B. After 10-min incubation on ice, the samples were centrifuged at 3000 g for 10 min to pellet the nuclear protein. After centrifugation, the supernatant was stored for use as the cytoplasmic fraction, and the nuclear fraction was lysed with 100 μl of lysis buffer A.

Actin staining

H9c2 cells were inoculated into 12-well plate. After treatments, cells were fixed with 4 % paraformaldehyde solution for 10 min at room temperature. After a rinse with PBS, cells were treated with permeation solution (0.5 % Triton X-100) for 10 min at 4 °C. Following wash with PBS, samples were first incubated with actin staining reagent containing Rhodamine-conjugated phalloidin (Life Technologies) with high affinity for actin. The cells were also stained with 1 μg/mL 4-,6-diamidine-2-phenylindole dihydrochloride (DAPI) for 30 min to detect cell nucleus using UV light microscopic observations (blue). Samples were analyzed in a drop of PBS under a fluorescence and UV light microscope with an excitation wavelength of 540 nm and an emission wavelength of 565 nm (red). The increasing cell size and intracellular actin polymerization were measured by Axio Vision LE-software. All measurements were acquired from at least three independent experiments performed in a blinded manner.

Statistical analysis

Each sample was analyzed based on results that were repeated at least three times and SigmaPlot 10.0 software and standard *t* test was used to analyze each numeric data. In all cases, differences at $p < 0.05$ were regarded as statistically significant, ones at $p < 0.01$ or $p < 0.001$ were considered higher statistical significances.

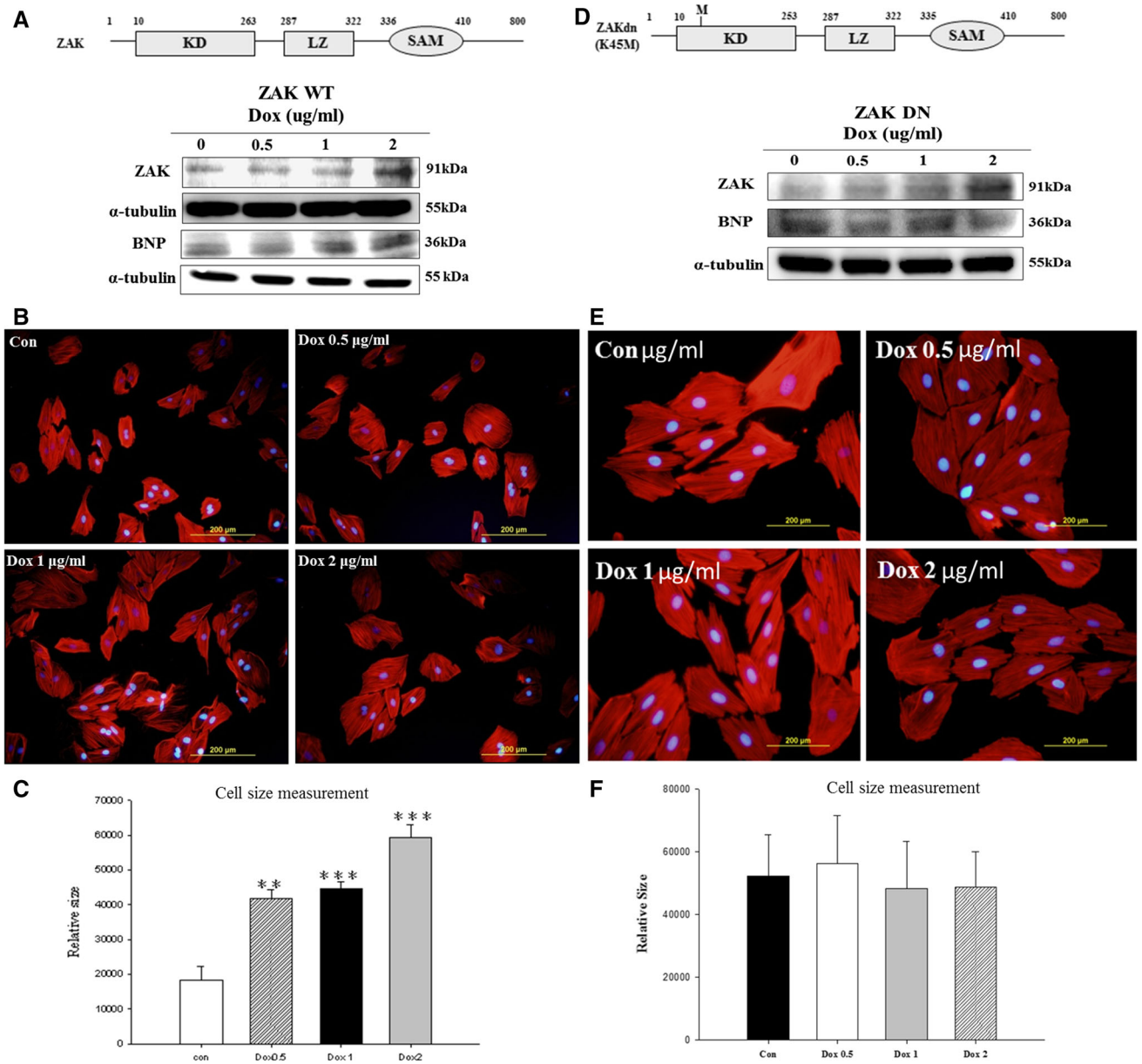

Fig. 1 ZAK induced pathological hypertrophy marker and cell size increase in H9c2 cardiomyoblast cells. **a**, **d** Cells were cultured in serum-free medium for 12 h, and then treated with doxycycline (Dox, 0, 0.5, 1, and 2 μg/mL) for 24 h. The expression levels of ZAK and BNP hypertrophic proteins were measured. **b**, **c**, **e**, **f** Cells were cultured in serum-free medium for 12 h, and treated with Dox (0, 0.5, 1, and 2 μg/mL) for 24 h, then stained with phalloidin-rhodamine to measure the cell number, DAPI staining was used to mark nuclei. The statistical results were shown from three independent experiment; mean ± SD, $n = 3$. $^{**}p < 0.01$ and $^{***}p < 0.001$, represent significant difference versus control)

Results

ZAK elevated pathological hypertrophy marker and cell size in H9c2 cardiomyoblast cells with Tet-on ZAK WT

To clarify whether Tet-on ZAK systems are well established, the cells were treated with Dox at different concentrations. In this result, ZAK protein expression level in wild-type ZAK and ZAK dominant-negative cell line was both increased by Dox in a dose-dependent manner. Moreover, from actin staining, we observed that ZAK expression increased the cell size of ZAK WT H9c2 (Fig. 1b, c, e, f). BNP hypertrophy marker protein was increased by treating with Dox at different concentration in ZAK WT H9c2; however, in ZAK DN H9c2 cells, the results show no change in BNP protein expression level (Fig. 1a, d).

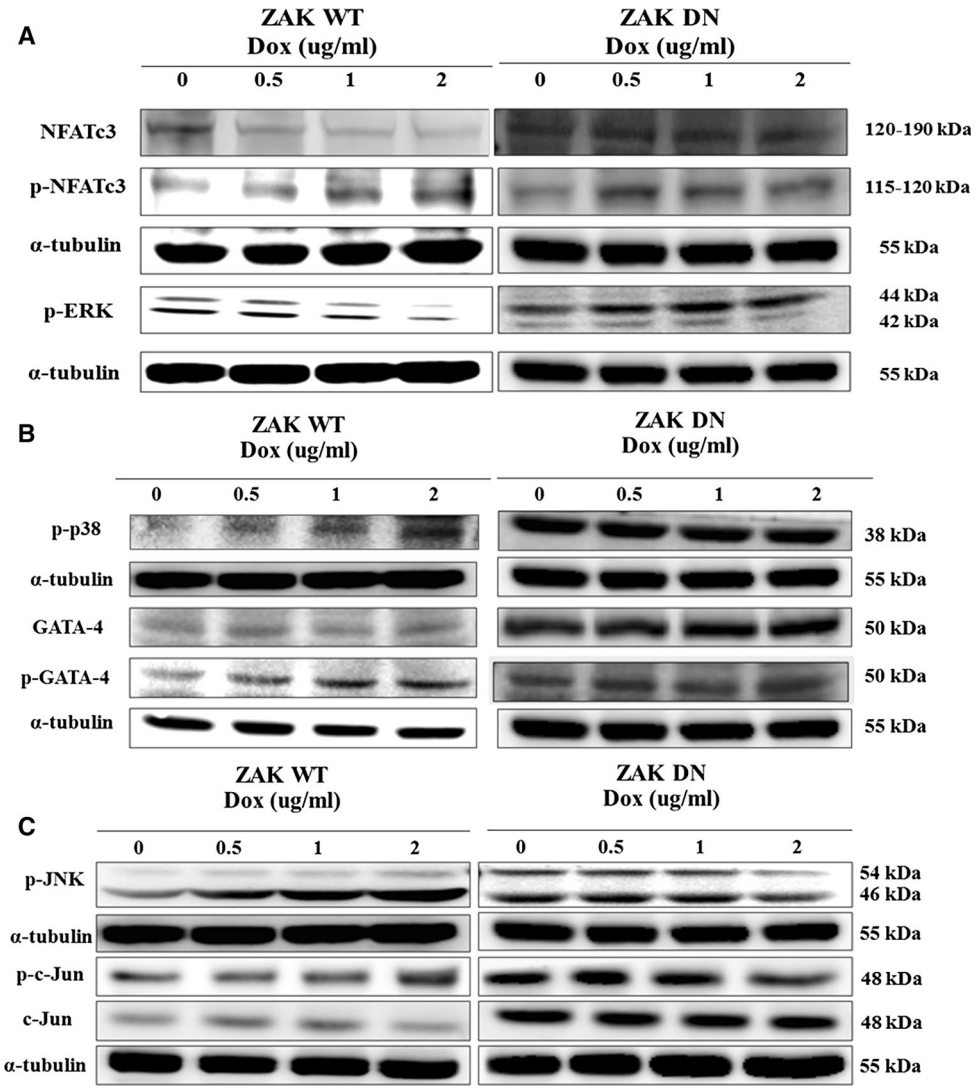

Fig. 2 The protein levels and activities of hypertrophy-related MAPK and transcription factors between ZAK WT and ZAK DN expressed H9c2 cell lines. **a–c** Cells were cultured in serum-free medium for 12 h and then treated with Dox (0, 0.5, 1, and 2 μg/mL) for 24 h. The expression of hypertrophy-related MAPK proteins and transcription factors in ZAK WT and ZAK DN H9c2 Tet-on cells were measured

The protein levels and activities of hypertrophy-related MAPK and transcription factors were compared between ZAK WT- and ZAK DN-overexpressed H9c2 Tet-on cell lines

To clarify whether the hypertrophy-related protein levels were induced by Tet-on ZAK system, we detected the phosphorylated and dephosphorylated type of ERK, NFATc3, p38, JNK, GATA-4, and c-Jun protein level expression. The results showed a dose-dependent increase in protein levels of p-JNK and p-p38 hypertrophy-related MAPK, and p-c-Jun and p-GATA-4 transcription factors in the ZAK WT H9c2 Tet-on cells. However, ZAK DN cells show no change of these mentioned proteins (Fig. 2b, c). Moreover, there is no change of p-ERK and NFATc3 protein levels in the Tet-on ZAK WT H9c2 cells (Fig. 2a).

ZAK expression enhances nuclear translocation of hypertrophy-related p-GATA4 and p-c-Jun transcription factors in Tet-on ZAK WT H9c2 cells

We further investigated whether ZAK overexpression-induced hypertrophy was associated with the transcription factors, p-GATA4, and p-c-Jun by using immunostaining and confocal microscopy. The results indicated that ZAK WT expression induces p-c-Jun and p-GATA-4 nuclear translocalization in ZAK WT H9c2 cells (Fig. 3a). Furthermore, examination of the nuclear and cytoplasmic fraction showed that ZAK overexpression triggers the p-c-

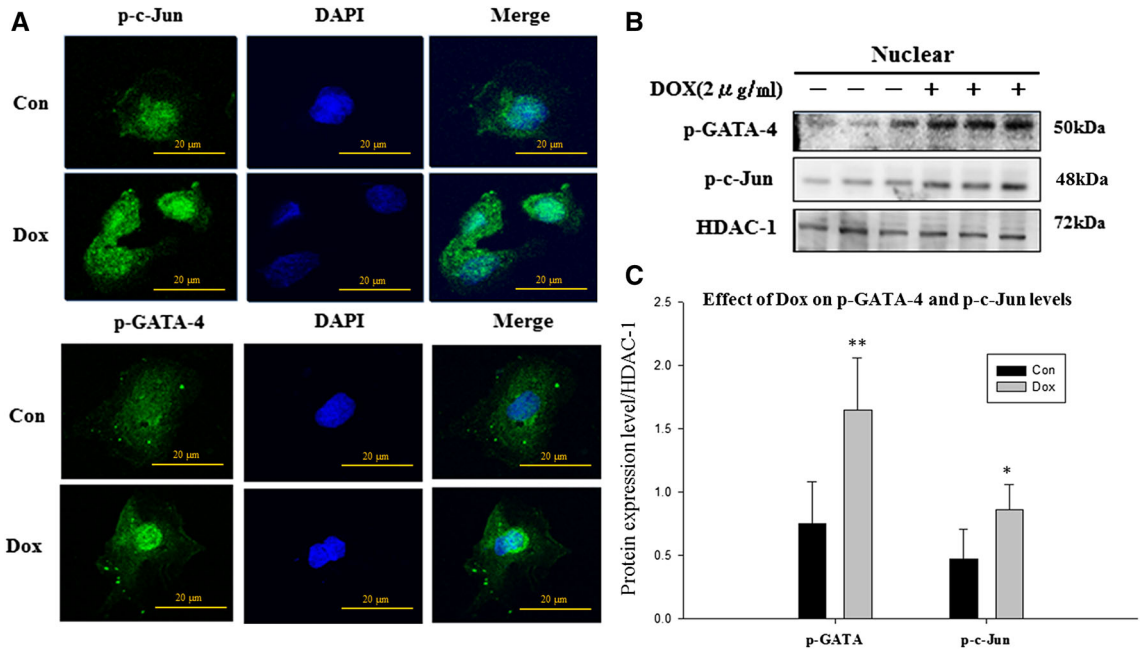

Fig. 3 Nuclear translocation enhancement of hypertrophy-related p-GATA4 and p-c-Jun transcription factors in Tet-on ZAK WT H9c2 cells. **a** Confocal microscopy assay was applied to identify the nuclear translocation of p-c-Jun and p-GATA-4 of ZAK-overexpressed Tet-on cells. **b** Nuclear isolation was used to identify the nuclear accumulation of p-c-Jun and p-GATA-4 of ZAK-overexpressed Tet-on cells. **c** The statistical results were shown from three independent experiments; mean ± SD, $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$ represent significant difference versus control

Jun and p-GATA-4 protein nuclear accumulation (Fig. 3b, c).

ZAK expression induces myocardial cell hypertrophy mediated through p38/GATA4 and JNK/c-Jun signaling pathways, without the involvement of calcineurin-NFATc3 and ERK pathways

To further identify the signal transduction pathway(s) involved in the mechanism behind ZAK-induced myocardial cell hypertrophy, selective inhibitors U0126 (ERK1/2 inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK1/2 inhibitor), CsA (Calcineurin inhibitor) were applied, respectively, to block specific pathways. The results showed that SP600125 and SB203580 significantly suppressed ZAK-induced BNP protein expression, whereas U0126 and CsA treatment did not show any suppressive effect (Fig. 4a). The findings suggest that JNK1/2 and p38 act as a key mediator of ZAK to induce BNP protein expression. In addition, siRNA (50 nM)-mediated inhibition of JNK and p38 also confirms the above result. ZAK overexpression after 24 h of siRNAs transfection inhibited cardiomyocyte hypertrophy-associated p-p38/p-GATA4 and p-JNK/p-c-Jun induced by ZAK (Fig. 4b). Further cell morphology was observed by actin staining and the change in the cell size was measured. The JNK and p38 siRNAs (50 nM) significantly suppressed the cell size increase of H9c2 cells with Tet-on ZAK WT (Fig. 4c, d). Further the effect of ZAK was also verified in neonatal cardiomyocytes. After 24 h of ZAK plasmid transfection, a significant increase in cell size was observed, whereas co-transfection with JNK and p38 siRNAs effectively blocked the cardiomyocyte hypertrophy induced by ZAK overexpression (Fig. 4e, f).

Discussion

In humans, sustained cardiac hypertrophy is a key factor in the development of heart failure [5, 24–26]. To understand the molecular mechanisms of cardiac hypertrophy, it is important to define the extracellular stimuli and specific signaling molecules that elicit the hypertrophic phenotype. In our previous study, results showed that the expression of a wild-type form of ZAK induces the characteristic hypertrophic growth features, including elevated atrial natriuretic factor expression and increased actin fiber organization [21]. In this study, we further overexpressed ZAK WT in the H9c2 cardiomyoblast cells and neonatal cardiomyocyte to reveal the hypertrophy-induced mechanisms. We found that ZAK directly up-regulated hypertrophic marker BNP protein

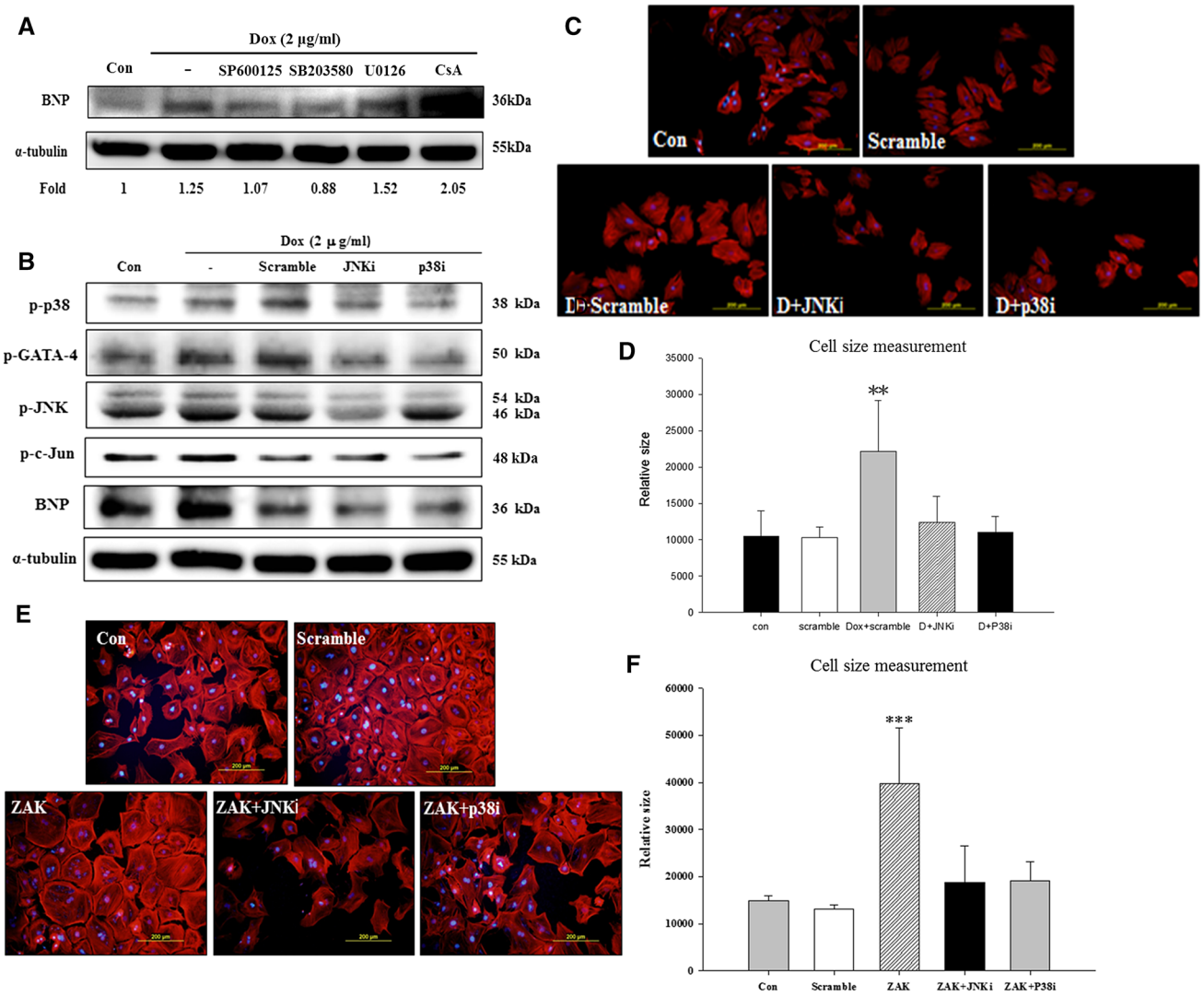

Fig. 4 ZAK expression induces myocardial cell hypertrophy mediated through the p38/GATA4 and JNK/c-Jun signaling pathways, without calcineurin-NFATc3 and ERK pathways involved. **a** Cells were cultured in serum-free medium for 12 h, then treated with doxycycline (2 μg/mL) with or without the one of the following inhibitors; SP600125 (15 μM, JNK1/2 inhibitor), SB203580 (3 μM, p38 MAPK inhibitor), U0126 (15 μM, ERK1/2 inhibitor), and CsA (Calcineurin inhibitor) for another 24 h to block specific pathways, and BNP was measured by Western blotting. **b** After H9c2 cells were transfected with JNK1 and p38–siRNA (50 nM) for 24 h, cells were cultured in serum-free medium for 12 h, and then treated with the Dox to induce ZAK gene expression for another 24 h, and non-specific siRNA was used as scramble control. The levels of indicated proteins were analyzed by Western blotting assay. **c**, **d** ZAK overexpression-induced H9c2 cardiomyoblast cell hypertrophy was abolished by p38 and JNK siRNA transfection, non-specific siRNA was used as scramble control and then stained with phalloidin-rhodamine. DAPI staining was used to mark nuclei. The statistical results were shown from four independent experiments; mean ± SD, $n = 3$. $^{**}p < 0.01$, represent significant difference versus control. **e**, **f** ZAK overexpression-induced neonatal cardiomyocyte hypertrophy was blocked by knock-down p38 and JNK with siRNA, non-specific siRNA was used as scramble control, and then stained with phalloidin-rhodamine. DAPI staining was used to mark nuclei. The statistical results were shown from four independent experiments; mean ± SD, $n = 3$. $^{***}p < 0.001$ represent significant difference with control

level, and induced the increase in cell size, however, ZAK DN shows no effect on BNP protein level and cell size (Fig. 1).

ZAK belongs to the mixed lineage kinase (MLK) family, which comprises a group of closely related serine/threonine kinases that function as MAP3K [16]. The MAPKs, ERK, JNK, and p38 MAPK, have been shown to be inducible by a variety of hypertrophic stimuli, including mechanical stretch, ET-1, and other GPCR (G protein-coupled receptor) agonists [27, 28]. Also previous report has identified that cardiac hypertrophy is induced by calcineurin, the calcium-dependent phosphatase, which dephosphorylates the transcription factor NF-AT3, enabling it to translocate to the nucleus to induce BNP expression, and

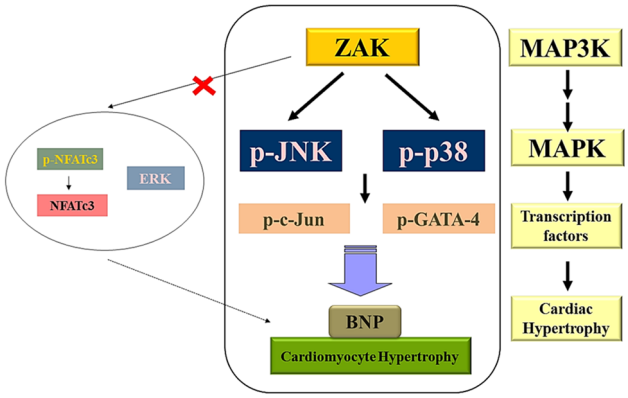

Fig. 5 ZAK induces JNK and p38 hypertrophic-related pathways, and c-Jun and GATA-4 protein expression and nuclear translocation in myocardial cells

to develop cardiac hypertrophy and heart failure [29]. However, our results strongly indicate ZAK overexpression leads to subsequent up-regulation in JNK and p38 MAPK signaling, and further phosphorylated the downstream transcription factors, c-Jun and GATA-4, to promote cardiomyoblast cell hypertrophy (Fig. 2). The previous reports also demonstrate that activation of p38 kinase is necessary for hypertrophic agonist-induced GATA-4 binding to B-type natriuretic peptide gene [13], and indicate that activation of JNK/c-Jun pathways, which in turn have a key role in the early activation of the hypertrophic genetic program by mechanical stress in cardiac myocytes [30]. We further identified these c-Jun and GATA-4 activations resulted from ZAK overexpression, including nuclear translocation examined by immunofluorescence assay and nuclear accumulation by nuclear isolation assay (Fig. 3).

However, overexpressed ZAK could induce JNK and p38, but not ERK and calcineurin activation, consequently increased c-Jun and GATA-4 nuclear translocation cause cardiomyocyte hypertrophy. The elevated level of BNP and cell size induced by ZAK was significantly reduced by SB203580 and SP600125, indicate that JNK and p38 are definitely the downstream of ZAK. The inhibitors SB203580 and SP600125 blocked p38 and JNK, to suppress the GATA-4 and c-Jun nuclear translocation and accumulation (Fig. 4a). All the above results suggest that ZAK induced BNP elevation and cell hypertrophy is not associated with ERK and calcineurin/NFATc3, but through p38/GATA4 and JNK/c-Jun signaling. Moreover, the treatments of JNK and p38 with siRNAs effectively abolished ZAK-induced hypertrophy effect both in H9c2 cardiomyoblast cells and neonatal primary cardiomyocyte (Fig. 4).

Our results provide the information about how ZAK induced cardiac hypertrophy and further implicated the possible mechanisms of the ZAK-induced cardiovascular disease. The crucial downstream events of ZAK implicated in the pathogenesis of hypertrophic cardiomyopathy are JNK and p38 phosphorylation, c-Jun and GATA-4 nuclear translocation, and then induced cell size increase (Fig. 5). Our results also portray ZAK and its downstream signal proteins, such as JNK and p38, as potential targets for therapies against ZAK-induced cardiomyocyte pathological hypertrophy and heart failure.

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Compliance with ethical standards This article does not contain any studies with human participants performed by any of the authors. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institutional animal care and use committee (IACUC) of China Medical University.

Conflict of interest No conflict of interest exists to be declared.

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