200710004A

厚生労働科学研究費補助金

創薬基盤推進研究事業：政策創薬総合研究

ヒトＥＳ細胞を用いた安全な人工血液の開発に関する研究

平成１９年度　総括研究報告書

主任研究者　湯尾　明

平成２０（２００８）年　４月

目　　次

Ⅰ．総括研究報告

ヒトＥＳ細胞を用いた安全な人工血液の開発に関する研究
湯尾　明

Ⅱ．研究成果の刊行に関する一覧表

Ⅲ．研究成果の刊行物・別刷

Ｉ．総括研究報告

ヒトＥＳ細胞を用いた安全な人工血液の開発に関する研究

湯尾　　明

厚生労働科学研究費補助金（創薬基盤推進研究事業：政策創薬総合研究）

総 括 研 究 報 告 書

ヒトＥＳ細胞を用いた安全な人工血液の開発に関する研究

主任研究者　　湯尾　明　　国立国際医療センター研究所血液疾患研究部長

研究要旨　病原体混入がないクリーンな環境で安定して血液細胞成分を生成する技術を開発していくことは極めて重要である。そこで、本研究では動物由来成分を少しでも排除するために、ヒトＥＳ細胞から無フィーダー環境において血液細胞生成を試みた。我々の分化培養系は、前半の細胞凝集塊形成（sphere 形成）の浮遊培養と、後半の平面単層培養（接着培養）の２段階から成り立っており、全体を通じて無フィーダー培養である。第１段階の sphere 形成は、６種類の増殖因子・サイトカイン（IGF-II, VEGF, SCF, Flt3-L, TPO, G-CSF）存在下に３日間行われ、その後、ゼラチンコート培養皿の上にて第２段階の接着平面培養に移行した。接着した sphere からは２週間程度で嚢状構造物（sac-like structure）が形成され、構造物の内部に球状細胞（血液細胞）が充満した。産生された血液細胞は、CD45 陽性、CD34 わずかに陽性、CD11b、CD33 陽性の比較的成熟した骨髄系（食細胞系）の血液細胞で、そのうち、形態、細胞組織化学染色、表面抗原などから 30-50%が好中球と考えられた。これらの好中球は、in vitro では遊走能、貪食能、活性酸素産生能を示し、in vivo でも遊走能を発揮した。このような成熟好中球が効率よく産生できるＥＳ細胞の分化誘導システムは、霊長類では世界初であり、顕著な成果が得られたと言える。

Ａ．研究目的

血液成分の輸注は現代医療において不可欠であるが善意のボランティアに供給を依存しているため供給量に限りがあり、また、感染症の媒体となる危険をかかえている。従って、病原体混入がないクリーンな環境で安定して血液細胞成分を生成する技術を開発していくことは極めて重要である。

骨髄の造血幹細胞から血液細胞成分を増幅する試みは長年研究されてきたが、造血幹細胞は体外では充分に増幅しないことも確認された。一方、ＥＳ細胞は無限増殖能と多能性分化能を持つために再生医療における優れた材料として近年注目されている。即ち、ヒトＥＳ細胞から安定して試験管内で血液細胞成分を作成することが可能になれば輸血医療・移植医療において革命的な進歩がもたらされる。とりわけ、寿命が短いために現行の輸血療法では効果が乏しい「顆粒球」に関してはその恩恵は大きい。尚、ヒトを含めた霊長類ＥＳ細胞はマウスＥＳ細胞とかなり異なる性質を持つので、医療応用を目指す研究は霊長類ＥＳ細胞においてなされる必要がある。さらには既存の霊長類ＥＳ細胞の培養技術ではマウスフィーダー細胞の混入が避けられず新しい培養技術の確立が要求されている。

これらのことを踏まえて、本研究ではヒトＥＳ細胞から無血清無フィーダー環境において未分化維持と血液細胞生成を試みる。特に、白血球産生のための培養条件の決定を目指すとともに、ヒトＥＳ細胞の未分化維持と血液細胞への分化を制御する分子を網羅的な手法も駆使して見出すことにより、一層効率的な分化システムの構築を目指す。当該研究における成果は、免疫抑制剤投与方法の地道な工夫、組織適合抗原の発現制御技術、間葉系幹細胞を用いた免疫不適合緩和技術、そして将来的にはヒトクローン胚由来ＥＳ細胞を用いた培養技術、などと組合わすことで、常時万人に血液細胞成分を安全に供給するシステムが確立されることとなる。

Ｂ．研究方法

１．細胞など研究材料

マウス胎児線維芽細胞（murine embryonic fibroblasts, MEF）はマイトマイシンＣ

（MMC）処理またはX線照射によって増殖を停止させて未分化維持用のフィーダー細胞として用いた。ヒトＥＳ細胞（KhES-1, KhES-2, KhES-3）は、MMC 処理 MEF 上で 20%KSR 存在下に無血清培養により継代した。継代は週２回、コラゲナーゼ処理にて行い、細胞密度を２－４倍に希釈した。OP9 細胞は、20%牛胎児血清存在下で継代培養した。

２．分化誘導プロトコール

未分化ヒトＥＳ細胞をコラゲナーゼ処理により MEF の混入を避けて回収した後に、2-methacryloyloxyethyl phosphorylcholine コート低接着培養皿にて３日間スフェア（sphere）形成させた。分化培養液には、15%牛胎児血清の他に、６種類のサイトカイン・増殖因子（insulin-like growth factor II（IGF-II), vascular endothelila growth factor（VEGF), stem cell factor（SCF), Flt3 ligand（Flt3-L), thrombopoietin（TPO), granulocyte colony-stimulating factor（G-CSF)）を添加した。その後、スフェアはゼラチンコート培養皿での平面培養に移行した。サイトカイン・増殖因子は同様の６種類である。平面培養後、２週間以内に敷石状の細胞が増殖して、スフェアが着地した箇所に嚢状構造物が形成され、その中に球状の細胞が充満した。

一部の実験においては、ヒト臍帯血由来 CD34 陽性細胞を、上記の６種類のサイトカイン・増殖因子（IGF-II, VEGF, SCF, Flt3-L, TPO, G-CSF）存在下で、フィーダー細胞（マウスストローマ細胞 OP9）と共培養して、好中球の分化誘導を行った。

３．形態学的組織化学的観察方法

生細胞は、培養皿や培養フラスコのまま倒立顕微鏡により形態観察した。浮遊状態の血液細胞はスライドグラス表面にサイトスピン固定した後に、ライトギムザ染色、ミエロペルオキシダーゼ染色、好中球アルカリフォスファターゼ染色を行い、正立顕微鏡により観察した。

４．フローサイトメトリー

細胞膜表面抗原の同定は、細胞を PBS 中で３０分間１次抗体と反応させた後に FACSCalibur を用いて解析した。解析した抗原は、CD34、CD45、CD11b、CD33、CD66、CD16、GPI-80 である。

５．遊走能

遊走能は、２４穴プレートン設置したケモタキセルを用いて測定した。遊走因子は、菌体成分由来走化性ペプチド formyl-methionyl-leucyl-phenylalanine（FMLP)、ケモカイン interleukin 8（IL-8）を用いた。

６．貪食能

貪食能は、FMLP 存在下でのザイモザンの貪食により定量した。細胞はライトギムザ染色し、正立顕微鏡により貪食を観察した。

７．NBT 還元能

NBT 還元能は、FMLP 存在下での活性を測定した。細胞浮遊液をマツナミスライドグラスに滴下させ、formazan blue-black deposits 陽性細胞を正立顕微鏡下で観察した。

８．ケミルミネッセンス

ルミノール結合粒子の貪食による蛍光発色を蛍光分光光度計により定量した。

９．空気嚢炎モデルによる in vivo 遊走能の測定

ヒトＥＳ細胞由来好中球の NOG マウスへの移植実験と、空気嚢炎の組み合わせで実験を行った。マウス皮下に空気嚢を作成して３日後に、$2x10^6$ の細胞を経静脈的に移植し、空気嚢内に起炎剤としてザイモザンを注入した。１６時間後に、空気嚢に集積してくる好中球を回収して、フローサイトメトリーによりヒトＥＳ好中球を同定した。

（倫理面への配慮）

本研究ではヒト検体は使用しないし、臨床研究もない。また、動物実験を行う計画はない。さらに、ヒトのクローンなどの生命倫理に抵触するような実験、研究はいっさい含まれない。

<u>ヒト ES 細胞研究を開始するための国立国際医療センターにおける生命倫理に対する取り組み</u>

１．主任研究者による使用計画書とその概要の作成
２．機関内倫理委員会（「ヒト ES 細胞研究倫理　審査委員会」）の人選と確定
３．当機関としての倫理規定、倫理委員会運営

規定などの作成
4．生命倫理に関する勉強会、講演会の開催と参加
5．主任研究者が提出した使用計画に対する機関内倫理審査員会の審査
第１回：平成１７年４月１８日
第２回：平成１７年５月３０日
第３回：平成１７年８月　２日
6．使用計画書一式、機関内倫理委員会審査経過を文部科学省に提出
7．文部科学省特定胚及びヒトＥＳ細胞研究専門委員会にて当機関の使用計画が審査され承認。（平成１７年９月３０日 ）

最終的には、平成１７年１１月９日に当機関長宛に文部科学大臣の確認の文書（１７諸文科振第７３４号）が送付され、ヒトＥＳ細胞使用が認められた。

その後、研究者の追加・削除と研究業績の変更、使用期間と使用の方法の変更、使用機関の基準に関する説明の変更についても平成１８年１１月２４日に文部科学大臣の確認（１８諸文科振第７４３号）を得た。

さらにその後、文部科学省指針の改定に伴う変更と使用の方法の変更についても平成１９年１２月１８日に文部科学大臣の確認（１９国文科振第２６号）を得た。

さらにその後、研究者の追加・削除について機関内倫理委員会と機関長の了承を得て、平成２０年３月１１日に文部科学省に届け出た。

C．研究結果

1．２段階からなる独自のヒトＥＳ細胞由来血液細胞分化誘導法

分化誘導実験は、当初は３株のうちで、KhES-3 株にて詳細に行ったので、先ずその結果を示す。

我々の分化培養系は、前半の細胞凝集塊形成（sphere 形成）の浮遊培養と、後半の平面単層培養（接着培養）の２段階から成り立っており、全体を通じて、フィーダー細胞との co-culture は一切含まれておらず、無フィーダー培養である。第１段階の sphere 形成は、６種類の増殖因子・サイトカイン（20 ng/ml IGF-II, 20 ng/ml VEGF, 100 ng/ml SCF, 100 ng/ml Flt3-L, 50 ng/ml TPO, 100 ng/ml G-CSF）存在下に３日間行われた。

その後、ゼラチンコート培養皿の上にて、第２段階の接着平面培養に移行した。接着した sphere は当初は次第に平坦になって行くが、その後、接着部位の中心付近で再度隆起してきて、２週間程度で嚢状構造物（sac-like structure）が形成された。嚢状構造物の内部には球状細胞が次第に充満してきて、これが血液細胞となった。この時点で、ステムセルナイフを用いて嚢状構造物を切除して内部の球状細胞を培養液中に放出させた。嚢状構造物の外に出た球状細胞は、浮遊状態もしくは接着細胞に緩やかに接触しながら、活発に増殖した。

2．ヒトＥＳ細胞から分化誘導されたヒト血液細胞の解析

培養開始後４－６週間後に、球状細胞の細胞表面抗原（特に血液細胞抗原）のフローサイトメトリー解析を詳細に行った。この時期が球状細胞の増殖の最盛期であった。球状細胞の殆ど全てが血液細胞抗原である CD45 を発現していた。一方、造血幹細胞抗原である CD34 は、10%以内に止まり、球状細胞は血液細胞であるが、比較的成熟した血球であることが示された。さらに、球状細胞の 90%以上が、骨髄系細胞インテグリン系接着分子である CD11b 抗原陽性であり、同じく骨髄系細胞に特異的な抗原 CD33 も 90%以上が陽性であったので、血液細胞の大部分が骨髄系（食細胞系）の血液細胞で有ることが示された。

以上のように、産生された血液細胞は骨髄系血液細胞であったが、どのような血球成分が含まれるかライトギムザ染色による形態観察を行った。その結果、約 30%が多型核好中球、約 30%がマクロファージ、その他の約 40%が未分化な骨髄系細胞であると考えられた。このような形態像に一致して、80-90%の細胞がミエロペルオキシダーゼ染色陽性で、30-50%が好中球アルカリフォスファーターゼ染色陽性であった。

さらに、球状血液細胞中の好中球含有率を評価するために、好中球特異的抗原のフローサイトメトリー解析を行った。その結果、好中球特異的とされる CD66b、CD16b、GPI-80 抗原は、概ね、20-70%陽性であった。従って、上記の形態像や細胞組織酵素染色なども総合的に鑑みて、30-50%が好中球と考えた。

3．ヒトＥＳ細胞から分化誘導されたヒト好中球の機能解析

まず、好中球機能の中で炎症反応の最初に発揮され最も重要な成熟機能である遊走能に関して、菌体成分由来走化性ペプチド

formyl-methionyl-leucyl-phenylalanine（FMLP）とケモカイン interleukin 8（IL-8）を遊走因子として検討した。その結果、いずれに因子に対しても遊走能を示した。FMLP についしてはより強い遊走能を示し、IL-8 に対してはより好中球特異的な遊走を示した。すなわち、IL-8 に対して遊走した血球は、主に分節核好中球から成り立ち、好中球アルカリフォスファターゼ陽性であった。

IL-8 に対して遊走した好中球を回収して、これを、準純化ヒトＥＳ細胞由来ヒト好中球と考えて、様々の成熟好中球機能の解析を行った。その結果、ザイモザン貪食能、活性酸素産生能（NBT 還元能）はいずれも陽性であり、蛍光粒子貪食による活性酸素産生も陽性有った。以上より、ヒトＥＳ細胞から分化誘導したヒト好中球は、成熟機能を十分に備えた機能細胞であることが明らかとなった。

４．ヒトＥＳ細胞由来ヒト好中球の遊走能の in vivo 解析

我々は以前より空気嚢炎を駆使した生体内での（in vivo の）遊走能の測定系を有し、しかも、ヒト造血幹細胞を移植した NOG マウスにおいて生着したヒト血液細胞がマウス体内で好中球に分化して空気嚢炎症巣に遊走することを確認してきた。本研究においてもこのような独自な系を駆使して、ヒトＥＳ細胞由来ヒト好中球の遊走能の in vivo 解析を遂行した。その結果、ヒト CD66 抗原陽性のヒト ES 細胞由来のヒト好中球が、炎症巣にわずかながらも（1%未満）遊走していることが確認できた。対照として用いられたヒト造血肝細胞由来ヒト好中球においても、空気嚢に有した好中球は 1%未満であったので、この数値は有意であると考えられた。

５．KhES-1 株、KhES-2 株を用いた血液細胞分化誘導

KhES-1 株、KhES-2 株を用いて、上記と同様の分化誘導を行ったが、血液細胞分化は困難であった。KhES-1 株ではわずかに血球分化が認められたが効率は極めて低く、KhES-2 株では、全く血球分化しなかった。ただし、KhES-2 株に関しては、無血清（KSR20%）条件下で分化誘導を行ったところ、接着平面培養に移行した後は、嚢状構造物は出現しないまま、紡錘形の接着細胞とともに球状細胞が大量に増殖し始めた。これらの球状浮遊細胞は、CD45、CD11b、CD33 は陽性になるものの CD66b、CD16b は陰性で、形態上も WG 染色でアズール顆粒が偏って染色され、非定形的な分化と考えられた。

D．考察

我々の行った分化培養によって、ヒトＥＳ細胞から極めて高効率に血液細胞が分化誘導できた。また、その中での好中球の比率も高く、霊長類ではこれまでに報告がない。世界的に見てもこれほど高効率での分化誘導は例が無く、極めて優れた技術開発に成功したと言っても過言ではない。

他の研究グループで達成できなかった高純度の血液分化誘導が我々の研究室で可能であった理由として、以下の点が推測される。我々の分化誘導系は前半の細胞凝集塊形成浮遊培養と後半の接着平面単層培養の２段階システムからなる分化誘導系であることである。血液細胞分化誘導などの中胚葉分化法においては、このような２段階培養は殆ど試みられておらず、我々の系の独自性が良好な結果につながった可能性が考えられる。

生体内での機能性好中球の評価システムは極めて重要である。我々の研究グループは従来より通常のマウスで試みられていた in vivo 炎症モデルの１種である空気嚢炎モデルを、移植実験で有用な NOG マウスにおいて成功させた。さらに、NOG マウスにヒト造血幹細胞を移植して生着させて、そこからマウス体内で分化してくるヒト好中球を、空気嚢炎内で検出することにも成功して、血液系移植後の機能性好中球産生評価システムに使用できることを証明した。今回、このシステムを駆使してヒトＥＳ細胞由来のヒト好中球がマウス体内で遊走能を発揮することを示した。今後は、ヒトＥＳ細胞からヒト造血幹細胞を作成して、その体内分化能を検証することにも応用可能であると考えられる。

今回の検討においては、ヒトＥＳ細胞株のうちの１株において成果が得られた。他の株における工夫を凝らして、無血清培養も含めた展開が期待される。また、今回は好中球の分化に成功したが、より未熟な造血幹細胞の誘導を目指したい。

本年度の成果により、ヒトＥＳ細胞から、無フィーダーの独自の分化システムによって、血液細胞を高純度に作成することに成功した。このような成果は、類似した性質を有するヒト iPS にも則応用可能であると考えられ、今後の発展が期待される。

E．結論

本研究では、異種動物由来の成分の混入を回避する培養法の開発のために、ヒトＥＳ細胞を用いて、無フィーダー分化誘導系による血液細胞の産生を試みた。培養法の基本は、前半のsphere形成浮遊培養と後半の平面培養であった。敷石状の細胞の増殖、中心部での嚢状構造物の形成を経て、血液細胞が産生された。産生された血液細胞は比較的分化した骨髄系の細胞で、好中球を多く含んでいた。これらの好中球は、in vitroでもin vivoでも十分な機能を発揮出来る成熟好中球であった。このような成熟好中球が効率よく産生できるＥＳ細胞の分化誘導システムは、霊長類では世界初であり、顕著な成果が得られたと言える。

F．健康危険情報

なし

G．研究発表

１．論文発表

1. Nakahara M, Saeki K, Yogiashi Y, Kimura A, Horiuchi A, Nakamura N, Yoneda A, Saeki K, Matsuyama S, Nakamura M, Toda T, Kondo Y, Kaburagi Y, Yuo A: The protein expression profile of cynomolgus monkey embryonic stem cells in two-dimensional gel electrophoresis: a successful identification of multiple proteins using human databases. J Electrophoresis 51:1-8, 2007.

２．学会発表

1. 中原正子、過足芳子、佐伯晃一、中村直子、松山さと子、米田麻子、佐伯久美子、湯尾　明：サル・ヒトＥＳ細胞からの無フィーダー培養による高効率な血管内皮細胞分化法の開発。第６９回日本血液学会・第４９回日本臨床血液学会・合同総会、２００７年１０月、横浜。
2. 中原正子、過足芳子、佐伯久美子、佐伯晃一、中村直子、松山さと子、米田麻子、小柳　真、近藤　靖、末盛博文、中辻憲夫、湯尾　明：霊長類（サル、ヒト）ＥＳ細胞からの無フィーダー培養による高効率な血管内皮細胞分化誘導法の開発－各サイトカインの役割の検討及びin vivoでの機能評価－。第３０回日本分子生物学会年会・第８０回日本生化学会大会・合同大会、２００７年１２月、横浜。
3. 中村直子、過足芳子、中原正子、佐伯久美子、小柳　真、松山さと子、小柳明美、八木田秀雄、湯尾　明：霊長類胚性幹（ＥＳ）細胞からの高効率な血管内皮細胞分化。第７回日本再生医療学会総会、２００８年３月、名古屋。

H．知的財産権の出願・登録状況

１．特許取得

国際特許

霊長類動物胚性幹細胞の培養及び継代方法、並びにその分化誘導方法

発明者：湯尾　明、佐伯久美子、佐伯晃一、中原正子、中村直子、過足芳子、松山さと子、米田麻子

出願人：国立国際医療センター、田辺三菱製薬株式会社

PCT/JP2007/71811

２．実用新案登録

なし

３．その他

なし

Ⅱ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

なし

雑誌

発表者氏名	論文タイトル	発表誌名	巻号	頁	出版年
Nakahara M, Saeki K, Yogiashi Y, Kimura A, Horiuchi A, Nakamura N, Yoneda A, Saeki K, Matsuyama S, Nakamura M, Toda T, Kondo Y, Kaburagi Y, Yuo A	The protein expression profile of cynomolgus monkey embryonic stem cells in two-dimensional gel electrophoresis: a successful identification of multiple proteins using human databases.	J Electrophoresis	51	1-8	2007

III. 研究成果の刊行物・別刷

[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

Masako Nakahara[1], Kumiko Saeki[1], Yoshiko Yogiashi[1], Akiko Kimura[1], Akiko Horiuchi[1], Naoko Nakamura[1], Asako Yoneda[1], Koichi Saeki[1], Satoko Matsuyama[1], Megumi Nakamura[2], Tosifusa Toda[2], Yasushi Kondo[3], Yasushi Kaburagi[4] and Akira Yuo[1]

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

[1] Department of Hematology, Research Institute, International Medical Center of Japan, Tokyo, Japan.
[2] Research Team for Molecular Biomarkers, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan.
[3] Advanced Medical Research Laboratories, Tanabe Seiyaku Co. Ltd., Osaka, Japan.
[4] Department of Metabolic Disorder, Research Institute, International Medical Center of Japan, Tokyo, Japan.
Correspondence address: Akira Yuo; Department of Hematology, Research Institute, International Medical Center of Japan, 1-21-1, Toyama, Shinjuku-ku, Tokyo 162-8655, Japan.

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.

(Received October 16, 2006, Accepted December 22, 2006, Published March 15, 2007)

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 allo-transplantation 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), 4×10^7 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-

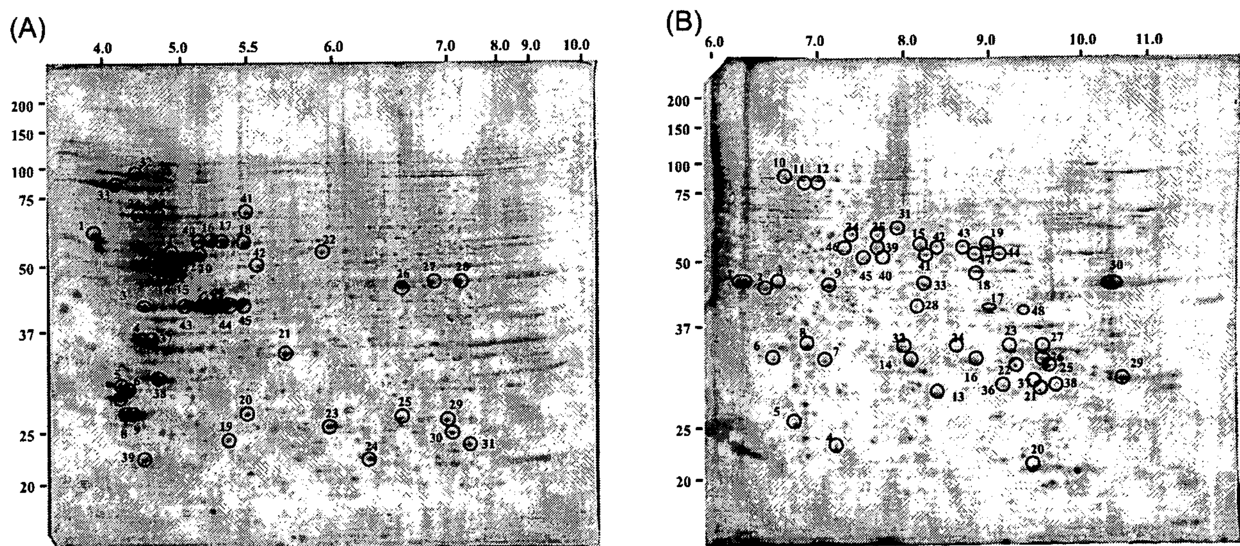

Fig. 1. Protein expression patterns of cynomolgus monkey ES cells in 2-DE.

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

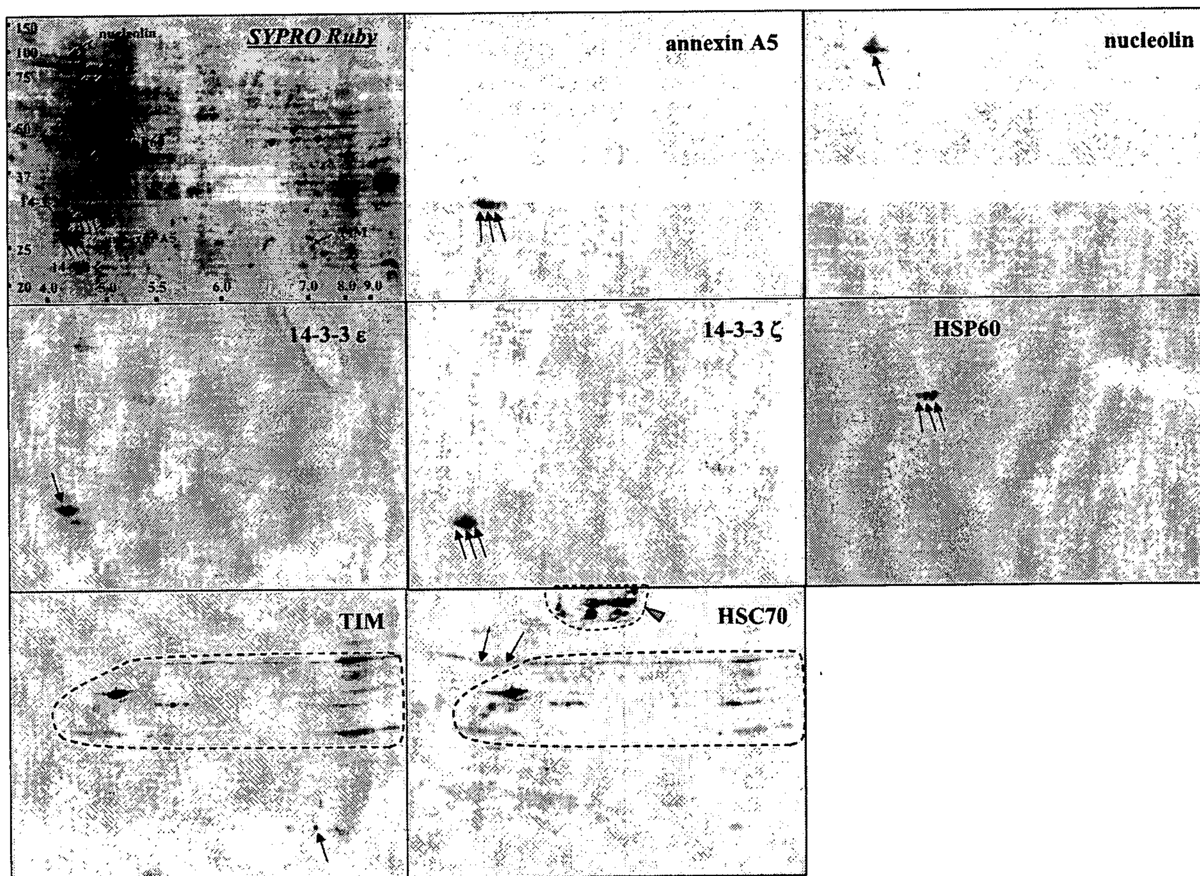

Fig. 2. Two dimensional western blotting after a pH 3–10 strip-using 2-DE gel.

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.

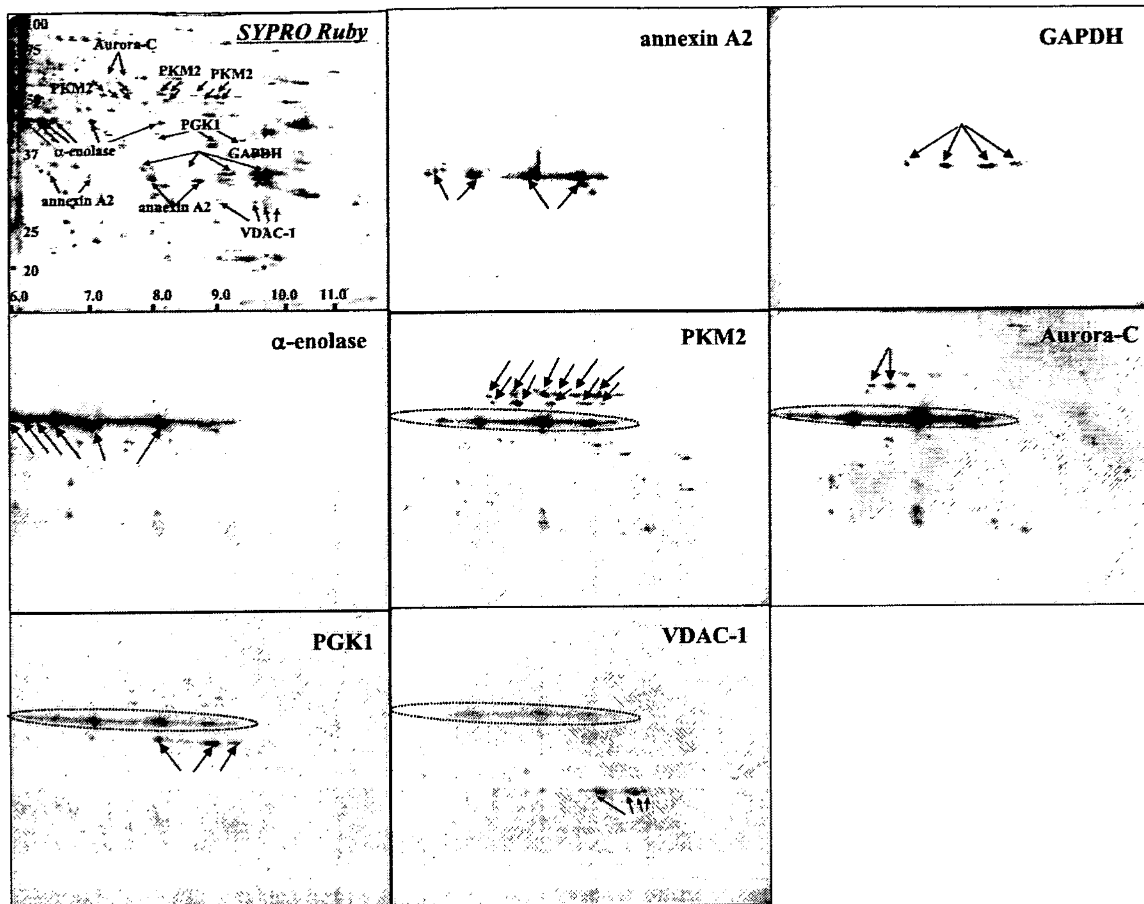

Fig. 3. Two dimensional western blotting after a pH 6–11 strip-using 2-DE gel.

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.

ACKNOWLEDGEMENTS

The authors greatly thank Etsuko Yasugi and Emiko Okuma for technical assistance.

REFERENCES

1) Pincus SE, Lukacher N, Mohamed N, Sesay M, Zabinski R, Ebelle R, Duncan L, Li J, Chen X, Peng W, Adaelu J, Casey L, Porter JP, Spitalny G, Nardone LL. Evaluation of antigen-based heteropolymer for treatment of systemic lupus erythematosus in a nonhuman primate model. Clin Immunol 2002;105:141–154.
2) Umeda S, Suzuki MT, Okamoto H, Ono F, Mizota A, Terao K, Yoshikawa Y, Tanaka Y, Iwata T. Molecular composition of drusen and possible involvement of anti-retinal autoimmunity in two different forms of macular degeneration in cynomolgus monkey (Macaca fascicularis). FASEB J 2005;19:1683–1685.
3) Sumi T, Fujimoto Y, Nakatsuji N, Suemori H. STAT3 is dispensable for maintenance of self-renewal in nonhuman primate embryonic stem cells. Stem Cells 2004;22:861–872.
4) Zwaka TP, Thomson JA. A germ cell origin of embryonic stem cells? Development 2005;132:227–233.
5) Takagi Y, Takahashi J, Saiki H, Morizane A, Hayashi T, Kishi Y, Fukuda H, Okamoto Y, Koyanagi M, Ideguchi M, Hayashi H, Imazato T, Kawasaki H, Suemori H, Omachi S, Iida H, Itoh N, Nakatsuji N, Sasai Y, Hashimoto N. Dopaminergic neurons generated from monkey embryonic stem cells function in a Parkinson primate model. J Clin Invest 2005;115:102–109.
6) Hering BJ, Wijkstrom M, Graham ML, Hardstedt M, Aasheim TC, Jie T, Ansite JD, Nakano M, Cheng J, Li W, Moran K, Christians U, Finnegan C, Mills CD, Sutherland DE, Bansal-Pakala P, Murtaugh MP, Kirchhof N, Schuurman HJ. Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. Nat Med 2006;12:301–303.
7) Suemori H, Tada T, Torii R, Hosoi Y, Kobayashi K, Imahie H, Kondo Y, Iritani A, Nakatsuji N. Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. Dev Dyn 2001; 222:273–279.
8) Toda T, Sugimoto M, Omori A, Matsuzaki T, Furuichi Y, Kimura N. Proteomic analysis of Epstein-Barr virus-transformed human B-lymphoblastoid cell lines before and after immortalization. Electrophoresis 2000;21:1814–1822.
9) Saeki K, Yasugi E, Okuma E, Breit SN, Nakamura M, Toda T, Kaburagi Y, Yuo A. Proteomic analysis on insulin signaling in human hematopoietic cells: identification of CLIC1 and SRp20 as novel downstream effectors of insulin. Am J Physiol Endocrinol Metab 2005;289:E419–E428.
10) Elliott ST, Crider DG, Garnham CP, Boheler KR, Van Eyk JE. Two-dimensional gel electrophoresis database of murine R1 embryonic stem cells. Proteomics 2004;4: 3813–2832.
11) Yan L, Ge H, Li H, Lieber SC, Natividad F, Resuello RR, Kim SJ, Akeju S, Sun A, Loo K, Peppas AP, Rossi F, Lewandowski ED, Thomas AP, Vatner SF, Vatner DE. Gender-specific proteomic alterations in glycolytic and mitochondrial pathways in aging monkey hearts. J Mol Cell Cardiol 2004;37:921–929.
12) Sirover MA. New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. Biochim Biophys Acta 1999;1432:159–184.