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Proteomic Analysis of Cardiomyocytes Differentiation in Mouse Embryonic Carcinoma P19CL6 Cells

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Abstract A clonal derivative named P19CL6 has been isolated from pluripotent P19 mouse embryonic carcinoma cells, and this subline efficiently differentiates into beating cardiomyocytes when treated with 1% dimethyl sulfoxide (DMSO). It offers a valuable model to study cardiomyocytes differentiation in vitro. In this study, comparative proteomic analysis was used to characterize the protein profiles associated with the DMSO-induced cardiomyocytes differentiation of P19CL6 cells. We demonstrated that P19CL6 cells indeed differentiated into cardiomyocytes after DMSO inducement as they expressed sarcomeric myosin heavy chain (MHC) as well as three cardiac-specific transcription factors (Csx/Nkx-2.5, GATA-4, and MEF2C). Image analysis of silver-stained two-dimensional gels was used to find protein spots that exhibited an at least 1.5-fold change in abundance after successful differentiation. Seventeen protein spots were selected for further analysis by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF-MS) and/or nano-electrospray ionization MS/MS (ESI-MS/MS), and 16 protein spots were identified. The identified proteins are involved in different cellular functions such as metabolism, signal transduction, and cellular organization. To confirm the expression changes of the identified proteins during differentiation, the mRNA levels of six identified proteins (including seven protein spots) were assessed by the real-time polymerase chain reaction and three showed a correlation between mRNA level and protein abundance. As an initial step toward identifying proteins involved in maintaining the differentiated state of cardiomyocytes derived from P19CL6 cells, our data provide some helpful information that may lead to a better understanding of the molecular mechanisms by which P19CL6 cells differentiate into cardiomyocytes after treatment with DMSO. J. Cell. Biochem. 102: 149–160, 2007.

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Heart is the first organ that becomes functional during vertebrate embryonic development. Elucidating the mechanisms of cardiomyogenesis has important implications in understanding the etiology of congenital heart disease as well as developing novel strategies for the

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regeneration of cardiac tissue. Significant progress has been made over the past decade in identifying genes that play key roles in vertebrate heart formation. Several genes that play important roles in cardiomyocyte differentiation have been identified, such as the transcription factors Csx/Nkx-2.5, GATA-4, MEF2C, and so on [Monzen et al., 1999]. A clonal derivative named P19CL6 has been isolated from pluripotent P19 embryonal carcinoma cells and P19CL6 cells efficiently differentiate into beating cardiomyocytes after treatment with 1% dimethyl sulfoxide (DMSO) [Habara-Ohkubo, 1996]. The in vitro differentiation of P19CL6 cells mimics the normal developmental program of the cardiomyocytes in vivo. For example, cardiomyocytes derived from P19CL6 cells express cardiac transcription factors such as Csx/Nkx-2.5, GATA-4, and MEF2C followed by the expression of cardiac contractile proteins such as cardiac isoforms of sarcomeric myosin heavy chain (MHC) and myosin light chain (MLC) [Monzen et al., 1999; Monzen et al., 2001]. Therefore, this model offers great advantages for the investigation of the molecular events that occur at each stage of the cardiomyocytes differentiation, that is, the precardioblast stage, cardioblast stage, and cardiomyocyte stage.

Peng et al. [2002] performed microarray analysis to examine global changes in gene expression during cardiomyocytes differentiation using the P19CL6 cell line, and found a number of novel candidate genes that may mediate cardiomyocytes differentiation. Nevertheless, protein expression and its changes under the influence of these genes are not well understood. mRNA expression patterns are necessary but insufficient for a quantitative description of the state of biological systems [Gygi et al., 1999; Lian et al., 2001], since proteins ultimately carry out function. In recent years, proteomics has provided unparalleled information in the understanding of the cellular biology. Systems analysis performed at the protein level has the advantage of being closest to their function. The application of high-throughput proteomics can systematically identify and characterize the protein expression profiles. In this study, we employed proteomic approaches using two-dimensional gel electrophoresis (2-DE), followed by MS to investigate the differential expression of proteins associated with maintaining the cardiac state of P19CL6 cells. Altogether, we have identified 16 protein spots that are clearly regulated obviously in response to DMSO. The differential expression of these proteins is expected to provide helpful information and an unbiased insight into the mechanisms involved in maintaining the cardiac state of P19CL6 cells in vitro.

MATERIALS AND METHODS

Cell Culture

P19CL6 mouse embryonic carcinoma cells were kindly provided by K Monzen (University of Tokyo Graduate School of Medicine, Tokyo, Japan). P19CL6 cells were cultured as described previously [Monzen et al., 1999]. Briefly, P19CL6 cells were maintained in α-MEM (Gibco) containing penicillin and streptomycin, supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 2 mM L-glutamine. To induce differentiation, P19CL6 cells were plated at a density of 3.7×10^5/ml in 175 cm^2 flasks with the growth medium containing 1% DMSO. The days of differentiation are numbered consecutively beginning after the day of the DMSO treatment (Day 0).

Immunofluorescence Staining

The ability of DMSO to induce the differentiation of P19CL6 cells was assessed by immunofluorescence analysis of sarcomeric MHC. The immunofluorescence staining procedure for MHC using an anti-MF20 antibody (Developmental studies Hybridoma Bank) was performed as described by Naito et al. [Bader et al., 1982; Naito et al., 2003]. Briefly, following treatment with DMSO-containing or DMSO-free media for 12 days, P19CL6 cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (pH 7.4). Cells were then incubated overnight with anti-MF20 antibody, followed by incubation with tetramethyl rhodamine isothiocyanate conjugated secondary antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Confocal microscopic analysis was performed using a confocal laser microscope (Carl Zeiss, Laser scanning microscope, LSM510).

Sample Preparation

Cells were washed three times with chilled salt-free buffer (10 mM Tris-HCl, 250 mM

sorbitol). The supernatant was discarded, and cell lysates were then prepared on ice using lysis buffer (8 M urea, 4% CHAPS, 40 mM Tris) containing a protease inhibitor cocktail (Roche Diagnostic). After 40 min of stirring at 4°C, cells were scraped and centrifuged at 14,000*g* for 40 min to remove debris. The supernatants were then collected, protein concentration was determined using the Bradford method, and lysates were aliquoted and stored at −80°C until use.

Two-Dimensional Electrophoresis and Image Analysis

Two-dimensional electrophoresis was performed as described in the manufacturer's instructions [Berkelman and Stenstedt, 1998] and modified by Gorg et al. [1988]. Briefly, total protein lysates (200 μg for silver staining and 800 μg for Coomassie Brilliant Blue G-250 staining) of different cell states were subjected to 2-DE. Isoelectric focusing (IEF) was performed with the IPGphor system (Amersham Pharmacia Biotech). After active rehydration for 12 h at 30 V, the strips (18 cm, pH 3–10, nonlinear) were focused at 0.05 mA/IPG strip for 80,000 Vh at 20°C. Once the IEF was finished, the IPG strips were immediately equilibrated in 10 ml equilibration solutions (6 M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS), 50 mM Tris-Cl pH 8.8, 1% dithiothreitol (DTT)) with gentle shaking for 15 min. The strips were then treated with the same solution containing 2.5% iodoacetamide instead of DTT. SDS–polyacrylamide gel electrophoresis (PAGE) was performed using 13% polyacrylamide gels without a stacking gel in the PROTEAN II XL system (Bio-Rad company). Following SDS–PAGE, gels were stained with 0.1% (w/v) Coomassie Brilliant Blue G-250 in 50% methanol and 10% acetic acid for preparative gels, or silver staining according to the protocol of Shevchenko et al. [1996] for analytical gels. The 2-DE images were captured using ImageScanner (Amersham Pharmacia Biotech). Spot detection, quantification, and matching were performed with Image Master 2-D Elite Version 3.01 software according to the manufacturer's instructions (Amersham Biosciences). Proteins were subjected to further analyses when the expression levels of given protein spots altered at least 1.5-fold. Each experiment was performed at least three times.

In-Gel Digestion and Peptide Mass Fingerprinting by MALDI-TOF-MS

In-gel digestion was performed using a modified version of a previously published protocol [Shevchenko et al., 1996; Gamble et al., 2000]. The selected protein spots, excised with a blade and transferred into microcentrifuge tubes, were destained by washing in 200 μl aliquots of 50 mM ammonium bicarbonate in 50% (v/v) acetonitrile for 30 min. A second wash was performed using acetonitrile for 15 min at room temperature. The gel pieces were then dried in a SpeedVac Vacuum (Savant Instruments, Holbrook, NY) and rehydrated at 4°C for 30 min in 10 μl digestion solution (25 mM ammonium bicarbonate and 10 ng/μl modified sequence-grade trypsin). Excess trypsin solution was removed and 4–10 μl of digestion solution without trypsin was then added to keep the gel pieces wet during the digestion. After an overnight incubation at 37°C, the digestion was stopped with 5% trifluoroacetic acid (TFA) for 1 h at 37°C and then by 20 μl 2.5% TFA/50% acetonitrile for 1 h at 37°C. The digestion buffer, now containing extracted peptides, was carefully removed and transferred into a clean microcentrifuge tube. All mass spectra of MALDI-TOF-MS were obtained on a Bruker REFLEX III MALDI-TOF-MS (Bruker-Franzen, Bremen, Germany) in positive ion mode at an accelerating voltage of 20 kV with the matrix of -cyano-4-hydroxy cinnamic acid. The spectra were internally calibrated using trypsin autolysis products. The obtained peptide mass fingerprint (PMF) was used to search through the NCBInr database using the Mascot search engine (http://www.matrixscience.co.uk) with a tolerance of 100 ppm and one missed cleavage site [Jin et al., 2003; Xia et al., 2005].

Peptide Sequencing by ESI-MS/MS

ESI-MS/MS experiments were performed on a Q-TOF2 hybrid quadrupole/TOF mass spectrometer (Micromass, UK) with a nanoflow Z-spray source. Peptide sequencing was performed using a palladium-coated borosilicate electrospray needle (Protana, Denmark) according to the method of Yan et al. [2000]. The mass spectrometer was operated in the positive ion mode with a source temperature at 80°C and a potential of 800–1000 V applied to the Nanospray probe. The amino acid sequences

of the peptides were deduced with the peptide-sequencing program MasSeq. The database search was finished with the Mascot search engine (http://www.matrixscience.co.uk) using the data processed through MaxEnt3 and MasSeq.

RNA Isolation and Real-Time PCR

RNA was isolated by using TRIzol (Invitrogen) as the manufacturer's protocol. Total RNA was briefly exposed to RNAase-free DNAase I and 5 μg total RNA was reverse transcribed to cDNA using Oligo(dT) and M-MuLV reverse transcriptase (Biolabs). Real-time PCR was run sequentially by using a 7500 Sequence Detector System (Applied Biosystems). Real-time PCR conditions were as follows: an initial step of 10 min at 95°C, followed by 15 s at 95°C and 1 min at 60°C for 40 cycles. The copy number for each transcript was normalized to β-actin. Fluorescence spectra were recorded during the elongation phase of each PCR cycle. The sequences of the primers used for real-time PCR are listed in Table I, and β-actin primers were from Edgar (2002). To confirm amplification specificity, the PCR products from each sample were examined by melting curve analysis. All the genes suited for the design of real-time PCR primer were evaluated, and genes having a high homology to unrelated genes were discarded after carrying out a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST).

TABLE I. Real-Time PCR Primers Used for Gene Expression Analysis

Gene	Primer sequence 5′ → 3′
β-actin	CAGAAGGAGATTACTGCTCTGGCT
	GGAGCCACCGATCCACACA
Nkx2.5	TGCAGAAGGCAGTGGAGCTGGA-CAAGCC
	TGCACTTGTAGCGACGGTTCTGG-AACCAG
MEF2C	TCTGTCTGGCTTCAACACTG
	TGGTGGTACGGTCTCTAGGA
GATA-4	AAAACGGAAGCCCAAGAACCT
	TGCTAGTGGCATTGCTGGAGT
Ybx1	TTACAGACCACGATTCCGAAGG
	TCTCTAGGCTGTCTTTGGCGAG
Cofilin1	AGTCTGGAGCCCCACCTACT
	CTCAGACTTGGGGCCAGTTA
Aldolase A	GTCCCTTCCCCCAAGTTATCA
	CCTTAATGCCCACAACACCAC
Stress-induced phosphoprotein	CAGATGTGCTCAAGAAGTGCCA
	AAGCGCTCCTGTTCCTTCAGA
Chaperonin subunit2 (beta)	AAGCCACAAAGGCAGCAAGAG
	TCAGAACCATGATCCACAGCG
WD repeat domain 58	TATTGTCTGGCGGTGAGGATG
	GGCTATACGGAGATCCCAAAGC

Statistical Analysis

Each experiment was performed at least three times. Statistical significance was determined using Student's t-test, and $P < 0.05$ was considered significant.

RESULTS

DMSO Stimulates P19CL6 Cells to Differentiate Efficiently into Cardiomyocytes

P19CL6 cells were treated with DMSO-containing or DMSO-free media for 12 days. The phenotype of differentiated P19CL6 cells was verified by immunofluorescence staining and real-time PCR analysis (Fig. 1A,B). The expression of sarcomeric MHC was detected only in differentiated P19CL6 cells using the MF20 antibody. The immunofluorescence results showed that P19CL6 cells differentiated efficiently into cardiomyocytes (Fig. 1A). The differentiation of P19CL6 cells into cardiomyocytes was characterized by detecting the expression of cardiac-specific transcription factors Nkx-2.5, GATA-4, and MEF2C. The real-time PCR results demonstrated that all of the three cardiac-specific transcription factors were significantly increased after treatment with DMSO (Fig. 1B). Our results showed that P19CL6 cells successfully differentiate into cardiomyocytes after treatment with 1% DMSO for 12 days.

Two-Dimensional Gel Analysis

Protein analysis of P19CL6 cells not treated or treated with 1% DMSO was performed using 2-DE coupled to MS to identify differentially expressed proteins. Representative 2-DE gel spot patterns are shown in Figure 2. The pH range for the first dimension was pH 3–10 NL and the separation range for the second dimension SDS–polyacrylamide gel was 10–100 kDa. After spot detection, background subtraction, and volume normalization, we reproducibly detected over $2{,}183 \pm 21$ spots/gel after silver staining. Overall, around 17 protein spots, which were found consistently up- or down-regulated by at least 1.5-fold in triplicate experiments after DMSO treatment for 12 days, with 14 spots were increased and 3 spots decreased in intensity. The location of each spot was labeled with a number and arrow (Fig. 2A,B). Figure 2C shows magnified comparison maps of labeled spots in 2-DE patterns

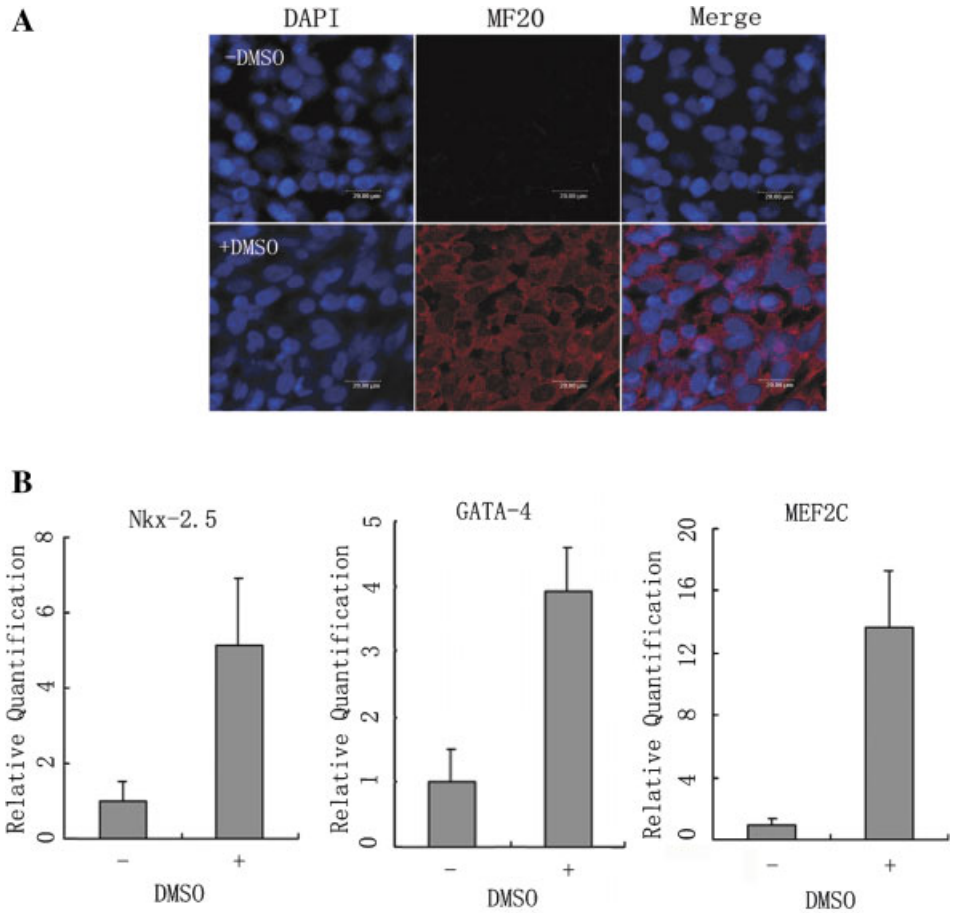

Fig. 1. DMSO stimulates P19CL6 cells to efficiently differentiate into cardiac myocytes. **A**: Immunostaining for sarcomeric MHCs in cultures treated (bottom) or not treated (top) with DMSO for 12 days. Cells were stained with an anti-MF20 antibody (red). Nuclei were counterstained with DAPI (blue). (Bar = 20 μm). **B**: Gene expression of three cardiac-specific transcription factors (CSX/Nkx-2.5,GATA-4, and MEF2C) before or after DMSO treatment were determined by real-time PCR, normalized to β-actin, and represented as relative copy number. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

between uninduced P19CL6 cells and DMSO-induced P19CL6 cells. The distinct expression patterns of these 17 spots imply that they have different functions during the cardiac differentiation of P19CL6 cells. Thus, the 17 spots selected were subjected to in-gel trypsin digestion and then analyzed by MALDI-TOF-MS and/or ESI-MS/MS.

Identification of Differentially Expressed Proteins by MALDI-TOF MS and Clustering of Identified Proteins

All of the 17 differentially expressed protein spots were taken from the Coomassie Blue G-250-stained gels and subjected to in-gel trypsin digestion and then analyzed by MALDI-TOF-MS. The reliability of the results identified by PMF was evaluated by MOWSE values and sequence coverage. However, no reliable results were obtained for spots 3 and 13. To confirm these two proteins, ESI-MS/MS was used to sequence peptides chosen from the corresponding PMF, and only spot 3 was identified successfully as Ybx1 (Y box binding protein 1) protein (Fig. 3A,B). Besides spot 3, another 16 protein spots were clearly identified and are listed in Table II, which include 12 proteins. A database search and functional exploration of these differentially expressed proteins showed that these proteins have different cellular functions (Table II) including metabolism, signal transduction, transcription, cellular organization, and protein synthesis.

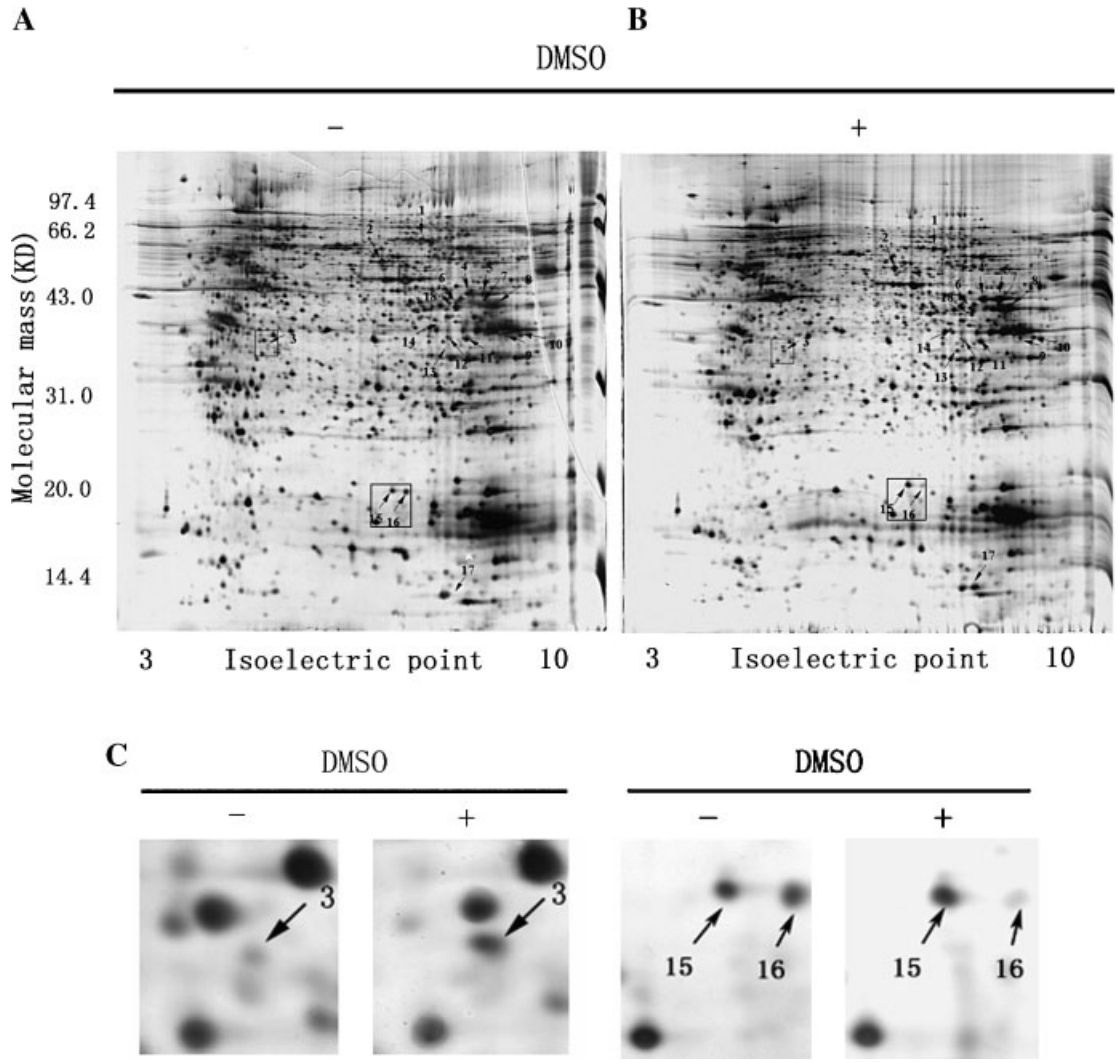

Fig. 2. Comparison of 2-DE maps between P19CL6 cells not treated (**A**) or treated (**B**) with 1% DMSO for 12 days. Differentially expressed proteins are marked with black arrows. Proteins from whole-cell extracts of P19CL6 cells were separated on a pH 3–10 NL IPG strip, followed by SDS–PAGE on a 13% gel. Proteins were visualized by silver staining. **C**: Close up sections showing boxed areas in figure A and B.

Validation of Differentially Expressed Proteins by Real-time PCR Analysis

Among the identified 12 proteins, 6 of them were selected for real-time PCR analysis. The upregulation of cofilin1, Ybx1, and WD repeat domain 58 agreed with the 2-DE and silver stain results ($P < 0.05$). The proteins chaperonin subunit 2 (beta) and stress-induced phosphoprotein 1 were upregulated at the protein level, whereas they showed no significant change at the mRNA level (Fig. 4). There was a discrepancy between changes in mRNA level and protein spot intensity for aldolase 1 (A isoform).

DISCUSSION

P19CL6 cells can be used as an excellent model for studying cardiac differentiation in vitro. In the presence of 1% DMSO, they can efficiently differentiate into cardiomyocytes that exhibit the biological features recapturing embryonic cardiogenesis in vivo [Monzen et al., 1999]. Proteomic analysis can provide us a panoramic view of changes in protein expression and represent as the first step to elucidate the molecular mechanism of cardiomyocytes differentiation. We selected P19CL6 cells as a model to investigate the global changes in

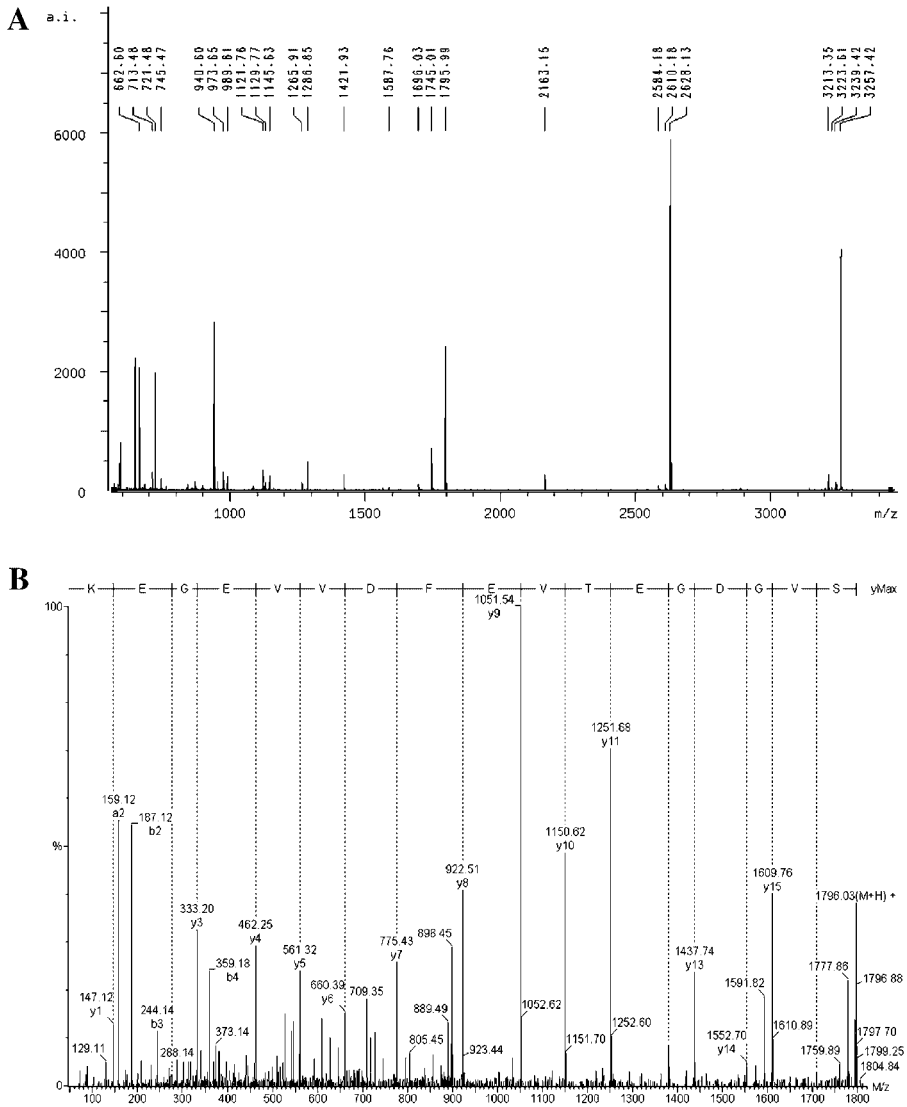

Fig. 3. Identification of the differentially expressed protein Ybx1 by 2-DE gels with MALDI-TOF MS PMF and nano-ESI–MS/MS. **A**: PMF identification of Ybx1 was shown. After database searching, the protein score is significant ($P < 0.05$). **B**: The 1795.99 peptide was chosen from the PMF and sequenced by nano-ESI–MS/MS. The deduced sequence is indicated.

protein expression between the control and differentiated P19CL6 cells using proteomic approaches. In this study, we identified a total of 16 protein spots that were consistently modulated in response to DMSO treatment. These identified proteins could be categorized into several classes according to their functions, with most belonging to the following categories: cellular metabolism, signal transduction, cellular organization, and heat shock protein/chaperones (Table II). Among the identified proteins, some proteins interested may play certain roles in maintaining the cardiac state of P19CL6 cells.

Aldolase 1 (A isoform), L-lactate dehydrogenase A chain (LDH-A) and phosphoglycerate kinase 1 are important glycolytic enzymes [Garfinkel and Garfinkel, 1985], and their expression was upregulated in the differentiated P19CL6 cells. Aldolase A is involved in fructose metabolism and catalyzes the reversible conversion of fructose-1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. It has been reported that aldolase 1 (A isoform) was expressed in cardiomyocytes and that its expression was upregulated in rectus abdominus muscle of obese women relative to lean controls [Mamczur and

TABLE II. Differentially Expressed Proteins Identified by MALDI-TOF MS

Spot no.	Protein name	NCBI accession number	Functional clustering	MW(kDa)	pI	Sequence coverage %	Score	Protein expression
1	Stress-induced phosphoprotein 1	13277819	Chaperon	63.2	6.40	44	110	↓
2	Chaperonin subunit 2 (beta)	13938629	Chaperon	57.8	5.97	62	270	↓
3	Ybx1 protein	29437175	Transcription	35.7	9.87	56	243	↑
4	Phosphoglycerate kinase 1	80477474	Energy metabolism	44.9	8.02	63	166	↑
5	Phosphoglycerate kinase 1	80477474	Energy metabolism	44.9	8.02	50	173	↑
6	Aspartate aminotransferase	871422	Energy metabolism	46.5	6.68	44	125	↑
7	Aldolase 1, A isoform	27695278	Energy metabolism	39.8	8.31	66	239	↑
8	Aldolase 1, A isoform	27695278	Energy metabolism	39.8	8.31	68	246	↑
9	Similar to heterogeneous nuclear ribonucleoprotein A3 isoform 1	82919177	Transcription	32.1	8.2	45	101	↑
10	Similar to heterogeneous nuclear ribonucleoprotein A3 isoform 3	82933226	Transcription	30.9	7.05	50	130	↑
11	L-lactate dehydrogenase A chain(LDH-A) (LDH muscle subunit)	535924	Energy metabolism	36.8	7.62	33	160	↑
12	LDH-A	535924	Energy metabolism	36.8	7.62	31	92	↑
13	Unidentified	—	—	34.7	8.76	—	—	↑
14	WD repeat domain 58	58477297	Signal transduction	37.9	6.67	24	90	↑
15	Cofilin 1, nonmuscle	37194891	Cellular organization	18.8	8.22	42	79	↑
16	dUTPase	8347092	—	17.6	5.74	37	67	↓
17	Macrophage migration inhibitory factor	56270610	Cell proliferation	12.7	6.79	29	63	↑

↑ means the protein level increased in response to treatment with 1% DMSO treatment; ↓ means the protein level decreased in response to treatment with 1% DMSO.
Spot no. was defined according to spot positions in the 2-DE gel as indicated in Figure 2.
Protein description: the name of each identified protein in the NCBInr database.
pI: theoretical isoelectric point of the matched protein, mass: theoretical molecular weight of the matched protein in kDa.
Expression level of protein: the relatively quantitative alterations of proteins were determined based on the average volume of protein.
All the information about function comes from the SWISS-PROT, NCBI database.

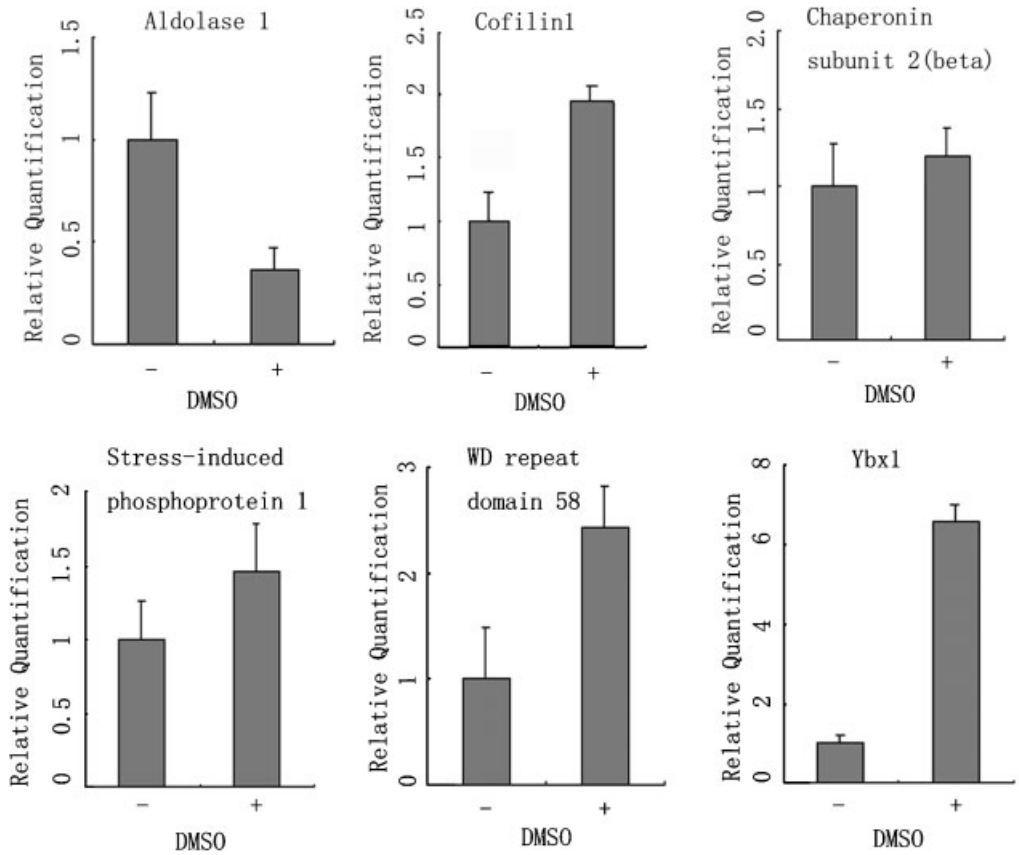

Fig. 4. Validation of six differentially expressed proteins in 2-DE gels by real-time RT-PCR analysis in P19CL6 cells not treated or treated with 1% DMSO for 12 days. Total cellular RNA was isolated and subjected to real-time RT-PCR analysis using gene-specific primers, normalized to β-actin. Fold expression levels are relative to that of genes.

Dzugaj, 2004; Hittel et al., 2005]. Deficiency of aldolase 1 was associated with predominantly myopathic symptoms, including muscle weakness and premature muscle fatigue [Kreuder et al., 1996]. In the rat, muscle-specific MEF-2 is involved in the muscle-specific induction of the aldolase A gene [Hidaka et al., 1993], and this report lends further credence to the fact that MEF2C expression is increased in our study. LDH-A catalyzes the reversible NAD-dependent interconversion of pyruvate to L-lactate. The significance of the increased level of LDH-A in response to DMSO treatment needs to be clarified. Phosphoglycerate kinase 1 is involved in ATP generation in the glycolytic pathway and plays a structural role through its association with microtubules [Acevedo et al., 2001]. Previous proteomic analysis showed that phosphoglycerate kinase 1 was also upregulated upon retinoic acid (RA) induction in P19 cells [An et al., 2005]. Therefore, the increase in phosphoglycerate kinase 1 may be necessary for differentiation during both RA-induced neural differentiation of P19 cells and DMSO-induced cardiac differentiation of P19CL6 cells. Combining our observations and those of others, we infer that glycolytic enzymes may be deeply involved in cardiac metabolism and play important roles in cardiomyocyte differentiation.

On 2-DE gels, aldolase 1 (A isoform), LDH-A, and phosphoglycerate kinase 1 were presented as multiple protein spots. This heterogeneity may be the result of similar isoforms, post-translational modifications such as phosphorylation, glycosylation, or proteolytic cleavage [Casey et al., 2005]. A quick check in Swiss-Prot (http://www.expasy.org/uniprot) indicates that LDH-A is N-terminally acetylated and has a predicted phosphotyrosine site. Aldolase 1 (A isoform) and phosphoglycerate kinase 1 also contain several phosphoserine or phosphotyrosine sites. In addition, our results showed that the levels of aspartate aminotransferase also increased. This enzyme is involved in the

tricarboxylic acid cycle [Perluigi et al., 2005], and its increase may imply some involvement of energy metabolism during cardiomyocytes differentiation.

Ybx1 functions in various biological processes, including transcriptional and translational control, DNA repair, drug resistance, and cell proliferation [Swamynathan et al., 2002; Shibahara et al., 2004]. Ybx1 is also required for normal late embryonic development and viability and optimal cellular stress [Lu et al., 2005]. Although Ybx1 was widely expressed, it was preferentially expressed in cardiac muscle. CARP is a cardiac ankyrin repeat protein, which is regulated by Nkx-2.5 at the transcriptional level, and the interaction of Ybx1 with CARP has been implicated in the function of Ybx1 in the control of the cardiac muscle gene programs [Zou et al., 1997]. Consistent with their result, we found that Ybx1 was highly abundant in differentiated P19CL6 cells, which suggests its involvement in the control of expression of cardiac-related proteins. Ybx1 may also play some other unknown roles during cardiomyogenesis.

The protein cofilin 1 is involved in cellular organization. It has two isoforms: A muscle-type and a nonmuscle-type. Cofilin 1 (nonmuscle) was found upregulated in P19CL6 cells after DMSO treatment. It has been reported that nonmuscle cofilin 1 was also expressed in cardiac muscles and is involved in the regulation of actin assembly during myofibrillogenesis [Mohri et al., 2000]. Its particular role in cardiomyogenesis needs further investigation.

Some known DMSO targets, such as Nkx-2.5, GATA-4, and MEF2C, which have been reported to be regulated during the cardiomyocytes differentiation of P19CL6 cells in previous studies, are absent in our list of upregulated proteins. This was partly due to the low abundance of these transcription factors. The specific characteristics of these proteins would also contribute to the inability to detect them by the 2-DE system. A quick search of Swiss-Prot indicates that Nkx-2.5 and GATA-4 have pIs > 9 and the pI of MEF2C is >8. In addition, the missing of these valuable information undoubtedly arose at least in part from the technical limitations of current methods of sample preparation and 2-DE, such as the loss of acidic and basic proteins that fall outside of the pI range of the IEF step, the loss of poorly soluble proteins as well as poor coverage of low abundance proteins [Xia et al., 2005]. In this case, we separated protein MEF2C by SDS–PAGE and performed Western blot to indicate that this protein has been upregulated (data not shown).

The real-time PCR results revealed that of the six differentially expressed proteins identified by proteomic analysis, three showed similar changes at the mRNA and protein levels. However, there were no obvious changes at the mRNA level for chaperonin subunit 2 (beta) and protein stress-induced phosphoprotein 1 after DMSO treatment, although their expression decreased at the protein level. There was also a discrepancy between changes in mRNA and protein spot intensity for aldolase 1 (A isoform). Several studies in which both mRNA and protein have been compared in the same sample have also shown that there is not a good correlation between mRNA and protein levels [Anderson and Anderson, 1998; Gygi et al., 1999]. The discrepancy between mRNA and protein levels may indicate the importance of post-transcriptional and post-translational processes during cell differentiation [Juan et al., 2002; Lian et al., 2002], including post-translational protein modification and selective degradation of proteins [Lian et al., 2001].

Protein WD repeat domain 58, dUTPase, and protein macrophage migration inhibitory factor have not been previously reported to be correlated with cardiomyocytes differentiation. WD repeat domain 40 has been associated with adaptor/regulatory modules in signal transduction, pre-mRNA processing, and cytoskeleton assembly [Smith et al., 1999]. dUTPase, which has been reported to be involved in nucleotide metabolism, was downregulated in our study .The enzyme hydrolyzes dUTP to dUMP and pyrophosphate, decreasing the intracellular concentration of dUTP so that uracil cannot be incorporated into DNA. The revelation of these differentially expressed proteins will allow further studies to focus on these proteins and their roles in the differentiation of P19CL6 cells to cardiomyocytes.

In conclusion, using 2-DE, we made a first attempt to clarify changes in protein abundance after the DMSO-induced differentiation of P19CL6 cells to cardiomyocytes. Proteins with different expression patterns may be involved in the DMSO-induced cardiac differentiation of P19CL6 cells through different mechanisms. The identification of these proteins provides some novel insights into the mechanism of

cardiomyogenesis, and further investigation is needed to characterize the functions of these differentially expressed proteins in detail.

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