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Molecules and Cells

Proteomic Analysis of Time-Dependant Difference of Protein Expression Profile Changes during Neuronal Differentiation of Mouse Embryonic Stem Cells

Jung Hee Shim[1,3], Sun A Cho[1,3], Min Ji Seo[1], Jung Hee Kim, Na Kyung Ryu[1], Kyung Hyun Yoo, Moon Hee Yang[1], Seyoon Kim[2], Young Yil Bahk[2,*], and Jong Hoon Park[1,*]

The study of ES cell-mediated neuronal differentiation allows elucidating the mechanism of neuronal development in spite of the complexity and the difficult accessibility. During the differentiation of embryonic stem cells into neuronal cell, the expression profiles in the level of protein were extensively investigated by proteomic analysis. These cells were analyzed for charges in proteome during the differentiation of ES cells by 2-dimensional electrophoresis (2-DE) and MALDI-TOF MS. Seven unique proteins were identified, some of which were differentially expressed at each stage. A complex system of neuronal differentiation can be activated in cultured embryonic stem cells and our two dimensional electrophoresis data should be useful for investigating some of the mechanism that regulates neuronal differentiation.

INTRODUCTION

Stem cells are the basic cells of animal tissues and organs; it has a unique capacity to renew itself for long periods and gives rise to special cell types under certain conditions. Pluripotent embryonic stem (ES) cells derived from inner cell mass (ICM) of 3.5-day blastocysts of preimplantation embryos. The isolation of mouse ES cells derived from blastocysts in culture was first done by Evans (Evans and Kaufman, 1981). Thereafter the isolation of human ES cells from human zygote was a success (Reubinoff et al., 2000; Thomson et al., 1998). ES cells undergo unlimited divisions without differentiation and can give rise to differentiate cell types that are derived from all three germ layers such as neuron, muscle, liver, bone, blood cells and insulin secreting cells via several differentiation methods *in vitro* (Odorico et al., 2001).

ES cells have been widely used in neurobiological model system (Kim et al., 2002) because they can be differentiated into neurons (Bain et al., 1995; Dinsmore et al., 1998; Sasai, 2002), neuronal supporting cells (Fraichard et al., 1995) and neurite formation under specific stimulus. Recently, stem cell biology has advanced rapidly, generating a rich and complex body of knowledge. It is becoming clear that especially ES cells can be an efficacious remedy of specific diseases (McNeish, 2004). However, although signaling pathway studies evolved from each of the gene products have produced vast amount of scientific literatures, the rich and complex body of knowledge generated from the traditional single signaling pathway approaches does not provide adequate understanding of ES cell differentiation. Thus, systematic approaches are needed to identify multiple global targets. Microarray experiment is one of the most popular screening techniques for the comparative study of gene expression profiles in pathogenic lesion (Alberts et al., 2005; Rouse and Hardiman, 2003). However, it is protein that executes the functions of genes and mRNA based gene arrays are unable to detect translational regulation. On the other hand, the newly emerging proteomics provides a potentially more comprehensive approach to analysis the signaling events of biological phenomenon. The proteomic techniques might enable evaluation of the specific proteins in the protein network attributable to genetic, physiological and environmental conditions differing from recent technologies for monitoring mRNA levels, which mRNA levels correlate poorly with the corresponding protein levels (Gygi et al., 1999). The field of proteomics aims to characterize dynamics in protein function on a global scale and this technique permit directly to monitor the changes in protein expression and the reflection of the protein function regulated by post-translational mechanism. For this reason, a functional proteomic analysis were performed for identify protein expression changes in neuronal differentiation (Hoffrogge et al., 2006). In this experiment, several neurotrophic factors were search at different time points through genomic and functional analysis. This study can provide that the proteome technology is very useful to understand the biological phenomena by identifying the new proteins and their modifica-

[1]Department of Biological science, Sookmyung Women's University, Seoul 140-742, Korea, [2]Present address: Protein Network Research Center, Yonsei University Seoul 120-749, Korea, [3]These authors contributed equally to this work.
*Correspondence: parkjh@sookmyung.ac.kr (JHP); bahk12@empal.com (YYB)

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tions in various cellular processes such as cell differentiation.

MATERIALS AND METHODS

Cell lines

D3 mouse ES cells (Doetschman et al., 1985) were obtained ATCC (CRL 1632) and STO blastocysts were obtained from ATCC (CRL-1503)

D3 (mouse embryonic stem cell) culture

ES cells were grown on STO feeder cells. When feeder cells filled up 0.1% gelatin coating tissue culture flasks, feeder cells were inactivated with treatment of mitomycin-C, at the concentration of 0.01 mg/ml for 90 min. ES cells were seeded on inactivated feeder cell layer. ES cells formed a colony of genetically identical cells after seeding. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, USA) supplement with 15% Fetal Bovine Serum (FBS) (Invitrogen, ES-Q), 1.5 g/L sodium bicarbonate (Sigma Mo. USA), 1% A.A (Invitrogen), 0.1 mM β-mercaptoethanol (Invitrogen), 1× MEM/NEAA (Invitrogen), LIF 1000 unit/ml (Ghemicon-ESG1107) in humidified air (CO_2 5%) at 37°C. LIF and β-mercaptoethanol used for preventing differentiation of the ES cells. The STO cells (ATCC CRL-1503; Fibroblast, Embryo, Mouse) used as feeder cell are cultured in DMEM supplement with 10% FBS, 1.5 g/L sodium bicarbonate (Sigma), 1% A.A (Invitrogen) in humidified air (CO_2 5%) at 37°C.

Neuronal differentiation of D3 cell

Neuronal differentiation of ES cells did to an 8-day induction progress (Bain et al., 1995; Gajovic et al., 1998). The procedure consisted of 4 days of culture as EBs without all trans retinoic acid (ATRA) (Sigma) followed by 4 days of culture in the presence of ATRA. First ES cell colonies and STO cells were trypsinized with 0.25% trypsin and 1 mM EDTA. The floated ES cells were selected among reattached ES cells and STO cells. Differentiation of ES cells was started at without LIF and β-mercaptoethanol into EBs on adhesive bacteria dish in serum-containing medium. Cell suspensions were cultured to form floating aggregates, EBs for 4 days. Cells were supplemented 1×10^{-6} M ATRA during the rest of 4 days. After 8day induction periods, EBs, which transferred to tissue culture dish, was changed media without LIF and β-mercaptoethanol.

Immunocytochemistry

ES cells were cultured on 0.1% gelatin coating circular glass coverlips, and fixed for 10 min with 4% paraformaldehyde in phosphate buffered saline (PBS) [PBS 40 ml, paraformaldehyde 1.6 g, 40 ul of 10 N NaOH, pH with HCl (pH 7)], rinsed with PBS, and permeabilized for 15 min in PBS containing 0.2% Triton X-100. To reduce non-specific background staining, the fixed cells were then blocked for 60 min with PBS containing 0.5% bovine serum albumin and incubated 30 min with polyclonal anti-DAPI (4′,6-Diamidino-2-phenylindole, Sigma), and polyclonal anti-Neurofilament-light (NF-L, NOVUS biologicals). After washing 3 times, peroxidase-conjugated affinipure goat anti-rabbit (Jackson Immuno Research) were treated for 30 min. Immunostained sections were analyzed using a Confocal Laser Scanning Microscope (Olympus).

Sample preparation and two dimensional polyacrylamide-gel electrophoresis

Cells were rinsed twice with ice-cold PBS and then harvested into lysis buffer (9 M urea and 4% CHAPS containing protease inhibitor cocktail from Roche) following 5 min incubation. The cell lysate was centrifuged at 100,000 × *g* (450,000 rpm) in a Beckman TL-100 table ultracentrifuge. The supernatant was taken as the total cell extract and stored at -70°C until use. Protein extracts were normalized by BCA assay from Pierce (USA) or Bradford protein assay from Bio-Rad (USA). Isoelectric focusing (IEF) was carried out using commercially available, dedicated apparatus: Multiphor II. IPG strips were used according to manufacturer's instructions. Samples containing appropriate amounts of protein were diluted to 350 ul with rehydration solution (9 M urea, 4% CHAPS, 100 mM DTT, 0.5% (v/v) IPG buffer, trace bromophenol blue), and applied to strips (pH 3-10 (NL), 4-7, 4.5-5.5, and 5.5-6.7) by overnight rehydration in a rehydration tray and 2-DE was performing using 9-16% second-dimension gels (200 × 250 × 1.0 mm) in an IsoDALT apparatus (Hoefer Scientific Instruments, USA) until the tracking dye reached the anode end of the gels.

Protein visualization and image analysis

Gels were silver-stained according to Yan et al. (2000). Briefly, the gels were fixed with methanol: acetic acid: water (40:10:50) for 30 min, followed by sensitizing in 30% methanol, 5% sodium thiosulfate, 6.8% (w/v) sodium acetate for 30 min. This was followed by three 5 min washes in deionized water. Proteins were stained in a solution 2.5% silver nitrate for 20 min, and washed twice in deionized water for 1 min. Subsequently, gels were developed with 2.5% (w/v) sodium carbonate, 0.04% formaldehyde. When the desired intensity was attained, the developer was discarded and stopped with 1.46% (w/v) EDTA solution for 10 min. Finally, the gels were washed with deionized water and stored in the sealed plastic bags at 4°C. Protein patterns in the gels were recorded as digitalized images using a high-resolution scanner (GS-800 Calibrated Imaging Densitometer:Bio-Rad). Scanned images were analyzed using 2-DE program, Melanie III.

MALDI-TOF-MS and database search

Mass analysis was performed on a PerSeptive Biosystem Voyager-DE STR MALDI-TOF-MS (USA) in reflector mode. The spectrometer was run with the settings: accelerating voltage, 20 kV; grid voltage, 65%; and a DELAY, 100 NS. Half of one μl tryptic peptide extracts were dispensed on to a MALDI sample plate along with 0.5 μl of matrix solution consisting of 10 mg/ml α-cyano-4-hydroxycinnamic acid, 0.1% TFA and 50% acetonitrile. External peptide calibrants, angiotensin I (monoisotopic mass, 1296.6853), rennin substrate (1758.9331), ACTH (18-39) (2465.1989), were used for mass calibration. Spectra were internally calibrated using autolytic fragments from trypsin. Under conditions where the auto-digested fragments of trypsin were abundant, the monoisotopic peak *m/z* 1020.5030, *m/z* 2163.0564 and *m/z* 2273.1594 were used as mass calibrants. For each sample, the average of 18-20 spectra was acquired in the delayed extraction and reflector mode. Proteins were identified by peptide mass fingerprinting with search program ProFound (http://129.85.19.192/profound_bin/WebProFound.exe, Rockefeller University, Version 4.10.5). All mass searches were performed using a mass window between 0 and 100 kDa and included *Mus musculus*, *Rattus* and *Rodentia* sequence. The search parameters allowed for N-terminal acetylation, carboxyamidomethylation of cysteine. The criteria for positive identification of proteins were set as follows; (i) at least 4 matching peptide masses, (ii) 50 ppm or better mass accuracy, and (iii) molecular weight and pI of identified proteins should match estimated values obtained from images analysis.

Western blotting analysis

A total 30 ug of proteins run on 12% SDS-PAGE, and transferred onto nitrocellulose membranes (Amersham Biosciences,

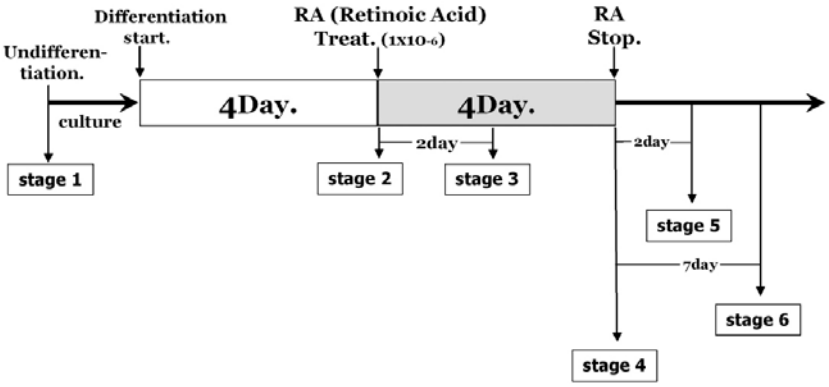

Fig. 1. Differentiation strategy of embryonic stem cell.

United Kingdom). After blocked with 5% skim milk/Tween 20/PBS for 1 h at room temperature, the membranes were incubated with the primary antibody; B-actin (mouse monoclonal IgG_1, Santa Cruz Biotechnology), Oct4 (Rabbit polyclonal-embryonic stem cell marker, abcam) (Ovitt and Scholer, 1998), Nanog (Rabbit polyclonal-embryonic stem cell marker, abcam) (Rodda et al., 2005), LDH (Rabbit polyclonal, Santa Cruz Biotechnology), EEF1D (Rabbit polyclonal, abcam), and Tcfap2c (Rabbit polyclonal, AVIVA systems biology) for 1 h at room temperature and then with either goat anti-mouse (Upstate) or goat anti-rabbit IgG (Upstate) as the secondary antibodies for 1 h at room temperature. Labled bands were detected by ECL plus western blotting detection system (Amersham Biosciences, United Kingdom), and images were captured. The intensity of the bands was quantitated with the Luminescent image analyzer (LAS-3000, Fusifilm life science). The relative expression of certain protein was determined by dividing the densitometric value of the test protein by that of the control B-actin.

RESULTS

Expression of marker for pluripotency in ES cells

ES cells were differentiated into neurons by 8 day-induction, the former mediated ATRA (Fig. 1). The *oct4* is acknowledged marker for pluripotency in ES cells and its expression lost upon differentiation. And *nanog*, the *oct4* target genes, has important roles in the proliferation of undifferentiated ES cells (Gajovic et al., 1998; Ovitt and Scholer, 1998). Real-time PCR and Western blotting data using Oct4 and Nanog showed that ES cells were maintained pluripotency (Fig. 2).

Expression of neuronal marker in neuronal differentiation of ES cells

Neurofilament is a kind of intermediated filaments and is composed of neurofilament proteins. These proteins are used as one of the neuronal markers generally, because it is only located in neurons. In order to confirm neuronal differentiation of ES cells, semi-quantitative RT-PCR was performed for detecting the expression of *neurofilament-medium* (NF-M), which is a subtype of *neurofilament*, as a milestone of neuronal phenotype. The gene expression of NF-M was not seen at undifferentiated state and induction periods, but its expression level was increased after induction by ATRA (Fig 3). And then, undifferentiation ES cells and neuronal differentiated cells were stained by another neuronal marker, neurofilament-light (NF-L). Undifferentiated ES cells tend to form colonies when they grow, but no neuron was found by immunochemistry. Otherwise, differentiated ES cells aggregate densely and undergo neuronal extension (Fig. 3). This result showed that ES cells were correctly differentiated into neurons with RA.

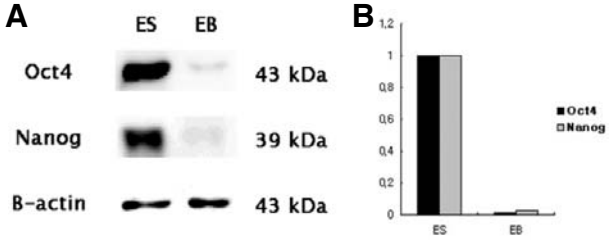

Fig. 2. The expression of marker for pluripotency by (A) Western blotting and (B) Real-time PCR. The expression levels of *oct4* and *nanog* were decreased during differentiation. ES; undifferentiated ES cells, EB; differentiated ES cells.

Proteomic analysis

The 2-DE gel system employed was IEF on 18 cm Immobile Dry Strips, pH ranges of 4-7, 4.5-5.5, and 5.5-6.7, in the first dimension and 9-16% linear-gradient SDS-PAGE in the second dimension using the total cell extracts from ES cells. Silver staining was used to detect proteins on the gels. Although it has some disadvantages, this method is one of the most sensitive and is compatible with MALDI-TOF mass spectrometer. More than 1,000 spots were detected on the two-dimensional gels. These data were analyzed two or more gels for each time point, and selected only those whose normalized volumes were similar for the two gels. Thirteen normalized protein spots that did not change with neuronal differentiation were selected for MALDI-TOF mass spectrometry analysis (data not shown) to provide a reference framework for comparison of molecular weights and pie, and theoretical values were calculated from amino acid sequences for each pH range. In most cases the theoretical and observed values were very similar.

Proteomic changes of neuronal differentiated ES cells pH 4-7

Approximately 350 ug of protein from the differentiated ES cells by time points were dehydrated using Immobilize Dry Strip pH 4-7. Silver staining was used to detect proteins on the gels. Thirty-six spots which were contained up-regulated, down-regulated and unchanged spots were detected by the Melanie III software with three different pH gradients. Among these spots, four proteins were identified. The *Galk1* and *Tubgct2* were up-regulated and *EEF1D* and *Ldh2* were down-regulated.

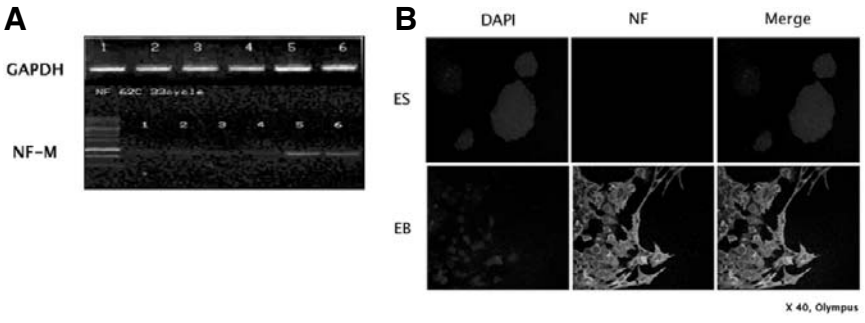

Fig. 3. (A) Semi-quantitative PCR and (B) Immunocytochemistry. To confirm whether ES cells were correctly differentiated into neurons with RA or not. Only neuronal markers were expressed in neuronal differentiated ES cell. Lane 1; undifferentiated, lane 2; 2 days after the neuronal induction with ATRA, lane 3; 2 days after the neuronal induction without ATRA, lane 4; 7 days after the neuronal induction with ATRA, lane 5; 7 days after the neuronal induction without ATRA. ES, undifferentiated ES cells; EB, differentiated ES cells; NF-M, Neurofilament-medium; DAPI, The blue fluorescent, nucleic acid stain; NF-L, Neurofilament-light.

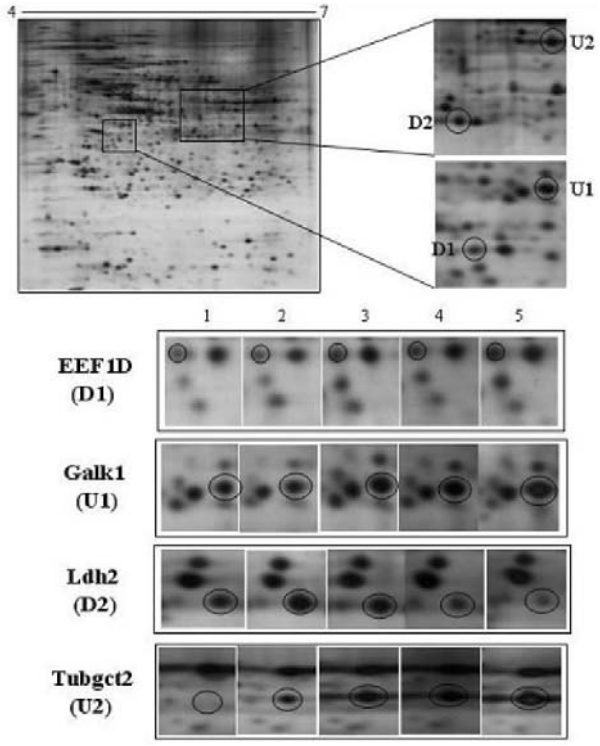

Fig. 4. Represent two-dimensional electrophoresis patterns of proteins from each stage in ES cells using Immobiline Drystrip pH 4-7. The same amount proteins were separated by 2-deminsional electrophoresis and stained with silver nitrate. U; up-regulated protein, D; down-regulated protein. Lane 1; stage 1, lane 2; stage 2, lane 3; stage 3, lane 4; stage 4, lane 5; stage 6.

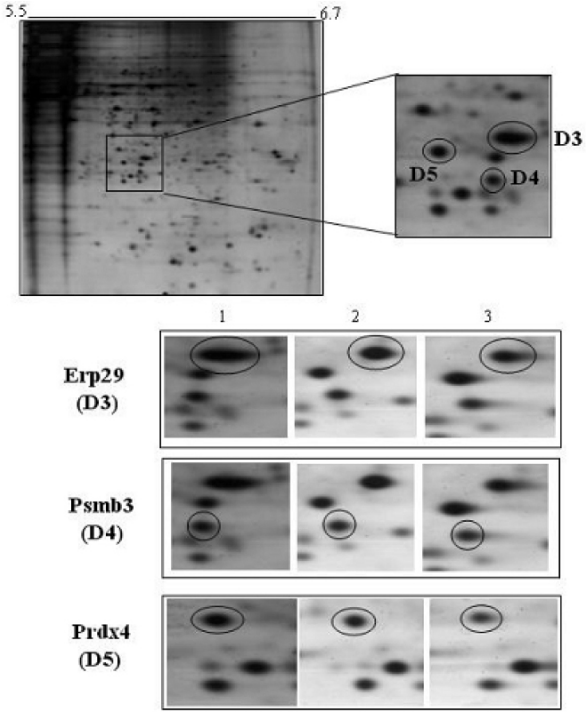

Fig. 5. Represent two-dimensional electrophoresis patterns of proteins from each stage in ES cells using Immobiline Drystrip pH 5.5-6.7. The same amount proteins were separated by 2-deminsional electrophoresis and stained with silver nitrate. U; up-regulated protein, D; down-regulated protein. Lane 1; stage 2, lane 2; stage4, lane 3; stage 5.

Otherwise, Tubgct2 was not expressed in undifferentiated state, but appeared since differentiation and was gradually increased. Increase of *Tubgct2* expression was caused by neuronal differentiation (Fig. 4).

Proteomic changes of neuronal differentiated ES cells pH 5.5-6.7

500 μg of protein from the differentiated ES cells by time points was dehydrated using Immobilize Dry strip pH 5.5-6.7. Total twenty spots were detected and selected by Melanie III software. Three differentially expressed proteins were identified. *Erp29*, *Psmb3* and *Prdx4* were down regulated (Fig. 5) Table 1 was the gene lists of changed proteins. Seven proteins were changed in differentiated ES cells. *Galk1*, a 42.79 kDa protein with pI 5.2 and *Tubgct2*, a 104 kDa protein with pI 6.3 were gradually increased. *EEF1D*, a 31.42 kDa protein with pI 4.9 and *Erp29*, a 28.88 kDa protein with pI 5.9 and *Psmb3*, 22.95 kDa with pI 6.1. Last one, *Prdv4*, which is a 31.03 kDa with pI 6.7 was decreased.

Confirmation of Protein expression patterns depends on neuronal differentiated ES cells

In order to confirm a change in protein expression pattern from undifferentiated to differentiated ES cells, proteins were collected and analyzed from both undifferentiated and neuronal differentiated ES cells. Among them, the expression level of *EEF1D* and *Ldh2* protein in neuronal differentiation was identi-

Table 1. Selected proteins in ES cells of each stage

	Accession number	Protein name	Seq. cov	pI	Mass
D1	gi|12963597	Eukaryotic translation elongation factor 1 delta [Mus musculus] (EEF1D)	31	4.9	31.42
U1	gi|29747831	Galactokinase 1 (Galk1)	16	5.2	42.79
D2	gi|6678674	Lactate dehydrogenase 2, B chain [Mus musculus] (Ldh2)	18	5.7	36.91
U2	gi|19526968	Tubulin, gamma complex associated protein 2 [Mus musculus] (Tubgct2)	15	6.3	104
D3	gi|19526463	Endoplasmic reticulum protein ERp29 precursor [Mus musculus] (Erp29)	24	5.9	28.88
D4	gi|6755202	Proteasome beta 3 subunit [Mus musculus] (Psmb3)	26	6.1	22.95
D5	gi|7948999	Peroxiredoxin 4; Prx IV [Mus musculus] (Prdx4)	28	6.7	31.03

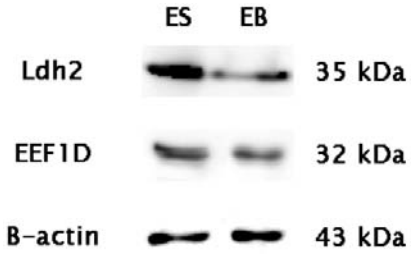

Fig. 6. Western blot analysis of ES cells. In other words, expression of *LDH* and *EEF1D* are decreased in EB. B-actin used as a control. Molecular weights (MW) of each protein are indicated.

fied by proteomic analysis and Western blot hybridization, as a down-regulated in differentiated ES cells. (Fig. 6)

DISCUSSION

The development of eukaryotic organism represents a complex interplay of numerous proliferation and differentiation events that proceeds in a highly ordered manner. In the CNS development, firstly most of the cells proliferate rapidly as the shape of neuroepithelium. These cells finally differentiate into neurons, astrocytes and oligodendrocytes at the late stage of development. The cell signaling pathways are pivotal in the specific set of the mechanism of neuronal differentiation as well as other cell fates. Recently, many researchers have studied these early mechanism using pluripotent cells (O'Shea, 1999). ES cells are developmentally pluripotent cells isolated from early embryos. ES cells can be spontaneously differentiated into multiple somatic cell types in response to some stimuli. The study of differentiation of the pluripotent ES cells may provide some clues for solving the mechanism that regulates the development and differentiation of ES cells *in vivo*. Differentiation of mouse ES cells can lead to search development, so will be able to apply to human ES cell research. Especially, ES cells are powerful tools for the studies of human CNS, because ES cells induced by ATRA after EBs formation give rise to neurons. To provide some clues for the signaling network of the neuronal differentiation, the expression profile changes were observed in the level of protein. It has recently been shown that high-resolution 2-DE is a useful technique for separating complex protein mixtures present in various tissues and cells. At the individual protein level was analyzed proteins with various MW ranges and pIs from 4.0 to 7.0 (pH 4-7, pH 4.5-5.5 and pH 5.5-6.7). By this reason, proteomic analysis was performed for functional studies. Proteomic technology provides more comprehensive method to identify neuronal differentiate processes. As a result, selected protein will be related to differentiate into neuronal cell. The first, *ERp29*, *Tubgct2, Galk1, Ldh2, Prdx4, Idh3a* and *EEF1D* were expressed in all the cell types in common. *ERp29* protein is present in all mammalian tissue with exceptionally high levels of expression in the secretory cells (Baryshev et al., 2006). Tubgct2 is cytoskeleton which maintains shape of cells and related to movement. *Galk1, Ldh2, Prdx4* and *Idh3a* participate in metabolism of cells (Poon et al., 2006; Timson and Reece, 2003). The genes related to metabolism except *Galk1*, were decreased expression levels of proteins by time points. The function of *EEF1D* is signal transduction, movement of signals from outside of the cell to inside. Until now, the study of these genes related to neuronal differentiation or ES cell differentiation to the neurons is still lack. But we are expecting to find out novel mechanisms associated with neural differentiation through studying of these unclear genes.

Embryonic stem (ES) cells have capacity that is differentiated into all cell types. If ES cells are used, the development mechanism will be identified. Especially the embryonic development of such CNS which is the core among all organs is regulated by specific genes. The study of ES cell-mediated neuronal differentiation allows elucidating the mechanism of neuronal development in spite of the complexity and the difficult accessibility. Recent research into the regeneration mechanism of the CNS has focused on the differentiation of ES cells into neurons and neuronal supporting cells. It has raised hopes that we can find ways to actually repair the damage in the nervous system.

In this paper, certain proteins were extensively investigated the details of an expression profiling in the level of protein using and proteomic techniques during the differentiation of ES cells into neuronal cell. These cells were analyzed changes in proteome during the differentiation of ES cells by 2-dimensional electrophoresis (2-DE) and the identification using MALDI-TOF MS. In conclusion, a complex system of neuronal differentiation can be activated in cultured ES cells and 2-DE data should be favorable for investigating some of the mechanism that regulates neuronal differentiation.

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