ORIGINAL

Identification of novel proteins differentially expressed in pluripotent embryonic stem cells and differentiated cells

Kei Enomoto[1], Kanako Watanabe-Susaki[2], Megumi Kowno[3], Hitomi Takada[2], Atsushi Intoh[4], Yuko Yamanaka[5], Hisashi Hirano[5], Hiromu Sugino[2], Makoto Asashima[2, 4, 6], and Akira Kurisaki[2, 3]

[1]*Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo, Japan,* [2]*Research Center for Stem Cell Engineering, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central 4, Higashi, Tsukuba, Ibaraki, Japan,* [3]*Graduate School of Life and Environmental Sciences, The University of Tsukuba, Tennodai, Tsukuba, Ibaraki, Japan,* [4]*Department of Life Sciences (Biology), Graduate School of Arts and Sciences, The University of Tokyo, Komaba, Meguro-ku, Tokyo, Japan,* [5]*Department of Molecular Biology, Yokohama City University Graduate School of Medicine, Kanazawa-ku, Yokohama, Kanagawa, Japan,* [6]*ICORP Organ Regeneration Project, Japan Science and Technology Agency (JST), Komaba, Meguro-ku, Tokyo, Japan*

Abstract : Mammalian pluripotent stem cells possess properties of self-renewal and pluripotency. These abilities are maintained by the strict regulation of pluripotent stem cell-specific transcription factor network and unique properties of chromatin in the stem cells. Although these major signaling pathways robustly control the characteristics of stem cells, other regulatory factors, such as metabolic pathways, are also known to modulate stem cell proliferation and differentiation. In this study, we fractionated protein samples from mouse embryonic stem (ES) cells cultured with or without the leukemia inhibitory factor (LIF). Protein expression was quantified by 2-dimensional differential gel electrophoresis (2D-DIGE). In total, 44 proteins were identified as being differentially expressed in the pluripotent stem cells and the differentiated cells. Surprisingly, half of the identified proteins were the proteins localized in mitochondria, which supply cellular energy and regulate cell cycle, development, and cell death. Some of these identified proteins are involved in the metabolic function and the regulation of pluripotency. Further analysis of the identified proteins could provide new information for the manipulation of pluripotency in ES cells. J. Med. Invest. 62 : 130-136, August, 2015

Keywords **:** *Proteomics, ES cells, pluripotency, differentiation, proteins.*

INTRODUCTION

Mammalian ES cells are derived from the inner cell mass of a blastocyst (1). ES cells have the ability to self-renew and differentiate into all three germ layers, namely, endoderm, mesoderm, and ectoderm. Mouse ES cells can self-renew and maintain pluripotency when cultured with LIF (2). It has been reported that pluripotency-specific transcription factor network controls the pluripotency of ES cells (3, 4). Moreover, the pluripotency of ES cells could be attributed to the unique characteristics of the chromatin in ES cells. For example, the chromatin in pluripotent stem cells has an "open" structure, which is characterized by the enrichment of active histone marks such as acetylation and methylation in a particular region ; in contrast, differentiated cells have relatively "compact" chromatin (5). This property is considered important for the maintenance of adequate gene expression necessary for pluripotency.

Recent reports have indicated that energy metabolism plays an important role in the regulation of stem cell characteristics (6). Pluripotent stem cells are mainly dependent on glycolysis, while differentiated cells, like fibroblasts, rely on mitochondrial respiration (7). Therefore, ES cell differentiation is characterized by a dramatic shift in metabolic pathways, from anaerobic glycolysis to aerobic mitochondrial respiration. Of greater interest is the report by Zhou *et al.*, which states that mouse ES cells and human ES cells are metabolically different. The latter cell type, which is similar to the epiblast stem cell (EpiSC) established from later-stage post-implantation epiblasts, is exclusively glycolytic, as opposed to a naïve pluripotent cell type, mouse ES cell, which is dependent on both glycolytic and mitochondrial respiration (8). This observation raises a possibility that not only transcriptional network but also metabolic pathway is important for the control of stem cell pluripotency.

In this study, we performed proteome analysis to explore the novel mechanisms underlying the maintenance of pluripotency in ES cells. For this purpose, 2D-DIGE was used to identify the differentially expressed proteins between two samples by comparing the intensities of differentially dye-labeled protein spots separated on a gel. We have identified several novel proteins that were highly expressed in pluripotent ES cells. Here, we discuss the manner in which these proteins are possibly involved in the pluripotency of ES cells.

MATERIALS AND METHODS

Cell culture

Mouse ES cell line D3 was purchased from ATCC (American Type Culture Collection). In order to maintain their pluripotency, ES cells were cultured on mitomycin-C-treated mouse embryonic fibroblast (MEF) feeder cells in Dulbecco's Modified Eagle's Medium with high glucose (Gibco, Grand Island, NY, USA), 15% heat-inactivated ES-qualified FCS (Gibco), 1×non-essential amino acids (Sigma-Aldrich, St. Louis, MO, USA), 0.1 mM 2-mercaptoethanol

Received for publication December 4, 2014 ; accepted January 16, 2015.

Address correspondence and reprint requests to Akira Kurisaki, Research Center for Stem Cell Engineering, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central 4, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8562, Japan and FAX : +81-29-861-2987.

(Wako Pure Chemical Industries, Osaka, Japan), 0.1 mg/ml penicillin/streptomycin (Gibco) and 1,000 U/ml LIF (ESGRO ; Chemicon International, Inc, Temecula, CA, USA) under 5% CO_2 at 37°C. For the preparation of chromatin-associated proteins, ES cells were cultured on gelatin-coated plates without MEFs in the presence or absence of LIF for 7 days.

Measurement of alkaline phosphatase activity

The ES cells cultured with or without LIF for 7 days were washed twice with phosphate buffered saline (PBS) and fixed in 3.7% formaldehyde/PBS for 10 min at room temperature (r.t.). The cells were then washed twice with PBS and incubated with BM purple AP substrate (Roche, Mannheim, Germany) for 30 min at r.t.

Preparation of protein samples

The ES cells cultured in the presence or absence of LIF were treated with 0.06 μg/ml colcemid (Wako) for 12 h before harvesting. They were then scraped from the dishes in polyamine buffer (15 mM Tris-HCl [pH 7.4], 0.2 mM spermine, 0.5 mM spermidine, 2 mM EDTA, 80 mM KCl, 0.1 mM phenylmethylsulfonyl fluoride) followed by centrifugation at 780×*g* for 10 min at 4°C. Subsequently, the cells were resuspended in the polyamine buffer supplemented with 0.1% (w/v) digitonin, and the cell membrane was destroyed by vortexing. After centrifugation at 190×*g* for 3 min at 4°C, the supernatant was collected as a crude chromatin fraction. The crude sample was purified by sucrose density-gradient centrifugation. Briefly, the sample was gently layered on the density gradient tube with a sucrose gradient generated by gradually mixing 5% and 20% sucrose in the polyamine buffer by using a peristaltic pump, followed by centrifugation at 2,380×*g* for 15 min at 4°C. The purified chromatin fraction was then treated using a nucleic acid affinity column (Vivapure D Maxi H ; Vivascience, Hannover, Germany) according to the manufacturer's protocol and desalted by centrifugation with Amicon ultra-15 (NMWL 10ka ; Millipore, Billerica, MA, USA) at 2,580×*g* for 90 min at 4°C three times in 7 M urea buffer (30 mM Tris-HCl [pH 8.5], 7 M urea, 2 M thiourea, and 4% CHAPS). The purified protein sample was subjected to 2D-DIGE or further purified by removal of DNA and RNA by enzymatic digestion and desalting with ultrafiltration. Briefly, 1 ml of the protein sample was mixed with 10 μl Nuclease Mix (GE Healthcare BioSciences, Piscataway, NJ, USA) and incubated for 45 min at r.t. followed by centrifugation as described above.

Western blotting

The protein sample was lysed in a lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM Na_3VO_4, 25 mM NaF, and 25 mM β-glycerophosphate) supplemented with a protease inhibitor cocktail (complete ; Roche), and rotated for 1 h at 4°C. After centrifugation at 17,000×*g* for 10 min at 4°C, the supernatants were collected and their protein concentrations were determined using the Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Subsequently, 10 μg of protein extract was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat skim milk in TBS/Tween-20 (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 0.1% Tween-20) and incubated overnight with a primary antibody at 4°C. After incubation with a horseradish peroxidase-conjugated secondary antibody, the blots were incubated with an enhanced chemiluminescent assay reagent (SuperSignal West Femto ; Pierce Biotechnology, Rockford, IL, USA) for 5 min at r.t., and the protein bands were visualized using a LAS-1000 lumino-image analyzer (Fuji Film, Tokyo, Japan). The antibodies used for western blotting were as follows : Nanog (RCAB0001P ; ReproCELL, Yokohama, Japan), Oct-3/4 (sc-9081 ; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Histone H3 (ab1791 ; abcam, Cambridge, MA, USA), FBL (ab4566 ; abcam), α-tubulin (T9026 ; Sigma-Aldrich), PHB1 (3805-100 ; BioVision, Milpitas, CA, USA), and PHB2 (sc-133094 ; Santa Cruz).

2D-DIGE

2D-DIGE was performed using Ettan DIGE (GE Healthcare BioSciences). Briefly, the protein samples were dissolved in 2D-DIGE sample buffer (30 mM Tris-HCl [pH 8.5], 7 M urea, 2 M thiourea, and 4% CHAPS), and the amount of protein was measured by Bradford protein assay kit (Bio-Rad). Approximately 50 μg of chromatin-binding proteins extracted from pluripotent ES cells were labeled with Cy3, while those from differentiated cells were labeled with Cy5, according to the manufacturer's protocol (GE Healthcare BioSciences). The fluorescent-labeled proteins with different colors were mixed and subjected to isoelectric focusing. Immobiline dry strips (pH : 3-11, linear, 24 cm) (GE Healthcare BioSciences) were rehydrated for 12 h at r.t. before isoelectric focusing for a total of 65 kVh with a Multiphor II apparatus. The second dimension was performed under a standard SDS-PAGE protocol using 12% (w/v) polyacrylamide gels. The gels were scanned using a Typhoon 9400 fluorescent scanner (GE Healthcare BioSciences) and analyzed using ImageMaster DIGE software (GE Healthcare BioSciences).

Protein identification by peptide mass fingerprinting

After electrophoresis, the gel was stained with Deep Purple (GE Healthcare BioSciences), and the images of protein spots were captured with the Typhoon 9400 scanner. The preparative gel image was matched to the master gel image in the analytical gel by using the DeCyder software (GE Healthcare BioSciences). The protein spots were picked up by Ettan Spot Picker (GE Healthcare BioSciences), an automated spot handling system. In-gel digestion was performed as follows. The gel plugs were washed with 50 mM ammonium bicarbonate containing 50% (v/v) acetonitrile, followed by washing with 100% (v/v) acetonitrile for dehydration, and dried completely in a SpeedVac evaporator. Following overnight digestion with trypsin (Promega, Fitchburg, WI, USA) at 37°C, peptides were extracted twice with 66% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. The extracted peptides were desalted using a ZipTip C18 column (Millipore) according to the manufacturer's protocol, and analyzed using a Micromass Q-Tof Mass Spectrometer (Waters Corp., Milford, MA, USA). Peptide mass fingerprints were analyzed using the Mascot software (Matrix Science Ltd., London, UK).

Immunofluorescent staining

Mouse ES cells were washed with PBS, fixed in 3.7% formaldehyde in PBS for 30 min at r.t., and then permeabilized with 0.5% Triton X-100 in PBS for 5 min at r.t. The cells were blocked with 5% FCS in PBS for 1 h at r.t., and incubated with primary antibodies. After washing 3 times with 5% FCS in PBS, the cells were incubated with fluorescently labeled secondary antibodies for 1 h at r.t., and cell nuclei were stained with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). The cells were observed under a fluorescence microscope (IX70 ; Olympus, Tokyo, Japan) equipped with CoolSNAP HQ2 (Photometrics, Tucson, AZ, USA) and images were processed using MetaMorph software (Molecular Devices, Sunnyvale, CA, USA). A confocal fluorescence microscope (FV1000 ; Olympus) was also used to analyze the cells. The antibodies used for the immunofluorescent staining were as follows : FBL (ab4566 ; abcam) and Nucleolin (ab70493 ; abcam).

Plasmid construction

Plasmids were constructed as previously described (9, 10). Briefly, the full-length coding regions of FBL and PHB2 (Flag-tagged at the C-terminus) were amplified by polymerase chain reaction

(PCR) and inserted into the pCAG-IP vector (11) or pPthC vector (12), respectively. FBL-expressing ES cell lines were established as follows : ES cells were transfected with pCAG-IP-FBL vector by using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA), and the cells stably expressing FBL were selected in the presence of 0.4 μg/ml puromycin. Tetracycline (Tc)-regulated PHB2-expressing ES cell lines were established according to the method of Masui *et al.* (12). An EB3 cell-derived mouse ES cell line, EBRTcH3, which contained a Tet-off cassette in the ROSA26 locus (12), was co-transfected with a targeting vector, pPthC-PHB2-Flag and a Cre recombinase expressing vector, pCAGGS-Cre, using Lipofectamine 2000 (Life Technologies) and cultured in the presence of 1.5 μg/ml puromycin and 1 μg/ml Tc.

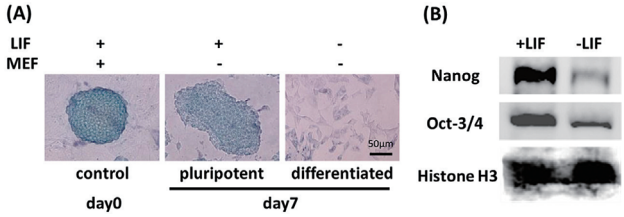

Fig. 1. Expression of pluripotency-specific markers in cells used for proteomic analysis. (A) Alkaline phosphatase activity in mouse ES cells cultured in the presence or absence of LIF for 7 days. Cells with alkaline phosphatase activity are stained in blue. (B) Western blotting analysis of chromatin-binding proteins extracted from mouse ES cells cultured in the presence or absence of LIF for 7 days. MEF : mouse embryonic feeder cells.

RESULTS

Preparation of pluripotent ES cells and differentiated cells

Initially, we explored the novel chromatin-associated proteins, which are highly expressed in pluripotent ES cells. Mouse ES cells were cultured under feeder-free conditions to avoid contamination by feeder cell-derived proteins. The pluripotency of ES cells was confirmed by monitoring alkaline phosphatase activity (13). After 7 days of culture without feeder cells, ES cells cultured without LIF almost completely lost their alkaline phosphatase activity, while the cells with LIF still maintained the strong activity (Fig. 1A). In addition, ES cells cultured with LIF exhibited a colony-like shape similar to that exhibited by pluripotent ES cells cultured on the feeder cells with LIF, indicating maintenance of pluripotency. Therefore, we defined the cells cultured for 7 days without LIF as "differentiated cells" and those cultured with LIF as "pluripotent ES cells" and prepared chromatin-associated proteins from each group.

Analysis of pluripotent markers on protein samples

The protein level of pluripotent markers Nanog and Oct-3/4 was analyzed with purified chromatin-associated protein samples. Western blotting analysis showed that both Nanog and Oct-3/4 were significantly down-regulated in the differentiated state, while the control Histone H3 protein did not show any difference between the two states (Fig. 1B). This result indicated successful completion of protein fractionation, with protein expression profile specific to each state. These protein samples were labeled with Cy3 (pluripotent ES cells) or Cy5 (differentiated cells) and analyzed by 2D-DIGE.

Identification of differentially expressed proteins between pluripotent ES cells and differentiated cells

Protein samples were separated in the isoelectric point (pI) range of 3.0-11.0 by isoelectric focusing followed by SDS-PAGE using 12% (w/v) polyacrylamide gels. As shown in Fig. 2, we selected more than 70 protein spots differentially expressed between two samples, and finally identified 39 differentially expressed protein spots (29 independent proteins) by mass spectrometry. However, most of the proteins distributed in the high pI range, i.e. basic proteins, were not well separated. Considering that chromatin is composed of DNA and DNA-binding proteins, majority of the chromatin-associated proteins are supposed to be basic proteins. Therefore, we further purified these proteins by treatment with nucleases to remove DNA and RNA that could hamper the separation of proteins by electrophoresis. These purified samples were then subjected to 2D-DIGE with the pI range of 7.0-11.0. The resulting electrophoresis successfully resolved many basic proteins. More than 30 differentially expressed protein spots were selected, and mass spectrometry identified additional 21 spots (15 independent proteins) as the differentially expressed proteins (Fig. 3). Surprisingly, majority of these differentially expressed proteins in the chromatin-associated protein fraction were known to localize in other organelles, such as mitochondrion (Table I, II).

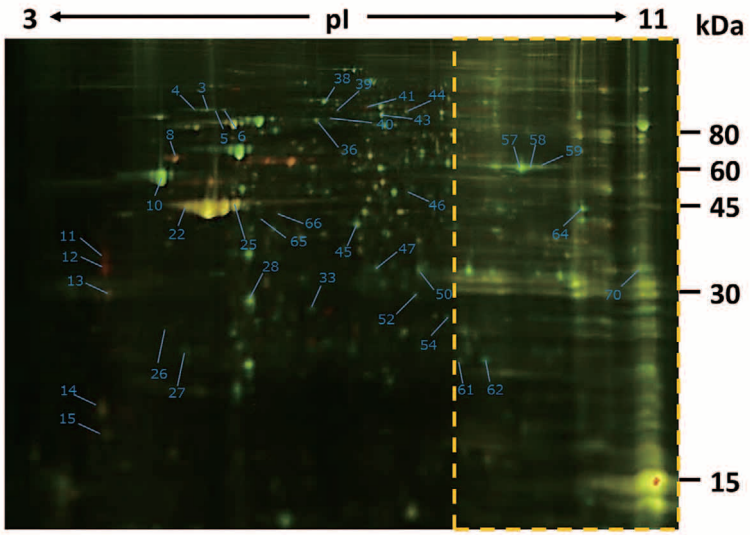

Fig. 2. 2D-DIGE of chromatin-binding proteins fractionated from pluripotent ES cells and differentiated cells. These proteins were separated in the pI range of 3.0-11.0. Only the differentially expressed protein spots identified by mass spectrometry were numbered. The region circled by orange dashed line was analyzed in Fig. 3.

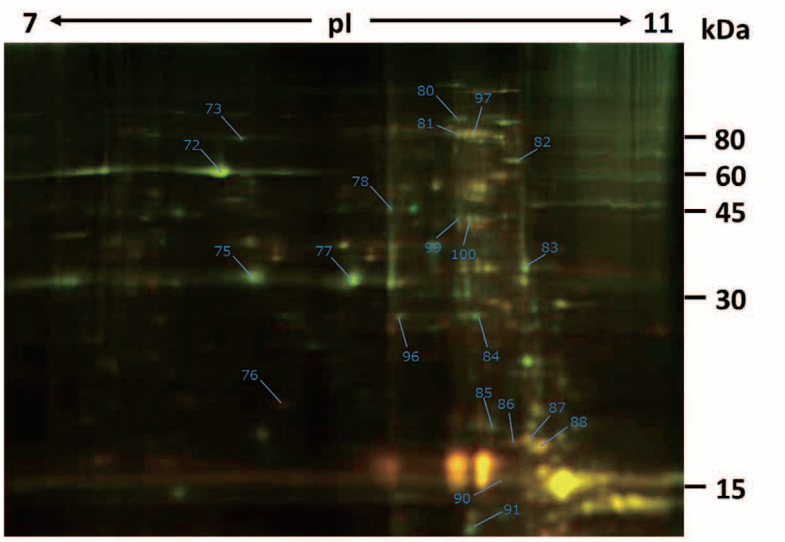

Fig. 3. 2D-DIGE of chromatin-binding proteins in the alkaline pH range. Proteins fractionated from pluripotent ES cells and differentiated cells were further purified with removal of nucleotides by enzymatic digestion, and separated by 2D-PAGE in the pI range of 7.0-11.0. Only the differentially expressed protein spots identified by mass spectrometry were numbered.

Table I. The list of proteins identified with the first 2D-DIGE (pI range : 3.0-11.0).

Spot No.	Ratio (+LIF/-LIF)	Protein name	Localization	pI	MW
62	2.89	Manganese superoxide dismutase	M	8.8	24662
54	2.64	Adenylate kinase 4	M	7.02	25046
38	1.82	Inner membrane protein, mitochondrial	M	6.18	83848
64	1.81	Ubiquinol cytochrome c reductase core protein 2	M	9.26	48205
3	1.79	NADH dehydrogenase (ubiquinone) Fe-S protein 1	M	5.51	79698
47	1.76	Voltage-dependent anion channel 2	M	7.44	31713
50	1.75	Pyrroline-5-carboxylate reductase 1	M	6.36	32353
39	1.73	Fragile-X-related protein 1 isoform c	N, Cy	5.93	72770
4	1.7	NADH dehydrogenase (ubiquinone) Fe-S protein 1	M	5.51	79698
5	1.69	NADH dehydrogenase (ubiquinone) Fe-S protein 1	M	5.51	79698
61	1.67	Ptrh2 protein	Cy, M	6.96	19514
36	1.66	TNF receptor-associated protein 1	M	6.25	80027
40	1.64	TNF receptor-associated protein 1	M	6.25	80027
27	1.62	NADH dehydrogenase (ubiquinone) Fe-S protein 8	M	5.89	23996
45	1.61	Stomatin-like protein 2	Cy, M	8.95	38361
52	1.6	SCO cytochrome oxidase deficient homolog 1	M	8.66	32098
33	1.6	Mitochondrial ribosomal protein L46	M	6.93	32112
6	1.58	NADH dehydrogenase (ubiquinone) Fe-S protein 1	M	5.51	79698
43	1.56	Glycerol-3-phosphate dehydrogenase 2	M	6.26	80932
28	1.52	Prohibitin	N, M	5.57	29802
70	1.52	Fibrillarin	N	10.25	34286
10	1.52	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	M	5.24	56632
59	1.52	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1	M	9.22	59716
58	1.51	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1	M	9.22	59716
57	1.5	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1	M	9.22	59716
13	-1.51	Tropomyosin 3, gamma	Cs	4.73	33129
65	-1.57	Proteasome 26S non-ATPase subunit 13	N, Cy	5.46	42782
25	-1.66	Actin, gamma 1	Cs	5.31	41766
66	-1.68	Actin, beta 1	Cs	5.78	39161
14	-1.71	RIKEN cDNA 2900073G15	-	4.76	19883
22	-1.84	Actin, gamma 1	Cs	5.56	40992
26	-2.19	Lamin B	N	5.14	66719
8	-2.54	Vimentin	Cs	5.06	53655
44	-2.71	Caldesmon 1	Cy	6.97	60417
46	-2.97	Calcium-binding transporter	M	7.02	52868
41	-4.02	Caldesmon 1	Cy	6.97	60417
15	-4.6	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	M	5.24	56632
11	-5.47	Actin, beta 1	Cs	5.78	39161
12	-8.26	Alpha tropomyosin	Cs	4.69	32661

N : nucleus, M : mitochondrion, Cy : cytoplasm, Cs : cytoskeleton

Table Ⅱ. The list of proteins identified with the second 2D-DIGE (pI range : 7.0-11.0).

Spot No.	Ratio (+LIF/-LIF)	Protein name	Localization	pI	MW
84	2.42	4-nitrophenylphosphatase domain and non-neuronal SNAP25-like protein homolog 1	M	9.48	33342
73	1.6	Programmed cell death 8	N, M	9.23	66724
75	1.51	Voltage-dependent anion channel 1	Cy, M	8.62	30737
77	1.48	Voltage-dependent anion-selective channel protein 1	Cy, M	8.55	32331
72	1.45	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1	N, M	9.22	59716
91	1.42	Ubiquinol-cytochrome c reductase binding protein	M	9.1	13553
96	1.4	Coiled-coil-helix-coiled-coil-helix domain containing 3	N, Cy, M	8.56	26318
82	1.38	Polyadenylate-binding protein 1	N, Cy	9.52	70598
85	1.36	Peptidyl-prolyl cis-trans isomerase B precursor	Extr	9.56	22699
83	1.26	Prohibitin 2	N, M	9.83	33276
81	-1.23	ATP-dependent RNA helicase DDX5	N	8.88	74579
78	-1.33	Chromosome condensation 1	N	8.34	44903
90	-1.34	Protease, serine, 1	Extr	4.75	26118
76	-1.41	Protease, serine, 1	Extr	4.75	26118
80	-1.42	ATP-dependent RNA helicase DDX5	N	9.06	69277
97	-1.43	Insulin-like growth factor 2, binding protein 1	N, Cy	9.26	63411
87	-1.43	Ribosomal protein S17	Cy	9.85	15383
99	-1.46	Ribonucleoprotein heterogeneous nuclear ribonucleoprotein A3	N, Cy	8.46	37063
88	-1.59	Ribosomal protein S17	Cy	9.85	15383
86	-1.62	Protease, serine, 1	Extr	4.75	26118
100	-1.69	Ribonucleoprotein heterogeneous nuclear ribonucleoprotein A3	N, Cy	8.46	37063

N : nucleus, M : mitochondrion, Cy : cytoplasm, Extr : extracellular matrix

Validation of identified proteins

Despite the unexpected results, we conducted a western blotting, in order to confirm the differential expression of the identified proteins. Among these proteins, Fibrillarin (FBL) was confirmed to be more highly expressed in pluripotent ES cells than in differentiated cells (Fig. 4A). FBL is known to localize in the nucleolus, and functions as a core subunit of small nucleolar ribonucleoprotein (snoRNP), which dynamically regulates protein synthesis and cell proliferation through ribosomal RNA (rRNA) biosynthesis (14). Immunofluorescent staining of FBL further confirmed a significant decrease of FBL protein in the nucleoli and this was associated with nucleolar morphology. Specifically, pluripotent ES cells with high FBL expression contained one or two large nucleoli while differentiated cells with lower FBL expression contained fragmented nucleoli, suggesting the possibility that FBL regulates nucleolar functions in the pluripotent ES cells (Fig. 4B). We therefore established a FBL-overexpressing ES cell line for further analysis. ES cells transfected with a control vector lost their pluripotency when cultured without LIF for 7 days. In contrast, the FBL-overexpressing ES cells maintained alkaline phosphatase activity even after the removal of LIF (Fig. 4C). These results suggested that FBL highly expressed in pluripotent ES cells has a positive effect on the maintenance of the undifferentiated state of ES cells.

We also confirmed high expression levels of mitochondrial membrane proteins Prohibitins (PHBs), which are involved in mitochondrial biogenesis and metabolism. The expression of PHBs was down-regulated in differentiated cells. Especially, PHB2 was much more decreased than PHB1 (Fig. 5A). To further investigate the regulatory effect of PHB2, we established a PHB2-overexpressing ES cell line. This cell line showed high alkaline phosphatase activity after 7 days of culture without LIF (Fig. 5B), suggesting that PHB2 could also positively regulate the maintenance of pluripotency in ES cells.

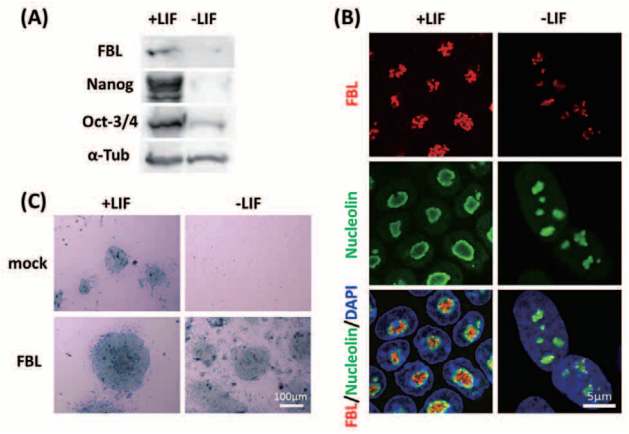

Fig. 4. The expression of FBL in pluripotent ES cells. (A) Proteins extracted from mouse ES cells cultured in the presence or absence of LIF for 7 days were analyzed by western blotting. (B) The subcellular localization of nucleolar proteins were analyzed by immunofluorescent staining. (C) Alkaline phosphatase activity in the control vector-transfected cells (mock) and FBL-overexpressing cells cultured in the presence or absence of LIF for 7 days was analyzed. Cells with alkaline phosphatase activity were stained in blue.

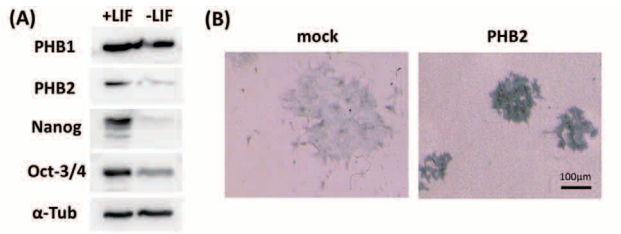

Fig. 5. The expression of PHB2 in pluripotent ES cells. (A) Proteins extracted from mouse ES cells cultured in the presence or absence of LIF for 7 days were analyzed by western blotting. (B) Alkaline phosphatase activity in the control vector-transfected cells (mock) and PHB2-overexpressing cells cultured in the absence of LIF for 7 days was analyzed. Cells with alkaline phosphatase activity were stained in blue.

DISCUSSION

In this study, we identified 44 proteins that are differentially expressed between pluripotent ES cells and differentiated cells. Some of these proteins are considered to be collateral evidence

of appropriate purification of the chromatin fractions as they are indicative of the properties maintained in cells in either state. One such protein is Lamin B1 (LMNB1), which is a major component of the interior structure of the nuclear envelope and regulates gene expression by interacting with chromatin (15). In this study, we observed increased LMNB1 levels in the differentiated cells. This is consistent with a previous report that showed a more robust interaction of LMNB1 with chromatin in differentiated cells than with chromatin in pluripotent ES cells (16). This phenomenon could be attributed to the detection of a greater amount of LMNB1 in the chromatin purified from differentiated cells.

We further investigated the possible functions of the identified proteins, and found that some proteins play significant roles in the maintenance of pluripotency in ES cells. One such example is FBL. We identified FBL as a protein that is up-regulated in pluripotent ES cells. Stable expression of FBL resulted in prolonged pluripotency after removal of LIF from culture medium. Moreover, FBL has a positive effect on the maintenance of pluripotency (Fig. 4C). In addition, we recently revealed that FBL inhibits neuronal differentiation of ES cells by controlling rRNA synthesis in a p53-dependent manner (9). These results suggested that proper rRNA production in nucleoli, which is responsible for dynamic regulation of protein synthesis and cell proliferation, may represent a novel factor that regulates pluripotency and differentiation of ES cells.

Despite the purification of chromatin-associated proteins, a considerable number of mitochondrial proteins were also identified. This phenomenon may be attributedto the technical difficulty caused by the high copy number of mitochondria associated with condensed chromosomes during mitosis. Similar observations were reported in a previous study (17). In this previous report, mitochondrial proteins identified from a purified chromosome-associated fraction were referred to as "chromosome coating proteins" (17).

Regardless of the physical coupling between the mitochondria and the nucleus, some mitochondrial proteins change their locations depending on the circumstances, and play important roles in the nucleus. In fact, some of the mitochondrial proteins identified in this study were demonstrated to have important functions in ES cells. For instance, programmed cell death protein 8, also known as apoptosis-inducing factor, mitochondrion-associated 1 (AIFM1), was identified to be highly expressed in pluripotent ES cells. It has been reported that AIFM1 is an apoptosis-inducing factor that localizes in mitochondria ; however, it translocates to the nucleus in response to stimuli and triggers cell death, which is indispensable for the early development of mouse embryo (18).

Other examples of the identified mitochondrial proteins highly expressed in pluripotent ES cells are PHBs. PHB1 and PHB2 have homologous amino acid sequences (more than 50% identity) and assemble into a heterodimer complex to form a ring-like structure in the inner membrane of mitochondria (19). PHBs are also known to localize in multiple intracellular compartments other than mitochondria and have multiple functions. They not only act as chaperones in the inner membranes of mitochondria, but also regulate gene expression by interacting with transcription factors in the nucleus (20). Apart from the cooperative function mentioned above, PHB1 and PHB2 have also been reported to have independent functions. For example, Heron *et al.* reported that PHB2 affects the regulation of skeletal muscle differentiation by binding to Akt2, but PHB1 does not (21).

In a mass spectrometry analysis, PHB1 showed a higher +LIF/-LIF expression ratio than PHB2 ; however, western blot analysis revealed that PHB2 expression changes more dynamically between pluripotent ES cells and differentiated cells than PHB1 expression (Table I, II, and Fig. 5A). Therefore, we focused our further analyses on PHB2. The PHB2-overexpressing ES cells cultured in the absence of LIF showed prolonged pluripotency similar to that shown by FBL-overexpressing ES cells (Fig. 5B). Indeed, a recent study from our group showed that PHB2 is mainly localized in mitochondria and indispensable for the maintenance of pluripotency in mouse ES cells (10). Moreover, PHB2 is essential for ATP synthesis and overexpression of PHB2 inhibited the differentiation of ES cells into neural and endodermal lineages. These data suggest that PHB2 is a crucial mitochondrial regulator for homeostasis and lineage-specific differentiation of ES cells. There might be other mitochondrial proteins in the list of identified proteins that regulate pluripotent stem cells, though their functions have yet to be defined.

In summary, we identified numerous proteins that are differentially expressed between pluripotent and differentiated states. Moreover, we validated that at least two of these proteins, FBL and PHB2, which are involved in the metabolic regulation such as rRNA biosynthesis and mitochondrial energy production, are novel regulatory proteins of pluripotency in ES cells. Further validation of the identified proteins in pluripotent ES cells could shed new light on the mechanisms underlying the maintenance of pluripotency, and contribute to future regenerative medicine and drug development using pluripotent stem cells.

CONFLICT OF INTEREST

None of the authors have any conflicts of interest to declare.

ACKNOWLEDGEMENTS

The expression vector of FBL was kind gift from Dr. Takahashi (Tokyo University of Agriculture and Technology, Japan). The expression plasmid for PHB2 was kindly provided by Dr. Endo (Jichi Medical University, Japan). The knock-in vector pPthC, Cre recombinase expression vector pCAGGS-Cre, and the mouse ES cell line EBRTcH3 were kind gifts from Drs. Niwa and Masui (RIKEN, Japan). This work was supported by JST PRESTO and Grant-in-Aid for Scientific Research (17510165 and 20610008). This work was also partially supported by grants from the Ministry of Health Labor and Welfare of Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

1. Evans MJ, Kaufman MH : Establishment in culture of pluripotential cells from mouse embryos. Nature 292 : 154-156, 1981
2. Nichols J, Chambers I, Taga T, Smith A : Physiological rationale for responsiveness of mouse embryonic stem cells to gp130 cytokines. Development 128 : 2333-2339, 2001
3. Niwa H : The pluripotency transcription factor network at work in reprogramming. Curr Opin Genet Dev 28C : 25-31, 2014
4. Martello G, Smith A : The nature of embryonic stem cells. Annu Rev Cell Dev Biol 30 : 647-675, 2014
5. Chen T, Dent SY : Chromatin modifiers and remodellers : regulators of cellular differentiation. Nat Rev Genet 15 : 93-106, 2014
6. Pereira SL, Rodrigues AS, Sousa MI, Correia M, Perestrelo T, Ramalho-Santos J : From gametogenesis and stem cells to cancer : common metabolic themes. Hum Reprod Update 20 : 924-943, 2014
7. Xu X, Duan S, Yi F, Ocampo A, Liu GH, Izpisua Belmonte JC : Mitochondrial regulation in pluripotent stem cells. Cell Metab. 18 : 325-332, 2013
8. Zhou W, Choi M, Margineantu D, Margaretha L, Hesson J,

Cavanaugh C, Blau CA, Horwitz MS, Hockenbery D, Ware C, Ruohola-Baker H : HIF1α induced switch from bivalent to exclusively glycolytic metabolism during ESC-to-EpiSC/hESC transition. EMBO J 31 : 2103-2016, 2012
9. Watanabe-Susaki K, Takada H, Enomoto K, Miwata K, Ishimine H, Intoh A, Ohtaka M, Nakanishi M, Sugino H, Asashima M, Kurisaki A : Biosynthesis of Ribosomal RNA in Nucleoli Regulates Pluripotency and Differentiation Ability of Pluripotent Stem Cells. Stem Cells 32 : 3099-3111, 2014
10. Kowno M, Watanabe-Susaki K, Ishimine H, Komazaki S, Enomoto K, Seki Y, Wang YY, Ishigaki Y, Ninomiya N, Noguchi TA, Kokubu Y, Ohnishi K, Nakajima Y, Kato K, Intoh A, Takada H, Yamakawa N, Wang PC, Asashima M, Kurisaki A : Prohibitin 2 regulates the proliferation and lineage-specific differentiation of mouse embryonic stem cells in mitochondria. PloS One 9 : e81552, 2014
11. Yoshida-Koide U, Matsuda T, Saikawa K, Nakanuma Y, Yokota T, Asashima M, Koide H : Involvement of Ras in extraembryonic endoderm differentiation of embryonic stem cells. Biochem Biophys Res Commun 313 : 475-481, 2004
12. Masui S, Shimosato D, Toyooka Y, Yagi R, Takahashi K, Niwa H : An efficient system to establish multiple embryonic stem cell lines carrying an inducible expression unit. Nucleic Acids Res 33 : e43, 2005
13. Pease S, Braghetta P, Gearing D, Grail D, Williams RL : Isolation of embryonic stem (ES) cells in media supplemented with recombinant leukemia inhibitory factor (LIF). Dev Biol 141 : 344-352, 1990
14. Gagnon KT, Zhang X, Agris PF, Maxwell ES : Assembly of the archaeal box C/D sRNP can occur via alternative pathways and requires temperature-facilitated sRNA remodeling. J Mol Biol 362 : 1025-1042, 2006
15. Kim Y, Sharov AA, McDole K, Cheng M, Hao H, Fan CM, Gaiano N, Ko MS, Zheng Y : Mouse B-type lamins are required for proper organogenesis but not by embryonic stem cells. Science 334 : 1706-1710, 2011
16. Peric-Hupkes D, Meuleman W, Pagie L, Bruggeman SW, Solovei I, Brugman W, Graf S, Flicek P, Kerkhoven RM, van Lohuizen M, Reinders M, Wessels L, van Steensel B : Molecular maps of the reorganization of genome-nuclear lamina interactions during differentiation. Mol Cell 38 : 603-613, 2010
17. Uchiyama S, Kobayashi S, Takata H, Ishihara T, Hori N, Higashi T, Hayashihara K, Sone T, Higo D, Nirasawa T, Takao T, Matsunaga S, Fukui K : Proteome analysis of human metaphase chromosomes. J Biol Chem 280 : 16994-17004, 2005
18. Joza N, Susin SA, Daugas E, Stanford WL, Cho SK, Li CY, Sasaki T, Elia AJ, Cheng HY, Ravagnan L, Ferri KF, Zamzami N, Wakeham A, Hakem R, Yoshida H, Kong YY, Mak TW, Zuniga-Pflucker JC, Kroemer G, Penninger JM : Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature 410 : 549-554, 2001
19. Tatsuta T, Model K, Langer T : Formation of membrane-bound ring complexes by prohibitins in mitochondria. Mol Biol Cell 16 : 248-259, 2005
20. Thuaud F, Ribeiro N, Nebigil CG, Desaubry L : Prohibitin ligands in cell death and survival : mode of action and therapeutic potential. Chem Biol 20 : 316-331, 2013
21. Heron-Milhavet L, Mamaeva D, Rochat A, Lamb NJ, Fernandez A : Akt2 is implicated in skeletal muscle differentiation and specifically binds Prohibitin2/REA. J Cell Physiol 214 : 158-165, 2008