Received: 1 November 2017 | Accepted: 24 May 2018

DOI: 10.1002/jcb.27199

RESEARCH ARTICLE

WILEY Journal of Cellular Biochemistry

ERβ targets ZAK and attenuates cellular hypertrophy via SUMO-1 modification in H9c2 cells

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Funding information
Ministry of Health and Welfare, Grant/Award Number: MOHW106-TDU-B-212-113004

Abstract

Aberrant expression of leucine zipper- and sterile α motif-containing kinase (ZAK) observed in pathological human myocardial tissue is associated with the progression and elevation of hypertrophy. Our previous reports have correlated high levels of estrogen (E2) and abundant estrogen receptor (ER) α with a low incidence of pathological cardiac-hypertrophy and heart failure in the premenopause female population. However, the effect of elevated ERβ expression is not well known yet. Therefore, in this study, we have analyzed the cardioprotective effects and mechanisms of E2 and/or ERβ against ZAK overexpression–induced cellular hypertrophy. We have used transient transfection to overexpress ERβ into the ZAK tet-on H9c2 cells that harbor the doxycycline-inducible ZAK plasmid. The results show that ZAK overexpression in H9c2 cells resulted in hypertrophic effects, which was correlated with the upregulation of p-JNK and p-p38 MAPKs and their downstream transcription factors c-Jun and GATA-4. However, ERβ and E2 with ERβ overexpressions totally suppressed the effects of ZAK overexpression and inhibited the levels of p-JNK, p-p38, c-Jun, and GATA-4 effectively. Our results further reveal that ERβ directly binds with ZAK under normal conditions; however, ZAK overexpression reduced the association of ZAK-ERβ. Interestingly, increase in ERβ and E2 along with ERβ overexpression both enhanced the binding strengths of ERβ and ZAK and reduced the ZAK protein level. ERβ overexpression also suppressed the E3 ligase-casitas B-lineage lymphoma (CBL) and attenuated

Wei-Wen Kuo and Chih-Yang Huang contributed equally to this paper.

CBL-phosphoinositide 3-kinase (PI3K) protein association to prevent PI3K protein degradation. Moreover, ERβ and/or E2 blocked ZAK nuclear translocation via the inhibition of small ubiquitin-like modifier (SUMO)-1 modification. Taken together, our results further suggest that ERβ overexpression strongly suppresses ZAK-induced cellular hypertrophy and myocardial damage.

KEYWORDS
estrogen receptor, hypertrophy, MAP3 kinase, 17β-estradiol, ZAK

1 | INTRODUCTION

Cardiovascular disease (CVD) is the most common cause of death worldwide[1]; the male population is generally seen to be at a higher risk for CVD than the female population,[2,3] and the average life expectancy is also known to be about 8 years lesser in males than in females. Sex differences in the manifestation of myocardial hypertrophy and the transition to heart failure have largely been attributed to the effects of sex steroid hormones, such as 17β-estradiol (estrogen, E2), which are mainly mediated by their nuclear receptors, estrogen receptor (ER) α and ERβ.[4,5] Previous research have demonstrated that physiological replacement of E2, the main circulating form of estrogen in premenopausal women and also in ovariectomized female mice, limits pressure-overload–induced left ventricular (LV) hypertrophy.[6,7] In addition, estrogen-replacement therapy contributes to a low incidence of heart disease after menopause.[8]

Accumulating evidence suggest that hypertrophy and apoptosis in cardiomyocytes contribute to the progression of heart failure and arrhythmias.[9] Additionally, our previous studies showed that E2 reduces cardiac hypertrophy by mediating the upregulation of phosphoinositide 3-kinase (PI3K)/Akt and by suppressing calcineurin/nuclear factor of activated T-cells (NF-AT3) signaling pathways in rats, indicating the possible signaling pathways and mechanisms for the cardioprotective effects of E2 administration.[10] In another study, we determined that E2 and ERα expressions exert their cardioprotective effects by inhibiting JNK1/2-mediated LPS-induced tumor necrosis factor α (TNF-α) expression and cardiomyocyte apoptosis through the activation of Akt.[11] E2 and its receptor ERβ also inhibit isoproterenol (ISO)-induced myocardial apoptosis by enhancing the degradation of calcineurin and by promoting PI3K/Akt/MDM2 signal transduction.[12] In another study, using human cardiovascular tissue array containing 10 normal heart and 10 myocardial scar tissues, we examined the expression level of the MAP3 kinases, namely, leucine zipper- and sterile α motif-containing kinase (ZAK) during myocardial remodeling. Among the tested myocardial scar samples, 8 showed a strong expression of ZAK (80%) and 2 showed moderate expression (20%).[13]

ZAK is a mixed-lineage kinase containing a sterile-α motif, a leucine-zipper region, and a kinase domain that shows 40% sequence similarity with the mixed-lineage kinase (MLK) family members like MLK1 or dual leucine zipper kinase (DLK). Upon activation of the molecular triggers such as protein kinase N1 (PKN1), ZAK undergoes autophosphorylation, resulting in the activation of mitogen-activated protein kinase (MAPK) pathways.[14] The leucine-zipper region of ZAK has been demonstrated to be involved in the oligomerization of the protein, autophosphorylation of ZAK activation loop residues T161 and S165, and activation of c-Jun N-terminal kinase (JNK) MAPK.[15] Elevated ZAK expression has been previously shown to induce cardiac hypertrophy and associated markers of hypertrophy; moreover, inhibition of ZAK was demonstrated to rescue H9c2 cells from TGF-β–induced cellular hypertrophy.[15,16] ZAK induces cardiomyocyte hypertrophy and associated brain natriuretic peptide expression by activating p38/JNK signaling and GATA4/c-Jun transcriptional factors.[17] Elevated ZAK induces matrix metalloproteinase-2 (MMP-2) activity via JNK/p38 signals and reduces matrix metalloproteinase-9 (MMP-9) activity by increasing tissue inhibitors of metalloproteinases-1/2 (TIMP-1/2) expression in H9c2 cardiomyoblast cells.[13]

Therefore, from the inference of our previous studies, we have investigated whether the biological effects of estrogen and ERβ confer protection against ZAK-induced cellular hypertrophy, and further, the respective molecular mechanisms involved in the effect of ERβ have been deduced.

2 | MATERIALS AND METHODS

2.1 | Construction of the tet-on gene expression system

The ZAKα form was used to investigate the effect of ZAK at the genomic level, and a wild-type (WT) ZAK tet-on

H9c2 system was used, as mentioned in our previous report.[17] The ZAK gene expression was turned on by the addition of doxycycline.

2.2 | Cell culture

H9c2 cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 mM pyruvate in 5% CO_2 at 37°C. During the treatment, after ERα transfection, cells were cultured in a serum-free medium for 12 hours, after which E2 was administered, and after 1 hour of incubation, the cells were treated with doxycycline to induce the expression of ZAK genes. After further incubation for 23 hours, the cells were harvested and extracted for analysis.

2.3 | Transient transfection

Cells were seeded in 10 cm dishes with DMEM containing 10% FBS; on the next day, the medium was replaced with fresh DMEM medium with penicillin 2 hours before transient transfection, and plasmid DNA carrying the ERβ gene was transferred using the PureFection (System Biosciences, Palo Alto, CA) nanotechnology-based transfection reagent according to the manufacturer's guidelines. Twelve hours later, the medium was replaced with serum-free DMEM medium with penicillin and the cells were prepared for appropriate treatments.

2.4 | Whole-cell extraction

Cultured H9c2 cells were scraped and washed once with phosphate buffered saline (PBS). Then, the cell suspension was spun down, and cell pellets were lysed for 30 minutes in lysis buffer (50 mM Tris [pH 7.5], 0.5 M NaCl, 1.0 mM ethylenediaminetetraacetic acid [EDTA)] [pH 7.5], 10% glycerol, 1 mM beta mercaptoethanol (BME), 1% IGEPAL-630, and proteinase inhibitor) and spun down in 12 000 rpm for 20 minutes. The supernatant was collected in a new Eppendorf tube (Fisher Scientific, Hampton, NH) and stored at −80°C.

2.5 | Western blot analysis

The protein concentrations were determined using Lowry's method of protein quantification.[18] Western blot analysis of protein expression was performed following previous reports with a slight modification.[19] Proteins were separated in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Nonspecific protein binding was stopped by soaking in blocking buffer (5% milk, 20 mM Tris-HCl [pH 7.6], 150 mM NaCl, and 0.1% Tween-20), followed by hybridization with specific antibodies diluted in a blocking buffer at 4°C overnight. After incubation with secondary antibody for 2 hours, the membranes were washed and images were obtained in a Fuji LAS 3000 imaging system (Fuji, Minato, Tokyo, Japan) using ECL substrate and densitometric analysis was performed. If necessary, the nitrocellulose membranes were stripped with Restore Western blot stripping buffer (Thermo Fisher Scientific, Waltham, MA) at room temperature for 5 to 10 minutes and hybridization was performed again.

2.6 | Actin staining

H9c2 cells were inoculated into a 12-well plate. After treatments, the cells were fixed with 4% paraformaldehyde solution for 10 minutes at room temperature. After a rinse with PBS, the cells were treated with permeation solution (0.5% Triton X-100) for 10 minutes at 4°C. Following the wash with PBS, samples were first incubated with actin staining reagent for 30 minutes containing rhodamine-conjugated phalloidin with high affinity for actin. The cells were also stained with 1 μg/mL 4-,6-diamidine-2-phenylindole dihydrochloride (DAPI) for 30 seconds to detect the cell nucleus using UV light microscopic observations (blue). Samples were analyzed in a drop of PBS under a fluorescence microscope. The increasing cell size and intracellular actin polymerization were measured by Axio Vision LE software (Carl Zeiss, Jena, Deutschland). All measurements were performed by at least 3 independent individuals in a blinded manner.

2.7 | Coimmunoprecipitation assay

First, 100 μL of cell lysis buffer (for co-IP) (1.5 mM $MgCl_2$, 1% Triton X-100, 50 mM pH 7.6 (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid , 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1 mM $NaVO_3$, 10 mM NaF, 10 mM β-glycerolphosphate, 5 protease inhibitor tablets, and 50 mL of distilled water [DDW]) per plate was added to lyse the cells, which were then scarped down and stored at −80°C overnight. The method of obtaining the total protein samples was the same as in Western blot analysis. Lowry assay was applied to obtain the protein concentration of each protein sample. Next, 500 μL of co-IP buffer (1.5 mM $MgCl_2$, 1% Triton X-100, 50 mM pH 7.6 (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1 mM $NaVO_3$, 10 mM NaF, 10 mM β-glycerolphosphate, and 500 mL of DDW) was mixed with 5 μL of protein G-agarose and 100 μg in each 1.5 mL microcentrifuge tube. Nonspecific

protein binding was blocked in a blocking buffer (5% milk in 1× Tris-buffered saline [TBS]) for 1 hour at room temperature. 1 × TBS was used to wash off with a blocking buffer, and the polyvinylidene difluoride (PVDF) membranes were rocked in 1:1000 primary antibody (Ab) solutions overnight or so depending on the protein concentration and the antibody binding affinity. Then, the primary Ab TBS solutions were recycled. The PVDF membranes were washed with 1 × TBS 3 times and soaked and rocked in 1:1000 secondary Ab solutions for 1 hour at room temperature. The PVDF membranes were washed with 1 × TBS 3 times, and the protein expressions were detected with Western blot analysis luminol reagent.

2.8 | Statistical analysis

Each sample was analyzed based on the results that were repeated at least 3 times, and comparisons among the groups were carried out using one-way Analysis of Variance with a Tukey posttest. In all cases, differences at $P < .05$ were regarded as statistically significant, and the ones at $P < .01$ or $< .001$ were considered higher statistical significances.

3 | RESULTS

3.1 | ERβ inhibits the hypertrophic effect and BNP expression in ZAK WT overexpressed tet-on H9c2 cells

To determine whether E2/ERβ can prevent ZAK-induced hypertrophy in cardiomyocytes, the H9c2 cardiomyoblast cells were treated either with or without E2, with or without the addition of Dox and E2 in addition to 3-(isopropylamino)-1-[(7-methyl-4-indanyl) oxy]butan-2-ol (ICI) (ERs antagonist); cell hypertrophy was measured using the actin staining method. The results show that the overexpression of ZAK by treating with Dox resulted in significant increase in the cell size; however, the changes in cell size were reverted by E2 (Figure 1). The antihypertrophic effect of E2 was however suppressed when treated with ICI (ER antagonist). Further overexpression of ERβ, either alone or in combination with E2, also suppressed ZAK-induced hypertrophy; when treated with ICI, the antihypertrophic effects of E2 in ERβ-overexpressing cells also were reverted (Figure 1A,B). The results thus indicate that E2 ameliorates ZAK-induced hypertrophic effects in an ERβ-dependent manner. The ZAK induced

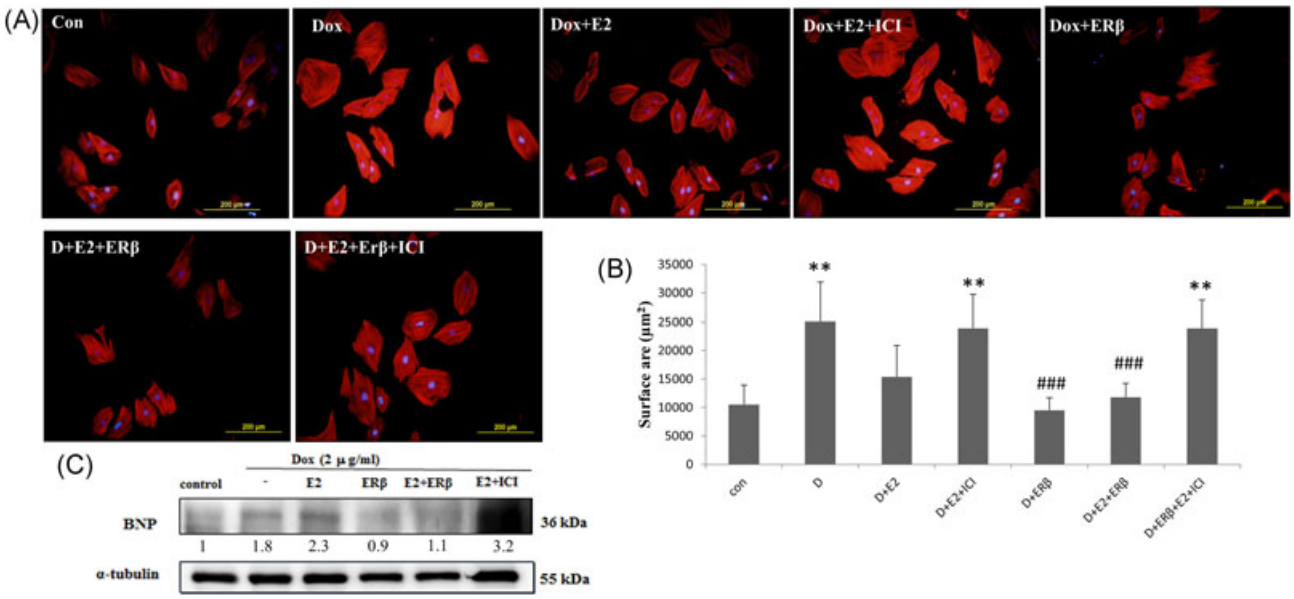

FIGURE 1 ERβ inhibited the cardiac hypertrophy effect and BNP expression of ZAK WT-overexpressing tet-on H9c2 cells. (A) ZAK tet-on cells were incubated with E2 (10^{-8} M) and E2 plus ER inhibitor (ICI, 1 μM) for 1 hour, and then treated with Dox (2 μg/mL) for another 23 hours; then, their actin filaments were stained with phalloidin-rhodamine. DAPI staining was used to stain the nuclei. (B) The statistical results representing the ratio of cell surface area calculated with respect to their perimeter found using imageJ software (National Institutes of Health, Bethesda, MD) are shown by 4 independent experiments; mean ± SD, $^{**}P < .01$ and $^{***}P < .001$ represent significant difference vs control H9c2 cells. $^{\#\#\#}P < .001$ represent significant difference vs ZAK-overexpressing H9c2 cells. (C) The expression of BNP protein as analyzed by Western blot analysis assay. All of the experiments were derived after performing 4 independent experiments. BNP, brain natriuretic peptide; DAPI, 4,6-diamidine-2-phenylindole dihydrochloride; ERβ, estrogen receptor β

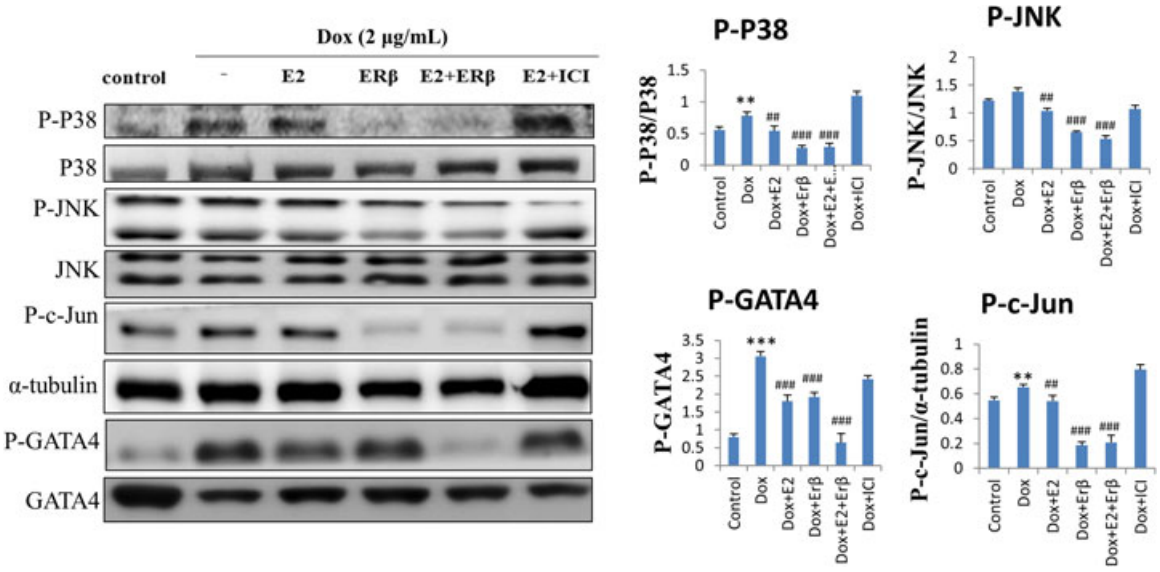

FIGURE 2 Effects of ERβ on the protein levels of p-p38, p-GATA-4, p-JNK, and p-c-Jun in the ZAK WT-overexpressing tet-on H9c2 cell system. The expressions of p-p38, p-GATA-4, p-JNK, and p-c-Jun proteins were analyzed by Western blot analysis of proteins from H9c2 cells that were transfected with ERβ (1.5 μg/mL), followed by treatment with or without E2 (10^{-8} M) and ER inhibitor (ICI, 1 μM) for 1 hour, and then treated with Dox (2 μg/mL) for 23 hours. ERβ, estrogen receptor β; SDS, sodium dodecyl sulfate

increase in cell size was also correlated with the elevated levels of the hypertrophy-associated marker protein, BNP, which was effectively reduced following ERβ overexpression and with E2 administration in ERβ-overexpressing ZAK WT cells. Furthermore, the ER inhibitor, ICI, reversed the ERβ effect exhibited against hypertrophy (Figure 1C).

3.2 | Effects of ERβ on the levels of p-p38, p-GATA-4, p-JNK, and p-c-Jun in the ZAK WT-overexpressed tet-on H9c2 cell system

Furthermore, the effect of E2/ERβ on the levels of downstream proteins of ZAK was determined in ZAK tet-on H9c2 cells. The data show that the increased levels of p-JNK, p-c-Jun, p-p38, and p-GATA-4 induced by ZAK overexpression were attenuated by ERβ overexpression and by E2 along with ERβ overexpression. Furthermore, ER inhibitor ICI reversed the effects of E2 and ERβ in ZAK tet-on H9c2 cells (Figure 2). The results therefore suggested that ERβ overexpression in both the presence and absence of E2 inhibits p-JNK, p-c-Jun, p-p38, and p-GATA-4 proteins that are associated with ZAK-induced cellular hypertrophy in H9c2 cells.

3.3 | ERβ physically interacts with ZAK and suppresses the protein level of ZAK

To understand the mode in which ERβ acts against ZAK-induced hypertrophy, the potential physical interaction of ZAK and ERβ was examined by the coimmunoprecipitation method. The results indicated that ERβ indeed binds with ZAK in normal condition (Figure 3A); however, the overexpression of ZAK following Dox treatment (Figure 3B) could effectively reduce ZAK–ERβ association and result in the elevation of free ZAK. On the contrary, overexpression

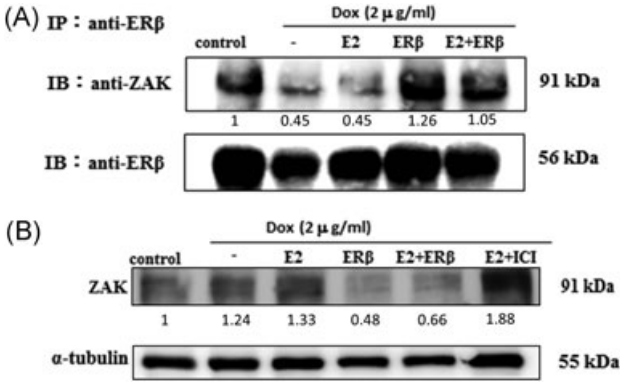

FIGURE 3 ERβ physically associates with ZAK to suppress the protein level of ZAK in ZAK WT-overexpressing tet-on H9c2 cells. (A) Tet-on/ZAK H9c2 cells were transfected with ERβ (1.5 μg/mL, 24 hours) followed by the treatment of E2 (10^{-8} M) for 1 hour, and then treated with Dox (2 μg/mL) for another 23 hours. ERβ was immunoprecipitated with anti-ERβ antibody, subjected to SDS-polyacrylamide gel, and detected using anti-ZAK antibody. (B) Expression of ZAK protein analyzed by Western blot analysis of ZAK protein levels in H9c2 cells that were transfected with ERβ (1.5 μg/mL, 24 hours), followed by treatment with or without E2 (10^{-8} M) and ER inhibitor (ICI, 1 μM) for 1 hour, and then treated with Dox (2 μg/mL) for another 23 hours. ERβ, estrogen receptor β; SDS, sodium dodecyl sulfate

of ERβ alone or in the presence of E2 enhanced the interaction between ERβ and ZAK (Figure 3A,B).

3.4 | ERβ reduced ZAK protein level is not ubiquitination-dependent, and ERβ inhibited CBL to prevent PI3K protein degradation

To determine whether ERβ suppresses the ZAK protein level and to find the possible role of ubiquitination in the effect of ERβ, H9c2 cell model with ZAK overexpression in combination with or without ERβ transient transfection was created, and the alteration in protein levels were measured by Western blotting assay. The results showed an increase in ZAK protein levels, without any change in E3 ligase-casitas B-lineage lymphoma (CBL), and ubiquitin levels after treatment with Dox. However, all these 3 protein levels were reduced by ERβ overexpressed in ZAK WT H9c2 cells (Figure 4A), and the protein level reduction was not restored by proteosome inhibitors (MG132, 0.5 to 2 g/mL). Further, the protein levels of ZAK and CBL were suppressed by different concentration of ERβ (5 to 15 g) treatment in a dose dependent manner (Figure 4B). Moreover, our results show that ERβ overexpression and E2 along with ERβ overexpression reduced the association of CBL and PI3K (Figure 4C). Reduction in the association of CBL and PI3K was also correlated with increase in Akt activation (Figure 4D).

3.5 | ERβ-repressed ZAK nuclear accumulation was mediated through the inhibition of SUMO-1 modification

The effect of ERβ in inhibiting ZAK nuclear translocation and ZAK-induced cellular hypertrophy was further examined by immunofluorescence staining to reveal ZAK accumulation in ZAK WT tet-on H9c2 cells. After Dox treatment of 24 hours, strong green fluorescence was measured in the nuclei, and the ZAK nuclear accumulation was apparent. The treatment of ERβ (1.5 μg/mL) or E2 plus ERβ blocked the accumulation of ZAK. However, the treatment of ER inhibitor (ICI, 1 μM) totally reversed the effect of ERβ (Figure 5A). In addition, we determined whether ERβ

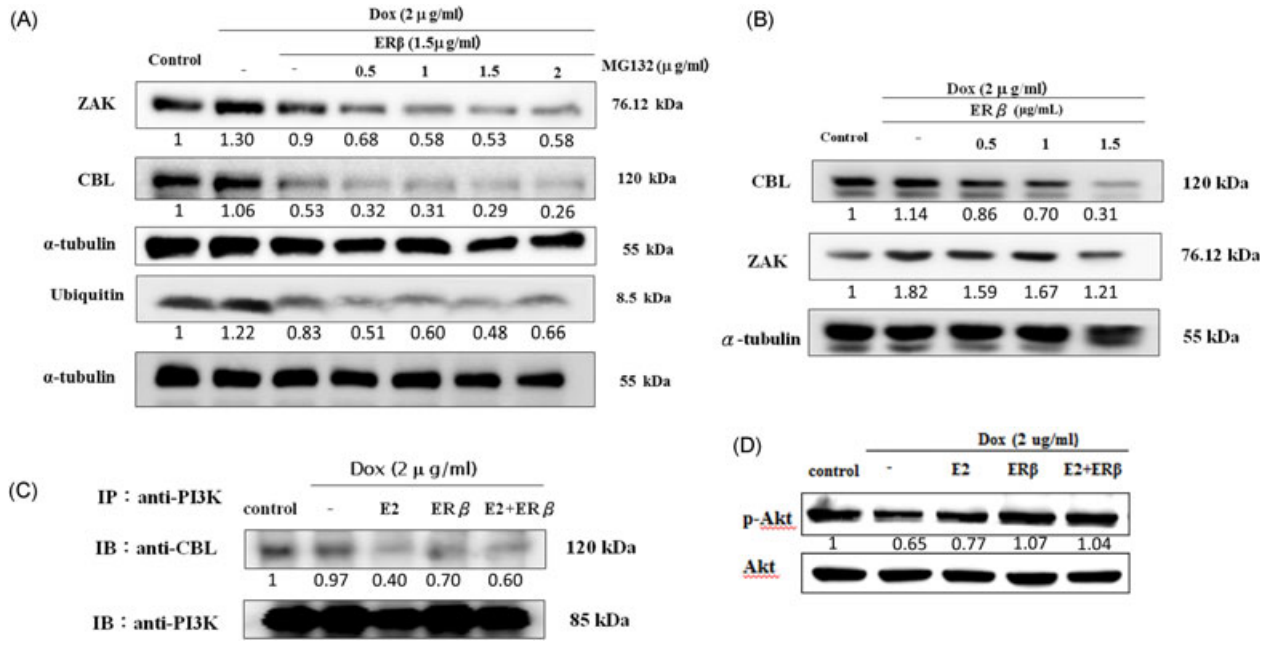

FIGURE 4 ERβ reduce ZAK protein level is not ubiquitination dependent, ERβ inhibits CBL to prevent PI3K protein degradation. (A) The expression of ZAK, CBL, Uubiquitin proteins were analyzed by Western blot analysis in H9c2 cells that was transfected with ERβ (1.5 μg/mL) for 24 hours, followed by the treatment of Dox (2 μg/mL) with or without proteosome inhibitor (MG132) for another 24 hours. (B) The expression of ZAK and CBL were analyzed by Western blotting analysis in H9c2 cells which were transfected with ERβ of different concentrations (0, 0.5, 1, and 1.5 μg/mL) for 24 hours, followed by the treatment of Dox (2 μg/mL) for another 24 hours. (C) Tet-on/ZAK H9c2 cells was transfected with ERβ (1.5 μg/mL) for 24 hours, followed by the treatment of E2 (10^{-8} M), E2 plus ERβ, and then treated with Dox (2 μg/mL) for another 24 hours. PI3K was immunoprecipitated with anti-PI3K antibody, and detected using anti-CBL antibody. (D) The expression of p-Akt and Akt proteins analyzed by Western blot analysis of proteins from H9c2 cells that were transfected with ERβ (1.5 μg/mL), followed by the treatment with or without E2 (10^{-8} M) and then treated with Dox (2 μg/mL) for 23 hours. CBL, casitas B-lineage lymphoma; ERβ, estrogen receptor β

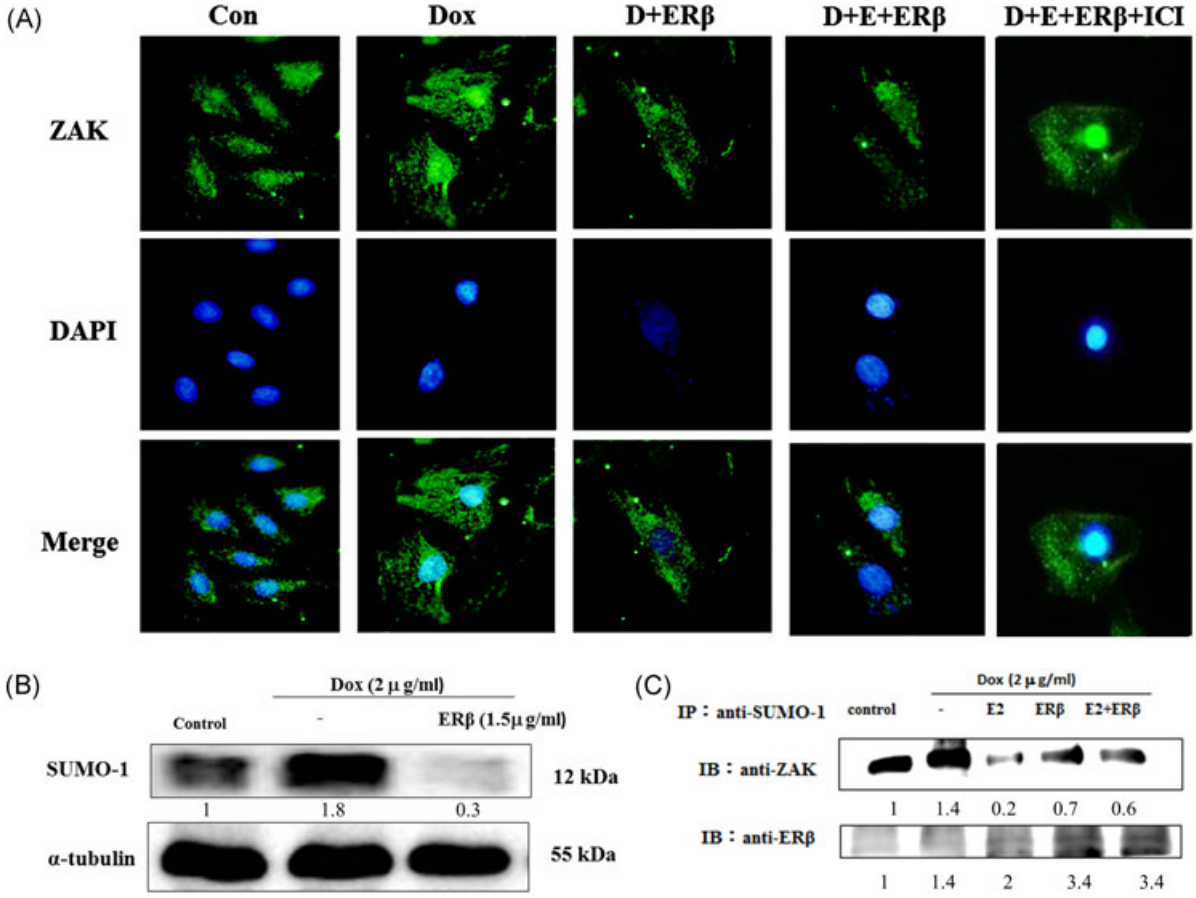

FIGURE 5 ERβ-repressed ZAK nuclear translocation was mediated through the inhibition of SUMO-1 modification. (A) Dox-induced ZAK overexpression promoted ZAK translocation, and both ERβ (1.5 μg/mL) overexpression and ERβ with E2 administration could inhibit ZAK nuclear translocation. However, ER inhibitor (ICI, 1 μM) treatment reversed the effect of ERβ. Indirect immunofluorescence was performed on ZAK WT tet-on H9c2 cells using Anti ZAK antibody (10 μg/mL, green) in all 5 group cells, followed by DAPI nuclear counterstaining (blue). (B) Expression of SUMO-1, analyzed by Western blot analysis of proteins from ZAK WT tet-on H9c2 cells transfected with ERβ (1.5 μg/mL) for 24 hours, followed by the treatment of Dox (2 μg/mL) for 24 hours. (C) Tet-on/ZAK H9c2 cells were transfected with ERβ (1.5 μg/mL) for 24 hours, followed by the treatment of E2 (10^{-8} M), E2 plus ERβ, and then treated with Dox (2 μg/mL) for another 24 hours. SUMO-1 was immunoprecipitated with anti-SUMO-1 antibody and detected using anti-ZAK and ERβ antibodies. ERβ, estrogen receptor β; SUMO-1, small ubiquitin-like modifier-1

repressed ZAK nuclear accumulation that was mediated through the inhibition of small ubiquitin-like modifier (SUMO)-1 modification. The results indicated that the amount of SUMO-1 protein was elevated after Dox treatment, but was inhibited by ERβ overexpressed in ZAK WT H9c2 cells (Figure 5B). Interestingly, coimmunoprecipitation assay of ZAK with SUMO-1 showed that ZAK overexpression elevated SUMOylation of ZAK, indicating its enhanced nuclear accumulation. However, treatment with ERβ (1.5 μg/mL) or E2 plus ERβ inhibited the effect, thereby suppressing the nuclear accumulation. Reduction in ZAK-SUMO interaction was accompanied by a corresponding increase in ERβ-SUMO-1 interaction. Collectively, our results show that ERβ interferes in the SUMOylation of ZAK to suppress its nuclear accumulation.

4 | DISCUSSION

It is well known that modulations in the sex hormones due to aging or illness potentially result in metabolic disorders, and these changes are commonly gender specific. Whereas testosterone levels are interpreted to provide protection from diabetes and aging, E2 levels are often correlated with partial protection from cardiovascular diseases.[20] Early response of hypertension or aortic stenosis such as cardiac hypertrophy further progresses to heart failure but at a different frequency and age among men and women.[21-24] The ejection fraction (EF) of women with heart failure is generally preserved; however, men undergo heart failure with deteriorated EF.[25,26] Moreover, women show a better recovery from reduced systolic function than do men.[27,28] The enhanced cardiac adaptation to hypertension stress among females is attributed to E2, as observed in animal

models and in humans.[29,30] Various studies on different models have shown that men develop heart disease or succumb to heart failure at an early age than do women.[31-33] At early stages of hypertension-related hypertrophy, women are associated with normal systolic function and men are likely to develop hypertrophy at a high frequency with difference in gene expression compared to their female counterparts.[34] In a recent report, it was shown that although the prevalence of hypertrophy in females may be higher than that in males, myocardial gene expression remains "close to normal."[35] Transcriptome analyses covering mRNA and miRNA from normal and pathological hearts show enormous sex difference, which would potentially alter cardiac homeostasis.[36]

From animal to human studies, estrogen has been shown to modulate vascular tone and arterial resistance, arterial dilation, and blood flow, thereby lowering blood pressure and preventing cardiac hypertrophy.[37] Myocardial hypertrophy frequently develops in older humans. With age, a sexual dimorphism appears, and the incidence of hypertrophy in postmenopausal women exceeds that n age-matched men.[38] The latter can be reversed in postmenopausal women by hormone replacement therapy,[39] suggesting that estrogen may oppose the developmental events in the cardiomyocyte and stroma that produce hypertrophy. Our previous reports indicated that E2 reduces pathological cardiac hypertrophy and heart failure by upregulating PI3K-Akt signaling and by suppressing calcineurin-NFATc3 signaling in ovariectomized rats,[10] and E2 and/or estrogen receptor α also has/have been demonstrated to suppress LPS-induced TNF-α expression and myocardial cell apoptosis.[11]

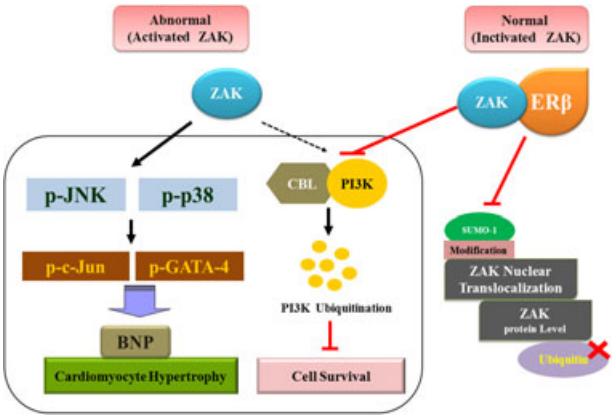

FIGURE 6 Estrogen receptor β physically interacted with ZAK to reduce the ZAK protein level and inhibited the nuclear translocation via SOMO-1 modification. The physical interaction with ERβ ameliorated ZAK-inhibited PI3K protein by CBL binding and also blocked ZAK-induced cardiohypertrophy effect. ERβ, estrogen receptor β; SUMO-1, small ubiquitin-like modifier-1

Here, we would like to evaluate whether E2 and/or ERβ show(s) inhibitory effect on ZAK-induced hypertrophy in H9c2 cells and further identify the role of ERβ in cellular hypertrophy. All our results indicate that ERβ overexpression and E2 along with ERβ overexpression display inhibitory effect on ZAK hypertrophy in the H9c2 cardiomyoblast. These suggest that ERβ enhancement could be a useful therapeutic approach against ZAK-induced cellular hypertrophy.

We further observed that the ERβ physically associated with ZAK and directly repressed the ZAK protein level. The results also indicated the increase of ZAK protein, but with no change of E3 ligase-CBL after ZAK overexpression. The results suggest that ERβ-mediated reduction in the ZAK protein level was not due to protein ubiquitination. Moreover, the CBL family of ubiquitin ligases is a negative regulator of nonreceptor tyrosine kinase and some activated signaling pathways,[40-43] and the tyrosine kinase-binding (TKB) domain of the CBL family proteins can interact with the p85 subunit of PI3K, resulting in their ubiquitination and degradation.[41,43,44] Our study demonstrated that treatment with ERβ effectively suppresses ZAK and CBL protein-level expressions. Our results further indicate that the overexpression of ERβ could reduce CBL to interact with PI3K, which may result in the inhibition of PI3K ubiquitination and degradation and could positively regulate the activity of Akt survival signals.

A previous study has indicated ZAK as a transcription factor containing a leucine-zipper domain.[45] In this study, we explored whether ERβ would attenuate ZAK-induced hypertrophy by inhibiting ZAK nuclear translocation. Our findings suggest that the overexpression of ZAK could enhance their translocation and accumulation in the nuclei. Furthermore, on treatment with ERβ and E2 plus ERβ, the translocation of ZAK from nuclei to cytoplasm was apparent. The findings suggest that ERβ blocked ZAK nuclear translocation to prevent ZAK-induced hypertrophy by modulating the SUMOylation of ZAK. In addition, the fact that the components of the SUMO conjugation pathway are abundant in the heart indicates the possibility that the SUMO pathway may be implicated in cardiovascular development via modifying the transcription factors indispensable for normal cardiovascular function and development.[46,47] The results show that Dox treatment to overexpress ZAK induced SUMO-1 protein expression; however, overexpressed ERβ could totally repress the SUMO-1 protein level induced by ZAK. SUMO-1 modification affected the transcription factor activity that might be involved in the mechanism to enhance ZAK nuclear translocation, and ERβ might mediate through the suppression of SUMO-1 modification to prevent ZAK-induced cellular hypertrophy.

Cardiomyocytes do not proliferate and differentiate in mature tissue, or the normal proliferation is extremely slow. Therefore, cardiac pathological hypertrophy along with the impaired repair mechanism results in severe damage to the heart. For this reason, strategies to prevent the occurrence of pathological hypertrophy have become increasingly important. Recently, ERβ has been shown to attenuate cardiac-hypertrophy and anticardiac-apoptosis effects,[48] and our results clearly suggest that ERβ overexpression even without estrogen supplementation strongly suppresses ZAK-induced cardiomyocyte hypertrophy (Figure 6). Therefore, direct ERβ activation might be an efficient alternative treatment strategy to estrogen replacement therapy in postmenopausal women to treat myocardial damage, which would potentially prevent side effects such as breast or vaginal cancer.

ACKNOWLEDGMENTS

This study was supported in part by Taiwan Ministry of Health and Welfare-Clinical Trial Center (MOHW106-TDU-B-212-113004).

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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How to cite this article: Pai P, Shibu MA, Chang R-L, et al. ERβ targets ZAK and attenuates cellular hypertrophy via SUMO-1 modification in H9c2 cells. *J Cell Biochem*. 2018;1-10.
https://doi.org/10.1002/jcb.27199