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All-trans retinoic acid regulates *c-jun* expression via ERK5 in cardiac myoblasts☆

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Abstract

Retinoic acid (RA) is an active metabolite of vitamin A and plays an important role in biological processes including cell proliferation. MAPKs play a pivotal role in regulating many critical cellular processes in the heart. The aim of the study was to determine whether all-trans RA (atRA) affects the proliferation of H9c2 rat ventricular cells and whether ERK family is involved in this process. H9c2 myocardial cells were cultured and subjected to MTT and ^{3}H-thymidine incorporation assays for proliferation detection. Luciferase reporter gene and Western blot assays were used to detect the transcription and protein expression of *c-jun*. In addition, the activities of ERK5 and ERK1 were determined by Western blot assay. The subcellular distribution of ERK5 and ERK1 was analyzed by confocal microscopy. It was shown that atRA (0.05 μM) facilitated the proliferation of H9c2 myocardial cells and increased the transcription and protein expression of *c-jun*. Inhibition of ERK5 significantly decreased atRA-induced pJluc expression ($P<.01$). The activity of ERK5 but not ERK1 was induced by atRA. Furthermore, atRA promoted the nuclear translocation of ERK5 but not ERK1. These results suggest that ERK5 pathway may be involved in the process that atRA regulates proliferation in the developing heart.

Keywords: All-trans retinoic acid; ERK5; ERK1; *c-jun*; Translocation; Proliferation

1. Introduction

Retinoic acid (RA) is an active metabolite of vitamin A and plays a crucial role in biological processes including cell proliferation, differentiation and morphogenesis, especially during heart development [1–5]. In different cells, the outcome of RA induction is different. For instance, RA can induce apoptosis of tumor cells [2], whereas atRA inhibits apoptosis of leukemic cells, mesangial cells and fibroblasts [1,6].

MAPKs play a pivotal role in regulating many critical cellular processes such as growth, differentiation, apoptosis and stress-related responses. MAPKs transduce various extracellular stimuli including growth factors, hormones, cytokines, antigens and many physical–chemical stimuli into distinct intracellular responses [7–11]. In mammals, four major MAPK pathways have been discovered. They are the extracellular signal-regulated kinases-1 and -2 (ERK1/2), the c-Jun N-terminal kinase (JNK), p38s and the big mitogen-activated protein kinase-1/ERK5 [12]. ERK5 is one member of the ERK family of protein kinases; its upstream kinase was identified to be MEK5. MEK5 and ERK5 interacted specifically with one another and did not interact with kinases in the MEK1/ERK1 signaling pathway [13].

ERK5 is involved in up-regulating *c-jun* and *c-fos* expression. The *jun* and *fos* gene families are immediate early genes whose expression can be induced by growth-promoting stimuli [14]. They are members of AP-1 family transcription factors. c-Jun and c-Fos heterodimers are some of the most

Abbreviations: ANOVA, one-way analysis of variance; atRA, all-trans retinoic acid; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ERK1/2, the extracellular signal-regulated kinases-1 and -2; ERK5, extracellular signal-regulated kinase 5; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; JNK, the c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; PI, propidium iodide; PVDF, polyvinylidene difluoride; RA, retinoic acid; VSMC, vascular smooth muscle cells.

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important forms of AP-1. Previous studies have demonstrated that these stable heterodimers, which bind to AP-1 consensus sequences, are present in numerous genes associated with cell proliferative response [15]. In vascular smooth muscle cells (VSMC), the up-regulation of *c-jun* expression is mediated, at least in part, via an ERK5-dependent event [16].

The effects of RA on cells are mostly related to RA regulating AP-1 activity, which the regulation of *c-jun* expression might also contribute to [17]. Here, our present work showed that atRA (0.05 μM) could induce H9c2 cell proliferation. And then, we mainly investigated whether atRA induced *c-jun* expression and which MAPK pathway was possibly involved in the proliferation of H9c2 cells. In our study, it was found that atRA might up-regulate *c-jun* transcription and c-Jun protein level. Moreover, it was also shown that the ERK5 signaling pathway but not ERK1 regulated atRA-induced *c-jun* expression in cardiac myoblasts.

2. Materials and methods

2.1. Antibodies and reagents

atRA and propidium iodide (PI) were obtained from Sigma (St. Louis, MO, USA). ^{3}H-Thymidine was from Amersham Pharmacia Biotech (Little Chalfont, UK). The rabbit anti-β-actin, mouse anti-c-Jun, rabbit anti-ERK5 and rabbit anti-ERK1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rabbit anti-phospho-ERK5 and anti-phospho-ERK1 antibodies were from Cell Signaling Technology (Beverly, MA, USA).

2.2. Cell culture and treatment

Rat cardiac H9c2 cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% fetal bovine serum (FBS, Hyclon Co.) and 2 mM glutamine, and maintained routinely in a humidified chamber at 37°C and 5% carbon dioxide. Once cells reached approximately 80% confluence, they were starved with DMEM containing 0.2% FBS overnight. Then cells were treated with 0.05 μM atRA or vehicle (DMSO) for different periods before being harvested. The final concentration of vehicle DMSO in all treatments did not exceed 0.1% (v/v) in the medium.

2.3. MTT assays

Cell proliferation was assayed by cell-mediated reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma).Viable cells can reduce MTT and therefore a direct correspondence relationship exists between MTT absorbance and cell number. The conversion of MTT to blue formazan by mitochondrial enzymes in cells is also considered as an indicator of mitochondrial activity. In brief, cells were cultured in a 96-well plate for 1 day, and then fresh medium was changed before treatment with atRA. Under sterile conditions, 10 μl of MTT stock solution [5 mg/ml in phosphate-buffered saline (PBS)] was added to each well reaching a final concentration of 0.5 mg MTT/ml. The plate was then incubated for 4 h at 37°C. At this time, the medium was removed from each well and 100 μl of DMSO was added. The absorbance was determined at a test wavelength of 570 nm on a mutiwell plate reader (Bio-Rad Microplate Reader, USA). And absorbance of medium in the absence of cells served as the blank [18].

2.4. ^{3}H-Thymidine incorporation

^{3}H-Thymidine incorporation was performed as previously described [19]. In brief, H9c2 cells were applied to 24-well plates in growth medium (DMEM plus 10% FBS). As indicated above, H9c2 cells were treated with or without atRA (0.05 μM) for 48 h, following starvation with DMEM containing 0.2% FBS overnight. During the last 6 h of the incubation with or without atRA, ^{3}H-thymidine was added at 1 μCi/ml (1 μCi, 37 kBq). Incorporated ^{3}H-thymidine was extracted in 0.2N NaOH and measured in a liquid scintillation counter.

2.5. Transient transfection and luciferase assay

Plasmid MEK5 (A), a negative form of MEK-5; the β-galactosidase expression plasmid called pCMV β-gal; and plasmids encoding a luciferase gene driven by the wild-type *c-jun* promoter (pJluc) were kindly provided by Dr. Jiahuai Han (The Scripps Research Institute, La Jolla, CA, USA).

All transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol in six-well plates. Twenty-four hours after plating, the reporter plasmid pJluc (1.0 μg) was co-transfected into cells along with an expression vector MEK5 (A) (0.6 μg) using Lipofectamine 2000 in the presence of Opti-MEM (Invitrogen). Plasmid pCMV β-gal was employed to control for transfection efficiency. The total amount of DNA for each transfection was kept constant using the empty vector pcDNA3. After 24 h, the medium was changed to normal growth medium. At 48 h after transfection, the cells were treated with or without 0.05 μM atRA. Cell extracts were prepared and the activities of β-galactosidase and luciferase were measured as described elsewhere [20]. Relative luciferase activity (firefly luciferase for reporter and β-galactosidase activity for normalization of transfection efficiency) was measured following manufacturer's instructions (Promega, Madison, WI, USA). To correct for transfection efficiency, the luciferase activity was divided by the β-galactosidase activity in every case. The data represent the means and standard deviations of at least three independent transfections.

2.6. Western blot analysis

After various treatments, cells were washed with PBS and lysed by cell lysis buffer containing 10 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM sodium fluoride, 0.2 mM sodium orthovanadate, 1 mM

PMSF and protease inhibitor cocktail. Protein concentrations were quantified by the Bradford method [21].

Each sample containing equal amounts of proteins was subjected to SDS–6% or 10% polyacrylamide gel electrophoresis. The proteins were subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% nonfat dry milk and incubated with each antibody (anti-β-actin, anti-c-Jun and anti-ERK1, 1:2000; anti-phospho-ERK5 and anti-phospho-ERK1, 1:1000) followed by incubation with HRP-conjugated secondary IgG (1:6000 dilution). Signals were detected by enhanced chemiluminescence (Cell Signaling Technology, Beverly, MA, USA).

2.7. Confocal microscopy

Cells plated on glass coverslips were washed with PBS, fixed in 2% *p*-formaldehyde and permeabilized with 0.1% Triton X-100 in PBS. All preparations were treated with 3% bovine serum albumin (BSA) to saturate nonspecific binding. Monolayers were then incubated with anti-ERK5 or anti-ERK1 antibody (1:200 dilution) overnight at 4°C. After three washes for 10 min each in PBS with 0.2% BSA, the coverslips were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (1:100 dilution) for 30 min and washed three times for 5 min each time in PBS with 0.2% BSA. Then PI was applied to the coverslips for 5 min at room temperature. After washes, samples were analyzed by confocal immunofluorescence microscopy using a Leica Microsystems equipment (Leica Microsystems Heidelberg GmbH).

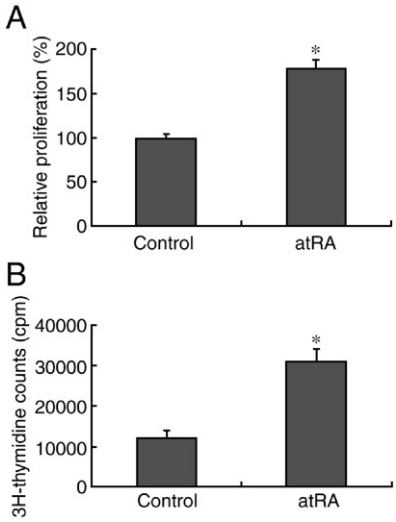

Fig. 1. atRA-facilitated proliferation of H9c2 cells. H9c2 cells were grown in the same density and then starved with DMEM containing 0.2% FBS overnight prior to exposure to 0.05 μM atRA for 48 h. (A) Cell proliferation was assessed by MTT assay. Each sample was repeated in six wells. The proliferation of control without atRA treatment was defined as 100%. (B) Measurement of ^{3}H-thymidine incorporation. ^{3}H-Thymidine incorporation into cellular DNA was determined. For (A) and (B), all data are means±S.D. of values from four experiments. *P<.01 vs. control cells cultured in the absence of atRA.

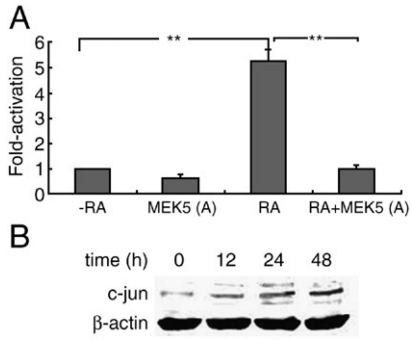

Fig. 2. ERK5 was required for atRA-induced *c-jun* expression. Three independent experiments were performed with comparable results. The results of one experiment are shown. (A) ERK5 upregulated *c-jun* transcription. H9c2 cells were cultured in six-well plates and transfected with the reporter plasmid pJluc (1.0 μg) and expression vector MEK5 (A) (0.6 μg) as indicated. After 24 h, cells were washed and left in growth medium. At 48 h after transfection, the cells were treated with or without 0.05 μM atRA for 48 h. Cell extracts were prepared and the luciferase activity and β-galactosidase activity were determined. The ratio of luciferase activity to β-galactosidase activity is presented as the mean±S.D. (n=3). **P<.01 compared to control. (B) H9c2 cells were treated with 0.05 μM atRA at different times. Western blots detected c-jun protein expression, which was performed as described in Materials and Methods.

2.8. Statistical analysis

All data are presented as mean±S.D. Differences between group means were assessed by a Student's t test for single comparisons or by ANOVA for multiple comparisons using SPSS 10.0. P values of <.05 were considered significant. All results are from at least three independent experiments.

3. Results

3.1. Effect of atRA on H9c2 cell proliferation

To study whether H9c2 cell growth is affected by atRA (0.05 μM), we treated cells with atRA for 48 h. Through an

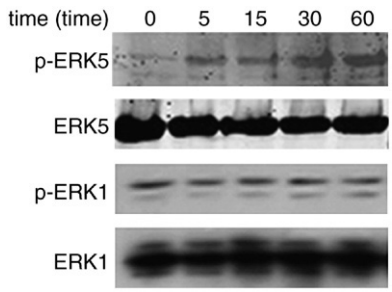

Fig. 3. atRA-activated ERK5 but not ERK1 in H9c2 cells. Serum-starved H9c2 cells were treated with 0.05 μM atRA in DMEM with 0.2% FBS for 0, 5, 15, 30 or 60 min. Protein extracts were subject to SDS–6% (for ERK5) or 10% (for ERK1) polyacrylamide gel electrophoresis. Proteins were then transferred onto PVDF membranes, and phosphorylated and total ERK5 and ERK1 were probed by the respective antibody and detected by enhanced chemiluminescence. All results represent at least three independent experiments.

inverted microscope, it was observed that atRA distinctly induced cell proliferation. Afterwards, MTT assay was performed. The results showed that there was a significant difference in cell proliferation between atRA group and control (Fig. 1A). Then, we also assessed cell proliferation by the ^{3}H-thymidine incorporation assay. It was found that atRA (0.05 μM) increased the incorporation of ^{3}H-thymidine into H9c2 cells by 1.6-fold compared with the control value for cells not exposed to atRA (Fig. 1B).

3.2. atRA induced c-jun transcription and c-Jun protein expression

In reporter gene assays, H9c2 cells were transfected with a luciferase reporter gene — pJluc — driven by the *c-jun* promoter (−225 to +150 bp) [20]. In our experiments, atRA stimulation led to about 6-fold induction of *c-jun* reporter gene expression (Fig. 2A).

To further test whether atRA induced c-Jun protein expression, Western blot assays were performed. We found that c-Jun protein expression was up-regulated distinctly in a time-dependent manner by atRA induction. Namely, at 12, 24 and 48 h, c-Jun protein expression increased 0.8-, 1.7- and 2.1-fold, respectively, relative to the control without atRA (Fig. 2B).

3.3. Involvement of ERK5 pathway in atRA-induced c-jun expression

To explore the role of ERK5 in the regulation of *c-jun* expression from the c-*jun* promoter, we employed MEK5 (A), a negative form of MEK-5, in luciferase reporter assays. It was shown that MEK5 (A) decreased pJluc expression with or without atRA. Most significantly, when ERK5 was inhibited, an 81.5% inhibition of atRA induction was observed (Fig. 2A). Also, previous studies have shown

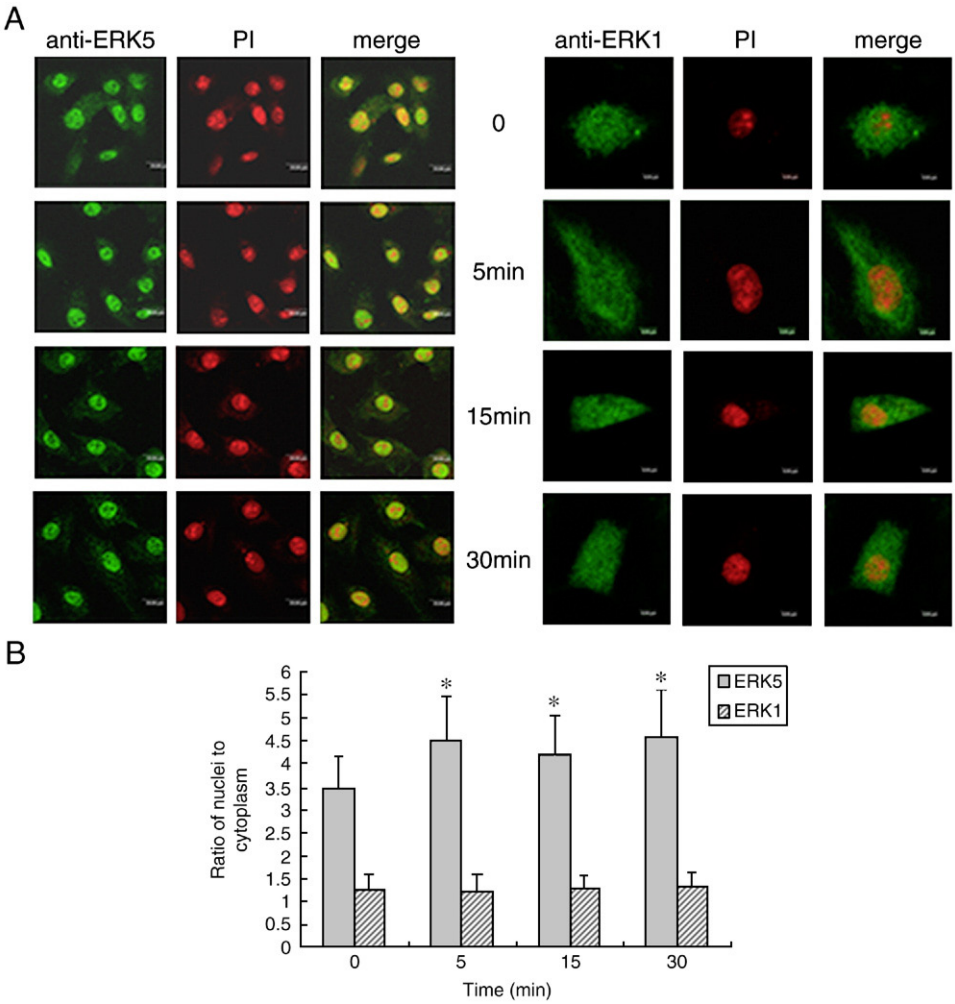

Fig. 4. Effects of atRA on the translocation of ERK5 and ERK1 in H9c2 cells. Where indicated, cells were treated with atRA for 0, 5, 15 or 30 min and then fixed and permeabilized. Immunofluorescence was carried out with the rabbit anti-ERK5 antibody, followed by FITC-labeled anti-rabbit antibody. Finally, PI was added to dye nuclei. (A) FITC, PI and merged images of ERK5 and ERK1. Double indirect immunofluorescence microscopy was performed on a Leica Scanning Confocal microscope. Bar, 20 μm (figure of ERK5); bar, 8.00 μm (figure of ERK1). (B) The ratio of ERK5 or ERK1 localized in the nuclei to cytoplasm. Error bars represent S.D. *P<.05 vs. vehicle control. All results represent three independent experiments, of which the data from each group were from at least 80 cells.

that MEK5 (A) prevents *c-jun* reporter gene expression in vascular smooth muscle cells [16].

To further prove whether ERK5 takes part in regulating *c-jun* expression, we performed a Western blot assay. Our results showed that atRA treatment induced phosphorylation of ERK5 in H9c2 cells. We found that ERK5 phosphorylation was induced by atRA as early as 5 min after treatment and peaked by 30 min. On the other hand, under atRA stimulation, the activity of ERK1 changed little from 0 to 60 min. There were no significant differences between groups. The results showed that although ERK1 and ERK5 belong to the ERK family, atRA could activate ERK5 but not ERK1 in H9c2 cells (Fig. 3).

3.4. Effects of atRA on the subcellular distribution of ERK5 and ERK1 in H9c2 cells

To determine the subcellular distribution of ERK5 and ERK1 under atRA treatment, we observed immunofluorescence staining of H9c2 cells by confocal microscopy. Cells were treated as described in the legend of Fig. 4. ERK5 or ERK1 was marked with green FITC. Red staining revealed the nuclei. It was observed that although endogenous ERK5 distributed most in the nuclei of H9c2 cells, ERK5 of atRA groups still translocated more into the nuclei than that of the control with vehicle. And there were no obvious differences in ERK1 distribution between cells with or without atRA treatment (Fig. 4A). The fluorescence ratio of nuclei to cytoplasm demonstrated that in atRA-stimulated cells, ERK5 translocated into the nuclei more than that in control cells, and there were significant differences between groups with atRA and the control at 5, 15, 30 min ($P<.05$). However, for ERK1, there were no significant differences among groups (Fig. 4B). Thus, the result indicated that atRA activated ERK5 but not ERK1 to translocate into the nuclei. This corresponds with the previous Western blotting result.

4. Discussion

In this study, we showed that atRA (0.05 μM) could activate ERK5 to increase *c-jun* expression and hasten the proliferation of H9c2 cells.

It is well known that the AP-1 transcription complex is most commonly composed of c-Jun homodimers or c-Jun/c-Fos heterodimers [22], which may lead to cell proliferation [16]. Here, we used H9c2 cells (rat cardiac myoblasts) as research subjects. Both the results of MTT assay and ^{3}H-thymidine incorporation measurement indicated that atRA enhanced the proliferation response of H9c2 cells. Furthermore, in a luciferase reporter assay, atRA stimulation increased *c-jun* reporter gene expression, and Western blots showed that atRA augmented c-Jun protein expression level in a time-dependent manner. These suggest that atRA might up-regulate *c-jun* transcription and c-Jun protein level to result in cell proliferation. This is similar to recent studies which show that atRA up-regulates expression of *c-jun/c-fos* and activity of AP-1 in certain cell types. For example, concentrations of atRA ranging from 10 nM to 10 μM increased AP-1 activity in B16 cells [22]. However, in contrast to these findings, it is also reported that RA induces apoptosis of tumor cells. Previous studies have shown that RA can down-regulate transcriptional activation by *c-jun* in HeLa and CV-1 cells and that atRA inhibits serum-induced activation of AP-1 in mesangial cells [17,23]. Taken together, the manner in which atRA mediates *c-jun* expression and cell proliferation varies in different cell lines.

On the other hand, MAPKs such as ERK5, p38 and JNK, can mediate the expression of *c-jun* gene in response to some stimuli [24–26]. As discussed at the outset, ERK5, as well as ERK1, is a member of the ERK family. For both, Thr-Glu-Tyr (TEY) sequence in kinase domain is in common. However, a large C-terminus and a unique loop-12 sequence distinguish ERK5 from ERK1 [27]. ERK5 and ERK1 both induce immediate early genes, such as *c-fos* and *c-jun*, and participate in the regulation of cell proliferation [28]. In our study, we found that in luciferase reporter assays, atRA stimulation increased *c-jun* reporter gene expression. When MEK5 (A), a negative form of ERK5 upstream kinase — MEK5, was added, the atRA-induced increase of pJluc expression decreased significantly. In addition, Western blots demonstrated that atRA activated ERK5. The results indicated that ERK5 enhanced *c-jun* transcription and c-Jun protein expression induced by atRA, which led to cell proliferation. This is similar to the phenomena from some previous studies. For instance, ERK5 activity is involved in serum-induced *c-jun* expression and leads to proliferation of VSMC [16]. Additionally, ERK5 is activated by epidermal growth factor (EGF), resulting in the proliferation of MCF10A cells and up-regulation of *c-jun* mRNA expression in mouse embryonic fibroblasts [25,26]. Zhao et al. [16] reported that the up-regulation of the *c-jun* transcription by ERK5 was through the phosphorylation of the members of the MEF2 family transcription factors. It may be a future task for us to explore the mechanism by which ERK5 phosphorylates MEF2 family to regulate *c-jun* expression in the heart.

As for ERK1, it was not found that it was activated and involved in the regulation of atRA on proliferation of H9c2 cells. This is different from such reports as ERK1 regulating EGF-induced HeLa cell proliferation [25]. This reveals that whether ERK1 plays a role in proliferation probably depends on the stimuli and cell types. In a word, ERK5 and ERK1 do not always cooperatively regulate cell proliferation, which relies on different triggers and cell lines.

MAPKs need to translocate from the cytoplasm into the nuclei to regulate transcription factors such as c-Jun in the nuclei [28]. Here, we found that endogenous ERK5 localized most in the nuclei in H9c2 cells, and the nuclear accumulation of ERK5 was enhanced by atRA stimulation. This is similar to ERK5 status with or without EGF induction in HeLa cells [29]. On the side, endogenous inactive ERK5 may localize either in the cytoplasm or

diffusively throughout the whole cell and translocate into the nuclei with stimulation in MCF7 and C2C12 cell lines [29,30]. Thus, endogenous ERK5 is cytoplasmic, nuclear or pancellular, lying on the cell types [29,31]. It has been proposed that ERK5 has a mechanism for the nucleocytoplasmic transport. The balance determines ERK5 localization between the nuclear localizing activity in ERK5 C-terminal region and the nuclear export activity [29]. In our results, atRA stimulation might make the nuclear localizing activity of ERK5 so strong as to bring on ERK5 nuclear translocation in H9c2 cells. In regard to ERK1, it was shown that endogenous ERK1 localized in both nuclei and cytoplasm in H9c2 cells. Here, atRA stimulation changed little on ERK1 subcellular distribution though ERK1 might translocate from the cytoplasm to the nuclei under some stimulation, for atRA could not induce ERK1 phosphorylation that resulted in the dissociation of ERK1 from MEK1. It has been described that MEK1 can retain ERK1 in the cytoplasm through direct interaction in quiescent cells [32]. The results from confocal microscopy also support the above conclusion that ERK5 but not ERK1 was activated by atRA.

In conclusion, these findings indicate that ERK5 pathway plays an important role in regulating atRA-induced *c-jun* expression, leading to the proliferation of cardiac myoblasts, which may give some clues as to how MAPKs regulate gene expression in the developing heart.

Acknowledgments

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