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Comparative Proteomic Analysis of Differentiation of Mouse F9 Embryonic Carcinoma Cells Induced by Retinoic Acid

Rui-Qing Jiao,[1] Guanwu Li,[2] and Jen-Fu Chiu[2,3]*

[1]*The State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing 210093, China*

[2]*Department of Biochemistry, Open Laboratory for Tumor Molecular Biology, Shantou University Medical College, Shantou 515041, China*

[3]*LKS Faculty of Medicine, Department of Anatomy, University of Hong Kong, Pokfulam, Hong Kong, SAR, China*

ABSTRACT

The multipotent mouse F9 embryonic carcinoma cell is an ideal model system to investigate the mechanism of retinoic acid (RA) in cell differentiation and cell growth control and the biochemical basis of early embryonic development. We reported here a proteomics approach to study protein expression changes during the differentiation of F9 cells into the visceral endoderm. F9 cells were incubated with or without RA at 0, 24, 48, and 72 h. Total proteins extracted were separated by two-dimensional electrophoresis (2-DE) and the protein patterns on the gels were comparatively analyzed by computer. Approximately 1,100 protein spots were detected in the F9 proteome, within the pH 3–10 range. Fourteen protein spots which the levels of expression were found to be altered dramatically during the F9 cells differentiating, and were identified by MALDI-TOF MS or ESI-MS/MS. These proteins included metabolism enzymes, HSP60s, RAN, hnRNP K, FUBP1, VDAC1, STI1, and prohibitin. These proteins are involved in cellar metabolism, gene expression regulation, stress response, and apoptosis, respectively. The data from proteomic analyze are consistent with the result obtained from Western blot analysis. This study increases our understanding of the proteomics changes during F9 cells differentiation induced by RA. J. Cell. Biochem. 113: 1811–1819, 2012.

KEY WORDS: F9 CELL; DIFFERENTIATION; PROTEOME ANALYSIS; TWO-DIMENSIONAL GEL ELECTROPHORESIS; MASS SPECTROMETRY

Retinoic acid (RA) is a member of the most biologically active forms of retinoid. In adult tissues, RA functions to maintain cell differentiation and is required for organ regeneration [McCaffery and Dräger, 2000]. In tissue culture, RA induces cell differentiation and suppresses cell growth in a wide spectrum of cell lines. RA have been successfully used for the chemoprevention and chemotherapy of various types of cancer, such as promyelocytic leukemia, breast, lung, head and neck, renal, and prostate cells [Sun and Lotan, 2002]. The mechanisms underlying the RA anti-cancer effects appear to be associated with its ability to prevent angiogenesis, modulate the growth, differentiation, and apoptosis [Vivaldi et al., 2009]. These actions are mediated by nuclear retinoid receptors (RARs and RXRs), and include pathways and proteins involved mainly in FOS, MAPK, PI3 kinase, AKT, cyclins, cyclin-dependent kinases and their inhibitors, bcl proteins, and caspase signaling [Dragnev et al., 2003].

The mouse F9 embryonic carcinoma cell is an ideal model system to investigate the mechanism of RA in cell differentiation and cell growth control and the biochemical basis of early embryonic development [Gajović et al., 1998]. When treatment with RA, F9 cells differentiate into primitive endoderm in monolayer cultures or into proximal (visceral) endoderm cells in aggregation culture. There are many species of proteins and mRNAs, such as alpha-fetoprotein (AFP), GATA-4, GATA-6, and Dab2, have been confirmed to take dramatic changes in their expression levels during the F9 cells differentiation [Morrisey et al., 2000]. However, most research focused on studying one or a few genes involved in the differentiation. The execution of this program is characterized by a specific spatial temporal expression profile of genes that control cell identity and function. To study this sophisticated process, it would be better to constitute a method that could comprehensively analyze the globally differential expression of genes in different

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*Correspondence to: Prof. Dr. Jen-Fu Chiu, Department of Biochemistry, Open Laboratory for Tumor Molecular Biology, Shantou University Medical College, Shantou 515041, China. E-mail: jfchiu@hkucc.hku.hk

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differentiation stages, in different tissues, or between normal and pathologic conditions. Traditional gene analysis, such as gene knockout, could only study one or a few genes once, and is costly and time-consuming. Because of this, the proteomics offers a promising alternative way for its capability of resolving many hundreds of proteins once.

In the present study, a proteomics approach is applied to understand biochemical alterations involved in F9 cells differentiation induced by RA. We were able to resolve more than 1,100 protein spots each in both treated and untreated F9 cells and have identified 14 differentially expressed protein spots as a result of RA treatment. These proteins were not been previously reported to be associated with differentiation of F9. This study used comparative proteomic analysis to investigate the protein profile of F9 cells during differentiation induced by RA.

MATERIALS AND METHODS

CELL CULTURE AND TREATMENT OF CELLS

Undifferentiated F9 cells were purchase from ATCC and maintained by culturing on tissue culture dish in α-MEM, supplemented with 10% fetal bovine serum and 1 mM glutamine. For experiments involving cell differentiation into visceral endoderm, F9 cells were seeded at 1×10^6 cells/190-mm bacteriological petri dish in a 1:1 mixture of Dulbecco's modifed Eagle Medium (DMEM) and Ham's F12 medium, supplemented with 10% fetal bovine serum and 1 mM glutamine. On the day after seeding, cells were treated with 50 nM all-trans RA. Cell aggregates were refed daily with the appropriate medium and RA concentration. Undifferentiated F9 cells were grown as in the same medium except that no RA was added. Mycoplamta testing was routinely utilized.

RNA ISOLATION AND RT-PCR

Trizol Reagent (GibcoBRL, Life Technologies) was used to isolate total RNA from frozen tissue samples according to protocol provided by supplier. Reverse transcription was performed using 3 mg of extracted total RNA mixed with reaction mixture in a final concentration of 0.5 mM dNTPs, 0.025 mg/ml oligo dT, 5 mM $MgCl_2$, 10 mM DTT, 2 U/ml RNaseOUTTM inhibitor, and 2.5 U/ml SSII RT. The reaction was achieved by incubating each sample at 65°C for 5 min, placing on ice for 1 min, incubating at 42°C for 2 min, mixing with SSII RT followed by incubating at 42°C for 1 h, and incubating at 70°C for 15 min to stop reaction. Following RT, PCR was carried out in a reaction volume of 25 μl with final concentration of 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.05 Taq DNApolymerase, and primer pair for AFP. Beta-actin was used for internal normalization. The primers used for AFP and beta-actin are listed as follows. The reaction was initiated at 95°C for 5 min followed by 30 cycles at 94°C for 45 s, 58°C for 50 s, and 72°C for 1 min, and final extension at 72°C for 10 min. AFP sense primer 5′-ACCTCAGCAGAGCT GATCGA-3′, antisense primer 5′-TTGCAGCAGTGGCTGATACCA-3′; beta-actin sense primer, 5′-GTGGGGCGCCCCAGGCACCA-3′, antisense primer 5′-CTCCTTAATGTCACGCACGATTTC-3′.

PROTEIN EXTRACTION

After treatment with RA for 24, 48, and 72 h, the F9 cells were washed with ice-cold washing buffer (10 mM Tris–HCl, 250 mM sucrose, pH 7.0). The cells were scraped in the buffer and spun down at 2,000 rpm for 5 min. After two washes with 1 ml of washing buffer, the cell pellet was lysed in 50 μl of lysis buffer (8 M urea, 4% CHAPS, 2% IPG buffer, 0.2 mg/ml PMSF) and centrifuged at 13,500 rpm for 10 min at 4°C. The supernatant were used directly for 2-DE analysis.

TWO-DIMENSIONAL GEL ELECTROPHORESIS

2-DE was performed with IPGphor IEF and electrophoresis units (GE Healthcare). Whole cell protein lysates (130 μg) were mixed with rehydration solution (8 M urea, 4% CHAPS, 1 mM PMSF, 20 mM DTT, and 0.5% IPG buffer) to a final volume of 250 μl. The precasted 13 cm IPG strips were rehydrated for 10 h at 30 V. IEF conditions were 500 and 1,000 V, 1 h each; 8,000 V to a total of 64 kVh. After IEF, strips were subjected to a two-step equilibration in buffer (6 M urea, 30% glycerol, 2% SDS, and 50 mM Tris–HCl, pH 6.8) with 1% DTT for the first step and 2.5% iodoacetamide for the second step. For SDS–PAGE, strips were transferred onto 1.5-mm thick 12.5% polyacrylamide gels at room temperature. All gels were visualized using silver staining. Stained gels were scanned with the Image Scanner and analyzed with ImageMaster 2D Elite software (GE Healthcare). Data were normalized, expressed as percentages of all valid spots to accounting for differences in protein loading and staining. Normalized volume differences were statistically calculated for all the 2D gels. Spots differed by >2-fold in silver stained gels was considered significant alternations. These spots were excised and analyzed by MALDI-TOF-MS or MS-MS.

TRYPTIC IN-GEL DIGESTION

Gel chips were destained in a 1:1 solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate and equilibrated in 50 mM ammonium bicarbonate to pH 8.0. After dehydrating with acetonitrile and drying in a SpeedVac, the gels were rehydrated in a minimal volume of trypsin solution (10 μg/ml in 25 mM ammonium bicarboate) and incubated at 37°C overnight. The supernatant was directly applied onto the sample plate with equal amounts of matrix.

MALDI-TOF/TOF MS AND MS/MS ANALYSIS

Mass spectra were recorded on an Applied Biosystems 4700 Proteomics Analyzer (Framingham, MA). Instrument setting was reflector mode with 20 kV accelerating voltage. Laser shots at 5,000 per spectrum were used to acquire the spectra with mass range from 600 to 3,000 Da. The peptides of interest were further analyzed by MS/MS, using an energy adjustable collision cell filled with pure argon. MS and MS/MS spectra from the ABI 4700 Proteomics Analyzer were processed by using the 4700 Explorer software. MASCOT was used in database searching for protein identification by incorporating MS and MS/MS data in the NCBI database. The search was restricted to one missed cleavage site, 50 ppm mass error tolerance for precursor ions, and 0.1 Da for MS/MS fragments. Duplicate or triplicate runs were made to ensure the accuracy of the analysis.

WESTERN BLOTTING

With reference to the verification of candidate proteins after peptide fingerprinting, the proteins of interest were selected for Western blotting to confirm the results of protein database searching. After SDS–PAGE, proteins were transferred onto PVDF membranes (Amersham Biosciences) at 0.8 mA/cm^2 for 1 h. After blocking in 5% non-fat milk in TBS-T containing 0.1% Tween 20 (Sigma) at 4°C overnight with gentle rocking, membranes were probed with antibodies. Primary antibodies involved in this study include heterogenous nuclear ribonucleoprotein K (hnRNP K; Santa Cruz Biotechnology), aldolase (Cell Signaling), prohibitin (Santa Cruz Biotechnology), and beta-actin (Sigma). Membranes were incubated with corresponding primary antibody for various durations according to the specificity and sensitivity of antibody. After incubation with corresponding secondary antibodies, immunoblots were visualized with the ECL detection kit (Amersham Biosciences). For reprobing membranes with another antibody, the membranes were stripped with stripping buffer (glycine 3.75 g/L, SDS 2 g/L, pH 2.0) before blocking.

STATISTICAL ANALYSIS

Statistical analysis was done using a two-tailed Student's *t*-test, and $P < 0.05$ was considered significant. Data were expressed as mean ± SD of triplicate samples, and reproducibility was confirmed in at least two independent experiments.

RESULTS

DIFFERENTIATION OF F9 CELLS

Using standard methods for inducing F9 cells differentiation, the cells were grown in suspension in bacteriological Petri dishes in medium containing 50 nM RA. As show in Figure 1, the cell aggregates to form embryonic bodies with a ring of outer cells which resemble visceral endoderm cells. After induction with RA for 24 h, these outerlayers of the embryonic bodies become rough while F9 cell aggregates grown in suspension under the same condition without RA treatment have a smooth surface. This morphologic change of F9 cell aggregates indicates that RA induces F9 cells differentiation.

To further confirm F9 cells differentiation, we detected expression of endoderm maker AFP at the different time points of treatment with or without RA by RT-PCR. As shown in Figure 1B, AFP mRNA began to be expressed at 48 h and obvious increasing at 72 h in RA-treated F9 cells; in contrast, there was no AFP mRNA detectable in non-treated F9 cells. These results shown that F9 cells differentiated into visceral endoderm cells in aggregation culture treated with RA.

COMPARISON OF PROTEOMIC PATTERNS OF F9 TOTAL PROTEINS TREATED WITH OR WITHOUT RA

For the purpose of determining changes in protein levels during F9 cells differentiation, we extracted the total proteins and compared the proteomic components of different time points. Figure 2 shows

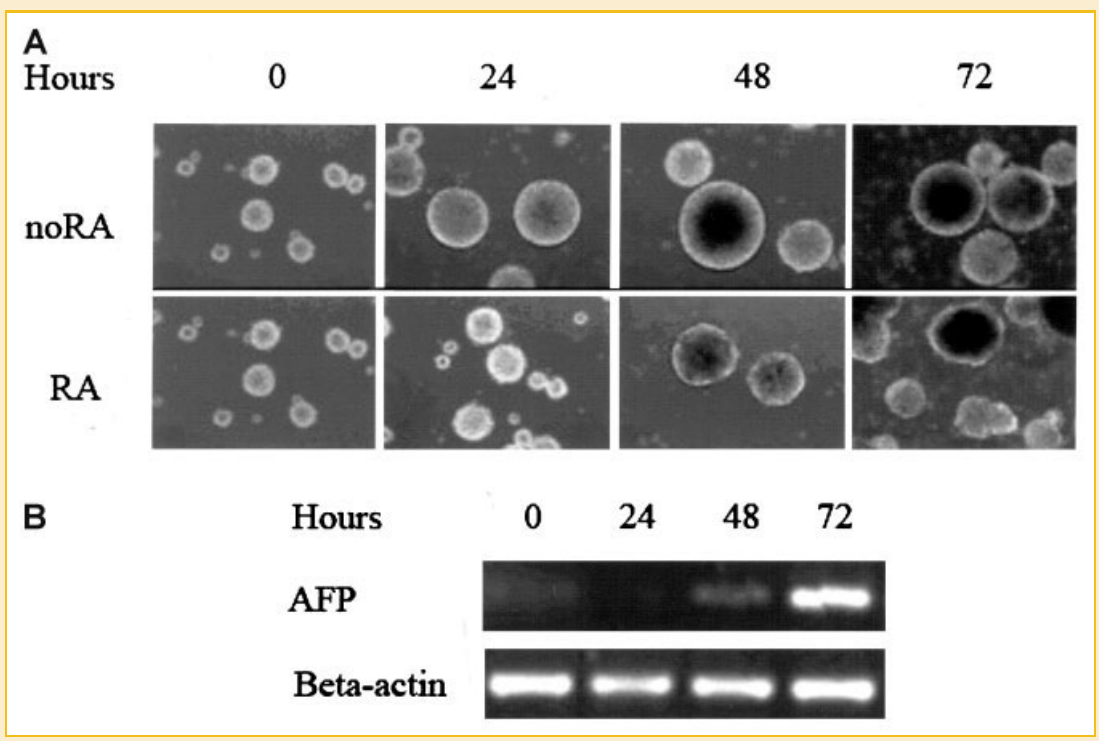

Fig. 1. Differentiation of F9 cells induced by AR morphology of F9 cells treated with RA (A). F9 cells were grown in suspension in the absence (noRA) or presence of 50 nM retinoic acid (RA) for 0, 24, 48, and 72 h. The aggregate were photographed under a microscope at ×200 magnification. B: RT-PCR results for F9 cells differentiation induced by AR, showing that altering of the AFP mRNA level at 0, 24, 48, and 72 h in RA-treated F9 cells.

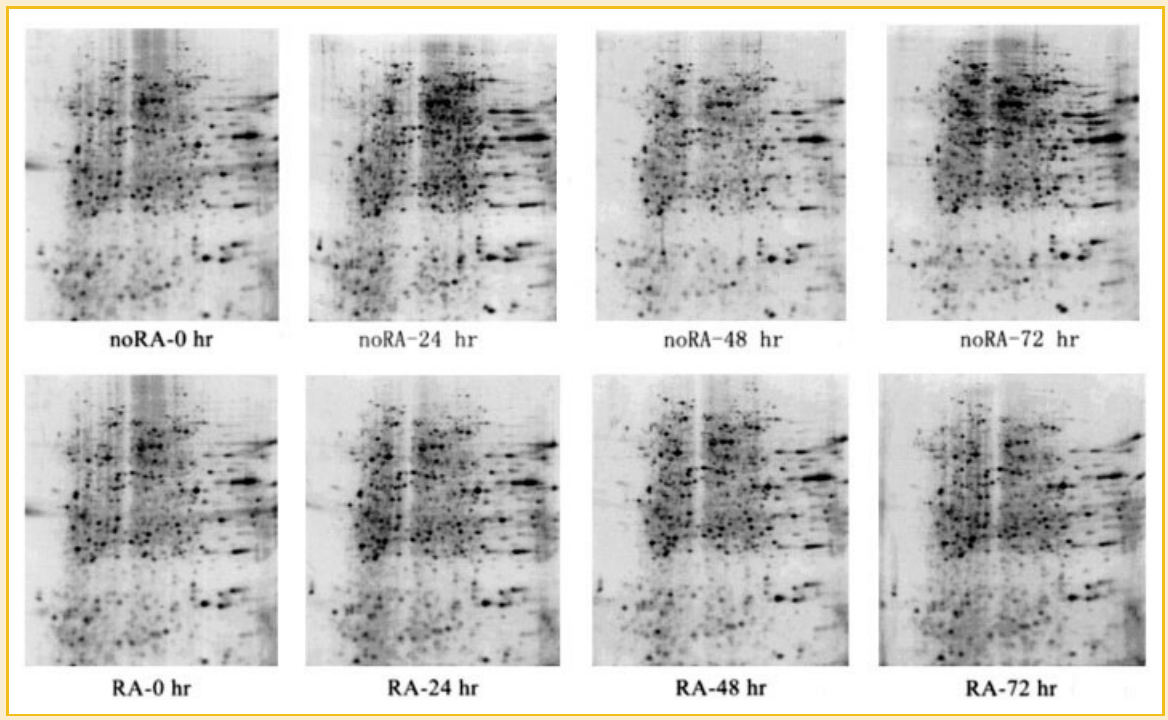

Fig. 2. SDS–PAGE gels of F9 cells silver-stained two-dimensional SDS–PAGE gels of F9 cells at 0, 24, 48, and 72 h after the cells were induced with or without RA. Eight independent batches (130 μg) were run on a 12.5% two-dimensional gel (pH 3–10) as described under "Materials and Methods Section".

analytical colloidal silver-stained 2-DE standard maps of F9 cells at 0, 24, 48, and 72 h after the cells were induced with or without RA. More than 1,100 spots unambiguously displayed on the 2-D gels according to the image analysis using software ImageMaster2-D Elite. We found that most of the spots correlated well between 2-DE maps of RA-treated and non-treated F9 cells. Two-tailed Student's *t*-test was utilized to select the protein spots that showed significantly and consistently differences in expression through the comparative analysis in ImageMaster. Fourteen protein spots were revealed to have differential expressions between RA-treated and non-treated F9 cells with *P*-values <0.05 (Table I). Among these protein spots, 3 spots (spot 95, 94, and 123) were significantly down-regulated at the all time points, 7 spots (spots 505, 1043, 667, 123, 286, 807, and 68) were up-regulated at 24 h and then down-regulated at 48 and 72 h, 2 spots (spots 666 and 110) were up-regulated at 24 and 48 h and then down-regulated at 72 h, 1 spot (spot 555) was down-regulated at 24 h and then up-regulated at 48 and 72 h, and 1 (spot 315) was down-regulated at 24 and 48 h and then up-regulated at 72 h in RA-treated F9 cells. The numbers denoted on the 2-DE maps in Figure 3 represent these protein spots.

TABLE I. Results of the Identified Protein Spots

			Method of identification					
			MALDI-TOF-MS			ESI-MS-MS		
Spot	NCBI number	Protein name	Score	Sequence cover (%)	Tol ppm	Score	Expect	MW/pI
286	31982178	Malate dehydrogenase, cytosolic (cMDE)	151	17	21.7			36.5/5.9
778	6671539	Aldolase 1 A isform (aldolase 1)				66	0.13	39.8/7.1
95	15214215	Bifunction purine biosynthesis protein (purH)	1,185	15	39			64.2/6.3
123	31559887	mTHF-dehydrogenase	127	21	29.6			87.7/7.9
315	7948999	Peroxiredoxid 4 (Prx4)				52	0.2	31.3/6.7
667	13384620	Heterogenous nuclear ribonucleoprotein K (hnRNP K)				90	0.00061	51.2/5.4
505	6678365	RAN, member RAS oncogene family (Ran)	3,804	22	11.5			24.4/7.0
807	16975504	Far upstream element binding protein 1 (FUBP1)	3.883e+04	26	19.1			68.5/7.7
1043	72957	Chaperonin groEL precursor (groEL)				57	7.70E−05	61.1/5.9
68	6671702	Chaperonin subunit 5 (epsilon) (Chape sub5)				54	0.01	60.0/5.7
110	91330	T complex polypeptide 1 b (TCP-1)				97	0.00032	57.9/6.1
94	13277819	Stress-induced phosphoprotein 1 (STI1)	68.1	31	35			62.6/6.4
555	6679299	Prohibitin	115	24	27.8			29.8/5.6
666	6755963	Voltage-dependent anion channel 1 (VDAC1)	4,058	33	25.2			30.8/8.6

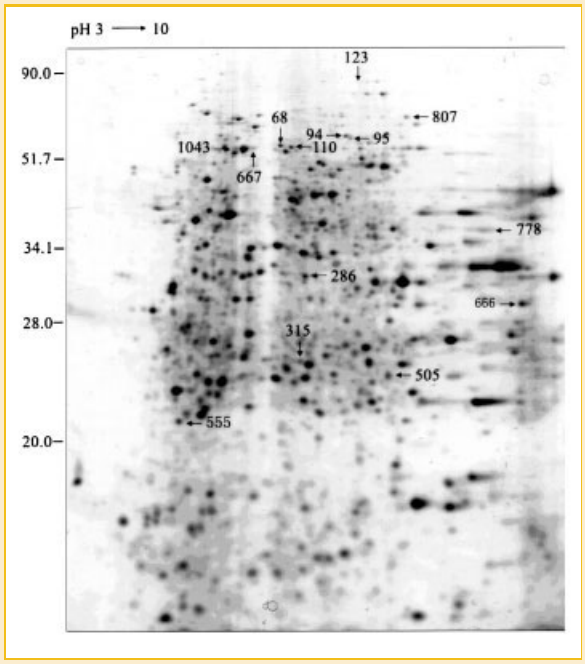

Fig. 3. Two-dimensional SDS–PAGE gel of F9 cells F9 proteins (130 μg) were separated by isoelectric focusing on a linear immobilized pH gradient from 3 to 10 and two-dimensional electrophoresis on a 12.5% gel as described under "Materials and Methods Section." Identified spots are marked by their number. Molecular mass markers are indicated on the left (kDa).

IDENTIFICATION OF PROTEINS ASSOCIATED WITH DIFFERENTIATION OF F9 CELLS BY MALDI-TOF MS OR MS-MS

Protein spots with statistically consistent and significant differences in protein expression were excised, subjected to in-gel tryptic digestion, MALDI-TOF mass spectral measurements, and PMF to obtain protein IDs. Table II lists the identified protein IDs, together with corresponding spot numbers, molecular weights and pI values, sequence coverage, total mass errors, and MOWSE scores. For most of the protein database matching, reasonable sequence coverage, low mass errors, and high MOWSE scores were obtained. These proteins can be classified into several categories based on their functions, including metabolism enzymes, gene expression regulation proteins, chaperoning proteins, and tumor suppressor proteins.

PROTEIN ALTERATIONS DURING F9 CELLS DIFFERENTIATION

As shown in Figure 4 and Table I, diverse protein alterations were first identified in RA-treated F9 cells compare to the non-treated cells at the same time points. Among the five identified enzymes, spots 95 (bifunctional purine biosynthesis protein, purH) and 778 (aldolase 1) were significantly down-regulated in RA-treated F9 cells at the all time points, spots 123 (methylene-THF dehydrogenase, mTHF-dehydrogenase) and 286 (malate dehydrogenase cytosolic, cMDH) increased expression at 24 h and then decreased expression at 48 and 72 h, while spot 315 (peroxiredoxin 4, Prx 4) down-regulated at 24 and 48 and then up-regulated at 72 h. We found three proteins, spot 505 (RAN, member RAS oncogene family), spot 667 (heterogenous nuclear ribonucleoprotein K, hnRNP K), and spot 807 (far upstream element binding protein 1, FUBP1) are related to gene expression regulation. All these proteins up-regulated at 24 h and then decreased at 48 and 72 h in differentiating F9 cells induced by RA. Another observation corresponding to the F9 cells differentiation is the alterations of chaperone protein, including spot 94 (stress-induced phosphoprotein 1, STI1), spot 1043 (chaperonin groEL precursor, groEL), spot 68 [chaperonin subunit 5 (epsilon)], and spot 110 (T complex polypeptide 1 b, TCP-1). STI1 is a HSP70/90-related protein and other three proteins belong to the numbers of HSP60 family. In RA-treated F9 cells, down-regulation of STI1 was evident from 24 to 72 h, expression of groEL and Chaperonin subunit 5 increased at 24 h and increase were found from 48 to 72 h, while TCP-1 up-regulated from 24 to 48 h and down-regulated at 72 h. In addition, spot 555 (prohibitin), a tumor suppressing protein, was found decreasing expression at 24 h and then increasing expression from 48 to 72 h in RA-treated F9 cells compared to the notreated cells. Finally, spot 666 (voltage-dependent anion channel 1, VDAC1) is an apoptosis-related protein, and this protein increased expression from 24 to 48 h and then decreased expression at 72 h in differentiating F9 cells induced by RA.

TABLE II. Protein Alterations After Treatment With RA (50 nM for 24, 48, and 72 h)

	Fold difference			
Protein name	24	48	72	Reported function
Malate dehydrogenase cytosolic (cMDE)	+0.36 ± 0.18	−0.21 ± 0.05	−2.7 ± 0.59	Glycolytic metabolism
Aldolase 1 A isform (aldolase 1)	−2.61 ± 0.38	−2.02 ± 0.24	−4.37 ± 3.23	Glycolytic metabolism
Bifunction purine biosynthesis protein (purH)	−0.54 ± 0.57	−1.83 ± 0.78	−6.00 ± 3.17	Nucleotide metabolism
mTHF-dehydrogenase	+3.42 ± 1.03	−0.54 ± 0.51	−0.48 ± 0.42	Nucleotide metabolism
Peroxiredoxid 4 (Prx4)	−0.31 ± 0.73	−0.16 ± 0.27	1.6 ± 0.49	Redox
Heterogenous nuclear ribonucleoprotein K (hnRNP K)	+0.79 ± 0.13	−1.34 ± 0.71	−1.95 ± 0.55	Nucleic acid metabolism
RAN, member RAS oncogene family	+0.55 ± 0.30	−0.04 ± 0.27	−2.13 ± 0.37	Gene regulation
far upstream element binding protein 1 (FUBP1)	+0.26 ± 0.19	−1.85 ± 0.21	−2.56 ± 0.66	Transcription regulation
Chaperonin groEL precursor (groEL)	+0.95 ± 0.29	−1.48 ± 0.77	−1.67 ± 0.53	Chaperon proteins
Chaperonin subunit 5 (epsilon)	+0.14 ± 0.09	−0.05 ± 0.41	−2.25 ± 0.40	Chaperon proteins
T complex polypeptide 1 b (TCP1)	+0.30 ± 0.34	+0.02 ± 0.23	−1.72 ± 0.38	Chaperon proteins
Stress-induced phosphoprotein 1 (STI1)	−2.1 ± 0.02	−2.36 ± 0.33	−2.84 ± 0.43	HSP-related
Prohibitin	−2.55 ± 0.13	+0.13 ± 0.23	+1.59 ± 0.39	Tumor suppressing
Voltage-dependent anion channel 1 (VDAC1)	+0.74 ± 0.37	+0.13 ± 0.26	−2.04 ± 0.32	Apoptosis-related

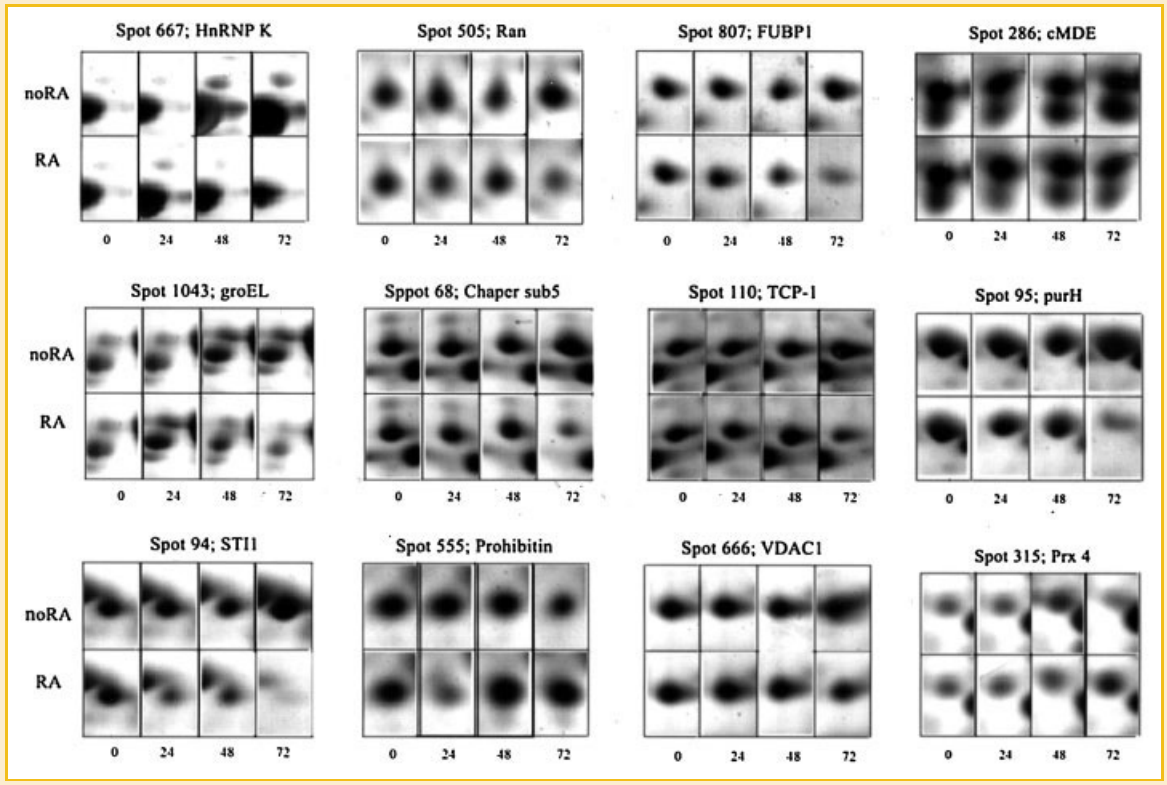

Fig. 4. Overview of protein spots that changed significantly upon differentiation. Each graph represents the change folds obtained from RA or noRA treatment at the difference time points. Representative gel images are displayed on top of each graph. Identified proteins are indicated.

PROTEIN EXPRESSION CONFIRMATION BY WESTERN BLOTTING

Western blotting was performed to verify six selected proteins, prohibitin, hnRNP K, aldolase 1, peroxiredoxin 4, chaperonin groEL precursor, and malate dehydrogenase cytosolic (cMDE), that may play functional roles in F9 cells differentiation. Figure 5 displays the representative gels of Western blotting, confirming the altered expressions of these six proteins in F9 cells treatment with or without RA at 24, 48, and 72 h.

DISCUSSION

It consists of two distinct phases in gene expression levels in RA-induced F9 cell differentiation [Harris and Childs, 2002]. The first phase involves the wave of detectable gene induction started after 4 h and peaked at 16 h, followed by a sharp decline at 24 h. The second phase started after 24 h and peaked around day 3 with the most genes showing an increase of steady expression levels compared to the non-differentiated F9 cells. Therefore, the time points at 24, 48, and 72 h treatment with RA are the reasonable time points for proteomic analysis. Here, we have identified 14 differentially expressed protein spots during F9 cells differentiation as a result of RA treatment.

In the present study, we identified five differentially expressed enzymes during F9 cells differentiation induced by RA. The cytosolic malate dehydrogenase (cMDH), an isoenzyme of MDH, has important roles in the citrate shuttle/malate–aspartate shuttle, one of the systems which link cytosol and mitochondria metabolisms [Goward and Nicholls, 1994]. We found that the decrease of cMDH in differentiating F9 cells was evident at 24 h RA treatment and was maintained from 48 to 72 h. The shift in cMDH expression patterns reflects glycolytic changes associated with F9 cells differentiation because the down-regulation of cMDH may lead to an increasing in cytosolic H^+, modifying activity of 6-phosphofructokinase, decreasing the energy production pathways via glycolysis [Vawter et al., 2004]. Interestingly, another glycolysis-related enzyme—aldolase 1 show similar expression patterns with cMDH in our study. Thus, RA down-regulates the cMDH and aldolase 1, and changed rate of glycolysis in F9 cells differentiation. The mechanisms of RA regulate cMDH and aldolase 1 gene expression is still unclear. Aldolase 1, unlike aldolases B and C, exhibits an additional level of complexity in regulating its expression during development and differentiation. To our knowledge, there is no report about the regulation of aldolase 1 gene expression during F9 cells differentiation induced by RA.

Our data also shows a continuous decrease of bifunctional purine biosynthesis protein (purH), which is a bifunctional enzyme that catalyzes the final two activities in de novo purine biosynthesis. Because cancer cells rely more heavily on the de novo pathway than normal cells, which favor the salvage pathway as their main purine source, purH is an attractive candidate for rational design of anticancer agents [Cheong et al., 2004]. A second enzyme associated with purine synthesis that was found to decrease in RA-treated F9 cells was mTHF-dehydrogenase, which is involved in the intercon-

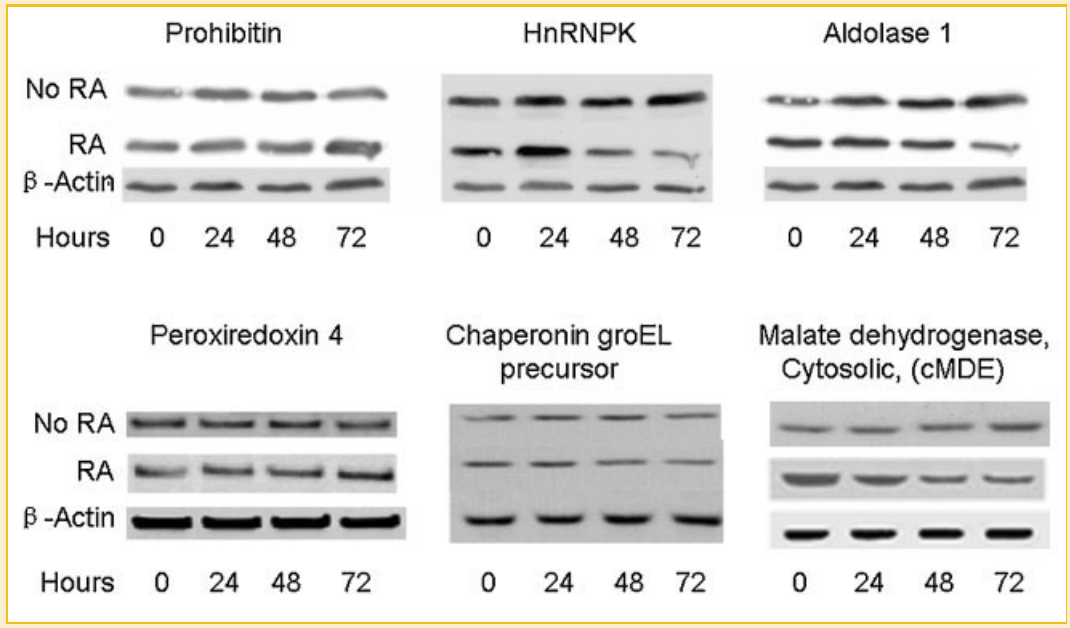

Fig. 5. Western blot analysis of prohibition, hnRNP K, and aldolase 1 at 0, 24, 48, and 72 h after treated with retinoic acid (RA) or without RA (noRA). For equal loading, beta-actin was used, and each sample was 50 μg total F9 protein. Beta-actin molecular weight was 42 kDa.

version of the one-carbon units carried by the biologically active form of folic acid. The activated one-carbon units are used in a variety of cellular processes, including de novo purine and thymidylate synthesis, serine and glycine interconversion, and methionine biosynthesis [Prasannan et al., 2003]. Together with the present data, the decreased expression of purH and mTHF-dehydrogenase result from the treatment with RA, and these decrease show a new mechanism that RA treatment suppress purine metabolism by decreasing purH and mTHF-dehydrogenase protein levels and that inhibit tumor cell growth.

Peroxiredoxin is a family of enzymes with an antioxidative function. Increased expression of Prx4 was found in various types of cancer, such as breast, prostate, brain, and stomach cells [Lin et al., 2007; Khalil, 2007]. A similar increase of Prx expression has been reported during the differentiation of embryonic stem cells to neural cells induced by RA [Guo et al., 2001]. However, in our study, we detected the decrease of Prx4 expression in differentiating F9 cells from 24 to 48 h of RA treatment, this decrease was sustained at 72 h.

In F9 cell differentiation system, we also detected altered expression of several proteins after treatment with RA. hnRNP K, an RNA-binding protein, showed the decreased expression level in RA-treated F9 cells. It is known that hnRNP K can regulate expression of the gene associated with cell growth and/or differentiation. Firstly, hnRNP K down-regulates expression of p21 that is particularly important in the initiation and maintenance of differentiation in mouse embryonic cells [Yano et al., 2005]. Secondly, hnRNP K down-regulates expression of c-myc both at the transcriptional and translational level which is a prerequisite for differentiation in many cell types [Tomonaga and Levens, 1995]. The down-regulation of Myc expression in F9 cells differentiation has also been confirmed [Griep and Westphal, 1988]. Thirdly, hnRNP K can regulate eukaryotic translation initiation factor 4E (eIF4E) increasing translation initiation and cell division in an eIF4E-dependent manner [Lynch et al., 2005]. The hnRNP K protein changes in the differentiation induced by RA have been reported. Harris et al. [2004] observed the decreased of hnRNP K protein in differentiating leukemia NB4 and HL-60 cells from 24 to 72 h of RA treatment. This result is consistent with our findings.

Another regulator of c-myc gene expression, FUBP1, was found to be strongly down-regulated from 48 to 72 h after treatment with RA in our present study. FUBP was named for its interaction with the far upstream element (FUSE) of the c-myc gene [Avigan et al., 1990; Duncan et al., 1994]. It was reported that degradation of FUBP led to down-regulate of c-myc during differentiation of alveolar type II cells [Kim et al., 2003]. From this, we speculate that decrease of FUBP1 is related to down-regulation of c-myc during F9 cells differentiation induced by RA.

Ran is a GTP-binding protein of the Ras superfamily that has been implicated in a large number of cellular processes, such as protein import and export, mRNA processing, cell cycle progression, and nuclear structure [Sazer and Dasso, 2000]. It was demonstrated that Ran is differentially expressed during mouse embryogenesis and early spermatogenesis suggesting a possible role during cell differentiation [López-Casas et al., 2003]. In this study, we demonstrated down-regulation of Ran in RA-induced F9 cells differentiation; it has also been observed in rat RPE-J cells differentiation induced by RA [West et al., 2001].

Heat shock proteins (HSPs) are ubiquitous and evolutionary conserved proteins. HSPs play an important role in embryonic development, cell cycle progression, apoptosis, and differentiation [Rodgers et al., 2009]. In our present study, three HSP60 protein Chaperonin groEL, Chaper sub5, and TCP-1 were found to have the same pattern of change in differentiating F9 cells treated with RA. The expression of these proteins in differentiating F9 cells increased

at 24 h after treatment with RA, and decreased at 48 and 72 h. A similar decrease of HSP60 expression has been reported during the differentiation of Caco-2. The function of HSP60 is to assist mitochondrial protein folding, unfolding, and degradation [Bukau and Horwich, 1998; White et al., 2005]. In addition, Hsp60 is also involved in apoptosis and tumorigenesis [Cappello et al., 2005]. Recent studies show that HSP60 may play a stress-protective role in RA-induced abnormal limb development [Zhu et al., 2010]. However, the regulation mechanism and role of HSP60s during differentiation need to be further studied.

Stress-induced phosphoprotein 1, a HSP-related protein, was found down-regulated in RA-treated F9 cells in this study. SIT1, as an adaptor protein, interacts with Hsp70 and Hsp90 and has an important function for proper protein folding and maturation [Hernández et al., 2002]. Up-regulation of Hsp70 and SIT1 have been observed in HepG2 cells treated with oridonin [Wang et al., 2010]. In addition, recent report demonstrated that SIT1 can stimulate the phosphorylation of eIF4E and may affect cell differentiation [Roffé et al., 2010]. Together with our data, it suggests that SIT1 may play an important role in F9 cells differentiation.

Interestingly, prohibitin, a tumor-suppressive protein, was found to increase in differentiating F9 cells from 48 to 72 h of RA treatment. Prohibitin is expressed in various cells and play important roles in the regulation of cell growth, proliferation, differentiation, aging, and apoptosis [Nijtmans et al., 2002; Piper and Bringloe, 2002; Fusaro et al., 2003]. There were conflicting experimental results regarding prohibitin regulation in relationship with cell proliferation and differentiation. Dixit et al. [2003] reported that prohibitin decreased markedly in proliferating cells while it increased markedly in differentiated cells. However, Sun et al. [2004] found that increase of prohibitin could repress muscle differentiation. These contradictory results show that the different regulation of prohibitin in different cell lines and differentiation stages might indicate bi-directional regulation, that is, prohibitin could control the overproliferation of cells and maintain cell survival. The changes of prohibitin in F9 cells differentiation have not been previously reported. Our present data suggest that prohibitin may promote F9 cells differentiation and suppress the cells proliferation.

VDAC, found in the outer mitochondrial membrane, is a key protein in mitochondria to mediate apoptosis, due to both its function in the release of apoptotic proteins located in the inter-membranal space and it being a target for the pro- and anti-apoptotic Bcl2-family of proteins [Tsujimoto, 2002; Shoshan-Barmatz et al., 2006]. In this study, the increase of VDAC1 expression in differentiating F9 cells at 24 and 48 h of RA treatment, and decrease of the expression was observed at 72 h. These changes show that the alternation in VDAC1 protein is associated with the apoptosis of F9 cells because retinoid-induced differentiation is accompanied by an apoptotic response [Clifford et al., 1996].

CONCLUSION

This study used comparative proteomics to profile F9 cells at different time point of treatment with RA. The expression level of proteins involved in cellular metabolism, gene expression regulation, stress response, and apoptosis was found to change during F9 cells differentiates induced by RA. We showed that the decreased expression of metabolism enzymes (cMDE, aldolase 1, purH, and mTHF-dehydrogenase) is associated with the retard of metabolism in RA-treated F9 cells. The proteins (Prx 4, Ran, and HSP60s) prior known to be associated with F9 cells differentiation were identified from 2-DE. The change in expression pattern of these proteins was comparable to previous observations. Most importantly, proteomics enabled us to reveal the identity of hitherto unknown proteins associated with F9 cells differentiation (purH, STI1, and VDAC1). The functionality of these proteins in terms of regulating or contributing to F9 cells differentiation is under investigation.

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