**[Full Paper]**

# The protein expression profile of cynomolgus monkey embryonic stem cells in two-dimensional gel electrophoresis: a successful identification of multiple proteins using human databases

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## SUMMARY

Global gene and protein expression analyses have had great impacts on scientific progresses in this new era of bioinformatics. Although studies using murine and human materials can fully exploit the large volume of their databases, there are quite a few inconveniences for an investigation on non-human primate materials due to still insufficient data collections. Here we examined the availability of human databases for the protein identification process using the two-dimensional electrophoresis-based proteomic study in cynomolgus monkey embryonic stem (ES) cells. Querying public human protein databases, we successfully identified multiple protein spots via mass spectrometric analysis using MALDI-TOF apparatus. The results of the protein identification were confirmed by western blotting using polyclonal antibodies raised against human epitopes. Interestingly, the results of western blotting further identified the existence of previously unreported multiple isoforms of common proteins including glycolytic pathway enzymes. Thus, combined analyses of the mass spectrometry querying the *Homo sapience* databases and the western blotting using polyclonal antibodies is highly effective in determining protein expressions in monkey cells. Our success in obtaining a draft protein expression profile of cynomolgus monkey ES cells will contribute to the promotion of non-human primate ES cell researches.

Key words: cynomolgus monkey, embryonic stem cells, proteomics, two-dimensional electrophoresis, mass spectrometric analysis.

## INTRODUCTION

Recent years, researches on non-human primates are of growing importance in the fields of life science. It has been emphasized that animal studies with clinical concerns such as a toxicological study of environmental factors and safety evaluation of newly invented drugs should be performed using primate, but not rodent, models. Although studies using rodents are feasible in technical and economical points of view, they do not always provide sufficient informations applicable to human cases. The differences in metabolism and tissue sensitivity of the drug between

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Abbreviations: ES, embryonic stem; 2-DE, two-dimensional gel electrophoresis; MEF, murine embryonic fibroblast; MMC, mitomycin C; SDS, sodium dodecyl sulphate; PMF, peptide mass fingerprinting; HSP60, 60-kDa heat shock protein; HSC70, heat shock cognate 71-kDa protein; TIM, triosephosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; VDAC-1, voltage-dependent anion-selective channel protein 1; PKM2, pyruvate kinase isozyme M2; PGK1, phosphoglycerate kinase 1.

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rodents and primates are not negligible. In addition, the short lifetime of rodents cannot afford the longtime, chronic effect of drugs. The primate study is also essential to understand the molecular mechanism of diseases. An integrated study on genome, transcriptome or proteome, which has become a powerful tool to elucidate the complicated mechanisms of human multi-factorial diseases, should better be performed using primate, but not rodent, models. Indeed, growing numbers of pedigrees of cynomolgus monkeys that provide excellent models for human diseases have been prepared[1, 2].

In addition to the living individuals, cell lines established from monkey tissues play beneficial roles. For example, embryonic stem (ES) cells, which are a valuable resource in regenerative medicine because of their high capacity to differentiate into a broad range of cell types, have particularly large impacts. It is known that primate ES cells show different characteristics from murine ES cells: they have different extracellular and intracellular signaling pathways for the maintenance of pluripotency[3] and distinctive differentiation capacities[4]. Thus, the promotion of researches on primate ES cells is now becoming exceedingly important. Although studies using human ES cells are essential for clinical application, basic researches using monkey ES cells still have importance because they can provide good allotransplantation models required for pre-clinical studies[5, 6]. Moreover, ethical regulation that is heavily imposed on the usage of human ES cells is remissive concerning the use of monkey ES cells. Thus biotechnological manipulation, including gene transfer, can immediately be applied to the usage of monkey ES cells, which will contribute to the further advance in our understanding of human ES cells.

Despite an increasing requirement to promote the monkey ES cell study, construction of integrated bioinformatics on non-human primates is still underway, and only a small volume of individually collected data is available at present. As a result, we often experience difficulties in constructing monkey polymerase chain reaction primers and the primers designed from human databases do not necessarily work in monkey samples. As compared with the informations on genes or messages, data on proteins are rather compressed due to three-to-one correspondence, where a train of three nucleotides corresponds to one amino acid and the last nucleotide in each train has a large redundancy. In addition, degrees of freedom in amino acid sequence are lower than those in nucleotide sequences due to functional requirement of proteins. Thus, in contrast to the genomic and transcriptomic studies, proteomic analysis of monkey samples might be effectively achieved querying human databases.

In the present study, we successfully determined a protein expression profile using the two-dimensional gel electrophoresis (2-DE) and human proteome databases in undifferentiated cynomolgus monkey ES cells. Our results will encourage the promotion of the monkey proteome study in the present situation, without waiting for the future accomplishment of the data construction of monkey bioinformatics.

## MATERIALS AND METHODS

### 1. Cells culture

Murine embryonic fibroblasts (MEFs), which had been treated with Dulbecco's modified Eagle's medium containing mitomycin C (MMC) for 3 hours, were seeded on the dishes coated with 0.1% gelatin. Cynomolgus monkey ES cells[7] were maintained on MMC-treated MEF-coated dishes in DMEM/F12 medium supplemented with 20% heat inactivated fetal bovine serum, 8 ng/ml fibroblast growth factor 2, 10 ng/ml recombinant human bone morphogenic protein 4, 1 mM β-mercaptoethanol, 1 mM L-glutamine, 10 U/ml penicillin and 10 μg/ml streptomycin. Monkey ES cells were passaged every 2 days using 0.25% trypsin treatment for one minutes and were seeded at split ratios of 1:2 to 1:4 on new MEF-coated dishes. For the collection of ES cells for 2-DE, ES cells were detached by 0.2% EDTA treatment to avoid the contamination of MEFs.

### 2. Two-dimensional gel electrophoresis (2-DE)

ES cells were collected by 0.2% EDTA treatment. After washing the cells with washing buffer (10 mM Tris-HCl buffer, pH 8.0, 5 mM magnesium acetate), $4\times10^7$ cells were suspended with 7 volumes of lysis buffer containing 2 M thiourea, 7 M urea, 4% (w/v) CHAPS and 1 mM Pefablc SC PLUS (Roche Diagnostics GmbH, Mannheim, Germany). The cell suspensions were kept for 10 minutes on ice, sonicated intermittently and centrifuged at 12,000 g for 10 minutes at 4°C, and then the supernatant fractions were collected. The protein concentration was determined in the lysis solution with a dye reagent from Amersham Biosciences using bovine serum albumin as a standard. The lysate was alkylated with Ready Prep™ Reduction-Alkylation Kit (Bio Rad Laboratories, Hercules, CA). The 120 μg protein lysate per gel were subjected to 2-DE. The first-dimensional isoelectric focusing was carried out using Immobiline dry strip (18-cm long, pH 3–10 non-linear or pH 6–11 linear) in a horizontal electrophoresis system, Ettan IPGphor (Amersham Biosciences) according to the manufacturer's instructions. After the first dimensional elecrofocusing, IPG gels were equilibrated with buffer containing 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulphate (SDS), 0.01% BPB and 0.5% dithiothreitol, followed by alkylation with equilibration buffer containing 4.5% idoacetamide instead of 0.5% dithiothreitol at room temperature for 15 minutes. The gels were subjected to the second-dimensional SDS polyacrylamide gel electrophoresis (10% SDS). Proteins were visualized in the gels by staining with SYPRO Ruby Protein Gel Stain (Bio Rad) for overnight. The florescence intensity of each protein spot was digitally recorded by

FluorImager 595 using Image QuaNT software and the protein expression was analyzed by PDQuest software.

### 3. Mass spectrometric analysis

The mass spectrometric analysis was performed according to the method reported previously[8] with minor modifications. Briefly, each protein spot in SYPRO Ruby-stained gels was picked by FluoroPhoreStar 3000 (Anatech, Tokyo, Japan). The pieces of gels were dehydrated in 50% acetonitrile and 50% ammonium bicarbonate, and then in 100% acetonitrile, and dried up. The proteins were digested with 5 μg/ml trypsin at 30°C. After the overnight protein digestion, peptide fragments in the digest were subjected to MALDI-TOF mass spectrometer (AXIMA-CFR, Shimazdu Corp., Kyoto, Japan) for peptide mass fingerprinting (PMF). Protein identification process was accomplished by a two-tiered approach using Mascot server (Matrix Science Ltd., Franklin St., Boston, MA) for selection of protein candidates and then using Protein Prospector (UCSF Mass Spectrometry Facility, San Francisco, CA) for its verification. In the former, molecular weights and pI values were taken into account as well as % coverage values during candidate protein selection. In the latter, verification was performed using MS-Digest software under a criterion that more than eight m/z values were detected in major peaks of PMF. The activated parameters used in Mascot server query were as follows: primate database of SWISS-PROT and NCBInr, peptide tolerance ±0.4 Da or ±1.0 Da, one missed cleavage and carbamidomethyl modification of cysteine. During MS-Digest software query, acetylation of N-terminal end or lysine and phosphorylation of serine, threonine or tyrosine was considered. Protein identification was repeated at least once with spots from different gels.

### 4. Two-dimensional Western blotting

The SYPRO Ruby-stained proteins on gels were resolubilized and transferred according to our previously reported method[9]. Briefly, the stained gel was incubated in resolubilization buffer (0.2% w/v SDS, 0.3% w/v Tris, 0.7% w/v glycine) for 10 minutes and mounted onto a PVDF membrane in a semi-dry blotting apparatus (Bio Rad). Electrotransfer was carried out at 4 V/cm$^2$ for one hour at room temperature using buffer containing 0.3% (w/v) Tris, 1.5% (w/v) glycine, 0.1% (w/v) SDS. The fluorescence images of the blotted PVDF membranes were scanned and recorded by FluorImager 595. The PVDF membranes were further subjected to the immunoblotting using polyclonal antibodies against 60-kDa heat shock protein (HSP60), annexin A5, heat shock cognate 71-kDa protein (HSC70), triosephosphate isomerase (TIM), 14-3-3 proteins, α-enolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology Inc.), annexin A2 (Abnova Corp., Taipei, Taiwan), voltage-dependent anion-selective channel protein 1(VDAC-1) (Abcam plc., Cambridge, UK), pyruvate kinase isozyme M2 (PKM2), phosphoglycerate kinase 1 (PGK1), serine/threonine-protein kinase 13 (Aurora-C) (Abgent Inc. San Diego, CA), nucleolin, thioredoxin reductase and GTP-binding nuclear protein Ran (Santa Cruz Biotechnology).

## RESULTS AND DISCUSSION

While the routine cell passage procedure was performed detaching ES cells by trypsinization, we collected the ES cells by EDTA treatment to prepare 2-DE samples to exclude the contamination by MEFs. The typical 2-DE protein expression patterns of "ES cells" using strips of pH 3–10 and pH 6–11 were shown in Figs. 1A and 1B, respec-

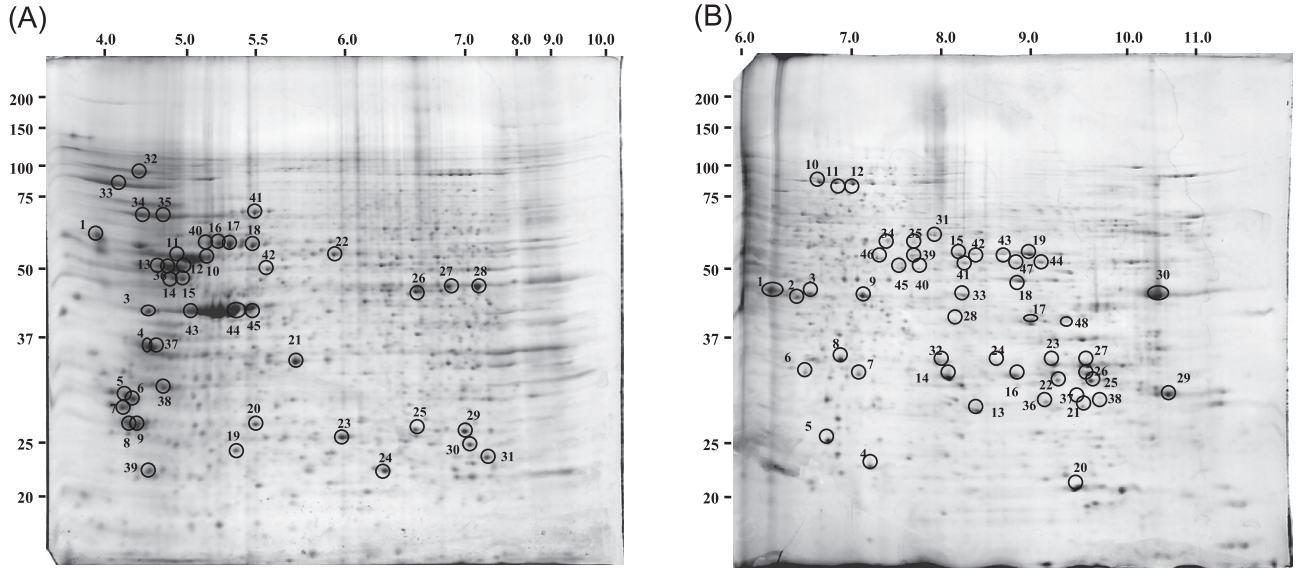


Fig. 1. Protein expression patterns of cynomolgus monkey ES cells in 2-DE.

The SYPRO Ruby staining patterns in the 2-DE using a strip of pH 3–10 non-linear (A) and a strip of pH 6–11 (B) in the first-dimensional isoelectric focusing. A typical result from four independent experiments for each was shown.

Table 1. Identification of the proteins detected in 2-DE gel using a pH 3-10 strip

| # | protein name | ID method | mass tolerance | % Coverage | Acc No | Funtion (as listed in Swiss-Prot) |
|---|---|---|---|---|---|---|
| 1 | calreticulin | MALDI | 1 | 21 | P27797 | calcium binding chaperone |
| 2 | 14-3-3 protein ζ | WB | | | P63104 | Adapter protein |
| 3 | 40S ribosomal protein SA | MALDI | 0.4 | 18 | P08865 | unknown |
| 4 | nucleophosmin | MALDI | 0.4 | 11 | P06748 | assembly and/or transport of ribosome. |
| 5 | tropomyosin-4 | MALDI, WB | 0.4 | 11 | P67936 | stabilizing cytoskeleton actin filaments |
| 6 | tropomyosin α-3 | MALDI, WB | 1 | 33 | P06753 | stabilizing cytoskeleton actin filaments |
| 7 | 14-3-3 protein ε | MALDI, WB | 0.4 | 24 | P62258 | Adapter protein |
| 8 | 14-3-3 protein ζ | MALDI, WB | 0.4 | 22 | P63104 | Adapter protein |
| 9 | 14-3-3 protein ζ | MALDI, WB | 0.4 | 22 | P63104 | Adapter protein |
| 10 | tublin α ubiquitous | MALDI | 0.4 | 29 | P68363 | major constituent of microtubules |
| 11 | vimentin | MALDI | 1 | 17 | P08670 | class-III intermediate filaments |
| 12 | tublin β-2 chain | MALDI | 0.4 | 11 | P07437 | the major constituent of microtubules |
| 13 | zinc-finger FYVE domain-containing protein 19 | MALDI | 1 | 10 | Q96K21 | unknown |
| 14 | ATP synthase β chain | MALDI | 0.4 | 12 | P06576 | Produces ATP from ADP |
| 15 | ATP synthase β chain | MALDI | 0.4 | 12 | P06576 | Produces ATP from ADP |
| 16 | 60-kDa heat shock protein (HSP60) | MALDI, WB | 0.4 | 13 | P10809 | mitochondrial protein import and macromolecular assembly |
| 17 | 60-kDa heat shock protein (HSP60) | MALDI, WB | 0.4 | 13 | P10809 | mitochondrial protein import and macromolecular assembly |
| 18 | T-complex protein 1 subunit ε | MALDI | 0.4 | 20 | P48643 | Molecular chaperone |
| 19 | ubiquitin carbonyl-terminal hydrolase isozyme | MALDI | 1 | 15 | P09936 | Ubiquitin-protein hydrolase |
| 20 | prohibitin | MALDI | 1 | 28 | P35232 | DNA synthesis inhibitor |
| 21 | L-lactate dehydrogenase B chain | MALDI | 0.4 | 23 | P07195 | CATALYTIC ACTIVITY: (S)-lactate+NAD+=pyruvate+NADH. |
| 22 | protein disulfide-isomerase A3 precursor | MALDI | 0.4 | 22 | P30101 | Catalyzes the rearrangement of -S-S- bonds |
| 23 | peroxiredoxin 6 | MALDI | 0.4 | 21 | P30041 | redox regulation of the cell |
| 24 | glutathione S-transferase π | MALDI | 0.4 | 48 | P09211 | Conjugation of reduced glutathione |
| 25 | phosphoglycerate mutase | MALDI | 0.4 | 20 | P18669 | Interconversion of 3- and 2-phosphoglycerate with 2,3-bisphosphoglycerate |
| 26 | elongation factor 1-γ | MALDI | 0.4 | 13 | P26641 | Probably plays a role in anchoring the complex to other cellular components |
| 27 | α-enolase | MALDI, WB | 0.4 | 29 | P06733 | Multifunctional enzyme that, as well as its role in glycolysis |
| 28 | α-enolase | MALDI, WB | 0.4 | 50 | P06733 | Multifunctional enzyme that, as well as its role in glycolysis |
| 29 | phosphoglycerate mutase | MALDI | 0.4 | 30 | P18669 | Interconversion of 3- and 2-phosphoglycerate with 2,3-bisphosphoglycerate |
| 30 | triosephosphate isomerase | MALDI, WB | 0.4 | 29 | P60174 | CATALYTIC ACTIVITY: D-glyceraldehyde 3-phosphate=glycerone phosphate |
| 31 | GTP-binding nuclear protein Ran | MALDI, WB | 1 | 37 | P62826 | GTP-binding protein involved in nucleocytoplasmic transport |
| 32 | nucleolin | MALDI, WB | 0.4 | 13 | P19338 | It induces chromatin decondensation by binding to histone H1 |
| 33 | thioredoxin reductase | MALDI, WB | 1 | 16 | Q16881 | CATALYTIC ACTIVITY: thioredoxin+NADP+=thioredoxin disulfide+NADPH |
| 34 | heat shock cognate 71-kDa protein (HSC70) | MALDI, WB | 0.4 | 17 | P11142 | Chaperone |
| 35 | heat shock cognate 71-kDa protein (HSC70) | MALDI, WB | 0.4 | 17 | P11142 | Chaperone |
| 36 | tublin β-2 chain | MALDI | 0.4 | 17 | P07437 | the major constituent of microtubules |
| 37 | annexin A5 | MALDI, WB | 1 | 24 | P08758 | an anticoagulant protein |
| 38 | annexin A5 | MALDI, WB | 1 | 24 | P08758 | an anticoagulant protein |
| 39 | annexin A5 | MALDI, WB | 1 | 24 | P08758 | an anticoagulant protein |
| 40 | 60-kDa heat shock protein (HSP60) | MALDI, WB | 0.4 | 13 | P10809 | mitochondrial protein import and macromolecular assembly |
| 41 | zinc-finger protein 192 | MALDI | 1 | 6 | Q15776 | May be involved in transcriptional regulation |
| 42 | pigment epithelium-derived factor | MALDI | 1 | 14 | P36955 | Neurotrophic protein |
| 43 | actin cytoplasmic 1 | MALDI | 0.4 | 19 | P60709 | involved in various types of cell motility |
| 44 | actin cytoplasmic 1 | MALDI | 0.4 | 19 | P60709 | involved in various types of cell motility |
| 45 | actin cytoplasmic 1 | MALDI | 0.4 | 19 | P60709 | involved in various types of cell motility |

The protein name listed in UniProt (SwissProt), identification method (ID method), mass tolerance used as activated parameter during MASCOT sever query, percent of the coverage (% coverage), accession number of the protein (Acc No), and function of the protein listed in UniProt (SwissProt) were shown.

Table 2. Identification of the proteins detected in 2-DE gel using a pH 6–11 strip

| # | protein name | ID method | mass tolerance | % Coverage | Acc No | Funtion (as listed in Swiss-Prot) |
|---|---|---|---|---|---|---|
| 1 | α-enolase | WB | | | P06733 | Multifunctional enzyme that, as well as its role in glycolysis |
| 2 | α-enolase | WB | | | P06733 | Multifunctional enzyme that, as well as its role in glycolysis |
| 3 | α-enolase | MALDI, WB | 0.4 | 23 | P06733 | Multifunctional enzyme that, as well as its role in glycolysis |
| 4 | GTP-binding nuclear protein Ran | MALDI, WB | 0.4 | 28 | P62826 | GTP-binding protein involved in nucleocytoplasmic transport |
| 5 | phosphoglycerate mutase | MALDI, WB | 0.4 | 30 | P18669 | Interconversion of 3- and 2-phosphoglycerate with 2,3-bisphosphoglycerate |
| 6 | annexin A2 | WB | | | P07355 | Calcium-regulated membrane-binding protein |
| 7 | annexin A2 | WB | | | P07355 | Calcium-regulated membrane-binding protein |
| 8 | septin 9 | MALDI | 0.4 | 18 | Q9UHD8 | Involved in cytokinesis |
| 9 | α-enolase | MALDI, WB | 0.4 | 23 | P06733 | Multifunctional enzyme that, as well as its role in glycolysis |
| 10 | elongation factor 2 | MALDI | 0.4 | 13 | P13639 | promotes GTP-dependent translocation |
| 11 | elongation factor 2 | MALDI | 0.4 | 13 | P13639 | promotes GTP-dependent translocation |
| 12 | elongation factor 2 | MALDI | 0.4 | 13 | P13639 | promotes GTP-dependent translocation |
| 13 | guanine mucleotide-binding protein β-subunit 2-like 1 | MALDI | 0.4 | 11 | P63244 | an intracellular receptor to anchor the activated PKC to the cytoskeleton |
| 14 | annexin A2 | MALDI, WB | 0.4 | 38 | P07355 | Calcium-regulated membrane-binding protein |
| 15 | pyruvate kinase isozyme M2 (PKM2) | MALDI, WB | 1 | 16 | P14618 | CATALYTIC ACTIVITY: ATP+pyruvate=ADP+phosphoenolpyruvate. |
| 16 | annexin A2 | MALDI, WB | 0.4 | 38 | P07355 | Calcium-regulated membrane-binding protein |
| 17 | phosphoglycerate kinase 1 (PGK1) | MALDI, WB | 1 | 33 | P00558 | glycolytic enzyme, polymerase alpha cofactor protein |
| 18 | ATP synthase alpha chain, mitochondrial precursor | MALDI | 1 | 14 | P25705 | Produces ATP from ADP in the presence of a proton gradient across the membrane |
| 19 | pyruvate kinase isozyme M2 (PKM2) | MALDI, WB | 1 | 23 | P14618 | CATALYTIC ACTIVITY: ATP+pyruvate=ADP+phosphoenolpyruvate. |
| 20 | peroxiredoxin-1 | MALDI | 0.4 | 25 | Q06830 | Involved in redox regulation of the cell |
| 21 | voltage-dependent anion-selective channel protein 1 (VDAC-1) | MALDI, WB | 1 | 33 | P21796 | Forms a channel through the mitochondrial outer membrane and also the plasma membrane. |
| 22 | transcription elongation factor A protein 2 | MALDI | 1 | 18 | Q15560 | Necessary for efficient RNA polymerase II transcription elongation |
| 23 | glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | WB | | | P04406 | CATALYTIC ACTIVITY: D-glyceraldehyde 3-phosphate+phosphate+$NAD^+$=3-phospho-D-glyceroyl phosphate+NADH. |
| 24 | glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | WB | | | P04406 | CATALYTIC ACTIVITY: D-glyceraldehyde 3-phosphate+phosphate+$NAD^+$=3-phospho-D-glyceroyl phosphate+NADH. |
| 25 | paired box protein pax-8 | MALDI | 0.4 | 17 | Q06710 | Transcription factor for the thyroid-specific expression of the genes |
| 26 | annexin A8 | MALDI | 1 | 22 | P13928 | an anticoagulant protein |
| 27 | glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | MALDI | 0.4 | 16 | P04406 | CATALYTIC ACTIVITY: D-glyceraldehyde 3-phosphate+phosphate+$NAD^+$=3-phospho-D-glyceroyl phosphate+NADH. |
| 28 | phosphoglycerate kinase 1 (PGK1) | WB | | | P00558 | glycolytic enzyme, polymerase alpha cofactor protein |
| 29 | heterogeneous nuclear, ribonucleoprotein A1 (herix-destabilizing protein) | MALDI | 0.4 | 30 | P09651 | Involved in the packaging of pre-mRNA into hnRNP particles, transport of poly(A) mRNA |
| 30 | elongation factor 1-α 1 | MALDI | 0.4 | 18 | P68104 | promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes |
| 31 | transketolase | MALDI | 0.4 | 8 | P29401 | CATALYTIC ACTIVITY: Sedoheptulose 7-phosphate+D-glyceraldehyde 3-phosphate=D-ribose 5-phosphate+D-xylulose 5-phosphate. |
| 32 | glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | WB | | | P04406 | CATALYTIC ACTIVITY: D-glyceraldehyde 3-phosphate+phosphate+$NAD^+$=3-phospho-D-glyceroyl phosphate+NADH. |
| 33 | α-enolase | WB | | | P06733 | Multifunctional enzyme that, as well as its role in glycolysis |
| 34 | serine/threonine-protein kinase 13 (Aurora-C) | MALDI, WB | 1 | 24 | Q9UQB9 | May play a part in organizing microtubules during mitosis |
| 35 | serine/threonine-protein kinase 13 (Aurora-C) | WB | | | Q9UQB9 | May play a part in organizing microtubules during mitosis |
| 36 | voltage-dependent anion-selective channel protein 1 (VDAC-1) | WB | | | P21796 | Forms a channel through the mitochondrial outer membrane and also the plasma membrane. |
| 37 | voltage-dependent anion-selective channel protein 1 (VDAC-1) | WB | | | P21796 | Forms a channel through the mitochondrial outer membrane and also the plasma membrane. |
| 38 | voltage-dependent anion-selective channel protein 1 (VDAC-1) | WB | | | P21796 | Forms a channel through the mitochondrial outer membrane and also the plasma membrane. |
| 39 | pyruvate kinase isozyme M2 (PKM2) | WB | | | P14618 | CATALYTIC ACTIVITY: ATP+pyruvate=ADP+phosphoenolpyruvate. |
| 40 | pyruvate kinase isozyme M2 (PKM2) | WB | | | P14618 | CATALYTIC ACTIVITY: ATP+pyruvate=ADP+phosphoenolpyruvate. |
| 41 | pyruvate kinase isozyme M2 (PKM2) | WB | | | P14618 | CATALYTIC ACTIVITY: ATP+pyruvate=ADP+phosphoenolpyruvate. |
| 42 | pyruvate kinase isozyme M2 (PKM2) | WB | | | P14618 | CATALYTIC ACTIVITY: ATP+pyruvate=ADP+phosphoenolpyruvate. |
| 43 | pyruvate kinase isozyme M2 (PKM2) | WB | | | P14618 | CATALYTIC ACTIVITY: ATP+pyruvate=ADP+phosphoenolpyruvate. |
| 44 | pyruvate kinase isozyme M2 (PKM2) | WB | | | P14618 | CATALYTIC ACTIVITY: ATP+pyruvate=ADP+phosphoenolpyruvate. |
| 45 | pyruvate kinase isozyme M2 (PKM2) | WB | | | P14618 | CATALYTIC ACTIVITY: ATP+pyruvate=ADP+phosphoenolpyruvate. |
| 46 | pyruvate kinase isozyme M2 (PKM2) | WB | | | P14618 | CATALYTIC ACTIVITY: ATP+pyruvate=ADP+phosphoenolpyruvate. |
| 47 | pyruvate kinase isozyme M2 (PKM2) | WB | | | P14618 | CATALYTIC ACTIVITY: ATP+pyruvate=ADP+phosphoenolpyruvate. |
| 48 | phosphoglycerate kinase 1 (PGK1) | WB | | | P00558 | glycolytic enzyme, polymerase alpha cofactor protein |

The protein name listed in UniProt (SwissProt), identification method (ID method), mass tolerance used as activated parameter during MASCOT sever query, percent of the coverage (% coverage), accession number of the protein (Acc No), and function of the protein listed in UniProt (SwissProt) were shown.

tively. Protein spots clearly visualized by SYPRO Ruby staining were picked from the gels and subjected to the mass spectrometric analysis using a MALDI-TOF apparatus. Table 1 and Table 2 show the summary of the results. We could identify totally 45 and 48 protein spots in 2-DEs using pH 3–10 and pH 6–11 strips, respectively. These proteins were either components of cytoskeleton (tropomyosin-4, β-tubulin, vimentin, β-actin), enzymes involved in energy regulation (ATP synthase, L-lactate dehydrogenase, α-enolase, transketolase, phosphoglycerate mutase, TIM, GAPDH, PKM2, PGK1), proteins involved in redox regulation (peroxiredoxin, glutathione S-transferase, thioredoxin reductase), chaperones (T-complex protein, heat shock proteins, calreticulin precursor), components of the translation machinery (elongation factors 1 and 2), regulators of dynamisms in nuclear events (prohibitin, nucleolin, GTP-binding nuclear protein Ran, Aurora-C), apoptosis-related proteins (VDAC-1, annexin A5) or adaptor proteins (14-3-3 ε and 14-3-3 ζ). These proteins are major actors required for cell survival in general. An exception is pigment epithelium-derived factor, a neurotrophic factor that induces extensive neuronal differentiation in retinoblastoma cells. Its expression in undifferentiated ES cells might explain, at least in part, the well-known characteristics of mammalian ES cells that they prone to undergo neuronal differentiation when signals required for the maintenance of undifferentiated states are eliminated.

We next performed the two-dimensional western blotting to confirm the results of mass spectrometry using commercially available polyclonal antibodies having wide cross-reactivity concerning HSP60, Annexin A5, HSC70, TIM, 14-3-3 ε and 14-3-3 ζ, α-enolase, GAPDH, annexin A2, VDAC-1, PKM2, PGK1 and Aurora-C. As shown in Fig. 2 and Fig. 3, each protein spot was eventually recognized by the corresponding antibody, proving the validity of the MALDI-TOF-based protein identification process using human databases. On the other hand, usage of non-human primate databases, in which cynomolgus monkey's data are included, provided no better information. They seldom gave us candidate proteins or only gave the same candidate as

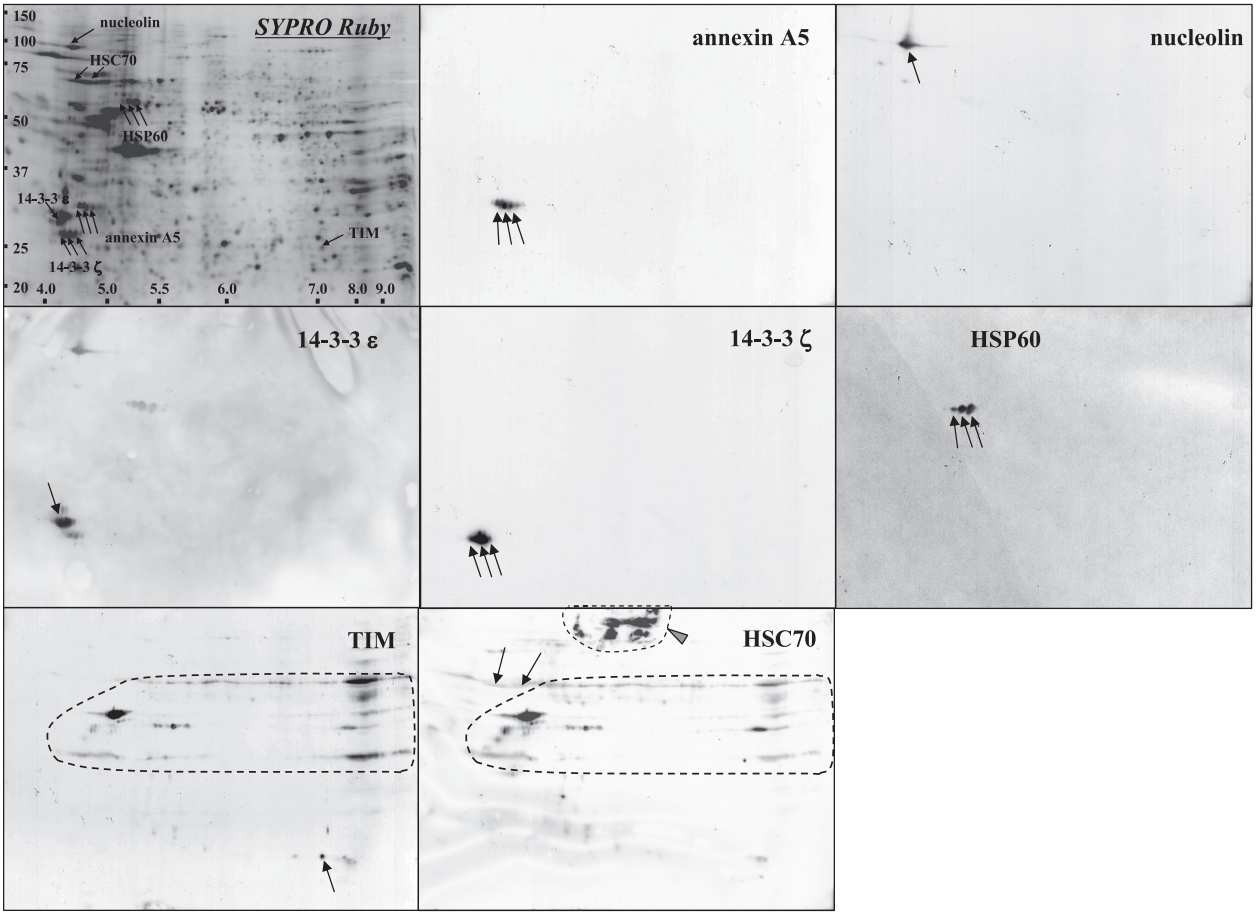


Fig. 2. Two dimensional western blotting after a pH 3–10 strip-using 2-DE gel.

The 2-DE gel was trimmed (an upper left panel) and transferred to the PVDF membrane. Western blotting was performed using indicated polyclonal antibodies, which are shown to have broad cross-reactivity among human, mouse and rat by the manufacturer. The PVDF membrane was re-used after stripping the previously used antibody. The multiple spots and slurs in an area surrounded by a dotted line in TIM and HSC70 antibody reactions are the background spots created during the anti-TIM antibody reaction. The irregular marks in the area shown by a dotted line with a gray arrowhead in anti-HSC70 antibody reaction are non-specific stains created during this antibody reaction.

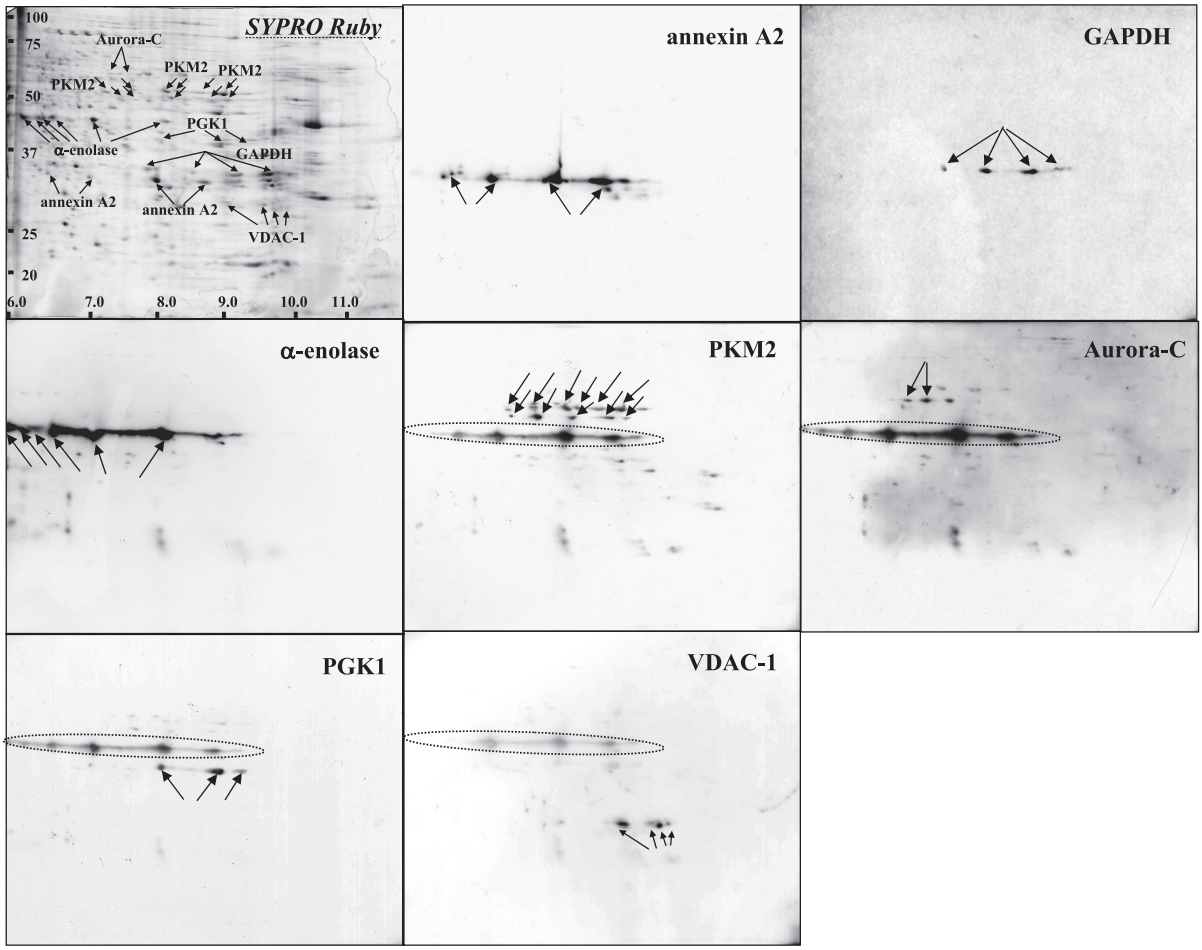


Fig. 3. Two dimensional western blotting after a pH 6–11 strip-using 2-DE gel.

The 2-DE gel was trimmed (an upper left panel) and transferred to the PVDF membrane. Western blotting was performed using indicated polyclonal antibodies, which are shown to have broad cross-reactivity among human, mouse and rat by the manufacture. The PVDF membrane was re-used after stripping the previously used antibody. The multiple spots and slurs in an area surrounded by a dotted line in PKM2, Aurora-C, PGK1 and VDAC-1 antibody reactions are the background spots stubbornly remained after the α-enolase antibody reaction.

human databases did with lower % coverage values (data not shown).

The results of the 2D western blotting further revealed that some of the proteins were expressed as multiple spots with similar molecular weights but different pI values, indicating that those proteins have multiple isoforms via modification such as phosphorylation and acetylation. Our study, in which a combined analysis of western blotting and mass spectrometry was performed, could identify more protein isoforms in monkey ES cells than a report did on mouse ES cells, where a sole mass spectrometry analysis was performed (Table 3). Although most of the proteins identified in monkey ES cells were also detected in mouse ES cells[10], we found that some proteins were unique to the monkey ES cells. For example, annexin A family proteins were not detected in mouse ES cells, while annexins A2, A5 and A8 were clearly detected in monkey ES cells. Because the 2-DE proteomic study on mouse ES cells[10] was extensively performed by mass spectrometry successfully identifying as many as 123 protein spots in pH 3–10 strip-using gel, it seems that annexin A family proteins are not expressed, or if any, in undifferentiated mouse ES cells. Other examples are Aurora-C and pigment epithelium-derived factor, whose murine homologues have not been reported so far. We conclude that the human proteome databases, consisting of a large volume of information with high quality organization, are of a significantly great service in identifying monkey proteins.

We showed here a draft protein expression profile of undifferentiated cynomolgus monkey ES cells by 2-DE proteomic studies. We have successfully identified multiple protein spots via a combined analysis of mass spectrometry using human databases and western blotting using polyclonal antibodies. We also found that non-human primate databases are not so useful as human databases in identifying monkey proteins. This finding coincides with a previous report, which is the only one report that has ever reported on the 2-DE-based proteomic analysis using monkey samples[11]. In this report, heart samples of individual cynomolgus monkeys were subjected to 2-DE, and the protein

Table 3. The protein isoform detected in monkey and mouse ES cells

| protein name | cynomolgus monkey | mouse |
|---|---|---|
| elongation factor 2 | 3 | 3 |
| glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | 4 | 1 |
| pyruvate kinase isozymes M2 (PKM2) | 10 | 2 |
| α-enolase | 7 | 3 |
| voltage-dependent anion-selective channel protein 1 (VDAC-1) | 4 | 1 |
| serine/threonine-protein kinase 13 (Aurora-C) | 2 | 0 |
| annexin A2 | 5 | 0 |

The number of the multiple spots detected by a combined study of mass spectrometry and western blotting in cynomolgus monkey ES cells (present study) and those detected by mass spectrometry in mouse ES cells reported elsewhere (reference No. 10) was listed.

identification was achieved by a MALDI-TOF-mediated mass spectrometry querying human databases. Thus, human databases are substantially available for identifying monkey protein spots in 2-DE.

By virtue of 2-DE western blotting analysis, we could identify multiple isoforms of common cytoplasmic proteins including enzymes involved in glycogenesis/glycolysis pathways such as GAPDH, α-enolase and PKM2. Among these, GAPDH is recognized as a multi-function protein, playing roles in endocytosis, microtubule bunding, phosphotrasnferase, nuclear RNA transport, DNA replication, DNA repair, vial pathogenesis, oncogenesis and apoptosis[12]. The multiple isoforms we identified in monkey ES cells might be related to its multiple functions. Other enzymes shown here to have multiple isoforms may also have multi-functions other than glycolytic regulation.

In conclusion, 2-DE–based proteomic studies using monkey samples can sufficiently be achieved via a combined analysis of mass spectrometry querying human databases and western blotting using polyclonal antibodies raised against human epitopes.

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