Institute of Pharmacology, Toxicology and Biochemical Pharmaceutics[1], College of Pharmaceutical Sciences, Zhejiang University; Department of Criminal Justice[2], Zhejiang Police Academy, Hangzhou, China

Involvement of PIKE in icariin induced cardiomyocyte differentiation from murine embryonic stem cells

Limin Zhou[1,2], Bei Zheng[1], Leilei Tang[1], Yujie Huang[1], Danyan Zhu[1]

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Dr. Danyan Zhu, Institute of Pharmacology, Toxicology and Biochemical Pharmaceutics, College of Pharmaceutical Sciences, Zhejiang University, No. 866, Yu Hang Tang Road, Hangzhou, 310058, China
zdyzxb@zju.edu.cn

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Icariin (ICA) has demonstrated to induce cardiomyocyte differentiation from murine embryonic stem (ES) cells *in vitro*, however, the mechanisms have not been fully elucidated. In the present study, we investigated whether phosphatidylinositol 3-kinase enhancer (PIKE) was involved in ICA induced cardiomyocyte differentiation of ES cells. Small interfering RNA (siRNA) of PIKE was applied to investigate the role of PIKE in ICA induced cardiomyocyte differentiation. The cardiomyocytes derived from ES cells were verified using immunofluorescence. The expressions of Troponin T, PIKE, phosphatidylinositol 3-kinase (PI3K), and nuclear factor-kappaB (NF-κB) were detected by western blot. The change of reactive oxygen species (ROS) generation was estimated using the fluorescent dye 2', 7' - dichlorodihydrofluorescein diacetate. The results showed that PIKE expression increased during cardiomyocyte differentiation. ICA markedly enhanced PIKE and PI3K expression in a time-dependent manner. Knockdown of PIKE by siRNAs blocked the differentiation of ES cells into cardiomyocytes expressing α-actinin for cardiac sarcomeric structures. Moreover, reduced ROS generation and NF-κB nuclear translocation were responsible for the inhibitory effect of si-*PIKE*. In conclusion, PIKE was involved in ICA induced cardiomyocyte differentiation, and ROS generation and NF-κB nuclear translocation were associated with PIKE activation.

1. Introduction

Icariin (ICA, $C_{33}H_{40}O_{15}$) is the active ingredient of plant herba *Epimedium*, which possesses biological actions, such as improving cardiovascular function, modulating hormone excretion and immunological functions, and displaying anti-tumor activities (He et al. 1995). Our previous study (Ding et al. 2008) demonstrated that ICA at a concentration of 1×10^{-7} mol/L promotes the directional differentiation of embryonic stem cells (ES) cells into cardiomyocytes. Reactive oxygen species (ROS) play a great role in regulating ICA induced cardiomyocyte differentiation. Recently, we found that mGluR5 (metabotropic glutamate receptor 5) stimulating Homer–PIKE (phosphatidylinositol 3-kinase enhancer) formation initiates ICA induced cardiomyogenesis by ROS (Zhu et al. 2012). However, the precise role of PIKE in ICA induced cardiomyocyte differentiation is still not understood.

PIKE was originally identified as a brain-specific nuclear GTPase, which binds phosphatidylinositol 3-kinase (PI3K) and enhances its lipid kinase activity in a GTP-dependent manner (Ye et al. 2000). The Homer/PIKE-L complex couples PI3K to mGluR1/5 and regulates a major action of mGluR1/5, prevention of neuronal apoptosis (Rong et al. 2003). Until now, research on PIKE is mainly focused on the brain, however, emerging evidence shows that the function of PIKE is not restricted to the brain. It is worthwhile to investigate the role of PIKE in cardiomyocyte differentiation from ES cells.

The mechanistic link between PIKE and ROS remains largely unexplored. We have shown that mGluR5 stimulates Homer-PIKE formation and regulates cardiomyocyte differentiation by activating ROS and nuclear factor-kappaB (NF-κB) (Zhou et al. 2013; Wo et al. 2008). However, the direct role of PIKE in ICA induced ROS generation and NF-κB nuclear translocation is not known. In the present study, small interfering RNA (siRNA) of PIKE was applied to investigate the role of PIKE in ICA induced cardiomyocyte differentiation. The changes of ROS generation and NF-κB nuclear translocation were also investigated.

2. Investigations, results and discussion

We found that PIKE migrated at an apparent molecular mass of ∼130 kDa and ∼90 kDa, consistent with the size predicted from the product data sheet of the antibody (molecular weight of PIKE-L: 130 kDa; molecular weight of PIKE-S: 90 kDa). Based on the fact that PIKE-L but not PIKE-S binds to Homer and activates cytosolic PI3K (Rong et al. 2003), leading to ROS generation, we choose ∼130 kDa in the following studies. As shown in Fig. 1A, PIKE expression increased during cardiomyocyte differentiation. In addition, PIKE was high expressed in the adult mouse heart.

ICA was added to the differentiation medium on day 5. As determined by western blot, from day 5 + 3 to 5 + 11, the protein expression of PIKE and PI3K p110α was increased in parallel with contracting cardiomyocyte maturation during differentiation. ICA significantly enhanced PIKE and PI3K p110α protein expression (Fig. 1B).

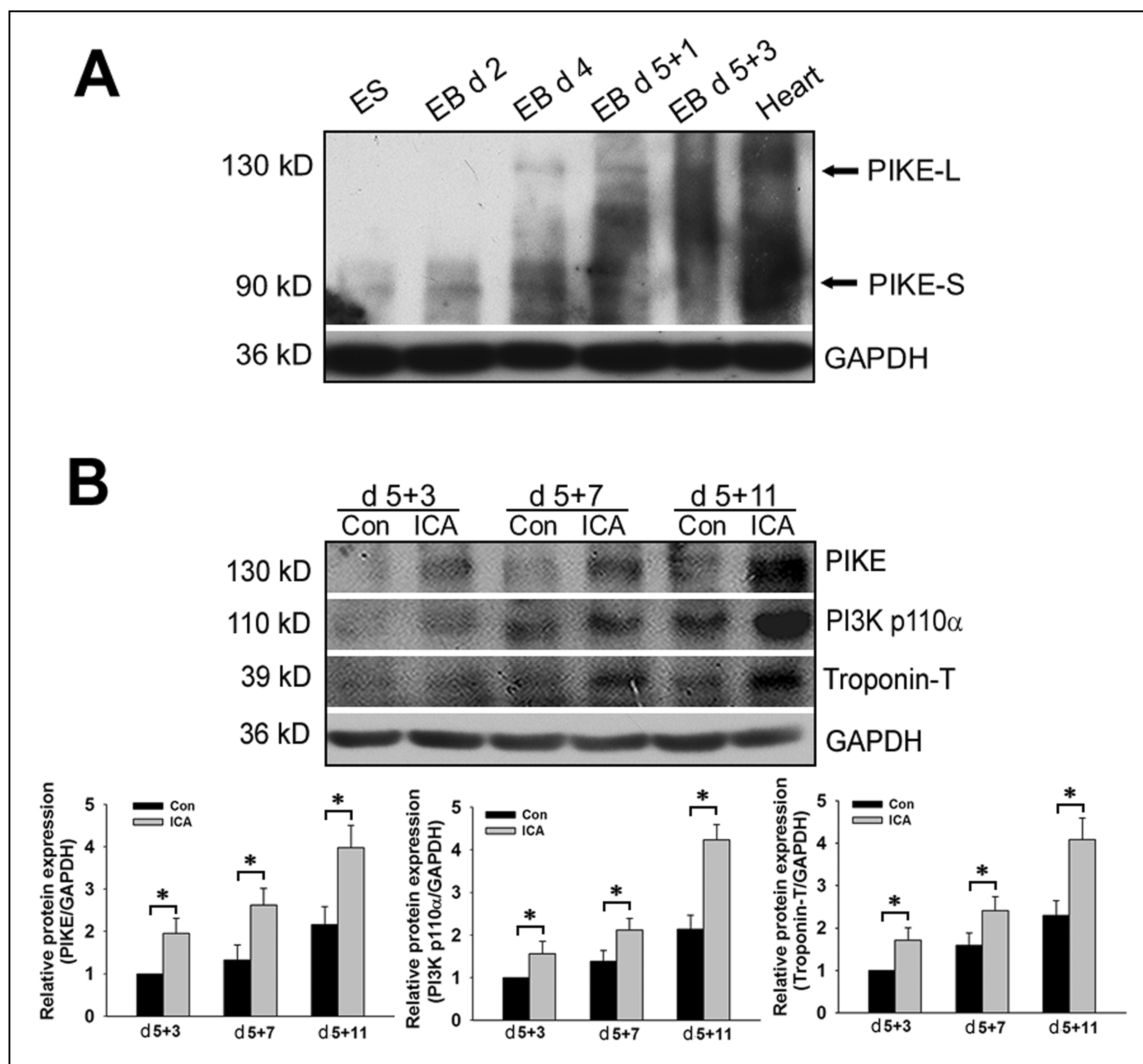

Fig. 1: ICA enhanced PIKE expression in cardiomyocyte differentiation. (A) Expression pattern of PIKE during cardiomyocyte differentiation. (B) ICA increased PIKE expression during cardiomyocyte differentiation. Similar data were obtained from three independent experiments.* $P < 0.05$ versus control.

The si-*PIKE*-1, si-*PIKE*-2 and si-*PIKE*-3 were transiently transfected into ES cells. The si-*PIKE*-2 was determined to substantially decrease the levels of PIKE, while si-*PIKE*-1 and si-*PIKE*-3 were not effective in knocking down PIKE (Fig. 2A), so we chose si-*PIKE*-2 in the next studies. We further investigated whether a transient down-regulation of PIKE in ES cells could block ICA induced cardiomyocyte differentiation. At day 5 + 7 of cardiomyocyte differentiation, the percentage of beating EBs was reduced to 20 ± 10% with si-*PIKE*-2, compared with that of si-*ctr* (45 ± 10%). ICA significantly increased the number of beating EBs to 90 ± 10%, which was abolished in the presence of si-*PIKE*-2 (28 ± 12%) (Fig. 2B). Moreover, si-*PIKE*-2 significantly reduced the protein expression of PI3K p110α and Troponin-T in EBs on day 5 + 3 (Fig. 2C). Moreover, si-*PIKE*-2 blocked the differentiation of ES cells into cardiomyocytes expressing α-actinin for cardiac sarcomeric structures (Fig. 2D). As shown in the higher magnification of rectangular frames in Fig. 3D, α-actinin, the sarcomeric protein, was disorganized in si-*PIKE*-2 treated ES cells-derived EBs, which suggested there was an abnormal cardiac myofibrillogenesis in these cells.

An increase in ROS generation was observed when EBs were treated with ICA for 2 h, which was inhibited in the presence of si-*PIKE*-2 (Fig. 3A). ICA administrated for 2 h up-regulated NF-κB p65 expression in nucleus fraction, meanwhile, its expression in cytoplasm fraction was down-regulated compared with the case in the control, indicating the nuclear translocation of NF-κB p65 after ICA treatment. ICA induced NF-κB nuclear translocation was abolished by si-*PIKE*-2 (Fig. 3B). The results suggested that PIKE played a pivotal role in ICA induced cardiomyocyte differentiation by ROS and NF-κB activation.

To date, three forms of PIKE have been characterized: PIKE-S, PIKE-L, and PIKE-A. PIKE-S is the initially identified shorter isoform (Ye et al. 2000). PIKE-L, a longer isoform of PIKE gene, differs from PIKE-S by C-terminal extension containing ADP ribosylation factor-GTPase activating protein (Arf-GAP) and two ankyrin repeat domains. In contrast to the exclusive nuclear localization of PIKE-S, PIKE-L occurs in both the nucleus and the cytoplasm (Rong et al. 2003). PIKE-A contains the same domains present in PIKE-L but lacks an N-terminal proline-rich domain (PRD), which binds PI3K and PLC-γ1 (Rong et al. 2003; Ahn et al. 2004; Ye et al. 2002). The all anti-PIKE antibodies from Santa Cruz Biotechnology clearly describe that the molecular weight of PIKE-L and PIKE-S is 130 kDa and 90 kDa, respectively. The anti-PIKE antibodies from other companies, such as Sigma-Aldrich and ProSci Incorporated, could only be used for detection of both PIKE-L and PIKE-A. Until now, there is no commercially available antibody against PIKE-L only (The information from the companies, such as Santa Cruz

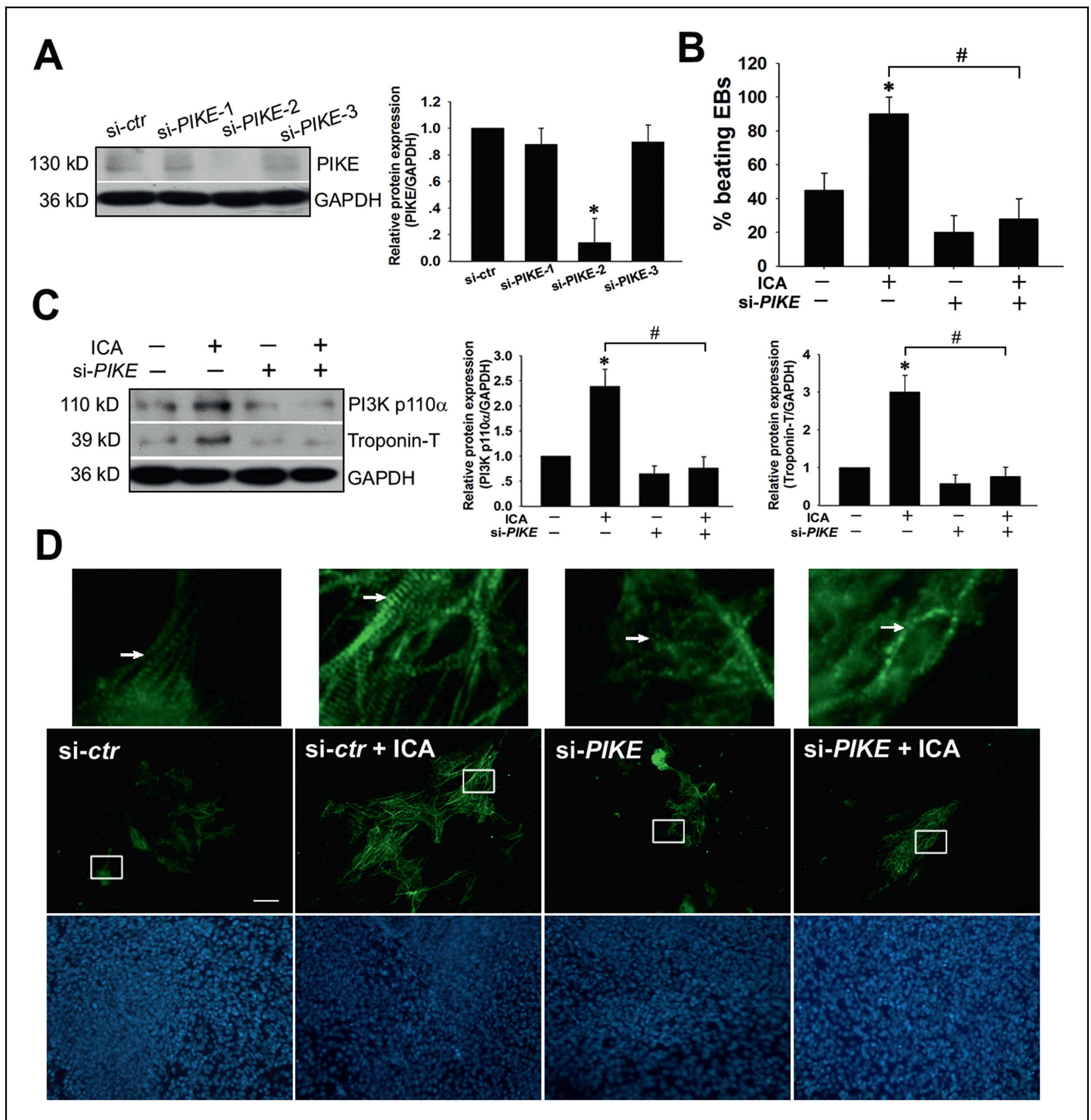

Fig. 2: Knockdown of PIKE impaired ICA induced cardiomyocyte differentiation. (A) Western blot analysis of PIKE protein expression in ES cells transfected with siRNAs, (B) Beating activity at day 5 + 7 in EBs generated after transient si-PIKE-2 transfection in the absence and presence of ICA. (C) Western blot analysis of PI3K p110alpha and Troponin T protein expression in EBs on day 5 + 3. (D) immunofluorescent analysis of alpha-actinin in EBs on day 5 + 7. Scale bar = 100 um. Similar data were obtained from at least three independent experiments. *$P < 0.05$ versus si-ctr. #$P < 0.05$ versus ICA.

Biotechnology, Cell Signaling Biotechnology, Abcam, Origene and Sigma-Aldrich). In the present study, we used the antibody both against PIKE-L and PIKE-S. Based on our former research on mGluR5 stimulating Homer-PIKE formation (Zhou et al. 2013), we focus on PIKE-L.

Since its discovery in 2000, PIKE has been recognized as a class of GTPase that controls the enzymatic activities of PI3K and Akt in the central nervous system (CNS). PIKE is important in regulating neuronal survival and development by substantiating the PI3K/Akt pathway (Chan et al. 2011). During development, PIKE knock-out ($PIKE^{-/-}$) neurons show reduced dendritic complexity, dendritic branch length, and soma size. These defects are due to the reduced PI3K/Akt activities in $PIKE^{-/-}$ neurons (Chan et al. 2011). However, our recent study suggested that the function of PIKE was not restricted to the CNS. PIKE was also involved in cardiomyocyte differentiation of ES cells and it was expressed in the adult heart. si-*PIKE* inhibited cardiomyocyte differentiation by repressing PI3K, which further confirmed the critical role of PIKE in regulating PI3K signaling.

In conclusion, the inducible mechanisms of ICA were related to PIKE signaling. Knockdown of PIKE blocked ICA induced cardiomyocyte differentiation via repressing ROS generation and NF-κB nuclear translocation (Fig. 4).

3. Experimental

3.1. Cell culture and treatment

The ES-D3 cells (American Type Culture Collection, CRL-1934) were differentiated into cardiomyocytes as described previously (Zhu et al. 2005). In brief, cultures of differentiating mouse ES cells were established by forming EBs in hanging drop culture for 3 days. Then EBs were transferred to float in the petri dishes for an additional 2 days. On day 5, the EBs were plated separately onto plates. At this time (day 5), ICA (Drug Biology Product Examination Bureau, Beijing, China) was added to the differentiation medium. In this experiment, day 5 + x referred to day x after the EBs were

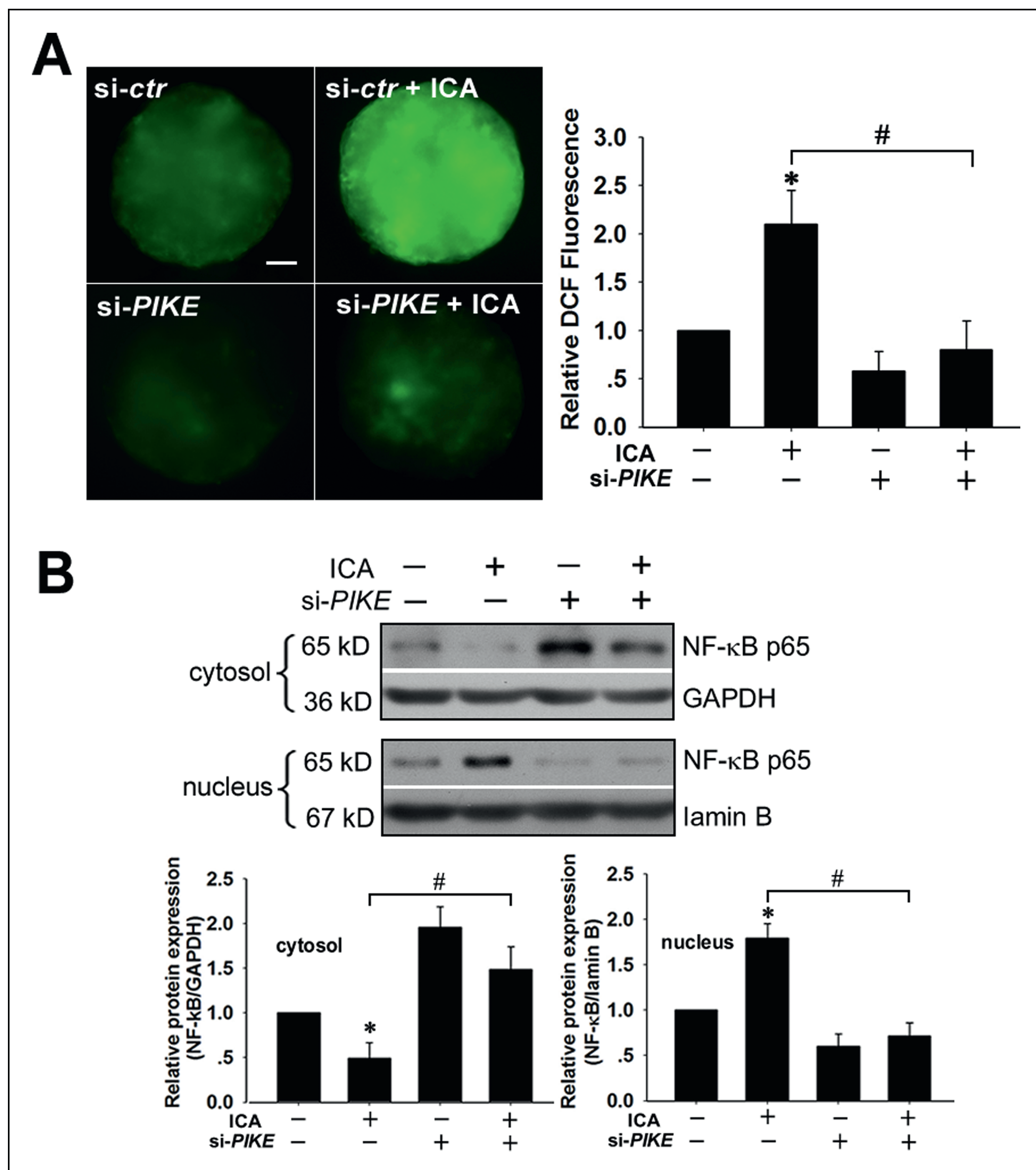

Fig. 3: Reduced ROS generation and NF-κB nuclear translocation were responsible for the inhibitory effect of si-PIKE. (A) Intracellular ROS generation (evaluated after 2 h of ICA) was significantly inhibited in EBs generated after transient si-PIKE-2 transfection. Scale bar = 100 um. (B) NF-κB nuclear translocation (evaluated after 2 h of ICA) was significantly inhibited in EBs generated after transient si-PIKE-2 transfection. GAPDH and lamin B expression in cytosol or nucleus was used to control the purity of the sub-cellular fractions studied. Similar data were obtained from at least three independent experiments. *$P < 0.05$ versus si-ctr. #$P < 0.05$ versus ICA.

plated onto gelatin-coated plates. At day 5 + 7, EBs containing two or more beating foci was considered beating EBs (Meyer et al. 2000; Sauer et al. 1999). This scoring technique is based on the fact that an EB containing only a single beating cluster, is usually very small and not representative of a typical beating EB in which several beating clusters are usually visible. The rhythmically beating EBs were considered to be spontaneously beating cardiomyocytes in EB outgrowths, and were defined as the phenotypic marker of successful differentiation.

3.2. Cell lysis and Western blot

Cells were collected in RIPA lysis buffer (containing 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 20 mM EGTA, 50 mM Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl), 20 mM sodium fluoride, 1 mM sodium orthovanadate, 0.1 mg/ml phenylmethanesulfonyl fluoride (PMSF), 10 μg/ml leupeptin and 2.0 μg/ml aprotinin) and lysed for 30 min on ice. Cytoplasmic and nuclear extracts were prepared according to the manufacturer's instructions (Applygen Technologies Inc, China). Proteins were loaded onto on a 7.5% SDS-polyacrylamide gel. Subsequently, proteins were transferred onto 0.45 μm pore size polyvinylidene fluoride (PVDF) membranes and blocked, followed by an overnight incubation at 4 °C with the appropriate antibodies: anti-PIKE, anti-PI3K p110α, anti-Troponin T, anti-NF-κB p65, anti-lamin B (1:500; Santa Cruz Biotechnology) and anti-GAPDH (1:10000). The membranes were then incubated with horseradish peroxidase (HRP)-conjugated antibodies (1:5000; Affinity Bioreagents, Golden, CO). The proteins were visualized autoradiographically with an enhanced chemiluminescent substrate (ECL; Pierce, Rockford, IL), and scanned using a bio-imaging analyzer (Bio-Rad). The product densities were quantitated using Quantity One version 4.2.2 software (Bio-Rad).

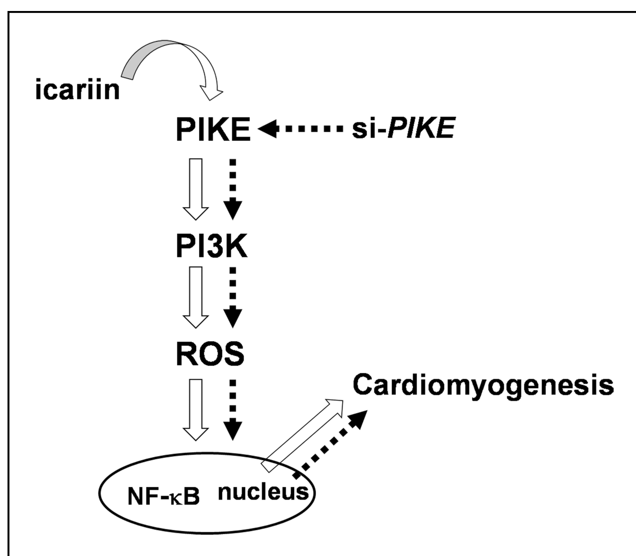

Fig. 4: Proposed model of the molecular mechanisms underlying ICA induced cardiomyocyte differentiation of ES cells involving the activation of PIKE/PI3K/ROS/NF-κB signaling pathway. The real arrow represents activation, while the broken arrow represents inhibition.

3.3. Short interfering RNAs transfection

The short interfering RNAs (siRNAs) targeting mouse PIKE mRNA (si-*PIKE*) and a validated negative control siRNA (si-*ctr*) were ordered from GenePharma (Shanghai, China) as follows:
(si-*PIKE*-1 forward): 5'-GCUGUGAUCUUCGUCUUCATT-3',
(si-*PIKE*-1 reverse): 5'-UGAAGACGAAGAUCACAGCTT-3';
(si-*PIKE*-2 forward): 5'-GCAUCGAGUGUUCUGGCAUTT-3',
(si-*PIKE*-2 reverse): 5'-AUGCCAGAACACUCGAUGCTT-3';
(si-*PIKE*-3 forward): 5'-CCAGCAUUAAUGACUACAUTT-3'
(si-*PIKE*-3 reverse): 5'-AUGUAGUCAUUAAUGCUGGTT-3';
(si-*ctr* forward): 5'-UUCUCCGAACGUGUCACGUTT-3',
(si-*ctr* reverse): 5'-ACGUGACACGUUCGGAGAATT-3'.
In brief, si-*ctr*, si-*PIKE*-1, -2, -3 (200 pmol) were combined with 2 μl siPORT NeoFX (Ambion) in a final volume of 200 μl in Opti-MEM medium (Invitrogen). The lipofection mixture was added to freshly passaged ES cells in suspension (15×10^4 cells/well of a 12 well plate). To identify the efficiency of si-*PIKE*-1, -2, -3, immediately after transfection, cells were plated onto gelatin-coated dishes with the presence of recombinant mouse leukemia inhibitory factor (LIF; Chemicon) and cultured for 48 h for western blot analysis. The transfected ES cells were harvested for EBs formation.

3.4. Immunofluorescence

Cells were fixed for 10 min in ice-cold methanol. Then, the fixed cells were permeabilized by incubation with 0.1% triton X-100 in phosphate buffered saline (PBS) for 30 min. After treatment with cattle serum for 30 min, specimens were incubated at 4 °C overnight together with the appropriate primary antibody anti-α-actinin (1:200; Sigma). Specimens were then incubated with the respective fluorescent secondary antibodies. 4, 6-diamidino-2-phenylindole (DAPI; Sigma) was used to dye the cell nuclei. Cells were then observed under a fluorescence microscope (Leica DMIL, Germany).

3.5. Intracellular ROS determination

Intracellular ROS levels were measured using the fluorescent dye 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes, Eugene, OR), which is a non-polar compound that is converted into a non-fluorescent polar derivative (H_2DCF) by cellular esterases after incorporation into cells. For the experiments, EBs on day 5 were transferred to 24-well culture plates and treated with 10^{-7} mol/L ICA for 2 h. Then EBs were incubated for 30 min with 5 μM H_2DCFDA in serum-free medium. At last, EBs were washed twice with PBS, and DCF fluorescence was evaluated in 5000 μm^2 regions of interest in the center of EBs. The 488-nm band of the argon ion laser of confocal setup was used for excitation and a long-pass LP505-nm filter set was used for record of emission.

3.6. Statistical analysis

Data are expressed as mean values with standard deviation (SD). At least three independent experiments were performed. Statistical analysis was performed with one-way ANOVA. P value of $P < 0.05$ was considered to be significant.

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