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Icariin promotes expression of junctophilin 2 and Ca^{2+} related function during cardiomyocyte differentiation of murine embryonic stem cells

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Junctophilin2 (JP2) is a critical protein associated with cardiogenesis. Icariin (ICA) facilitated the directional differentiation of murine embryonic stem (ES) cells into cardiomyocytes. However, little is known about the effects of ICA on JP2 during cardiac differentiation. Here, we explored whether ICA has effects on the expression and Ca^{2+} related function of JP2 during cardiomyocyte differentiation of ES cells *in vitro*. Embryonid bodies (EBs) formed by hanging drop were treated with 10^{-7} mol/L ICA from day 5 to promote the cardiac differentiation. Percentage of beating EBs and number of beating area within EBs were monitored. Cardiomyocytes were purified by discontinuous percoll gradient centrifugation from EBs. The expression of JP2, α-actinin and troponin-T within EBs or isolated cardiomyocytes were analyzed by immunocytochemistry, western blot and flow cytometry. The transient Ca^{2+} release was characterized in cardiomyocytes treated with/without 10 mmol/L caffeine and 8 mmol/L Ca^{2+}. Our results showed that ES cell-derived cardiomyocytes were well characterized with JP2 proteins. ICA promoted cardiomyocyte differentiation as indicated by an increased percentage of beating EBs and number of beating area within EBs. The expression of JP2, α-actinin and troponin-T were up-regulated both in EBs and isolated cardiomyocytes from EBs. Furthermore, ICA-induced JP2 expression was accompanied by a remarkable increase of the amplitude of Ca^{2+} transients in cardiomyocytes before/after caffeine and Ca^{2+} stimulating. In conclusion, ICA promotes in cardiac differentiation partly through regulating JP2 and improved the Ca^{2+} modulatory function of cardiomyocytes.

1. Introduction

Regenerative medicine offers new approach for developing effective therapies for heart diseases (Cho et al. 2014). Embryonic stem (ES) cells are hallmarked by their ability of differentiate into cardiomyocytes in the presence of physical-inducing and biological-inducing factors as a cell source for cell replacement therapy (Kingham and Oreffo 2013), as well as a useful test system for investigating the effects of drugs on heart function. So far, many promising studies and significant progress had been made toward increasing the efficiency of ES cell differentiation into cardiomyocytes, and shown the therapeutic potential of differentiated derivatives of ES cells in ameliorating heart failure in animal models (Menasche 2012). Either suitable models for drug discovery or cell-based therapy for cardiac repair would be highly attractive. However, before the drugs and cells can be used for these proposes, it will be necessary to understand the mechanisms that affect the differentiation and maturation of ES cells and their progeny.

Protein components contributing to cellular Ca^{2+}-handling are useful targets for various research purposes, and ES cell derived cardiac cells are functionally characterized by expression of Ca^{2+} modulatory proteins. Junctophilins are a novel protein family that contributed to normal formation of the junction membrane complexes between the plasma membrane and the endoplasmic/sarcoplasmic reticulum in excitable cells (Beavers et al. 2014; Landstrom et al. 2014; Takeshima et al. 2000). Junctophilin 2 (JP2) is a unique subtype abundantly expressed in the heart and essential for the physiological communication between caveolin channels and ryanodine receptor 2 in cardiac excitation-contraction coupling, mediating Ca^{2+} release for cellular Ca^{2+} homeostasis (Minamisawa et al. 2004, Wang et al. 2007). It was reported that mutant mice lacking JP2 exhibited

embryonic lethality (Takeshima et al. 2000). In addition, several human genetic diseases such as hypertrophic and dilated cardiomyopathies were believed to be caused by mutations at the JP2 genes (Beavers et al. 2013, 2014; Chen et al. 2013). Thus, JP2 was considered to play an important role in cardiogenesis/cardiomyopathies, and could be a useful target in drug discovery.

Herba Epimedii is a traditional Chinese herb used to ameliorate erectile function, osteoporosis, and other related conditions. Interestingly, icariin (ICA), the dominant active ingredient of *Epimedium*, possesses many kinds of biological actions such as improving cardiovascular function, immunological function modulation, and antitumor activity (Sun et al. 2011; Zhou et al. 2011). Using a model system comprised of ES cells, our previous work demonstrated that ICA facilitated the directional differentiation of murine ES cells into cardiomyocytes by enhancing and accelerating differentiation into beating cardiomyocytes, as well as increasing the expression of cardiac developmental-dependent genes and proteins (Ding et al. 2008; Wo et al. 2008). Nevertheless, little is known about the effects of ICA on protein components contributing to cellular Ca^{2+}-handling such as JP2. Therefore, the present study focused on the characterization of ICA on JP2 expression during cardiomyocyte differentiation and Ca^{2+} modulatory function of cardiomyocytes.

2. Investigations and results

2.1. Immunofluorescent analysis of JP2 expression on day 17

To examine on whether JP2 was specifically present in cardiomyocytes derived from ES cells, immunofluorescence microscopy was employed to reveal the distribution of JP2, probing for merging of JP2 and sarcomeric α-actinin or troponin-T in small clusters. In each case, fields full of cells were selected so that a comparison can be made between the level of staining in cells positive for the marker and those which were negative. As a result, cardiomyocytes were stained positively with anti-α-actinin and troponin-T antibody. There was a well matched correlation between JP2 and α-actinin or troponin-T during differentiation, while negative areas were negative for JP2, and cross striations were observed at higher magnification (Fig. 1), noting that cardiomyocytes expressing troponin-T also expressed high levels of JP2. It is suggested that mES-derived cardiomyocytes were well characterized with JP2 proteins.

2.2. Effects of ICA on cardiac differentiation and exprssion of JP2

To evaluate the effect of ICA on cardiac differentiation, EBs were treated with 10^{-7} mol/L ICA (Fig. 2A) from day 5 when EBs were attached to 0.1% gelatin-coated 24-well plates. Percentage of beating EBs was monitored on day 8, 11, 14 and 17 (Fig. 2B, left panel) and numbers of beating areas were inspected within EBs on day 17 (Fig. 2B, right panel). As a result, ES cells were induced into rhythmically beating EBs by ICA in a time-dependent manner. The effect of ICA on the differentiation of ES cells into cardiomyocytes was marked in comparison to control. The percentage of beating EBs with ICA treatment reached a peak level of 87%, and the numbers of beating area were about 7 on day 17, in contrast to 55% of the beating EBs and about 3 beating areas in control ($P < 0.05$). The representative staining results for the JP2 protein after ICA treatment showed that, in the area having the same number and density of cells (DAPI staining), EBs treated with ICA displayed more JP2 positive immunostaining cells (Fig. 2C). Taken together, the results indicated that ICA promoted the cardiac differentiation with increasing more JP2 positive cardiomyoctyes.

2.3. Analysis of JP2 and cardiac marker proteins after ICA treatment

To evaluate ICA treatment on the expression of JP2 and cardiac marker proteins α-actinin and troponin-T, the overall level of protein expression on day 17 EBs of cardiomyocyte differentiation was analyzed. As a result, the protein levels of JP2, α-actinin and troponin-T was remarkably increased compared to control (Fig. 2D). Furthermore, JP2 and α-actinin expression were quantified by flow cytometrs (FCM). On day 17, $9.995 \pm 0.715\%$ of cells stained positively for JP2 and $2.646 \pm 1.621\%$ for α-actinin in spontaneous differentiation population, the proportion of cells induced by ICA was increased to $24.743 \pm 3.024\%$ for JP2 ($P < 0.05$) and $20.360 \pm 1.428\%$ for α-actinin ($P < 0.001$). RA was used as positive control, the pecentage of JP2 positive cells was $21.750 \pm 0.988\%$ and $21.273 \pm 2.364\%$ for α-actinin (Fig. 2E). The proportion of cells co-expression of JP2 and α-actinin induced by ICA was $15.403 \pm 1.268\%$ in contrast to of $5.231 \pm 1.763\%$ in control. The results indicated that JP2 expression showed an analogy with the expression of α-actinin and troponin T, suggesting that JP2 may be critical to normal cardiac development *in vitro*.

2.4. Analysis of JP2 and cardiac marker proteins in isolated cardiomyocytes

Since the cultures of differentiated EBs were heterogeneous, to evaluate the expression of JP2, α-actinin and troponin-T in purified cardiomyocytes, we isolated the cardiomyocytes within day 17 old EBs by centrifugation through a discontinuous percoll gradient, the protein expression were investigated by western blot and FCM. As a result, the protein levels of JP2, α-actinin and troponin-T in the purified cardiomyocytes were increased in contrast to control (Fig. 3A). The FCM analysis showed that mean fluorescence intensity of JP2 in cardiomyocytes from ICA treated EBs was about 86.92 ± 21.73 in contrast with the solvent control case of 29.28 ± 5.58 ($P < 0.05$) (Fig. 3B), while the proportion of cells induced by ICA that contained positive α-actinin and troponin-T was increased to 83.65 ± 14.64 or 91.39 ± 30.03 in contrast with the case of 32.25 ± 4.70 ($P < 0.05$) or 35.33 ± 7.14 in control($P < 0.05$). Taken together, the results showed that ICA-induced cardiomyocytes expressed more JP2 and cardiac marker proteins than control, implying that ICA induces cardiogenesis not only through increasing the percentage of beating EBs during differentiation and the level of cardiac related proteins within EBs, but also up-regulates these proteins in isolated cardiomyocytes.

2.5. Characteristics of Ca^{2+} transients in cardiomyocytes under ICA treatment

To characterize the function of ICA-induced cardiomyocytes, we focused on the transient Ca^{2+} release in cardiomyocytes under ICA treatment, in the absence and presence of caffeine and Ca^{2+}, since Ca^{2+} is a critical regulator of cardiac function. Here we discriminated two kinds of cardiomyocytes, identified by displaying Ca^{2+} transient. The results showed that ICA remarkbly increased the basal Ca^{2+} transients in amplitude before caffeine and Ca^{2+} stimulating, the amplitude was 1.6 times higher than that in solvent-treated cells (Fig. 3C). Both caffeine (10 mmol/L) and Ca^{2+} (8 mmol/L) increased the amplitude in ICA and solvent-treated cells, and the amplitude was 1.8 times higher in ICA-treated cells than in solvent-treated cells, indicating that

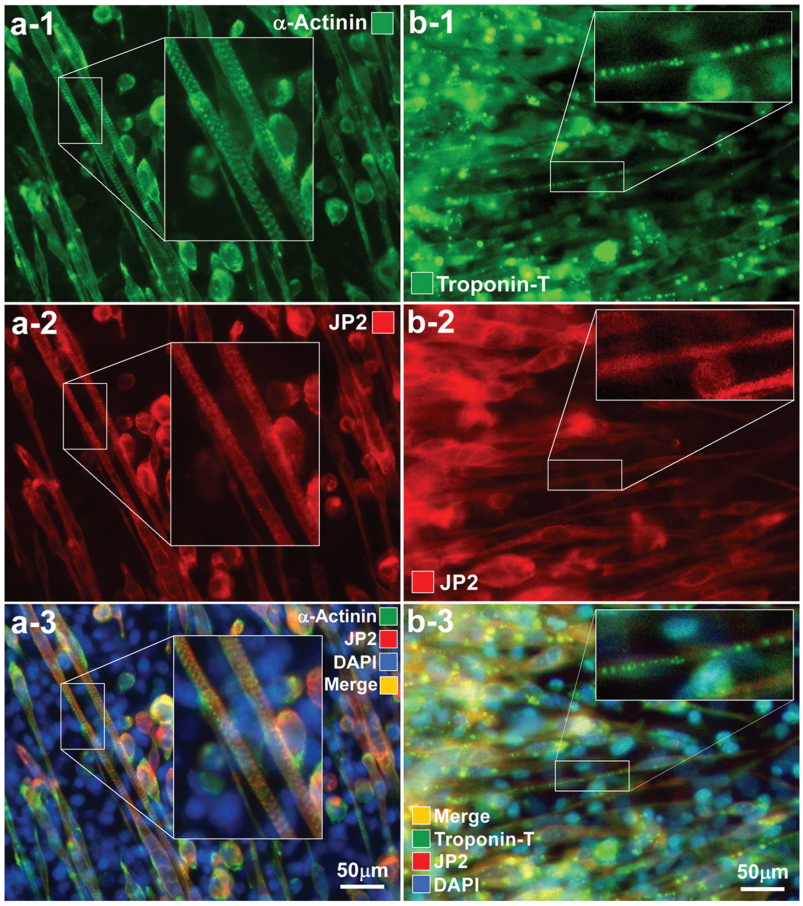

Fig. 1: Expression of JP2 and cardiac marker protein a-Actinin or Troponin-T on d 17 EBs in the differentiation of mouse ES cells into cardiomyoctye. EBs on day 17 were double stained with antibodies against JP2 (red) and a-Actinin or Troponin-T (green), DAPI staining (blue) showed nuclear morphology. The rectangle box indicated the higher magnification of sarcomeric a-Actinin or Troponin-T staining. Scale bar = 50 um.

SR Ca^{2+} content increased in cardiomyocytes derived from ICA-induced differentiation. Altogether, these data suggested that ICA-induced JP2 expression in ES cells derived cardiomyocytes affected caffeine and Ca^{2+} induced Ca^{2+} transients, identified as both RyR mediated rapid raising Ca^{2+} release, and Ca^{2+} induced Ca^{2+} transients, an amplification mechanism of Ca^{2+} signals, indicating that ICA promoted JP2 expression and beating activity by regulating the dynamics of Ca^{2+} transients level that may modulate the normal physiological function of cardiomyocytes.

3. Discussion

In this study, we explored the effects of ICA on JP2, which played an important role in cardiogenesis, and hypothesized that the cardiac inducible effects of ICA may be associated with promoting JP2 expression. Our results showed that JP2 is expressed in the mES cells differentiated cardiomyocytes, partly indicating the integrated Ca^{2+} modulatory function of these cells. The expression of JP2 was up-regulated both in ES cells differentiated day 17 EBs and cardiomyocytes isolated from day 17 EBs after ICA treatment, showing an analogy with the expression of cardiac marker proteins α-actinin and troponin T, implying that ICA regulated JP2 may be critical to cardiac differentiation *in vitro*.

It had been demonstrated that the expression of JP2 was greatly increased in the developing mouse heart (Takeshima et al. 2000). Physiological functions of Ca^{2+} release *via* junctional membrane complexes constituted by junctophilins in excitable cells. Under pathological conditions, expression of mutant but not wild-type *JP2* in H9c2 cells caused cullular hypertrophy and over-expression of mutant *JP2* in HL-1 cardiomyocytes attenuated the amplitude of Ca^{2+} transients (Landstrom et al. 2007; Matsushita et al. 2007). Moreover, human heart diseases such as hypertrophy are caused by mutations of the *JP2* genes (Reynolds et al. 2013; Zhang et al. 2014), indicating that JP2 is an important target in physiology and pathobiology, and could even be a drug therapy target in pharmacology.

The present study showed that JP2 had emerged as a potentially important regulator of excitation contraction coupling in either cardiomyocytes or cardiogenesis. ICA could promote cardiac differentiation from mouse ES cells *in vitro*, partly through regulating the expression of JP2. What is more important, ICA-induced JP2 expression affected caffeine and Ca^{2+} induced Ca^{2+} transients, displaying remarkably increased the basal Ca^{2+} transients in amplitude before and after caffeine and Ca^{2+} stimulating. It is well known that caffeine and Ca^{2+} identified as both RyR mediated rapid raising Ca^{2+} release and Ca^{2+} induced Ca^{2+} transients, an amplification mechanism of Ca^{2+} signals (Satin et al. 2008), indicating that ICA-induced cardiomyocytes expressed JP2 that improved the normal physiological function of these cells. Therefore, targeting JP2 and its binding partners may represent a new therapeutic strategy, and ICA could be used as an effecitve cardiac differentiation promoter in modulating stem cells. Also, JP2 plays a role in ES cell-derived progeny, and could be a useful target for drug discovery and pharmaceutical development, as well as for the treatment of heart diseases. ES cell-derived cardiomyocytes could be used as an *in vitro* model for the effect investigation of small molecules on target proteins

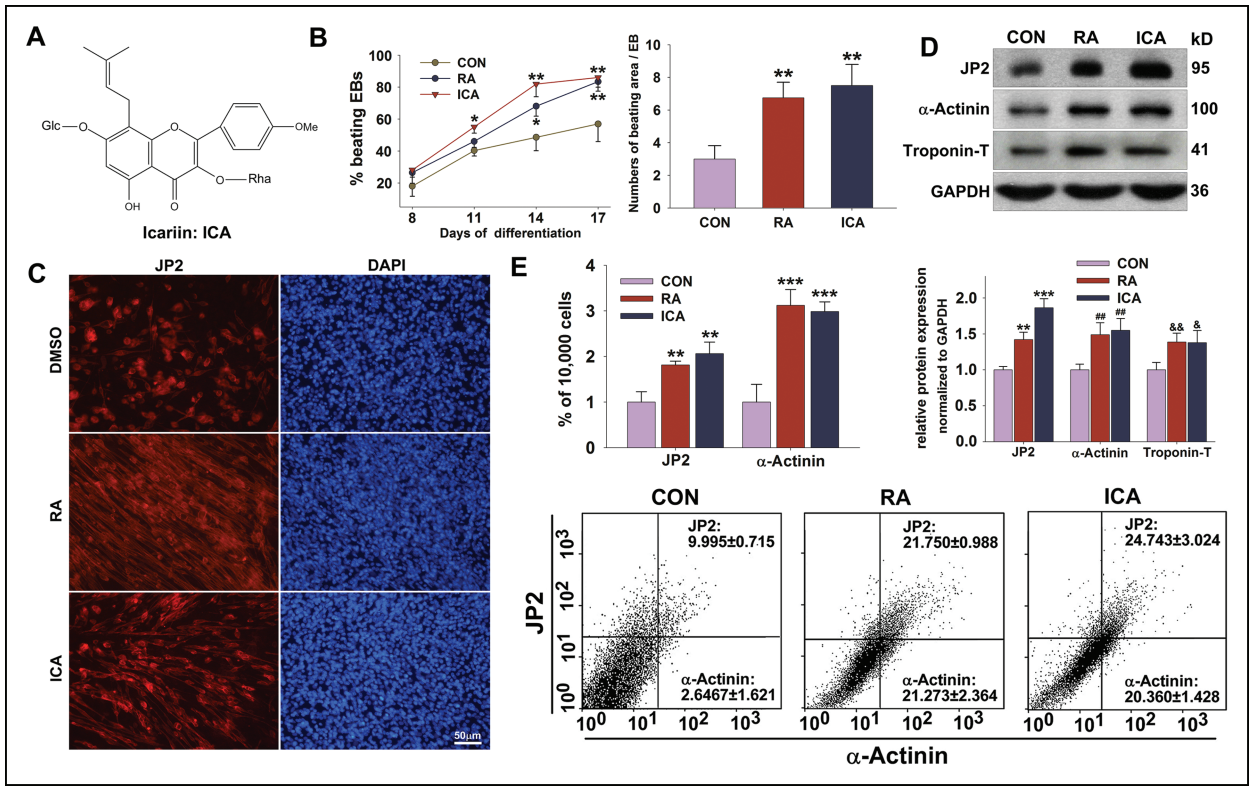

Fig. 2: Icariin promoted the expression of JP2 during cardiac differentiation of ES cells. (A) Molecule structure of ICA. (B) Effects of ICA on cardiac differentiation. Percentage of beating EBs was monitored on day 8, 11, 14 and 17 (left panel), n = 100. Inspect number of beating areas within EBs on day 17 (right panel). n = 20, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control. (C) Immunostaining with JP2 after treatment of ICA. DAPI staining (blue) showed the number and density of cells. (D) Protein expression of JP2, a-Actinin and Troponin-T on day 17. n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control. (E) Quantification of JP2 and cardiac marker protein a-Actinin expression by intracellular FCM. Representative histogram statistics analyses of anti-JP2 and anti-a-Actinin stained 17 day old Ebs (bottom panel). Quantification of JP2 and a-Actinin expression by FCM analysis (top panel). n = 3, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control. DMSO or RA served as negative and positive controls, respectively.

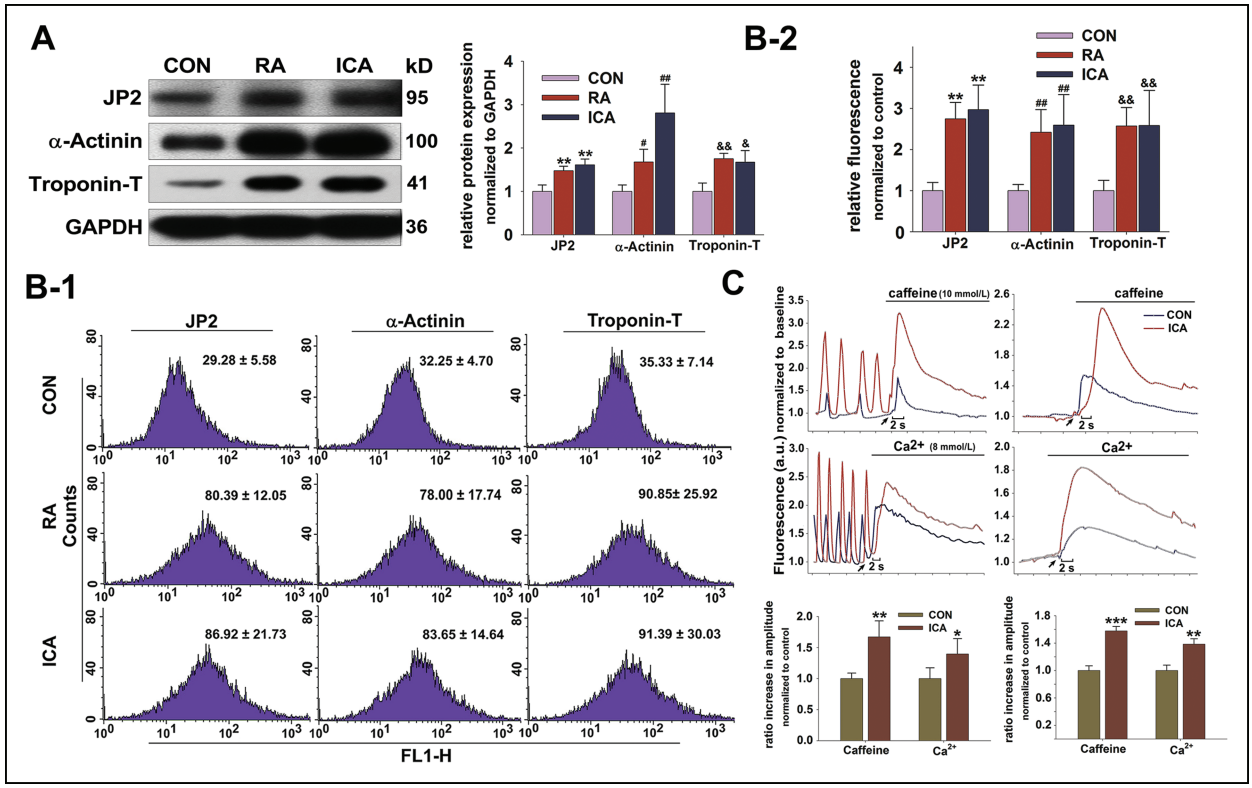

Fig. 3: Icariin promoted the expression of JP2 and ameliorated CICR activity in the percoll gradient centrifugalization isolated cardomyocytes derived from ES cells. (A) Protein expression of JP2, a-Actinin and Troponin-T in cardiomyocytes isolated from day 17 old EBs treated with 10-7 mol/L ICA. (B) Quantification of JP2 and a-Actinin expression by FCM to evaluated the effects of ICA on mES cells derived cardiomyocytes. Representative histogram statistics analyses of stained cardiomyocytes isolated from day 17 old EBs. The X-axis corresponds to fluorochrome FITC detected at 530 nm, and the Y-axis corresponds to number of cell per channel (bottom panel). Quantification of JP2 and a-Actinin expression by FACS analysis (top panel). DMSO and RA served as vehicle control and positive control, respectively. n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control. (C) Representative tracings of caffeine-evoked Ca2 + transients and Ca2 + -induced Ca2 + transients of cardiomyocytes treated with ICA. A total of 10 mm.

such as JP2. However, further study is necessary to characterize the JP2 and other Ca^{2+} modulatory proteins in mES cell-derived cardiomyocytes with ICA treatment and provide direct evidence for the normal function.

The significant promoting effects of some nature prenylflavonoids such as ICA, icaritin, and desmethylicaritin, on cardiac differentiation of mouse ES cells *in vitro* have been investigated previously (Ding et al. 2008; Sun et al. 2011; Wo et al. 2008). Protein isoprenylation is an important step in the intracellular signalling pathway conducting cell growth and differentiation (Duque et al. 2011) Therefore, wheter the promoting effect of ICA was affected by the isopentenyl on the 8-substituent isopentenyl or not, and protein prenylation in cardiac differentiation of ES cells will be investigated in further studies.

In summary, in this study, the JP2 expression we detected in ICA-induced cardiomyocytes can explain why ICA was able to induce cardiomyocytes with mature Ca^{2+} physiological function and normal beating function as adult cardiomyocytes. ICA treatment up-regulated the expression of JP2, acted as part of mechanisms for the inducible effect of ICA on cardiac differentiation. ICA-induced cardiomyocytes may have potential in cardiac cell therapy or tissue engineering, and ICA-based small molecules may find application in cardiac differentiation.

4. Experimental

4.1. Culture of ES cells and cardiomyocyte differentiation

Mouse ES cells (CRL-1934, American Type Culture Collection, USA) were routinely cultured on primary cultured mouse embryonic fibroblasts (MEF) cells in Dulbecco's modified Eagle's minimal essential medium (DMEM, Gibco, USA), supplemented with 10% fetal bovine serum (FBS, Gibco, USA), non-essential amino acids (NEAA, 1:100, Gibco, USA), β-mercaptoethanol (β-ME, Sigma, USA) and recombinant mouse leukemia inhibitory factor (LIF, Chemicon, USA)-conditioned medium in a humidified 5% CO_2 atmosphere at 37 °C. To induce differentiation of cardiomyocytes, a hanging drop method was used to initiate EB formation in differentiation medium. Briefly, drops (30 μL) containing approximately 600 ES cells were cultured for three days in hanging drops, then EBs were transferred to agar-coated Petri dishes and cultured in suspension for another two days. On day 5, EBs were plated on 0.1% gelatin-coated 24-well plates in differentiation medium with 10^{-7} mol/L ICA (National Institute for the Control of Pharmaceutical and Biological Products, China) to induce cardiac differentiation. A culture treated with 0.1% dimethylsulfoxide (DMSO, Sigma, USA) and 10^{-8} mol/L retinoic acid (RA, Sigma, USA) served as a solvent control and positive control, respectively. Daily microscopic observations were conducted to determine the beating rate.

4.2. Immunocytochemistry analysis

Immunofluorescence was performed with whole outgrown EBs plated on 24-well culture plates. Samples were washed with PBS and fixed with methanol at -20 °C for 15 min, followed by permeabilization in 0.5% Triton X-100 in PBS. EBs were incubated in blocking solution (10% FBS in PBS) for 1 h at room temperature, then placed into PBS containing anti-troponin T (dilution 1:200, Santa Cruz, USA) or anti-α-actinin (1:400, Sigma, USA) or anti-JP2 (1:400, Invitrogen, USA), respectively, and incubated overnight at 4 °C. Subsequently, EBs were washed and incubated in blocking solution containing Alexa fluor 488-conjugated anti-mouse IgG (1:300) or Alexa fluor 594-conjugated anti-rabbit IgG (1:300, Invitrogen, USA) for 1 h, avoiding light. Then the culture was incubated in 2 μg/mL DAPI solution in PBS for 3 min to label the nuclei. Fluorescence image was performed by fluorescence inverted microscope (Leica, Germany).

4.3. Isolation of cardiomyocytes derived from ES cells

The EBs were detached from culture surfaces by incubating them with 0.05% trypsin-EDTA for 1 min at 37 °C, then dispersed into cells with 1 g/L collagenase II (Worthington, USA)) for 30 min. After digesting into separated cells, ES cells derived-cardiomyocytes were isolated by centrifugation at 1500 × g for 30 min through a discontinuous percoll (GE Healthcare Life Sciences, USA) gradient and collected at the interface of the two layers (40.5% and 58.5%). After removal of the top layer, cardiomyocytes were collected and washed twice in PBS and adhered onto glass coverslip coated with 1% gelatin for further study.

4.4. Western blot analysis

The Cells were lysed in RIPA buffer for 30 min on ice. The protein concentration was quantified by modified Lowry assay. Briefly, equal amounts of protein were loaded per lane on a sodium dodecyl sulphate (SDS)-polyacrylamide gel. Subsequently, the proteins were transferred onto PVDF membranes and blocked in 5% nonfat milk in Tris buffer containing 0.1% Tween-20 (TBST). at room temperature for 1 h. The specific dilutions of the primary antibodies were as follows: JP-2 (1:1000); α-actinin (1:500); Troponin-T (1:300); GAPDH (1:10000). The blots were challenged with primary antibody in TBST overnight at 4 °C, followed by washing with TBST, and incubated with HRP-conjugated secondary antibody (1:5000) with gentle agitation for 1 h at room temperature. Then, the blots were washed with TBST and exposed to a chemiluminescent detection system using the Super Signal West Pico Substrate (Rockford, USA). Digital images of appropriate films were captured and quantified using the bio-imaging system.

4.5. Flow cytometry analysis

The EBs were dissociated to a single-cell suspension by 0.25% EDTA/trypsin treatment. For intracellular staining, cells (3×10^5) were fixed with 4% paraformaldehyde PBS for 1 h and treated with 10% FBS to block non-specific antigens. Then EBs were incubated with mouse anti-troponin-T (1:200) or mouse anti-α-actinin (1:500) or rabbit anti-JP2 (1:500) overnight at 4 °C, followed by Alexa fluor 488-conjugated anti-mouse IgG (1:300) or phycoerythrin-conjugated anti-rabbit IgG (1:300) for 1 h, then suspended in 0.5 mL PBS/1% BSA and analyzed on a FACScan (Becton Dickinson, Germany). The fluorochrome was detected at 530 nm in the FL-1 and FL-2 channel. Each plot represented 10,000 viable cells. Untreated cells and cells lacking primary antibodies were used as controls. Isotype controls were used to assess the level of non-specific antibody binding. All data analyses were carried out using CellQuest software. Differentiation was determined by comparing the percentage of positive cells and fluorescence intensity of the treated cells to that of solvent control.

4.6. Transient Ca^{2+} signal measurement

A spectrofluorometric method with Fluo-4/AM as the Ca^{2+} indicator was used for measuring $[Ca^{2+}]_i$. ESC-CMs were rinsed twice with bath solution (mmol/L): 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES (pH 7.4), incubated in bath solution containing 5 μmol/L Fluo-4/AM and 0.02% Pluronic F-127 with 5% CO_2, 95% O_2 at 37 °C for 30 min, and then rinsed twice with bath solution. The temporal resolution of the line scan was 439 Hz. 10 mmol/L caffeine and 8 mmol/L Ca^{2+} were added as stimulators, respectively. A cooled CCD camera mounted on the microscope equipped with a polychromator (IX S1, Olympus) was used to capture the fluorescence data with excitation at 488 nm and emission at 510 nm at room temperature. All analyses of $[Ca^{2+}]_i$ were processed at a single-cell level and expressed as the relative fluorescence intensity.

4.7. Statistic analysis

Student's *t*-test and one-way analysis of variance (ANOVA) were used to determine the statistical significance of differences between values for various experimental and control groups. The value $P < 0.05$ was considered statistically significant.

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