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Involvement of Ubiquitin–Proteasome System in Icariin-Induced Cardiomyocyte Differentiation of Embryonic Stem Cells Using Two-Dimensional Gel Electrophoresis

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ABSTRACT

Icariin has been shown to significantly facilitate the differentiation of embryonic stem (ES) cells into cardiomyocytes in vitro. However, the mechanism underlying the icariin-induced cardiomyocyte differentiation is still not fully understood. In the present study, 52 differentially displayed proteins selected from two-dimensional electrophoresis gels were identified by MALDI-TOF mass spectrometry analysis. More than half of proteins could be assigned to six main categories: (1) protein synthesis, metabolism, processing and degradation, (2) stress response, (3) cytoskeleton proteins, (4) energy metabolism, (5) carbohydrate metabolism/transport, and (6) RNA/other nucleic acids metabolisms and transport, nuclear proteins. MALDI-TOF/MS showed that icariin treatment resulted in the induction of five ubiquitin–proteasome system (UPS)-related proteins, such as ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), ubiquitin-conjugating enzyme E2N, proteasome 26S, proteasome subunit-alpha type 6, and proteasome subunit-alpha type 2 in the differentiated cardiomyocytes. These results implied that UPS might play an important role in the control of cardiomyocyte differentiation. Epoxomicin (a proteasome inhibitor) significantly reduced the cardiomyocyte differentiation rate of ES cells and proteasome activities, as well as inhibited NF-κB translocation into the nucleus, which were evidently reversed by presence of icariin. Meanwhile, icariin could significantly reverse the reduction of four proteins (proteasome subunit-alpha type 6, proteasome subunit-alpha type 2, UCH-L1, and ubiquitin-conjugating enzyme E2N) expressions owing to application of epoxomicin. These suggest UPS could be a means by which icariin may regulate expressions of key proteins that control cardiomyocyte differentiation. Taken together, these results indicated that UPS played an important role in ES cell differentiate into cardiomyocytes induced by icariin. J. Cell. Biochem. 112: 3343–3353, 2011.

KEY WORDS: UBIQUITIN–PROTEASOME SYSTEM; EMBRYONIC STEM CELL; CARDIOMYOCYTES; ICARIIN; TWO-DIMENSIONAL GEL ELECTROPHORESIS

The ubiquitin–proteasome system (UPS) is known to participate in many biological activities in vivo, such as cell cycle, division, differentiation, development, neural networks, morphogenesis, and cell surface receptors, as well as ion channels in the regulation of proteins [Miler and Gordon, 2005]. The UPS is an important pathway for the degradation of proteins and its mechanisms [Ravid and Hochstrasser, 2008] are: (1) by covalent binding of ubiquitin to mark the target protein; (2) the marker protein for proteasome degradation. Several studies [Sakurai et al., 2006; Szutorisz et al., 2006; Jian et al., 2007; Friehs, 2008] suggest that ubiquitin can promote the differentiation of embryonic stem (ES) cells, while blocking the activity of proteasome can activate the transcription regions of the genome only in the early differentiating stages of ES cells and inhibit the activity of NF-κB. Ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) plays an extremely important role in the differentiation of neural progenitor cells [Sakurai et al., 2006; Naujokat and Saric, 2007; Cord, 2009]. UCH-L1 has also been believed to play an important role in the differentiation of ES cells into cardiomyocytes.

Icariin 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 studies [Zhu and Lou, 2006; Ding et al., 2008; Wo et al., 2008] have shown that icariin can significantly improve the differentiation rate and advance the differentiation phase of

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cardiomyocytes from ES cells in vitro. Although the molecular mechanism by which icariin mediates its effects are not well understood, it is known to promote the development of myocardial dependent genes such as bone morphogenetic protein 2 (BMP2), GATA4, NKx2.5, cardiac-specific-myosin heavy chain (α-MHC), myosin light chain-2v (MLC-2v), and β-adrenoceptor (β-AR), as well as cardiac-specific sarcomeric proteins (e.g., α-actinin and troponin T). Recently, it is shown that the generation of reactive oxygen species (ROS) and the subsequent activation of p38MAPK are essential for the inducible function of icariin on cardiomyocyte differentiation of ES cells in vitro [Ding et al., 2008]. Experimental evidences also validate that IκBα phosphorylation and NF-κB p65 translocation to the nucleus appear rapidly when embryoid bodies(EBs) exposed to icariin, and the expression of IκBα or NF-κB p65 in cytoplasm is decreased concomitantly, which indicate NF-κB signaling can be activated during the promotion of the cardiac differentiation by icariin treatment [Wo et al., 2008]. However, the signaling pathways leading to changes in ES cell differentiation by icariin are still not fully understood. In this study, we used a proteomics approach to identify proteins regulated by icariin treatment.

Proteome-wide screening may identify unique markers and elucidate interconnections between different cellular signaling pathways. Furthermore, it can comprehensively analyze the differential expression of proteins in different developmental stages, or different compounds treatment. Until now, proteome analyses of ES cells have been mainly applied to reveal mechanisms underlying cellular differentiation, proliferation, and self-renewal [Unwin et al., 2003; Prudhomme et al., 2004; Baharvand et al., 2006; Van Hoof et al., 2006; Bendall et al., 2007]. Only a few articles have investigated the proteomic techniques combined with the directional differentiation of ES cells induced by drug in vitro. Guo et al. [2001] reported that the protein profiles associated with the early-stage differentiation of ES cells into neuronal cells are induced by all-*trans* retinoic acid in vitro using proteomic techniques.

This is the first report using a two-dimensional electrophoresis (2-DE) proteome profile of ES cells differentiating into cardiomyocytes by icariin. Proteins expressed at different levels were identified by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF/MS) and we found 52 differentially expressed protein spots in ES cells-derived cardiomyocytes by icariin. These proteins included those related to the ubiquitin–proteasome system known to be involved in cellular differentiation. We further investigated how each of these may shed new light on the mechanisms of icariin action and create new targets of regulation in the cellular response to icariin.

MATERIALS AND METHODS

CULTURE OF ES CELLS

ES-D3 cells (obtained from the American Type Culture Collection, CRL-1934) were maintained in an undifferentiated state by culturing on a monolayer of mouse embryonic fibroblast (MEF) cells treated with 1 mg/L mitomycin C as a feeder in high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Life Technologies, Germany), supplemented with 10% FCS, 0.1 mM β-mercaptoethanol, nonessential amino acids (NEAA), and 1×10^6 U/L leukemia inhibitory factor (LIF).

ES cells were differentiated into beating cardiomyocytes treated with icariin (Drug Biology Product Examination Bureau, Beijing, China, batch no 0737-200011, purity 99%), at a final concentration of 100 nM, as described previously [Zhu and Lou, 2006]. ES cells treated with dimethyl sulfoxide (DMSO) solvent or retinoic acid (RA, Sigma) was used as negative control or positive control. For evaluating the influence of proteasome on the icariin-induced cardiomyocyte differentiation, epoxomicin (Sigma) [Meng et al., 1999] at a final concentration of 10 nM was added to differentiation medium together with icatiin on day 5 and maintained until day 5 + 7 (i.e., 7 days after EBs were plated onto gelatin-coated culture plates on day 5). The rhythmically beating EBs were considered to be spontaneously beating cardiomyocytes in EB outgrowths, and were defined as the marker of successful differentiation [Metzger et al., 1995; Scholz et al., 1999]. The cardiomyocytes were determined by detecting the sarcomeric proteins for troponin T and α-actinin according to the previous report [Zhu and Lou, 2006].

SAMPLE PREPARATION

On differentiating day 5 + 7, EBs was detached from culture surfaces by incubation with 0.05% trypsin–EDTA for 1 min at 37°C. Cells were dissociated with 1 mg/ml collagenase (CLSII, Worthington Biochemical, Lakewood, NJ) and 0.25% mg/ml pancreatin in a buffer containing 117 mM NaCl, 20 mM HEPES, 1.2 mM NaH_2PO_4, 5.4 mM KCl, 1 mM $MgSO_4$, 5 mM glucose at pH 7.35. The EB-derived cardiomyocytes were separated by centrifugation through a discontinuous Percoll gradient and collected at the interface of the two layers.

ES cells, embryoid bodies (EBs), and separated cardiomyocytes at day 5 + 7 were harvested, washed twice with cold PBS (4°C), and then rapidly frozen in liquid nitrogen, thawed in water and repeated for a total of three times. Cell pellets were lysed by addition of a lysis buffer (containing 8 M urea, 2% w/v CHAPS, 1% DTT, and 2% biolyte 30–10), followed by centrifuged at 16,100*g* for 15 min at 4°C to obtain the supernatants. The protein concentration was determined by the Bio-Rad microprotein assay using bovine serum albumin as a standard and then stored at −80°C until used.

TWO-DIMENSIONAL ELECTROPHORESIS

Whole cell proteins (100 μg/aliquot) were separated by silver-stained gels and 1 mg for colloidal Coomassie-stained gels, were suspended in 300 μl rehydration buffer containing 8 M urea, 4% w/v CHAPS, 2% biolyte pH 3–10 buffer, and 70 mM DTT. The samples were passively rehydrated overnight followed by the isoelectric focusing (IEF) procedure on 17 cm nonlinear pH 3–10 immobilized gradient strips (ReadyStrip IPG; BioRad) using the Protean IEF Cell (BioRad) at 100,000 Vh. After IEF, the immobilized pH gradient (IPG) strips were equilibrated in the rehydration buffer plus 2% DTT for 20 min at room temperature. The IPG strips were then alkylated with 2.5% iodoacetamide in the rehydration buffer for 20 min. Subsequently, the IPG strips were washed in running buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS, and loaded onto 12.5% Gels with ReadyPrep overlay agarose containing trace bromophenol blue (Bio-Rad). The 2-DE was conducted at 200 V

for 6 h. The gels were stained by silver or colloidal Coomassie solution, and scanned using a GS-800 Calibrated Imaging Densitometer (Bio-Rad). The computer analysis of the 2-DE image was carried out using PDQUEST 7.0 (Bio-Rad) software. Samples were run three times in three independent cell culture experiments.

IN-GEL TRYPTIC DIGESTION

Protein spots were excised from the gels and placed into a 96-well microtiter plate. Gel pieces were destained with a solution of 15 mM potassium ferricyanide and 50 mM sodium thiosulfate (1:1) at room temperature for 20 min. After complete destaining, gels were washed twice with deionized water, shrunk by dehydration in ACN. The samples were then swollen in a digestion buffer containing 20 mM ammonium bicarbonate and 12.5 ng/μl trypsin at 4°C. After 30 min incubation, the gels were digested for >12 h at 37°C. Peptides were then extracted twice using 0.1% TFA in 50% ACN.

MASS SPECTRUM AND DATABASE SEARCH

The peptide extracts were dried under the protection of N_2. For MALDI-TOF-MS, peptides were eluted onto the target with 0.7 μl matrix solution (α-cyano-4-hydroxy-cinnamic acid in 0.1% trifluoroacetic acid, 50% ACN). Samples were allowed to air dry before inserting them into the mass spectrometer. Positive-ion mass spectra were recorded on a home-built linear time-of-flight mass spectrometer using 39 keV of total acceleration energy. Data from MALDI-TOF MS/MS were analyzed using MASCOT (Matrix Science) search software. The following parameters were used in the search: trypsin digest with one missing cleavage, peptide tolerance of 0.2, MS/MS tolerance of 0.6 Da, and possible oxidation of methionine. A MOWSE score of >80 was regarded as a reliable protein or homolog.

BIOINFORMATICS ANALYSIS OF PROTEIN FUNCTIONS

Peptides were identified using the ProFound-Peptide Mapping search engine (http://www.proteometrics.com/profound_bin/WebProFound.exe), and subsequently searched against the SWISS-PROT (http://www.expasy.org/). The proteins identified from different sets of two-dimensional gels were grouped into categories according to their functions as documented in SWISS-PROT and the National Center for Biotechnology Information (NCBI) databases.

REAL-TIME RT-PCR

Total RNA was extracted from ES cells, EBs, cardiomyocytes derived from EBs treated with DMSO or icariin on day 5 and day 9 after plating (D5 + 5, D5 + 9) using the Trizol reagent (Gibco, BRL) in accordance with the manufacturer's instructions. The reverse transcription reactions and polymerase chain reactions were done as described previously [Zhu and Lou, 2006]. The sense and antisense primers, the cycles of amplification and the temperature of annealing were as follows (Table I). And cDNA synthesis was performed using 3 mg RNA with MMLV RT (Invitrogen). Primer concentration for PCR was 10 pM. Amplifications were performed in a Mastercycler ep Realplex (Eppendorf, Germany) using iQTM SYBR Green Supermix (Bio-Rad). Following programs were used: Cycle 1: Step 1: 95°C for 3 min. Cycle 2: Step 1: 95°C for 45 s; Step 2: specific annealing temperatures for 45 s; Step 3: 72°C for 30 s. Cycle 3: Step 1: 72°C for 10 min. Annealing temperatures were: 60°C for *OCT-4*, 54°C for *NKX2.5*, 66.4°C for *α-MHC*. CT values were automatically obtained. The amount of gene was calculated and normalized by the standard curve. Relative expression values were obtained by the amount of the tested genes in comparison with the amount of the housekeeping genes.

For semi-quantitative RT-PCR, the total RNA extracted was denatured for 3 min at 94°C, followed by the amplification in the reaction with Ampli Taq DNA polymerase: 45 s denaturation and 45 s elongation at 72°C. PCR products were analyzed by 1.5% agarose gel electrophoresis, visualized with ethidium bromide staining, and then quantified using a bio-imaging analyzer (Bio-Rad).

IMMUNOCYTOCHEMISTRY

Cells were fixed with cold methanol for 10 min. After treatment with fetal calf serum for 30 min, specimens were incubated at 4°C overnight together with the primary antibody: troponin T (1:100 dilution; Sigma), α-actinin (1:200 dilution; Sigma). Specimens were then incubated with the fluorescein isothiocyanate isomer I (FITC) or rhodamine-conjugated affinity-purified anti-mouse IgG (1:200 dilution, Santa Cruz Biotechnology) at 37°C for 1.5 h. Images were captured by fluorescence microscope (Leica).

FLOW CYTOMETRY

EBs treated with either DMSO or icatiin on day 5 + 7 were dissociated to a single-cell suspension by treatment with EDTA/trypsin. Cells were suspended in 0.5 ml PBS/1% BSA and assayed using a flow cytometer (FACSCalibur; Becton Dickinson). For detection of troponin T and α-actinin, cells were stained as described above. Each plot represents 10,000 viable cells (nonviable cells were excluded from FACS analysis by appropriate gating). Untreated cells and cells lacking primary antibody were used as controls. In addition, isotype controls were used to assess the level of nonspecific antibody binding. All data analysis was carried out using CellQuest software (Becton Dickinson). Differentiation was determined by comparing the fluorescence intensity of the treated cells to that of untreated cells obtained from a solvent control plate. The results

TABLE I. Primers and Conditions for RT-PCR

Genes	Primers	Size (bp)	Annealing temperature (°C)	Cycles
Nkx2.5	5′-CAAGTGCTCTCCTGCTTTCC-3′ 5′-GGCTTTGTCCAGCTCCACT-3′	136	54	35
OCT-4	5′-AGGGATGGCATACTGTGGAC-3′ 5′-CCTGGGAAAGGTGTCCTGTA-3′	702	60	35
α-MHC	5′-CTGCTGGAGAGGTTATTCCTCG-3′ 5′-GGAAGAGTGAGCGGCGCATCAAGG-3′	301	66.4	40
β-actin	5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′	660	55	30

were expressed as the percentage of the fluorescence intensity of the control group.

WESTERN BLOTTING

The cells were lysed in radioimmunoprecipitation assay RIPA buffer (containing 0.2% Triton X-100, 5 mmol/L EDTA, 1 mM phenylmethanesulfonyl fluoride PMSF, 0.01 g/L leupeptin, and 0.01 g/L aprotinin) on ice for 30 min. The protein concentration was determined using the Bio-Rad microprotein assay using bovine serum albumin as the standard. Forty micrograms of each sample was separated by electrophoresis on a 10% SDS–polyacrylamide gel and electroblotted onto nitrocellulose membranes (Pall). The membranes were blocked for 1 h at room temperature in PBS containing 5% nonfat dry milk plus 0.1% Tween-20 (PBST) and incubated overnight at 4°C with primary antibodies to proteasome 26S (1:500, Santa Cruz Biotechnology), proteasome subunit-alpha type 6 (PSMA6) (1:1,000, Cellsignaling), proteasome subunit-alpha type 2 (PSMA2) (1:500 dilution, Santa Cruz Biotechnology), ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) (1:1,000, Cellsignaling), ubiquitin-conjugating enzyme E2N (1:1,000, Cellsignaling), ubiquitin antibody (1:500 dilution, Cell Signaling Technology), NF-κB (1:1,000 dilution, Santa Cruz Biotechnology), and α-actinin (1:1,000 dilution, Sigma). After incubation, the membranes were washed three times with PBST and then incubated with secondary antibody (1:5,000 dilution, Affinity Bioreagents, Golden, CO) for 1 h at room temperature. Proteins were visualized by enhanced chemiluminescence (ECL, Pierce) detection system using the SuperSignal West Pico Substrate (Thermo). Digital images of appropriate films were captured and quantified using the bio-imaging system (Bio-Rad).

PROTEASE ASSAYS

The samples were repeatedly frozen in liquid nitrogen and quickly melted in 37°C water (5 times). Protein was removed by centrifugation at 5,000*g* for 30 min. The 26S protease was assayed using fluorogenic peptides. Spectrofluorometric assays consisted of 10 μM fluorogenic peptide in 25 mM HEPES, pH 7.5, 0.5 mM EDTA, 0.05% NP-40, and 0.001% SDS. The reaction (600 μl final volume) was initiated by adding enzyme and incubating at 37°C for 1 h prior to measurement. Fluorescence was measured on a JASCO fluorometer using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The following fluorogenic peptide was used Suc-Ala-Ala-Phe-MCA (Sigma).

RESULTS

IDENTIFICATION OF ES CELLS-DERIVED CARDIOMYOCYTES BY ICARIIN

Cardiac differentiation was initiated by EB formation from ES cells, which adhered and continued to proliferate and differentiate into beating cardiomyocytes by treatment with icariin at day 5, as shown in Figure 1A–D. The ES cell-derived cardiomyocytes showed

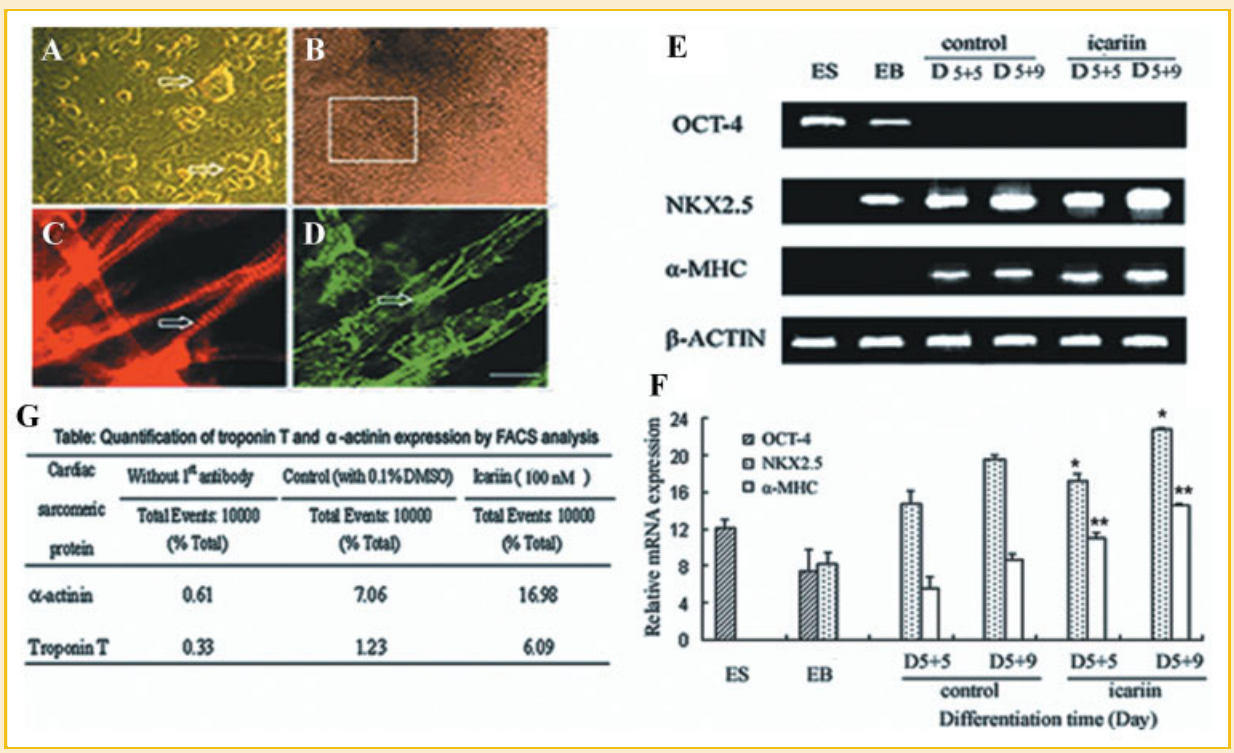

Fig. 1. Identification of the cardiomyocytes differentiated from ES cells. A: Colonies of ES cells (arrow) on a feeder layer of mouse embryonic fibroblasts. B: Synchronously contracting, functional syncytium of cardiac clusters (rectangular frame). C: Expression of cardiac-specific sarcomere α-actinin (red) in the derived cardiomyocytes. D: Expression of cardiac-specific sarcomere troponin T (green). E: Expression of ES cell-derived cardiomyocytes related genes by RT-PCR analysis. F: Quantity analysis of *OCT-4*, *NKX2.5*, *α-MHC* mRNA levels in relation to *GAPDH* identified by real-time PCR. G: Quantification of troponin T and α-actinin expression by FACS analysis. Bar = 100 μm (A,B), 50 μm (C,D). $^{*}P < 0.05$, $^{**}P < 0.01$ versus DMSO group. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

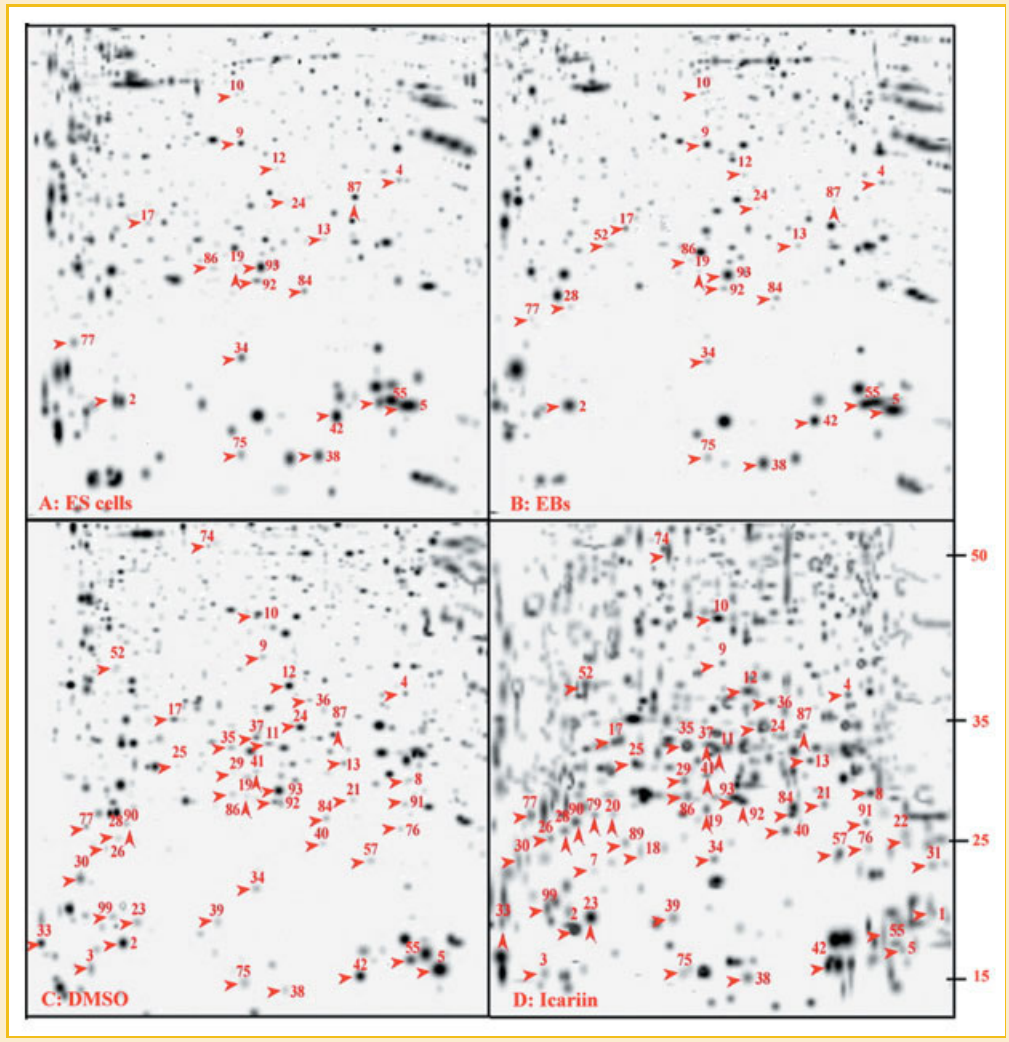

Fig. 2. Two-dimensional electrophoresis gels analysis of proteins extracted from ES cells, 5-day EBs, ES cell-derived cardiomyocytes treated with DMSO or icariin on day 5 + 7. Proteins were visualized using silver nitrate and arrows represent proteins identified by mass spectrometry. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

spindle, round, and tri- or multi-angular morphology with characteristic striations of sarcomeric structures of cardiomyocytes. On the other hand, mRNA levels of cardiac transcription factor *Nkx2.5*, cardiac-specific α-*MHC* and *MLC-2v* were significantly elevated by icariin in a time-dependent manner by real-time RT-PCR analysis (Fig. 1E, F).

Additionally, immunocytochemistry revealed the presence of Z-disc-specific protein α-actinin and troponin T in the ES cell-derived cardiomyocytes (Fig. 1C, D). Furthermore, cardiac-specific proteins troponin T and α-actinin expression were investigated by flow cytometry. On day 5 + 7, only 1.23% of cells stained positively for troponin T in spontaneous differentiation population, while 6.09% of cells treated with icariin stained for troponin T. The proportion of cells induced by icariin that contained positive sarcomeric α-actinin protein labeled sarcomeres was significantly increased to 16.98% in contrast with the case of 7.06% in the control (Fig. 1G).

COMPARISON OF PROTEOME PATTERNS WITH SILVER STAINED 2-DE AND PROTEIN IDENTIFICATION

We conducted a 2-DE analysis of ES cells, 5-day-old EB, and the ES cell-derived cardiomyocytes treated with either DMSO or icariin on day 5 + 7, as shown in Figure 2. For each group, triplicate 2-DE silver stained gels were integrated and PDQUEST (version 6.0; Bio-Rad) software was used to analyze the 2-DE image. For mass spectrometry analysis, 68 proteins were excised from Coomassie-stained gels and were subsequently digested. The protein spots were analyzed by MALDI-TOF-TOF MS/MS on the basis of a combined peptide mass fingerprinting and MS/MS analysis, leading to the identification of 52 proteins (Table II). The identified proteins were classified into 8 functional categories based on the information from the Gene Ontology database and additional information from ExPASy (http://www.expasy.org/sprot/). More than half of the proteins could be assigned to six main categories: energy metabolism (17%), protein synthesis/

TABLE II. Differentially Expressed Proteins in Cardiomyocytes Derived From ES Cells by Icariin

No	Protein name	Accession no.	Theoretical MW/PI	Matched peptides	Score	Score CI %
Cytoskeleton						
19	Endoplasmic reticulum protein ERp29 precursor	gi|19526463	28805.1/5.9	10	338	100
10	Keratin complex 1, acidic, gene 19	gi|6680606	44514.7/5.28	23	347	100
23	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform (MLC-2v)	gi|1709058	18870.4/4.71	6	73	99.435
74	Vimentin	gi|2078001	51533.1/4.96	4	238	100
25	Capping protein (actin filament) muscle Z-line, alpha 1 (Cap-Z)	gi|33468887	32919.3/5.34	7	369	100
33	Myosin light chain-2 isoform MLC-2a—mouse	gi|627919	18252.9/4.48	6	193	100
Energy metabolism						
13	Voltage-dependent anion channel 2 (VDAC2)	gi|6755965	31712.6/7.44	5	107	100
89	Adenylate kinase 4	gi|6753022	25046.1/7.02	10	364	100
1	ATP synthase, H + transporting, Mitochondrial F0 Complex, subunit alpha	gi|21313679	18737.6/5.52	9	259	100
87	Electron transferring flavoprotein, alpha polypeptide	gi|31981826	34928.4/8.42	14	218	100
9	Isocitrate dehydrogenase 3 (NAD+) alpha	gi|18250284	39613.1/6.27	9	417	100
52	Malate dehydrogenase 1, NAD (soluble)	gi|31982178	36445.1/5.92	2	91	99.992
55	Nucleoside-diphosphate kinase	gi|6679078	17351.9/6.97	5	99	99.999
28	Alpha-globin	gi|1162945	15117.8/7.96	7	80	99.895
35	3-Mercaptopyruvate sulfurtransferase (MST)	gi|90110410	33002.5/6.11	18	444	100
Protein synthesis/metabolism/processing/degradation						
77	Ubiquitin carboxy-terminal hydrolase L1 (UCH-L1)	gi|6755929	24821.5/5.33	5	180	100
2	Ubiquitin-conjugating enzyme E2N	gi|4507793	17127/6.13	8	413	100
17	Proteasome (prosome, macropain) 26S subunit, non-ATPase	gi|6754724	36517.2/6.29	10	447	100
91	Proteasome subunit, alpha type 2 (PSMA2)	gi|6679497	25909.3/8.39	5	272	100
20	Proteasome subunit, alpha type 6	gi|6755198	27354.8/6.34	6	253	100
86	Caseinolytic protease, ATP-dependent, proteolytic subunit homolog	gi|8393156	29781.3/7.05	5	144	100
3	Fatty acid binding protein 5, epidermal	gi|6754450	15127.4/6.14	3	106	100
29	Phosphatidylethanolamine binding protein	gi|1517864	20847.3/5.19	4	133	100
76	Rho GDP dissociation inhibitor (GDI) alpha	gi|31982030	23392.8/5.12	8	345	100
RNA/other nucleic acids metabolism and transport, nuclear proteins						
11	Heterogeneous nuclear ribonucleoprotein D-like	gi|7710036	33538.1/6.85	9	196	100
38	40S Ribosomal protein S12	gi|54039306	14515.6/6.82	2	110	100
84	5′,3′-Nucleotidase, cytosolic (5′-nucleotidase)	gi|7657031	23061.8/5.31	4	236	100
22	hprt Gene product	gi|309315	24553.6/6.51	5	78	99.817
26	Nudix (nucleoside diphosphate linked moiety X)-type motif 5	gi|13435414	23996.1/5.34	2	74	99.552
7	Deoxyuridine triphosphatase	gi|21281687	17373.7/5.74	4	304	100
Carbohydrate metabolism/transport						
24	Aldose reductase (AR)	gi|1351911	35709.4/6.71	7	132	100
36	Lactate dehydrogenase 2, B chain	gi|6678674	36549/5.7	10	179	100
79	6-Phosphogluconolactonase	gi|13384778	27237.4/5.55	3	201	100
93	Phosphoglycerate mutase 1	gi|10179944	28918.9/6.19	5	184	100
4	Glyceraldehyde-3-phosphate dehydrogenase	gi|55153885	35751.1/7.59	4	120	100
37	Pdhb protein	gi|12805431	34813.8/5.63	4	209	100
8	Carbonic anhydrase 2	gi|33243954	29023.5/6.52	10	513	100
90	Triosephosphate isomerase	gi|54855	26678.8/6.9	7	269	100
Stress response						
57	Heat shock protein HSP27	gi|424145	21961.2/6.45	7	304	100
30	Heat-shock protein beta-1 (HSPB1)	gi|547679	22999.7/6.12	9	442	100
34	Peroxiredoxin 4	gi|7948999	31033.1/6.67	7	197	100
92	Crystallin, alpha B (HSPB5)	gi|6753530	20056.4/6.76	10	430	100
42	Superoxide dismutase 1, soluble (SOD1)	gi|45597447	15932.8/6.02	7	427	100
75	Peroxiredoxin-6 (antioxidant protein 2)	gi|3219774	24855/5.71	15	626	100
5	Superoxide dismutase 2, mitochondrial	gi|31980762	24587.5/8.8	7	261	100
Transcription						
99	Basic transcription factor 3 isoform B	gi|20070130	17688.2/6.85	7	263	100
12	PKCq-interacting protein PICOT	gi|6840949	37758.4/5.42	3	143	100
Others						
39	es1 Protein	gi|20070420	28072.8/9	5	190	100
40	Chain B, Chimeric HumanMOUSE CARBONMONOXY	gi|18655687	15607.1/7.27	6	202	100
31	Aplysia ras-related homolog A2	gi|16923986	21768.1/5.83	5	216	100
21	Hypothetical protein LOC68045	gi|13386026	28134.8/6.4	10	346	100
18	Cytokine induced protein 29 kDa	gi|13384730	23518.3/6.29	5	89	99.986

metabolism/processing/degradation (17%), stress response (13%), RNA/other nucleic acids metabolisms and transport, nuclear proteins (12%), cytoskeleton proteins (12%), and carbohydrate metabolism/transport (15%). Each of the remainder did not comprise more than 10% of total identified proteins (Fig. 3). Table II showed the differentially expressed proteins being identified and sorted according to protein function. The differential expression levels of proteins were analyzed during the whole progression of cardiomyocytes derived from ES cells induced by icariin.

EXPRESSION OF PROTEINS ASSOCIATED WITH THE UBIQUITIN–PROTEASOME SYSTEM IN CARDIOMYOCYTES DERIVED FROM ES CELLS

Among these proteins identified, five proteins (e.g., ubiquitin carboxy-terminal hydrolase L1, ubiquitin-conjugating enzyme E2N, proteasome 26S, proteasome subunit-alpha type 6, and proteasome subunit-alpha type 2) were found to be associated with ubiquitin–proteasome system (Table II), which may be involved in the cellular differentiation by icariin. Five proteins of interest were selected for

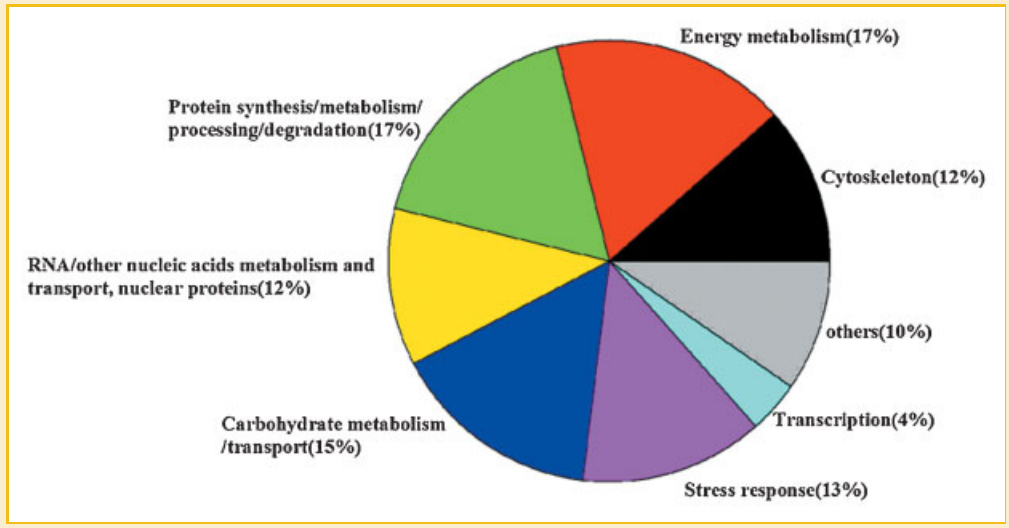

Fig. 3. Relative quantitative distribution of functional protein categories. The functional protein categories based on individual proteins (n = 52) and largely assigned according to Gene Ontology database and ExPASy information. More than 65% of the proteins were spread on the six largest functional groups. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

further validation by Western blotting analysis. It had been confirmed that expressing levels of five proteins were significantly increased in icariin-induced cardiomyocytes derived from ES cells (Fig. 4). These results were in accordance with the observations based on the comparative proteomic analyses.

EFFECT OF PROTEASOME INHIBITOR ON ICARIIN-INDUCED CARDIOMYOCYTE DIFFERENTIATION

Epoxomicin, proteasome inhibitor [Meng et al., 1999], was employed to evaluate the possible function of the proteasome in the cardiomyocyte differentiation. According to our preliminary experiment, 10 nM epoxomicin did not affect on ES cells proliferation or apoptosis over 48 h (data not shown); this concentration was used for the subsequently experiments.

Epoxomicin was applied to EBs from day 5, and the percentage of EBs containing beating cardiomyocytes was evaluated during differentiation. Figure 5A showed that epoxomicin significantly decreased the percentage of EBs containing beating cardiomyocytes in a dose-dependent manner. Simultaneously, expression of α-actinin was also markedly decreased, suggesting that the formation of sarcomeric proteins was interrupted by the specific antagonist epoxomicin. Moreover, the activity of proteasome may be closely related to the differentiation of ES cells into cardiomyocytes.

Induction of the number of beating EBs by epoxomicin was significantly decreased by approximately 35% compared with control group (i.e., DMSO) on day 5 + 7. When EBs were exposed to epoxomicin combined with icariin, the differentiation rate was increased up to approximately 52% ($P < 0.05$) compared with epoxomicin alone (Fig. 5B). Furthermore, it was shown that the icariin could reverse the down-regulation of α-actinin by epoxomicin treatment (Fig. 5B). This result indicated that icariin could prevent the inhibition of cardiomyocyte differentiation by epoxomicin, which correlated with icariin regulating the activity of proteasome.

PROTEASOME ACTIVITY ANALYSIS

In order to better understand the inducing mechanism of icariin, we further determined the proteasome activity in cells. Figure 5C showed that proteasome activity was decreased to 106 U in EBs after exposure to epoxomicin alone compared with the negative control. When icariin was added into epoxomicin-treated EBs, the proteasome activity was significantly increased (approximately 136 U, $P < 0.05$) and similar to control levels (Fig. 5C), indicating that icariin can reverse the inhibition of proteasome activity by epoxomicin. These results were also consistent with the differentiating rate change of EBs treated by icariin together with epoxomicin (Fig. 5B).

EFFECT OF PROTEASOME INHIBITOR ON UBIQUITIN–PROTEASOME SYSTEM RELATED PROTEINS EXPRESSIONS

Ubiquitin (Ub) cannot dissociate from Ub-conjugated proteins because of blocking proteasome activity by epoxomicin, which leads to high expression of Ub-conjugated proteins. Our experiments showed that icariin was able to increase proteasome activity in EBs by exposure to epoxomicin. Therefore, we assumed that the Ub-conjugated proteins should be lower in EBs treated with both icariin and epoxomicin than that in epoxomicin-treated EBs. As expected, Ub-conjugated proteins expressions were much less in EBs after treated with icariin and epoxomicin than that of EBs treated with epoxomicin alone (Fig. 5D).

On the other hand, we suggest that epoxomicin inhibited translocation of NF-κB into the nucleus that might result in increased expression of Ub-conjugated proteins. Contrary, icariin reversed the effect of epoxomicin on EBs and down-regulated the level of Ub-conjugated proteins, then facilitated the translocation of NF-κB into the nucleus and triggers the differentiation of cardiomyocytes (Fig. 5D). These results suggest that ubiquitin might play a key role in ES cell differentiation, and icariin could

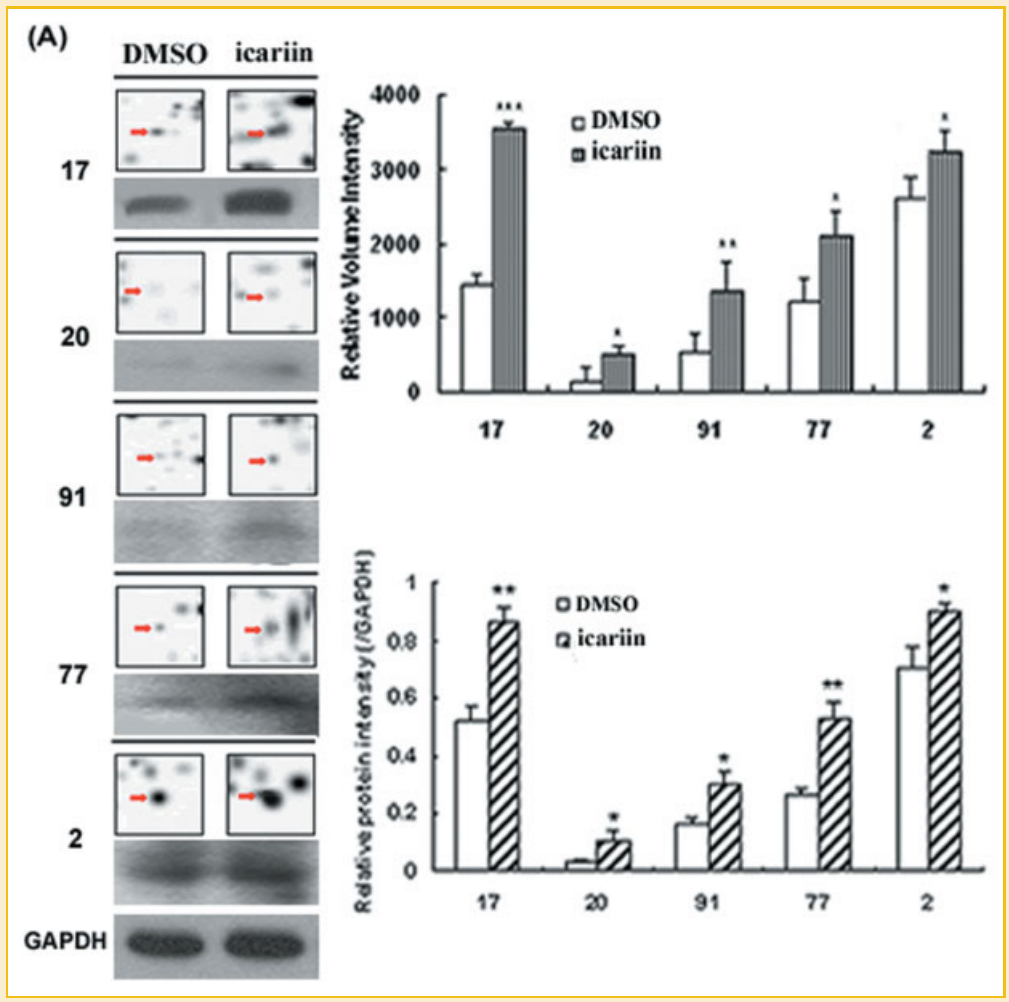

Fig. 4. Proteins showing expression levels after cardiomyocyte differentiation induced by icariin. A: Quantitative analysis of two-dimensional electrophoresis separated proteins compared with the results of Western blotting analysis for proteasome 26S (17), proteasome subunit-alpha type 6 (20), and proteasome subunit-alpha type 2 (91), ubiquitin carboxy-terminal hydrolase L1 (77), ubiquitin-conjugating enzyme E2N (2). B: The spot proteins images analyzed using the PDQuset Bio-Rad software program. Similar data were obtained in three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.01$ versus DMSO group. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

activate the NF-κB signaling pathway through regulating the UPS during the differentiation of cardiomyocytes from ES cells.

Meanwhile, we also investigated that the expression variation of ubiquitin–proteasome related proteins selected from 2-DE analysis when epoxomicin was added. The expressions of proteasome subunit-alpha type 6, proteasome subunit-alpha type 2, UCH-L1, and ubiquitin-conjugating enzyme E2N were decreased after epoxomicin treatment. Icariin could significantly reverse the reduction of the four proteins expressions compared with control (Fig. 5E). These results indicated these ubiquitin–proteasome related proteins might be involved in the cardiomyocyte differentiation from ES cells induced by icariin.

DISCUSSION

In present study, we have profiled the protein expression in cardiomyocytes differentiated from ES cells induced by icariin for the first time. Using a proteomic approach, the first two-dimensional reference map was developed for the cardiomyocytes derived from ES cells treated with icariin, which would facilitate further studies on the unique targets and mechanisms of icariin regulating ES cells differentiation. Moreover, the information obtained from proteomic profiling will help us elucidate inherent links between the broader cellular pathways/molecules that were neither apparent nor predictable through traditional biochemical analysis in the past. Proteomic tools [Prudhomme et al., 2004; Kim et al., 2010] are valuable for studying stem cell differentiation and elucidating the underlying molecular mechanisms by drugs. In the present study, we used a proteomic approach, a two-dimensional reference map, to identify protein expression in cardiomyocytes derived from ES cells treated with icariin. Interestingly, we identified that the expression of ubiquitin–proteasome system related proteins, such as ubiquitin carboxy-terminal hydrolase L1, ubiquitin-conjugating enzyme E2N, proteasome 26S, proteasome subunit-alpha type 6, and proteasome subunit-alpha type 2, were markedly different between icariin and

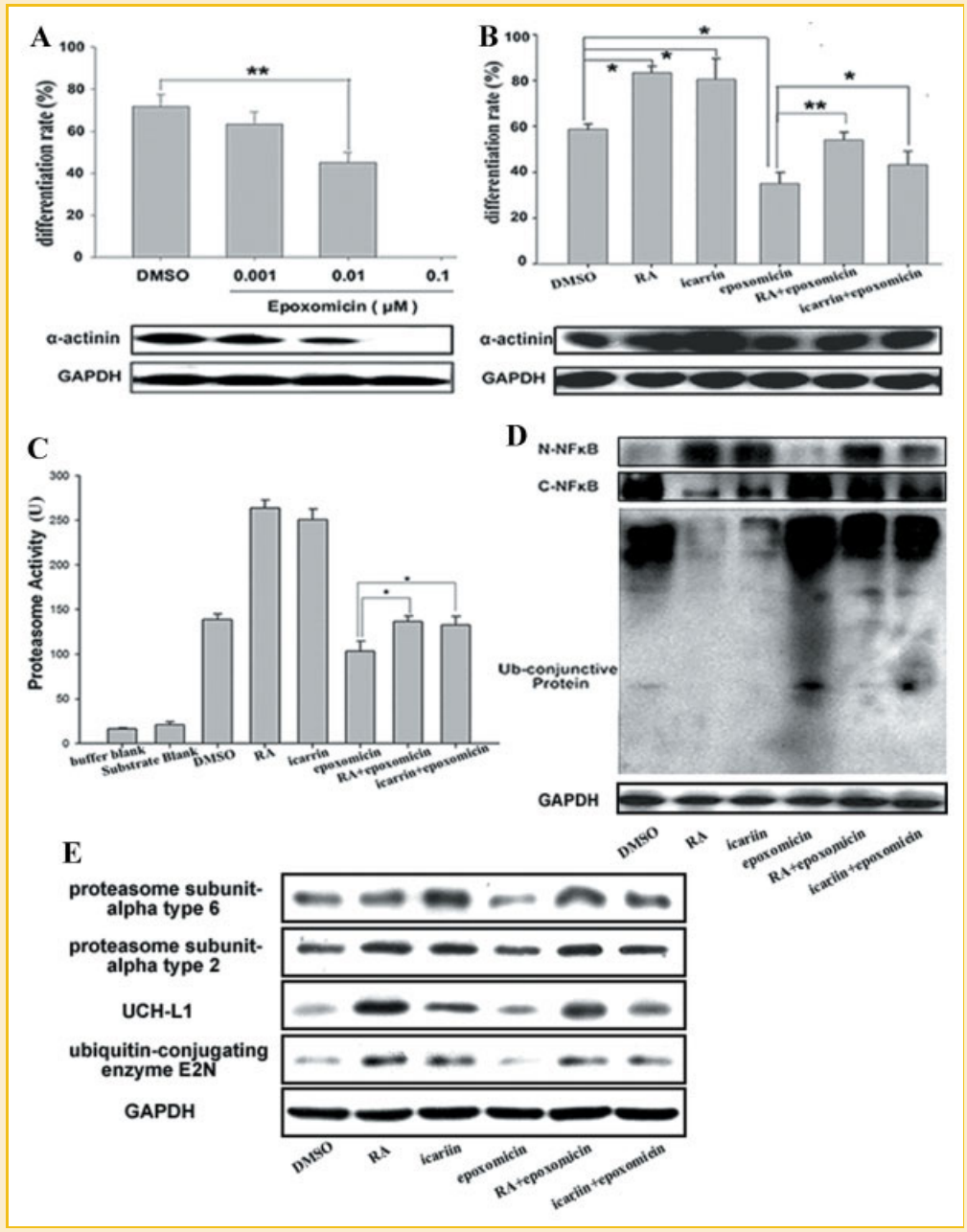

Fig. 5. Effect of epoxomicin on the cardiomyocyte differentiation together with icariin in EBs. A: Effects of epoxomicin on the beating number of EBs and expression of α-actinin. B: Influence of epoxomicin together with icariin on the cardiomyocyte differentiation of EBs. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control. C: Proteasome activity assay for cardiac differentiation after application of epoxomicin together with icariin in EBs. $^{*}P < 0.05$ versus epoxomicin. D: Western blot detection of the ubiquitin-conjuctive protein expression and NF-κB translation. E: The proteins expressions for proteasome subunit-alpha type 6, proteasome subunit-alpha type 2, ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), and ubiquitin-conjugating enzyme E2N after application of epoxomicin together with icariin in EBs.

DMSO-treated cells. The expressions of various proteins above mentioned were all increased by icariin treatment. The results of the present study indicated that proteomic analysis were in favor of detecting the unique targets and the inducing mechanisms of icariin. Moreover, proteomic profiling would help us elucidate inherent links between the broader cellular pathways/molecules.

Proteasome plays central roles in the proteolysis of ubiquitinated cellular proteins and is responsible for cleaving many regulatory proteins, such as cyclins, members of NF-κB family, and oncogenic products [Auld and Silver, 2006; Szutorisz et al., 2006]. They play important roles in transcriptional regulation, cell cycle, and apoptosis. Transcription factor NF-κB has been implicated in the inducible expression of a variety of genes directing differentiation toward cardiomyocytes [Jones et al., 2003]. NF-κB is normally present in cytoplasm in an inactive form, while NF-κB is translocated into the nucleus in an active form and may participate

in myocardial gene regulation throughout development [Norman et al., 1998]. Our previous results showed that I-κB was rapidly phosphorylated, then resulted in its own rapid degradation and NF-κB p65 nuclear translocation in EBs treated by icariin [Wo et al., 2008]. These indicated NF-κB signaling pathway could be activated during the promotion of the cardiac differentiation induced by icariin. However, whether icariin regulating the ubiquitin–proteasome system result in activating the NF-κB signaling was still not known. In the present investigation, our results revealed that epoxomicin (a proteasome inhibitor) significantly decreased the differentiation rate of ES-derived myocardial cells via inhibiting the proteasome activity and increasing the Ub-conjugated proteins expressions. Conversely, icariin could reverse the inhibiting effects of epoxomicin on the differentiation of cardiomyocytes, and activate NF-κB nuclear translocation. These results suggest that ubiquitination-mediated proteolysis of NF-κB by the proteasome was a critical step in most pathways leading to NF-κB activation of icariin in the transition from cardiogenesis to cardiomyocytes during differentiation. On the other hand, proteasome subunit-alpha type 6 is one of the alpha-type subunits of the 20S proteasome core complex. It participates in the ubiquitin–proteasome pathway and it can regulate cell proliferation and cell cycle control [Zhang and Wei, 2011]. Proteasome subunit-alpha type 2 is also a subunit of the 20S proteasome core complex. Each protein is an important part of UPS and participants in the cell cycle and cell growth. In our study, icariin reversed the inhibiting effect of epoxomicin on the differentiation of cardiomyocytes through increasing the two subunits of proteasome expressions. The increasing expressions of proteasome subunit-alpha type 6 and proteasome subunit-alpha type 2 were essential to the cardiomyocyte differentiation. The mechanism of icariin inducing differentiation might be related with regulating the expression and activity of the proteasome related proteins.

Except for three proteasome related proteins (namely, proteasome 26S, proteasome subunit-alpha type 6, and proteasome subunit-alpha type 2), MALDI-TOF mass spectrometry revealed that other ubiquitin related proteins, ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) and ubiquitin-conjugating enzyme E2N, also showed different levels between icariin and DMSO treated cells. UCH-L1 is characterized as a deubiquitinating enzyme, it also functions as a ubiquitin ligase and a mono-Ub stabilizer [Setsuie and Wada, 2007]. Ubiquitin-conjugating enzyme E2N is a protein whose modification with ubiquitin is an important cellular mechanism for targeting abnormal or short-lived proteins for degradation [Andersen et al., 2005]. UCH-L1 has a fundamental role in regulating various biological events, including cell-cycle progression, specific gene transcription, membrane protein trafficking, reversal of stress damage, and intracellular signaling [Cord, 2009]. Furthermore, accumulated evidence has shown that UCH-L1 seems to regulate the morphology of neural progenitor cells and modulate their differentiation [Sakurai et al., 2006; Naujokat and Saric, 2007; Cord, 2009]. UCH-L1 is also involved in the pathogenesis of the neurodegenerative diseases [Setsuie and Wada, 2007]. However, the expression and function of UCH-L1 in cardiomyocytes differentiated from ES cells treated with icariin was not fully understood. Our results revealed that UCH-L1 and ubiquitin-conjugating enzyme E2N were expressed in differentiated cardiomyocytes and were higher in icariin-induced cardiac differentiation than in controls (i.e., DMSO-treatment). Icariin could increase the expression of them to reduce the accumulation of Ub-conjugated proteins and reverse the inhibiting effects of epoxomicin on the differentiation of cardiomyocytes. Thus, UCH-L1 and ubiquitin-conjugating enzyme E2N are important for the facilitation of the cardiogenesis and sarcomeric formation induced by icariin.

In conclusion, icariin stimulated activation of the proteasome activity and the decreased Ub-conjugated protein expression resulted in activated NF-κB signaling for promoting the cardiac differentiation process, suggesting that the UPS may make an important contribution to icariin-induced cardiomyocyte differentiation.

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REFERENCES

Andersen PL, Zhou H, Pastushok L, Moraes T, McKenna S, Ziola B, Ellison MJ, Dixit VM, Xiao W. 2005. Distinct regulation of Ubc13 functions by the two ubiquitin-conjugating enzyme variants Mms2 and Uev1A. J Cell Biol 170:745–755.

Auld KL, Silver PA. 2006. Transcriptional regulation by the proteasome as a mechanism for cellular protein homeostasis. Cell Cycle 5:1503–1505.

Baharvand H, Hajheidari M, Zonouzi R, Ashtiani SK, Hosseinkhani S, Salekdeh GH. 2006. Comparative proteomic analysis of mouse embryonic stem cells and neonatal-derived cardiomyocytes. Biochem Biophys Res Commun 349:1041–1049.

Bendall SC, Booy AT, Lajoie G. 2007. Proteomic analysis of pluripotent stem cells. Curr Protoc Stem Cell Biol Chapter 1 Unit 1B.1.

Cord Naujokat. 2009. Role of ubiquitin ligases in neural stem and progenitor cells. Arch Immunol Ther Exp 57:177–188.

Ding L, Liang XG, Hu Y, Zhu DY, Lou YJ. 2008. Involvement of p38MAPK and reactive oxygen species in icariin-induced cardiomyocyte differentiation of murine embryonic stem cells in vitro. Stem Cells Dev 17:751–760.

Friehs I. 2008. Proteasome inhibition in hypertrophied myocardium. Am J Physiol Heart Circ Physiol 295:1385–1393.

Guo XX, Ying WT, Wan JH, Hu Z, Qian X, Zhang H, He F. 2001. Proteomic characterization of early-stage differentiation of mouse embryonic stem cells into neural cells induced by all-trans retinoic acid in vitro. Electrophoresis 22:3067–3075.

He W, Sun H, Yang B, Zhang D, Kabelitz D. 1995. Immunoregulatory effects of the herb *Epimediia* glycoside icariin. Arzneimittelforschung 45:910–913.

Jian R, Cheng X, Jiang J, Deng S, Hu F, Zhang J. 2007. A cDNA-based random RNAi library for functional genetic screens in embryonic stem cells. Stem Cells 25:1904–1912.

Jones WK, Brown M, Ren X, He S, McGuinness M. 2003. NF-kappaB as an integrator of diverse signaling pathways: The heart of myocardial signaling. Cardiovasc Toxicol 3:229–254.

Kim HR, Han RX, Wakayama T, Park CS, Jin DI. 2010. Aberrant protein expression in the placenta of cloned mouse derived from embryonic stem cell. Placenta 31:853–859.

Meng L, Mohan R, Kwok BH, Elofsson M, Sin N, Crews CM. 1999. Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo anti-inflammatory activity. Proc Natl Acad Sci USA 96:10403–10408.

Metzger JM, Lin WI, Johnston RA, Westfall MV, Samuelson LC. 1995. Myosin heavy chain expression in contracting myocytes isolated during embryonic stem cell cardiogenesis. Circ Res 76:710–719.

Miler J, Gordon C. 2005. The regulation of proteasome degradation by multi-ubiquitin chain binding proteins. FEBS Lett 579:3224–3230.

Naujokat C, Saric T. 2007. Role and function of the ubiquitin–proteasome system in mammalian stem and progenitor cells. Stem Cells 25:2408–2418.

Norman DA, Yacoub MH, Barton PJ. 1998. Nuclear factor NF-kappa B in myocardium: Developmental expression of subunits and activation by interleukin-1 beta in cardiac myocytes in vitro. Cardiovasc Res 39:434–441.

Prudhomme W, Daley GQ, Zandstra P, Lauffenburger DA. 2004. Multivariate proteomic analysis of murine embryonic stem cell self-renewal versus differentiation signaling. Proc Natl Acad Sci USA 101:2900–2905.

Ravid T, Hochstrasser M. 2008. Diversity of degradation signals in the ubiquitin–proteasome system. Nat Rev Mol Cell Biol 9:679–690.

Sakurai M, Ayukawa K, Setsuie R, Nishikawa K, Hara Y, Ohashi H, Nishimoto M, Abe T, Kudo Y, Sekiguchi M, Sato Y, Aoki S, Noda M, Wada K. 2006. Ubiquitin C-terminal hydrolase L1 regulates the morphology of neural progenitor cells and modulates their differentiation. J Cell Sci 119:162–171.

Scholz G, Pohl I, Genschow E, Klemm M, Spielmann H. 1999. Embryotoxicity screening using embryonic cells in vitro: Correlation to in vivo teratogenicity. Cells Tissues Organs 165:203–211.

Setsuie R, Wada K. 2007. The functions of UCH-L1 and its relation to neurodegenerative diseases. Neurochem Int 51:105–111.

Szutorisz H, Georgiou A, Tora L, Dillon N. 2006. The proteasome restricts permissive transcription at tissue-specific gene loci in embryonic stem cells. Cell 127:1375–1388.

Unwin RD, Gaskell SJ, Evans CA, Whetton AD. 2003. The potential for proteomic definition of stem cell populations. Exp Hematol 31:1147–1159.

Van Hoof D, Mummery CL, Heck AJ, Krijqsveld J. 2006. Embryonic stem cell proteomics. Expert Rev Proteomics 3:427–437.

Wo YB, Zhu DY, Lou YJ. 2008. Involvement of NF-κB and AP-1 activation in icariin promoted cardiac differentiation of mouse embryonic stem cells. Eur J Pharmacol 586:59–66.

Zhang W, Wei Q. 2011. Calcneurin stimulates the expression of inflammatory factors in RAW 264.7 cells by interacting with proteasome subunit alpha type 6. Biochem Biophys Res Commun 407:668–673.

Zhu DY, Lou YJ. 2006. Icariin-mediated expression of cardiac genes and modulation of nitric oxide signaling pathway during differentiation of mouse embryonic stem cells into cardiomyocytes in vitro. Acta Pharmacol Sin 27:311–320.